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Master of Biotechnology
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I would firstly thanks my co-supervisor Tore Brembu to gave e the opportunity to assist his research on diatoms, his passion for diatom molecular biology is acknowledge. The previous research on *Coscinodiscus wailesii* made by my co-supervisor were as helpful as his mentoring.

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Abstract

Diatoms are known for their numerous shapes and forms given by their cell wall made of silica named frustule. They also differ significantly in size. The research on diatom genetics has mainly focused on small diatoms for practical reasons, such as genome size, but also the convenience of shorter generation time of smaller cells. Despite having a longer doubling time, big diatoms perform comparatively in terms of biomass density and growth, making them good candidate species for industrial scale production. Those advantages could make the giant diatoms good candidate species to scale-up production. It is necessary to develop methods and knowledge on the giant diatom gene expression to unleash their industrial potential in the near future. This master thesis aimed to apply genetic engineering in *Coscinodiscus wailesii* by trans-kingdom conjugation from *Escherichia coli*.

As attempts to operate conjugations in Coscinodiscacae are hitherto unreported, cultivation and selection processes had to be designed. Furthermore, due to the inability to grow *C. wailesii* on agar, a new in-agar culture protocol was developed.

From the published genomic sequence of *C. wailesii*, highly expressed promoters from the gene CwLHCF4-like and CwLHCFNew-1 were used to design the episomes. The plasmids for conjugation are composed of a pPtPUC3Mut backbone associated with the promoters, a terminator and a selective marker gene. The selective gene inserted in the plasmid, the commonly use ble (zeocin). Previous experiments shown that *Coscinodiscus wailesii* cannot tolerate a concentration of 50 µg/mL of zeocin.

As of today, the conjugative DNA transfer between bacteria and *C. wailesii* could not being demonstrated. The document aims to provide an explanatory protocol on how to develop molecular tools in diatoms and encourage other researcher to take over the project using the information discovered during the master thesis.

Útdráttur

Þróun Sameindalíffræði tooes í kísilþörungar *Coscinodiscus Wailesii* kísilþörungars eru þekktir fyrir fjölmörg form og eyðublöð sem gefin eru af frumuveggnum sínum úr kísil sem heitir frustule. Þeir eru einnig mjög mismunandi í stærð. Rannsóknir á erfðafræðilegum erfðafræðilegum köflum hefur aðallega lagt áherslu á litla kísil af hagnýtum ástæðum, svo sem genamengi, en einnig þægindi af styttri kynslóð minni smærri frumna.

Þrátt fyrir að hafa lengri tvöföldunartíma, sinna stórum köflum tiltölulega hvað varðar lífmassaþéttleika og vöxt, sem gerir þeim góðan frambjóðandi tegundir til framleiðslu á iðnaðar mælikvarða. Þessir kostir gætu gert risastórt dísir góðar frambjóðandi tegundir til að mæla upp framleiðslu. Nauðsynlegt er að þróa aðferðir og þekkingu á risastórt kísilgen tjáningu til að gefa lausan tauminn iðnaðar möguleika sína í náinni framtíð. Þessi aðalritgerð sem miðar að því að beita erfðafræðilegum verkfræði í *C. wailesii* með samskiptum í *E. coli*.

Þar sem tilraunir til að starfrækja samtengingar í Coscinodiscacae eru hingað til unreported, þurfti að hanna ræktun og valferli. Enn fremur, vegna vanhæfni til að vaxa *C. wailesii* á þýðingar, var nýtt í þýðingar rækun samskiptareglur þróað. Frá birtu erfðamengjafræði röð *C. wailesii*, mjög gefið upp verkefnisstjórar frá gen CwLHCF4-like og CwLHCFNew-1 voru notuð til að hanna episomes. Plasmíðin fyrir samtengingu samanstanda af pCwPUC3Mut burðarás í tengslum við verkefnisstjóra, terminator og sértækur merkisgen. Vandlátur genið er sett í plasmíðið, almennt ble (zeocin). Fyrri tilraunir sýndu að *C. wailesii* getur ekki þolað styrkleika 50 g / ml af zeocin.

Frá og með í dag var ekki hægt að sýna samskipt DNA flytja milli baktería og *C. wailesii*. Skjalið miðar að því að veita skýringarmynd um hvernig á að þróa sameindaverkfæri í kísilum og hvetja aðra rannsóknaraðila til að taka við verkefninu með því að nota upplýsingarnar sem uppgötvast á aðalritgerðinni.

Preface

Year after year, more and more organism have their genome and molecular biology studied. Some species are being more studied than others. For example, for the case of the microalgae most of the research is done on *Thalassiosira pseudonana* and *Thalassiosira pseudonana Phaeodactylum tricornutum*, historical and biological reasons explains why those species became model species for the diatom, their studies was used by many scientists to pierce the secrets of the diatoms and of the photosynthetic microorganism. It is, however, thought that some aspect of the richness of the microalgae diversity could be missed out if the scientist are working on only two species.

There is a strong motivation to expand the array of species that could be used in lab as new molecular biology methods have been developed. In the past, the two usual methods to change the gene expression of diatoms were electroporation (Miyara et al., 2013) and bombardment (Apt et al., 1996). Those two methods are applicable on many different species with different level of results Velmurugan and Deka (2018) but they have the disadvantage to be damageable for the cells as the molecular tool are forced to penetrate the cells in both methods. In **?**, Karas et al. published an innovative study where they show it is possible for bacteria to transmit genetic materials to diatoms. The experiment was carried on *T. pseudonana* and *P. tricornutum*. Their results formed the basis of this master project.

Aims

The studies aimed to develop molecular tools to change the gene expression in the giant diatom *Coscinodiscus wailesii* by conjugative DNA transfer. Based on the work of (Karas et al., 2015) who created the first protocol of conjugative DNA transfer in diatom, the project would begin by designing episomes that would be expressed by the *C. wailesii* cells. Along the way, culture optimization was superficially studied to help the team to supplant the challenge to bring *C. wailesii* up in cell concentration and total cell number to carry out the molecular biology experiments. The figure below is a flowchart that shows a simplification of the distinct steps that were planned to achieve the goal of the study. It is important to note that most activities were done simultaneously during the research. It was intended to be written as a case study on how to extend the number of microalgae species that can be genetically changed.

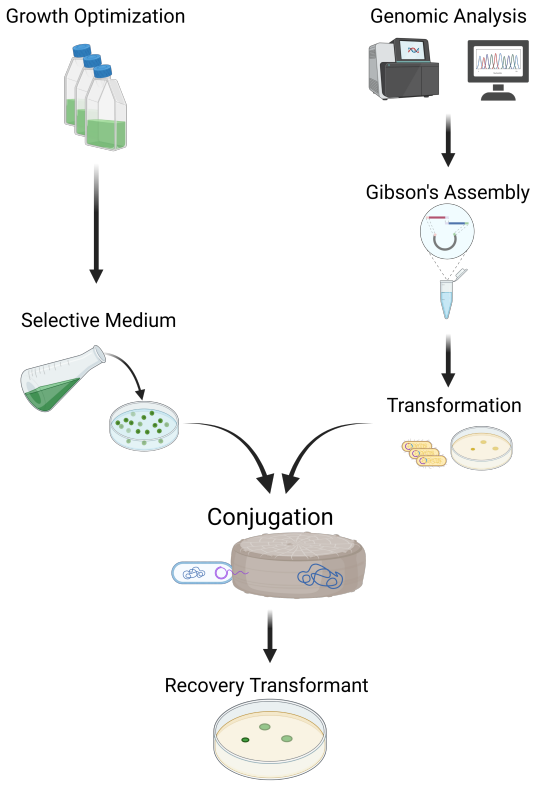

Figure: Flowchart illustrating the different steps that were carried in that studied. Created on biorender.com

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Chapter 1

Introduction

1.1 Diatoms

As defined by Seckbach and Kociolek in The Diatom World (2011) "Diatoms are microscopic unicellular or colonial (in the shape of filaments or ribbons, or tube dwelling) eukaryotic algae. Their cell walls are with silica shells, and they are ubiquitously distributed in aqueous habitats." Diatoms are part of the stramenopile. Doubts subsist about their origins. The commonly accepted theory claims that diatoms are the results of an heterokont engulfing a red alga, the engulfment are represented in Figure 1.1 (Prihoda et al., 2012). However, the paleontological observations draw of the engulfing timeline, do not match with genetic observations (Medlin, 2011). Diatoms are under the class Bacillariophyceae and in the super group Choromophyta. The specificity of diatoms autotrophy, being unicellular organisms with a special cell wall named frustule made of two silica valves.

Diatoms are found in a great variety of environments, from the global ocean to the sporadic spot of mist forming in dry terrestrial environment (Armbrust, 2009; Johansen, 1983). The number of diatoms specie is widely discussed. The most generous estimates propose that up to 200,000 species of diatoms exist, though more recent estimates suggest a range of 30,000 to 100,000 with 12 000 described and characterized (Mann and Vanormelingen, 2013). Molecular-clock-based estimation states that diatoms arose at around 250 millions years ago in the Triassic by estimation made on although the oldest fossils of diatoms are 190 million years ago (Sims et al., 2006).

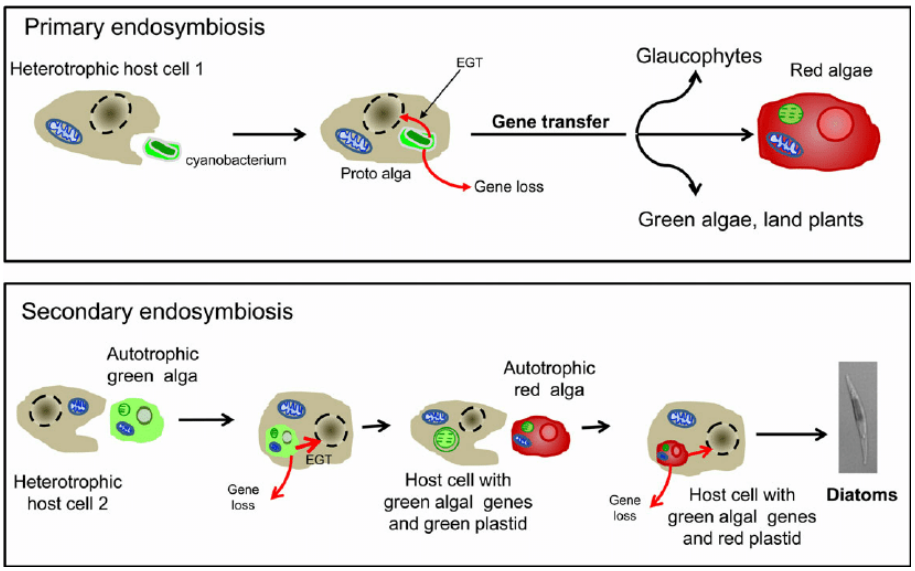

Figure 1.1: Scheme of the commonly accepted evolutionary history of diatoms. The primary endosymbiosis that lead to green and red algar are represented on the top part of the figure. The double endosymbiosis with the engulfment of a green algae by and an heterokont, later followed by the engulfment of a red algae is describe in the bottom of the figure. Retrieved from Prihoda et al. (2012).

1.1.1 Diatom Frustule

Many organisms have an external and internal silica skeleton, such as higher plants, protozoans, and sponges (Simpson and Volcani, 1981). However, the diatoms are the typical example of organisms with that structure. The diatoms are divided in three different groups depending on the general shape given by their frustule, namely the radial centric, the polar centric, and pennates (Kröger and Poulsen, 2008), the illustration in Figure 1.2 shows the diversity in shape and size given to the diatoms by their frustules.

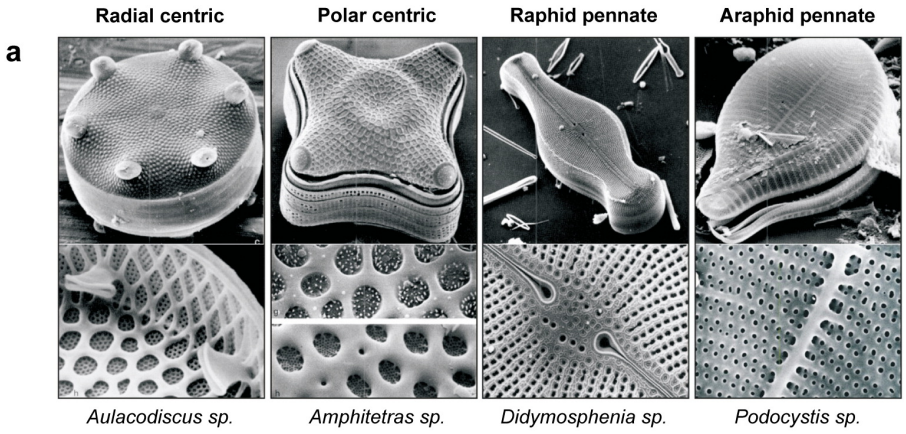

Figure 1.2: Illustration of the diversity of shape given to the diatom by their silica structure. Retrieved from Kröger and Poulsen (2008).

A frustule is composed of two complementary valves: the hypotheca and the epitheca. The two valves complement each other like the valves of a common Petri dish as illustrated in Figure 1.3. In the process of vegetative reproduction the mother cell will be divided in two by giving a theca to each daughter cell, the inherited theca ultimately becomes the epitheca of the newly born cell. This limits the number of asexual division a diatom can do without undergoing sexual reproduction. This issue will be addressed in Chapter 2.3.

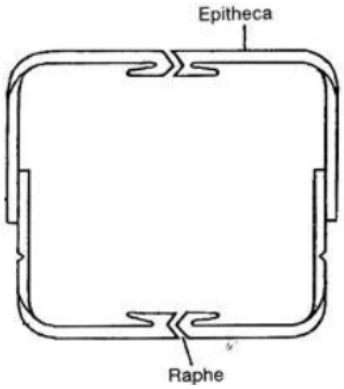

Figure 1.3: Scheme of the assembly of the the two theca composing the frustule of a diatom. The epitheca is represented on the superior part of the figure and the hypotheca is on the lower one. Modified from Neelesh (n.d).

It is thought that the diatom cell wall has evolutionary advantages on different aspect. One of them states that the use of inorganic material like silica is less costly to build a cell wall (Raven, 1983). Also, frustule could be a defense mechanism against predators, the shape and hardness of the diatom makes them less attractive for predator (Smetacek, 1999). Due to their frustules, diatoms have limited motility, which is a liability for the search for light. Therefore, diatoms mostly rely on current and on density modification to move through the water column when they are not simply laying on the bottom of shallow waters. Besides, the frustules of diatoms seems to act at

photonic crystals that would select certain wavelenght of light to be taken up by the diatoms, the functioning of the photonic crystals would be explain in section 1.2.2.

1.2 *Coscinodiscus wailesii* Gran & Angst

Coscinodiscus wailesii (Figure 1.4) is a centric diatom, it is a widely spread in temperate water present from the European coast to the considered as a giant diatom, it has an observed diameter of 320-350 µm in the natural environment but diatoms above 500 µm have been reported (Laing and Gollasch, 2002). The mantle, which is the downturned side of the diatom, is 30-40 µm high (Schmid, 1990). For the context, *Thalassiora pseudonana* another diatom have a diameter of only 2.5–15 µm (Muylaert and Sabbe, 1996).

C. wailesii can survive in a resting state in the sediment for up to 15 months. When in vegetative state its doubling time has been estimated at 70 hours (Rick and Dürselen, 1995).

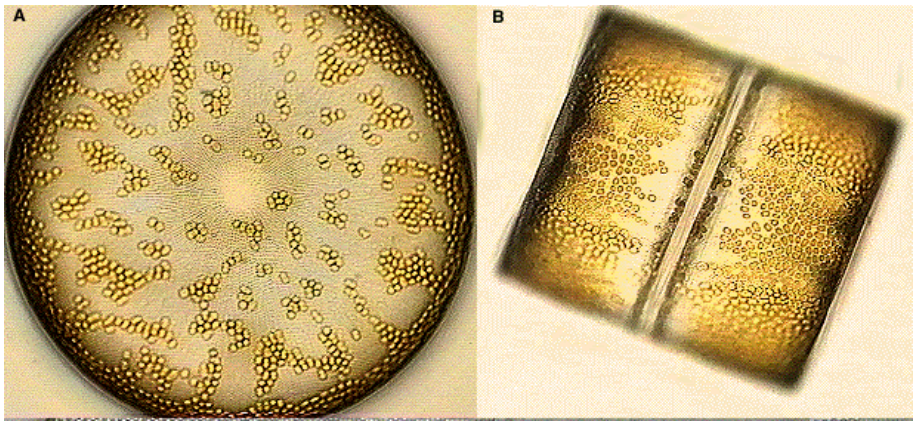

Figure 1.4: This species originally coming from the warm seas is invasive in many parts of the world such as in the European Atlantic coast line as early as 1978. It is thought that it has been imported in Europe by ballast water or by importation of oysters. Retrieved from (Reise et al., 1998).

C. wailesii has a tendency to bloom, the concentration of *C.wailesii* was measured up to 90% of the total phytoplankton biomass during blooms in the North sea (Rick and Dürselen, 1995). One of the direct effect of the *C. wailesii* blooms is the intense production of mucilage. Mucilage is a substance produced by plants and microorganisms, mostly composed of transparent exopolymer particles (TEPs). When *C. wailesii* is blooming, mucilage appears like a slime that can clog the fishing nets and the cages of fish farms. Consequently, the collapse of the bloom can create a sudden anoxia in the environment. Gómez (2008) suggested that *C. wailesii* bloom have been mostly reported between 1977 and 1990, it seem to correlate with the global cooling period of 1970 and the Great Salinity Anomaly of the end the 1980's but the causality of those events was not fully established. It is interesting to notice that the blooms usually occur during cold winters

1.2.1 Reproduction of *C. wailesii*

As the other diatoms, *C. wailesii* can have a sexual and asexual reproduction. The asexual reproduction of the diatom is made by a cellular division that separates the valves of the mother cell, resulting in two daughter cells with one valve from the mother each. A smaller new valve, the hypotheca, is produced to complete the old one, epitheca. This has for effect to reduce the diameter of diatom cells generation after generation. *C. wailesii* can undergo this asexual reproduction until reaching 50 µm in diameter (Hasle and Lange, 1992). Sexual reproduction involves the formation of auxopores by oogamous reproduction. Fertilization results in a zygote that develops into a full-grown diatom(Mann and Vanormelingen, 2013; Mann, 1993). A simplification of the full cycle of the reproduction of diatom can be seen in the figure 1.5 below.

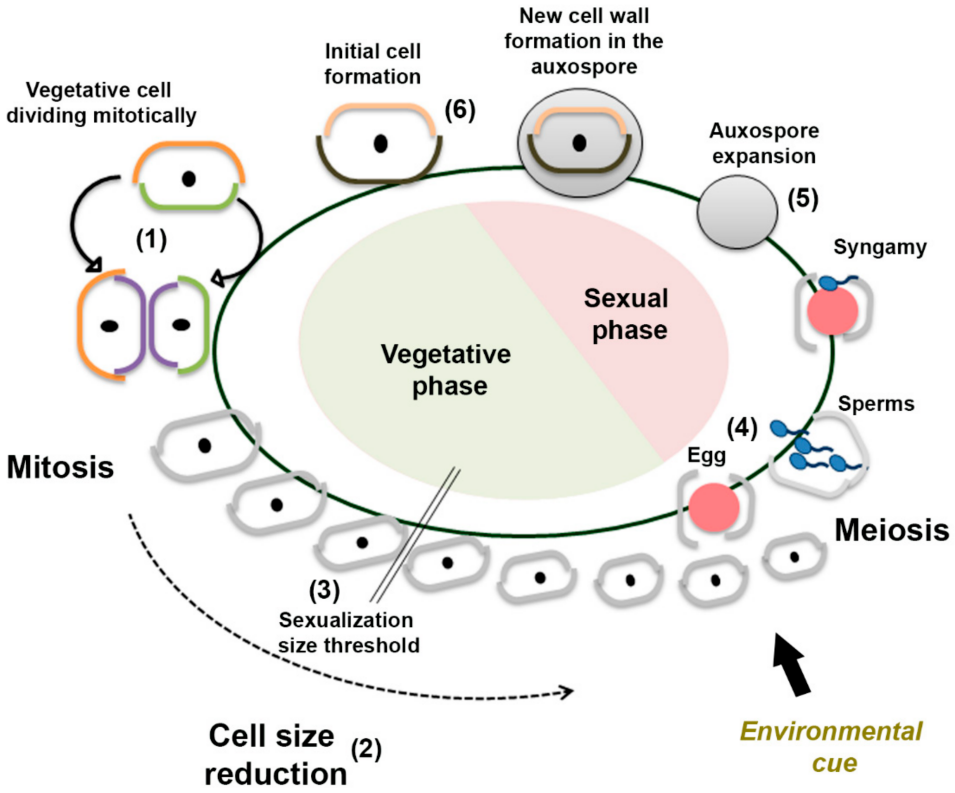

Figure 1.5: Full representation of the reproductive cycle of of diatoms. The asexual reproduction is on the (1) part of the figure with the reduction of the cell size in (2). Retrieved from Ferrante et al. (2019).

1.2.2 Industrial Interest of *Coscinodiscus wailesii*

Since the second half of the 20th century, diatoms have received considerable academic interests beyond mere scientific curiosity, as they have been identified as an underutilized source for compounds and materials for various industries (Zhang, 2019).

In material sciences, the intense focus on bioproduction of semiconductors, metals or polymers has placed diatoms in the cross-hairs/focal points of multiple research groups (Losic et al., 2009). One example would be the production of anode material for Li-ion batteries (Norberg et al., 2019). The current processes employed to manufacture pure grade silica is costly and energy consuming. It could potentially be a deal changer to use diatoms for the fabrication of silica material, to the point of of being carbon negative if it would be bio-produced by diatoms (Norberg et al., 2019). Another example is the use of silica structure based on diatom to deliver drugs inside an organism (Maher et al., 2018).

Through the last decade, nanotechnologists have had a growing interest in the biophotonic proprieties of diatom frustules. Photonic crystals are nanostructures that redirect light (selectively) redirect light depending on its bandwidth, a property observed in certain diatom frustules which exhibit selectivity/preference towards specific light frequencies. Other products, comprising an emerging market in which research towards the reduced cost of production of photonic crystals by the use of diatoms is receiving substantial attention. There is an emerging market that is closely researching at how to produce photonic crystals at inexpensive cost from diatoms (Mishler et al., 2014).

Being the giants of the diatom world, *Coscinodiscus sp.* are considered suitable candidates for research on photonic and other nanomaterial properties for industrial application.

1.2.3 Known Difficulties of *Coscinodiscus wailesii* Cultivation

The cell concentration of *C. wailesii* ranges up to a maximum 1000 cells/mL (Talgatti et al., 2010). In comparison, *Thalassiosira weissflogii*, a small diatom, can have a concentration of 1 million and 3 million cells/mL (Daniel and Srivastava, 2017). The low cell density combined with a long doubling time in nature of 70 hours when it is only of 8 hours for *Thalassiosira fluviatilis*, another centric diatom (Werner, 1970). Although the exact doubling time in monoculture is not known.

At first glance, the long doubling time in lab conditions of *C. wailesii* it would appear as an unlikely candidate for any biotechnological application. But as *C. wailesii* tends to bloom and therefore over perform other algae at sea, it is possible that some part of their biology are not understood well enough to be exploited.

1.3 Horizontal Gene Transfer

Bacteria are able to do different Conjugation is a mechanism developed by prokaryotes to transfer DNA from one donor cell to a recipient cell; the transfer is mediated by a pilus, a pilus is a bacterial appendage made of protein that penetrate other cells for the transfer (Madigan et al., 2019). The first observation of a conjugation between bacteria (*Escherichia coli*) was made in 1946 (Lederberg and Tatum, 1946).

This mechanism relies on three essential genes, tra, mob, and oriT. Those genes determine if a plasmid is self-transferable or not. To be self-transferable, all the three loci need to be present. The oriT is the origin of transfer of the plasmid, the locus tra codes for the expression of the pilli which connect the donor and recipient cell, and the locus mob is the one responsible for the linkage of the replicated plasmid to the transfer mechanism. In nature many plasmids are not self-transferable, they are known has mobilizable plasmids. Those plasmids rely on self-transferable plasmid like the F factors to be transmitted. Because the mobilizable plasmid can interact with the expression of mob and tra of the F plasmid. The oriT, is a mandatory component of plasmids. The process of conjugation is illustrated in Figure 1.6.

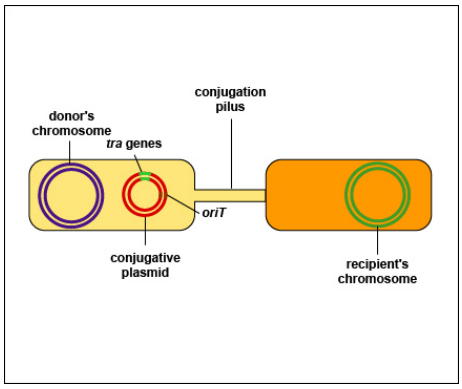

Figure 1.6: Representation of the conjugation process with the donor cell on the left and the recipient cell on the right. The plasmid is replicated and being sent through the pillus in the recipient cell. Retrieved from Kaiser(2021).

The scientific community has a growing concern toward the capacity of bacteria to transfer antibiotic resistance gene to other strains. This is considered an issue of public health (Brolund, 2014).

Conjugation is a popular method to genetically modify non-bacterial cells as it is possible to have a conjugation from a bacterial to a cell from other kingdoms.

1.3.1 Transkingdom Conjugation

Transkingdom gene transfers are horizontal gene transfers that occurs between organisms from different kingdoms. It has been thought to only occur between bacteria. The discovery of T-DNA in *Agrobacterium tumefaciens* during research on the cause of tumors in higher plants shown that the possibility of transmitting DNA from a bacteria to eukaryotes. A.alphaproteobacterium has a special plasmid that can be transmitted to a plant cell and then inserted into its genome by the action of the vir genes (Madigan et al., 2019).

In 1989 an experiment shown that the horizontal transfer of DNA between bacteria and yeast was possible. Heinemann and Sprague (1989) used episomes that would be both active in bacteria and in yeast. Their results showed that the yeast *Saccharomyces cerevisiae* had a gain of function after interacting with *E.coli*. Successful conjugations have been operated between bacteria and animal cells (Waters, 2001).

Conjugation from bacteria to diatom has been successfully conducted in 2015 by Karas et al.. The team designed an episome that would replicate both in *E.coli* and *Phaeodactylum tricornutum* by inserting a yeast maintenance region CEN6-ARSH4-HIS3 on a bacterial plasmids.

1.4 Previous Work

1.4.1 First Protocol of Conjugative DNA Transfer to Diatom (Karas et al., 2015)

The objective of achieving a successful transkingdom gene transfer from bacteria to eukaryote has been inspired by Karas et al. (2015). In that publication, they reported having successfully transformed plasmids from bacteria to diatoms by conjugation. The study focused on two diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*. The aim of the study was to find an alternative to the "inefficient genetic tools" such as particle bombardment that tends to destroy the bombarded cells (Falciatore et al., 1999) . As different studies highlighted the possibility to transfer gene from bacteria to eukaryotes (Waters, 2001), the scientist behind the publication decided to test the possibility to achieve conjugation between bacteria and diatoms.

To design an episome that would be self replicative in *E.coli* and in the diatoms, they inquired different maintenance region. The most efficient tweak was the yeast maintenance region named CEN6-ARSH4-HIS3. In *P. tricornutum* the presence of the yeast region multiply the efficiency of conjugation by 30 times when it was present in the plasmids. The CEN6-ARSH4-HIS3 is composed of yeast centromer (CEN6), a sequence for autonomous replication (ARSH4), and a histidine gene (HIS3) (Diner et al., 2016).

For both species, the conjugation happened in three steps : at first the *E.coli* and diatoms were mixed together and plated on agar for 90 to 120 minutes at 30°C, then the plate were incubated at 18°C for a time period of 4 hours to 48 hours depending on the specie. For the final step, 1 mL of L1 media was added and they scraped the cells to collect them, the retrieved cells were plated again on agar with antibiotic to obtain colonies. The transformed diatom cells were able to maintain their plasmids in absence of selective marker, after a period of 30 generations, 35% of the cells had the p0521s plasmid maintained. In light of the results, the authors suggested the use of pPtPuc3 plasmids as advantageous backbones for Gibson assembly.

1.4.2 Episome Design

One of the most essential step to achieve the insertion of a plasmid by conjugation in a species as *C. wailesii* that had not been used before is to ensure that the episome used would correctly express the wanted gene and be able to replicate. Meaning, that the antibiotic resistance gene to select the transformed bacteria should be expressed in the bacteria to select the transformant on a selective medium and the same should occur for the eukaryotes that successfully transformed the episome by conjugation. It is necessary that the episome has an origin of replication for the prokaryotic vessel and an origin of replication for the eukaryote cells to allow a multiplication of the plasmid and maintained its presence in sufficient number to have a successful expression. The following plasmids were designed by Tore Brembu with my assistance.

1.4.3 Design of the Three Plasmids used in that Experiment

To increase the chance of success of the conjugation, we created three different plasmids. All derived from ptPuc3 (Karas et al., 2015), the plasmids were named pCwPUC1, pCwPUC2, and pCwPUC3. The amplifications were executed by PCR (Section 2.2.2 and the fragments were assembled using Gibson Assembly (Section 2.2.3.

- **pCwPUC1**

This plasmid obtained from pPtPuc3 is shown in Figure 1.7. The episome exhibits the promoter pCwLHCFN (582 base pairs) and the terminator CwLHCFN 3 UTR (214 base pairs) derived from the ortholog version of the FcLHC1 gene. The functions of the FcLHC1 were discussed in [related work]. It is between the two fragments that the selective marker an BleoR (375 base pairs) an antibiotic resistance gene to zeocin is placed. The rest of plasmid has the same composition as the ptPuc3.

The plasmid was constructed by the assembly of four fragments:

◦ Linearized vector of pPtPuc3Mut amplified by the primers Vector.FOR and Vector.REV. The vector has the CEN6-ARSH4-HIS3 complex, an origin of replication and the selective marker in bacteria is KanR. [Annexe]

◦ pCwLHCFN: Generated using bioinformatic tools (SnapGene) from the likely promoter of the ortholog gene FcLHC1 in *C. wailesii.*

◦ BleoR : Resistance gene for zeocine, amplified from pPtPuc3.

◦ CwLHCFN 3 UTR: Generated using bioinformatic tools (SnapGene) from the likely promoter of the ortholog gene FcLHC1 in *C. wailesii.*

- **PCwPUC2a**

pCwPUC2a, illustrated in Figure 1.8 is an episome based on the ptPuc3 plasmid as a backbone from (Karas et al., 2015). The plasmid has a total size of 7323 base pairs. The eukaryotic promoter is PCwLHCF4a-1 (1000 base pairs) and the terminator is tCwLHCFN4a-1 (125 base pairs). BleoR is the selective marker (375 base pairs) that give the Zeocin resistance gene.

This episome was attempted from the Gibson Assembly of four fragments:

◦ Linearized vector of pPtPuc3Mut amplified by the primers Vector.FOR and Vector.REV. The vector has the CEN6-ARSH4-HIS3 complex, the origin of replication and the selective marker in bacteria is KanR.

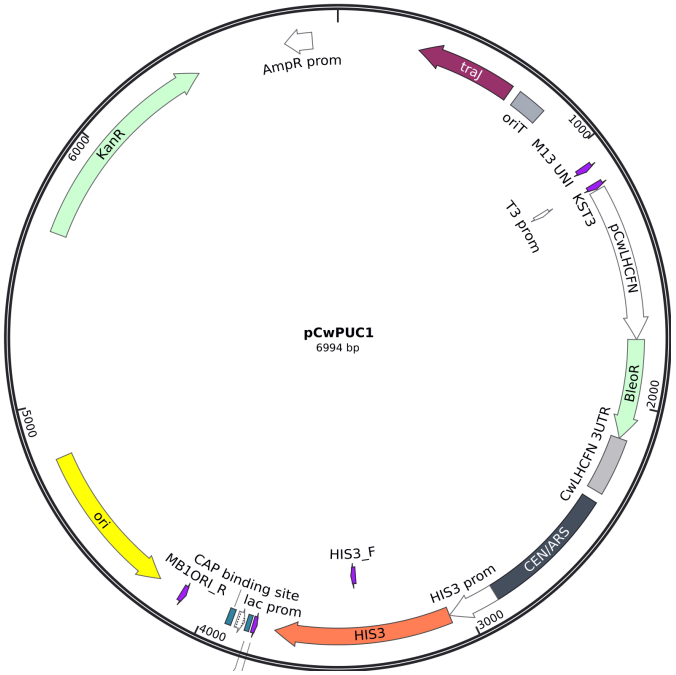

Figure 1.7: pCwPUC1, derived from pPtPuc3, the plasmid uses pCwLHCFN as promoter, CwLHCFN 3 UTR as terminator, and BleoR as selective marker. As the backbone comes from pPtPuc3 the CEN6-ARSH4-HIS3 complex is present and the origin of replications. The selective gene for bacterial growth is a kanamycin resistance gene.

◦ PCwLHCF4a-1: Created with bioinformatic tools from the the likely promoter of the orthologue gene TpLHCF4 of *C. wailesii*.

◦ BleoR: Resistance gene for zeocine, amplified from pPtPuc3.

◦ CwLHCFN4a-1: Created using bioinformatic tools (SnapGene) from the likely promoter of the ortholog gene TpLHCF4 of *C. wailesii*.

- **PCwPUC3**

PCwPuc3, presented in figure 1.9, has the same architecture as PCwPUC1, only the promoter differs. PCwPuc3 uses the promoter pCIP1 (503 base pairs), which is is a viral promoter that is usually overexpressed when used with eukaryotic cells.

The plasmid was constructed by the assembly of four fragments:

◦ Linearized vector (5844 base pairs) of pPtPuc3Mut amplified by the primers Vector.FOR and Vector.REV. The vector has the CEN6-ARSH4-HIS3 complex, the origin of replication and the selective marker in bacteria uKanR.

◦ pCIP1 : It is a viral promoter from a diatom-infecting virus (DIVs). It has been successfully used to promote gene expression in *P. tricornutum* (Kadono et al., 2015). ◦ BleoR : Resistance gene for zeocine, amplified from pPtPuc3.

◦ CwLHCFN 3 UTR : Ordered at generated using bioinformatic tools (SnapGene) from the likely promoter of the ortholog gene FcLHC1 in *C. wailesii*.

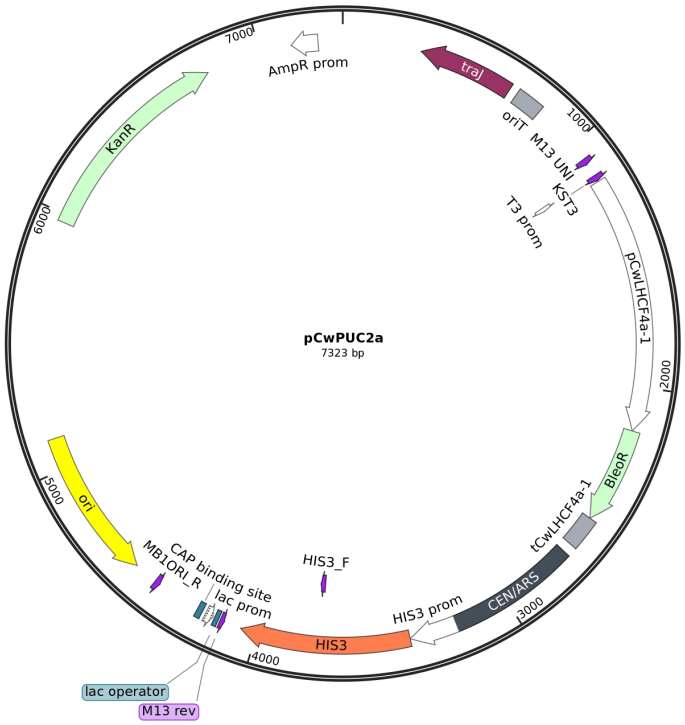

Figure 1.8: pCwPUC2a, derived from pPtPuc3, the plasmid uses PCwLHCF4a-1 as promoter, tCwLHCFN4a-1 as terminator, and BleoR as a selective marker. As the backbone comes from pPtPuc3 the CEN6-ARSH4-HIS3 complex is present as well as the origin of replications and the resistance gene for bacterial expression is a kanamycin resistance gene.

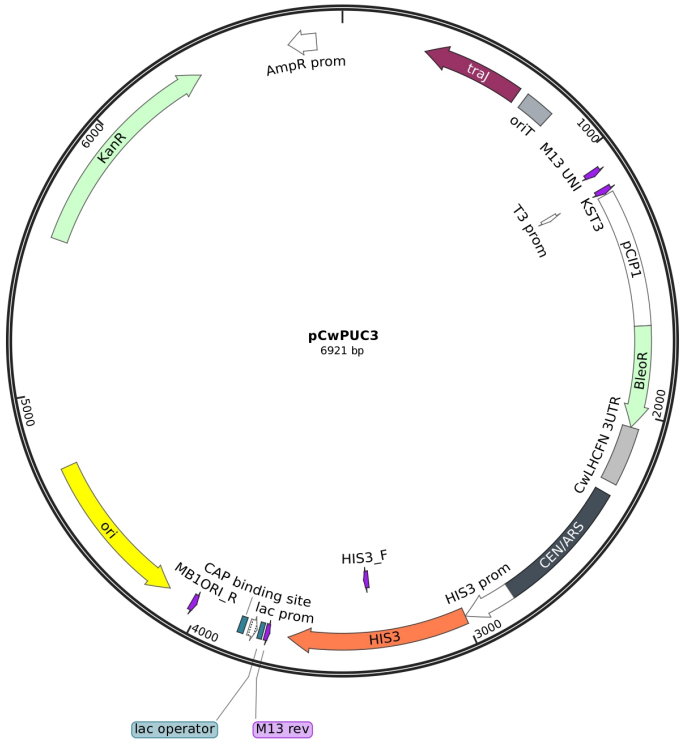

Figure 1.9: pCwPUC3, derived from pPtPuc3, the plasmid uses pCIP1 as promoter, CwLHCFN 3 UTR as terminator, and BleoR as selective marker. As the backbone comes from pPtPuc3 the CEN6-ARSH4-HIS3 complex is present and the origin of replications and the kanamycin resistance gene.

Chapter 2

Material and Methods

2.1 *Coscinodiscus wailesii* Cultivation

2.1.1 Culture

The *Coscinodiscus wailesii* strain used in this project is referred as CCMP2513 and comes from the Marine Algae and Microbiota (NCMA). Cultures were kept in L1 medium as described in section 2.1.2. The cultures were stored on the bench with a 10:14 hours day:night at 23 °C or in a cabinet with 14:6 hours day:night in a 24 hours period at 20 °C.

2.1.2 Medium L1 Preparation

The L1 medium is based on the f/2 medium (Guillard and Ryther, 1962). The recipe used is the same as the corrected version found in "Manual on harmful marine microalgae" (Hallegraeff and Anderson, 2003).

Filtered seawater collected in the Trondheim fjord was the basis of the L1 medium. With NaNO3 as nitrogen source, the vitamin are the same as for the f/2 vitamin solution medium. The full list can be seen in the tables of the Appendix A.

Collected seawater was stored in a 1 m^3 tank, and vacuum filtered in bulk. Filtered seawater was stored in 1.8 L batches in Schott bottles at 4 °C until needed. Under a laminar flow cabinet, each Schott bottle got the corresponding dose of stock solutions for 2 L. The content of the stock solution was sterile filtered. Once all the components added to the medium, the volume was completed to 2 L, and the pH is adjusted to the value between 8 and 8.2.

2.1.3 Growth Measurement by Sedgwick-Rafter Cell Counting Chamber

The growth rate of *Coscinodiscus wailesii* cultures were monitored by the use of a Sedgwick-Rafter cell counting chamber. As *C. wailesii* is a diatom species of big size, it is not possible to measure its growth by spectrometry. It would be possible to use automated cell counters, but it would require specific tip size to accommodate the 100 µm cells.

In this project, we used a Sedgwick-Rafter cell counting chamber (Chandler, 1899). This is a counting chamber of a volume of 1 mm^3 composed of 1000 squares of 1 mm^2 each. The operation started by defining how many counts from the same culture would be realized. Three counting is often seen as a minimum to have reliable counts. Gently mix the culture by hand shaking, sample the equivalent number of sample in mL plus one (e.g. three counts means 4 mL). Those samples were poured in the labeled falcon tube of 15 mL. The tube was agitated by hand to suspend the cells and 1 ml of the sample was transferred to the chamber. The chamber is closed by a rotation of the glass cover-slip at the same time as the sample is pipetted out. Once closed the cell can be counted using a binocular microscope.

With the help of a click counter, four stripes of 50 quadrants were counted. The quadrants to count were spaced throughout the counting chamber. The density in cells per milliliter of the sample can be estimated by then multiplying the count by five.

To be reliable, the counting is reproduced three times to get reliable results.

2.1.4 Optimization of Culture

Optimization of Culture Bottles

Four triplets of culture were divided in :
1 C1 : standing up no shaking
2 C2 : laying down no shaking
3 C3 : standing up shaking
4 C4 : laying down shaking

The bottles were maintained in the designated position from there group description. The shaking table was set at 50 rpm. The bottles were filled with 30 mL of *C. wailesii* culture based on L1 with a starting density of 380 cells/mL.

Each of the 12 culture vessels was filled with 35 mL of the appropriate medium and added 5 mL of *C. wailesii* inoculum (290 cells/mL). Culture growth rate was measured/monitored for 16 days by (Sledgedick-Laughter) cell counting as described in Section 2.1.3.

2.1.5 Cultivation of *Coscinodiscus wailesii* Cells for Conjugation

Ten culture bottles of 2 L each were prepared with fresh L1 medium three weeks before the expected date of each conjugation experiment. Firstly, the bottle received 1/10 of *C. wailesii* culture and 9/10 of fresk L1 medium. The volume of the 2 L culture bottle was gradually increased until reaching 2 L. The bottles were let to grow at normal growth condition and were annually agitated every 48 to 72 hours.

2.1.6 Selective Medium for Transformation

Production of Super Clean Agar Protocol

To get an optimal quality of agar it has to be treated as described in Waterbury and Willey (1988).

Agar (100 g, Bacterial agar (A5306), Sigma-Aldrich) was added to water (5 L) and stirred for 30 min. After settling, surplus water was siphoned off and the remaining sediment was filtered (Whatman F4). The filtrate was washed by stirring in ethanol (95%, 3 L) for 30 minutes, filtered, and washed again by stirring in acetone for 30 min. After the final filtration the purified agar was transferred to a glass tray, covered with aluminum foil and dried in an incubator for three days.

In-Agar (0.25%) Pourring Technique for *Coscinodiscus wailesii* Protocol

Six 20 mL batches of MQ water and 0.10 g of agar was sterilized/autoclaved in Falcon tubes before addition of 20 mL preheated (37°C) L1 medium. The tubes were subsequently reheated to 37°C before being placed on a shaking table at room temperature to gradually cool down while temperature was closely monitored with a laser thermometer. At 32-33 °C the agar was poured onto Petri dishes and maintained in liquid state by slow circular agitation by hand until *C. wailesii* culture (0.5 ml, 280 cell/mL) was added. The plates were subsequently incubated for four days at 20 °C at a 16:8 light regimen.

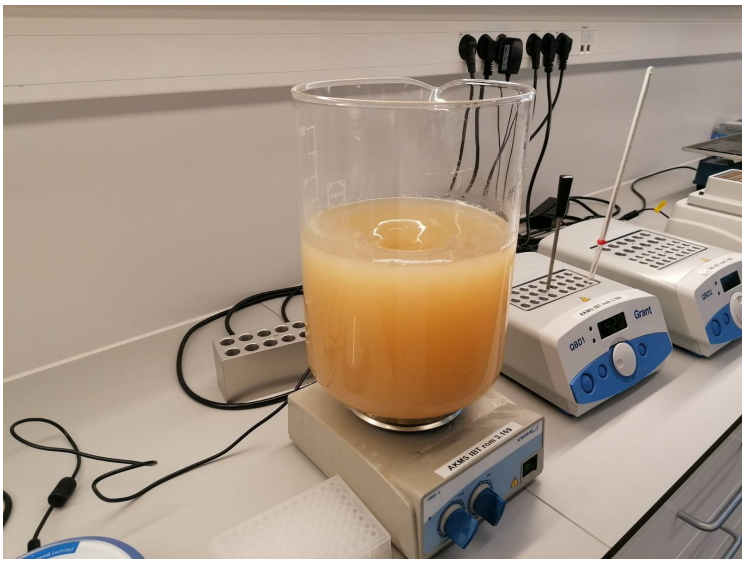

Figure 2.1: Washing of agar with ethanol.

Establishment of the Optimal Concentration of Antibiotic in the Selective Medium

To ensure that the production of the selective medium would be cost effective, different concentration of zeocin were investigated. Three concentrations were tested: 0 µg/mL, 50 µg/mL, and 100 µg/mL.

The plates were prepared following the protocol for in-agar pourring as described in 2.2.3, the antibiotic were added when the medium was at 37 °C to limit their degradation as zeocin is thermolabile. The plates were maintained four days in normal growth conditions.

Picking Method of the Potential Transformant

At first, a cut was operated by a sterile scalpel blade. The pipette tip was placed on micropitte and used under a microscope to aim for the right colonies. When a colony was pipetted, it was deposed in a 16 well plate filled with fresh L1 medium and the cultures were cultivated until growth was established.

2.1.7 Axenisation Protocol of Coscinodiscus wailessi culture

Axenisation Protocol of Diatoms (Mönnich et al., 2020)

The original protocol reported by Mönnich et al. (2020) was followed for the design of that experiment, with the exception of using L1 medium in lieu of ESAW as well as using an 8 µM pore filter instead of (the recommended) 3 µM.

Forty milliliters of concentrated *C. wailesii* culture were filtered through the filter. Those cells were then successfully washed with 150 mL sterile L1, then 50 mL sterile L1 20 µg/mL Triton-X 100, de powerful detergent, succeeded by a lat wash of 150 mL sterile L1. A Falcon tube containing the antibiotics streptomycin (50 µg/mL), gentamicin (67 µg/mL), ciprofloxacin (20 µg/mL), chloramphenicol (2.2 µg/mL), and ampicillin (100 µg/mL) was prepared in advance in a 40 mL to receive the membrane filters which was vortexes to separate the cells from the filter for a two days incubation in normal culture conditions. After the second day, the cells were filtered and treated again following the same process. At last, the cells are filtered with a clean membrane and sterile L1 before to be moved to a 40 mL culture bottle with 20 mL L1 a place at normal culture condition. A sample of 5 mL was placed in a 15 mL tube with L1 medium with peptone (5%) to

verify that no bacteria would be observed, confirming that the culture became axenic.

Axenisation Protocol of *Coscinodiscus wailesii* after Nagai et al. (1998)

Two rows of ten Petri dishes were numbered and aligned under a sterile hood. All Petri dishes were filled with L1 medium except the dishes 1, 2, 7, 10, and 13 which got a 1/20 dilution of AM9 antibiotic medium (Provasoli et al., 1959), see table in appendix. The Petri dishes 17 were filled with a five times dilution of AM9 medium.
Approximately two hundred cells of *C. wailesii* were harvested by filtration and placed equally in two sieves. The sieves were placed in the first Petri dish for 30 minutes then to the next Petri dish for the same time except for the session in the dish 17 were the sieves stayed for 90 minutes. The set up can be seen in the figure 2.2.
At the end of the transfers, the remaining cells were transferred in two culture bottles with sterile medium and placed in a culture cabinet at normal growth conditions to recover. Three days after after, a sample was taken to be put in incubation in a culture tube with L1 + trypton medium to test the axenuity of the culture.

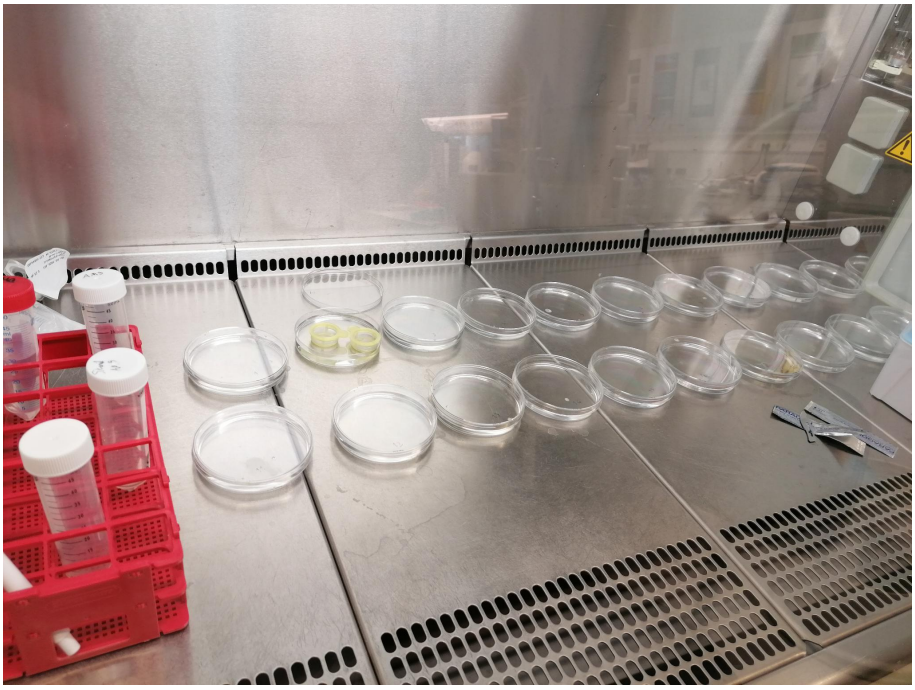

Figure 2.2: Axenisation set up of *C. wailesii* by Nagai et al. (1998). On the picture two sieve with the diatom cells are in the second petri dish (aligned from left to right starting from the ligne the farest from the user.

2.2 Molecular Biology Tools

2.2.1 Episomes Design Strategy

The episomes used in that experiment were created in collaboration by Tore Brembu using the Snapgene™ software and using previously isolated fragment. The episomes are describe in Section 1.4.2.

The primers used for the amplification by PCR were created using the special integrated function of SnapGene™ that creates Gibson Assembly compatible primers. A list of the primers used for the amplification with the annealing temperature can be found in table 2.1, table 2.2, and table 2.3. The sequence of the primers is detailed in the appendix Table B.1.

2.2.2 Fragments Amplification

The protocol used in that experience to realize a Gibson Assembly can be found in [Gibson Assembly]. The primers used for the amplification were created using the special integrated function of SnapGene™® that creates Gibson Assembly compatible primers. A list of the primers used for the amplification with the annealing temperature can be found in Table 2.1, Table 2.2, and Table 2.3. The sequence of the primers is detailed in the appendix Table B.1. The two PCR reaction used were the Platinum™ SuperFi™ and Phusion Hot Start II High Fidelity, the set-up of the reaction are in Appendix Table C.2 and C.3.

Table 2.1: pCwPUC1 fragments and their primers for amplification. The annealing temperature used for each combination of product and primers is detailed in the last column.

Product	Forward	Reverse	Size [bp]	Annealing [°C]
LHCFN prom	PUC-pLHCFN-FN	pLHCFN-BleR	604	60
BleoR	pLHCFN-Ble-F	Ble-tLHCFN-R	398	60
LHCFN term	Ble-tLHCFN-F	tLHCFN-PUC-RN	238	60
Vector	tLHCFN-PUC-FN	PUC-pLHCFN-RN	5844	63

Table 2.2: pCwPUC-2a fragments and their primers for amplification. The annealing temperature used for each combination of product and primers is detailed in the last column.

Product	Forward	Reverse	Size [bp]	Annealing [°C]
LHCF4a prom	PUC-pLHCF4L-F	pLHCF4-BleR	1000	60
BleoR	pLHCF4-Ble-F	Ble-tLHCF4-R	375	60
LHCF4a term	Ble-tLHCF4-F	tLHCF4-PUC-R	125	60
Vector	tLHCF4-PUC-F	PUC-pLHCF4L-R	5823	63

Table 2.3: pCwPUC3 fragments and their primers for amplification. The annealing temperatures used for each combination of product and primers are detailed in the last column.

Product	Forward	Reverse	Size [bp]	Annealing [°C]
CIP1 viral prom	PUC-pCIP1-F2	pCIP1-Ble-R	527	60
BleoR	pCIP1-Ble-F	Ble-tLHCFN-R	398	60
LHCFN term	Ble-tLHCFN-F	tLHCFN-PUC-RN	244	60
Vector	tLHCFN-PUC-FN	PUC-pCIP1-R	5848	63

2.2.3 Transformation & Gibson Assembly

Gibson Assembly Protocol

The Gibson Assembly is a molecular method that assembles DNA fragment efficiently. The kit used for that experiment was the Gibson Assembly Protocol (E5510) (NEB). In this kit, the supplier recommends different amounts of DNA depending on the number of different fragments per assembly. The volume of PCR product added to the mix was dependent of the concentration of PCR product measured with the NanoDrop. In our case, the three plasmids were composed of four fragments. The reactions were performed in Eppendorf tubes on ice following the composition found in the appendix Table C.4. The tubes were then incubated in a tube heater set at 50 °C for 60 minutes and was then stored on ice; later the reaction was then used for bacterial transformation.

2.2.4 Transformation

The *Escherichia coli* strains used in this project were DH5 and DH10B.

Chemically Competent Cells Protocol

From a culture of DH5 was selected to make a 1% inoculum in 50 mL Psi medium in a Falcon tube. The inoculum of DH5 in Psi was placed in an incubator at 37 °C until the OD600 was equal to 0.4. When the density reached 0.4, the culture was placed on ice for 15 minutes. The culture was then spun in a centrifuge at 1.200g for five minutes. The supernatant was discarded, and the pellet was resuspended in 40 mL of cold TFB1 and incubated for 5 minutes on ice. A second centrifugation was done at 1200 g for minutes. Afterward, the supernatant TFB1 was discarded, and the pellet was resuspended in 3 mL of cold TFB2. The suspension was divided into 30 Eppendorf tube with 100 µl of the suspension each. Finally, the Eppendorf tube containing the chemically competent DH5 were snap frozen using a liquid nitrogen bath and stored at -80°C until needed for a transformation.

Transformation Protocol

The Gibson Assembly products were transformed into DH5 *E. coli* cells following the following protocol. For one transformation, a tube of DH5 *E. coli* is taken from the 80°C freezer. The cells are let to thaw on ice. When the DH5 are liquid, 5 µL of Gibson Assembly products was added to the tube by pipetting. Then the tube was put on ice for 30 minutes for a cold incubation, in the meantime an Eppendorf tube with 1 mL of LB medium (Bertani, 1951), composition in appendix Table A.4, was placed on an incubator to be pre-warmed at 37 °C. After that, the cell were heat-shocked at 42 °C for 45 seconds before being incubated on ice for 2 minutes. At that point, the heat-shocked cells were mixed with the pre-warmed LB medium, and maintained at 37°C and 220 rpm shaking for one hour. The shocked cells were collected by centrifugation using a micro-centrifuge at 500 g and resuspended in 100 µL of LB medium (composition in appendix). The content of the resuspension was plated on a pre-heated LB agar plate (1.5% agar) with 50 µg/mL kanamycin. Finally, the plate was placed was sealed using parafilm to prevent desiccation and placed in an incubator at 37 °C overnight.

Selection of Potential Transformant by Colony PCR

The amplified fragment would have an expected length of 1432 base pairs. This fragment will be amplified by the forward primer “M13 Forw” and the reverse primer “CEN-ARS-R” [see annexe for detail]. A plasmid map showing the fragment is diplayed on figure 2.3. The colony PCR screen was performed using RedTaq DNA Polymerase Master Mix 2x (VWR) following the proportion found in table C.1.

Each selected colony picked with pipette tips under sterile hood were first plated on LB (Bertani, 1951) agar 1.5% 50 µg/mL kanamycin on a marked area, then the pipette tip was rinsed in the PCR tube with the reaction mix and discarded.

After the amplification, the PCR product lengths were compared with the length of the expected fragment by gel electrophoresis (1% agarose in Tris-acetate-EDTA buffer). GeneRuler1 kb Plus DNA Ladder (Thermo Scientific). A 5 µL sample from each product was directly added in the gel with GelRed® (final concentration 0.05 M, Biotium). The colonies that led to the amplification of a fragment of around 1432 bases pairs were transferred to culture tubes with 3 mL LB medium (Bertani, 1951), and 50 µg/mL kanamycin for overnight incubation at 37°C. The following day, plasmids were extracted from the overnight cultures using the GeneJET Plasmid Miniprep Kit (Thermo Scientific).

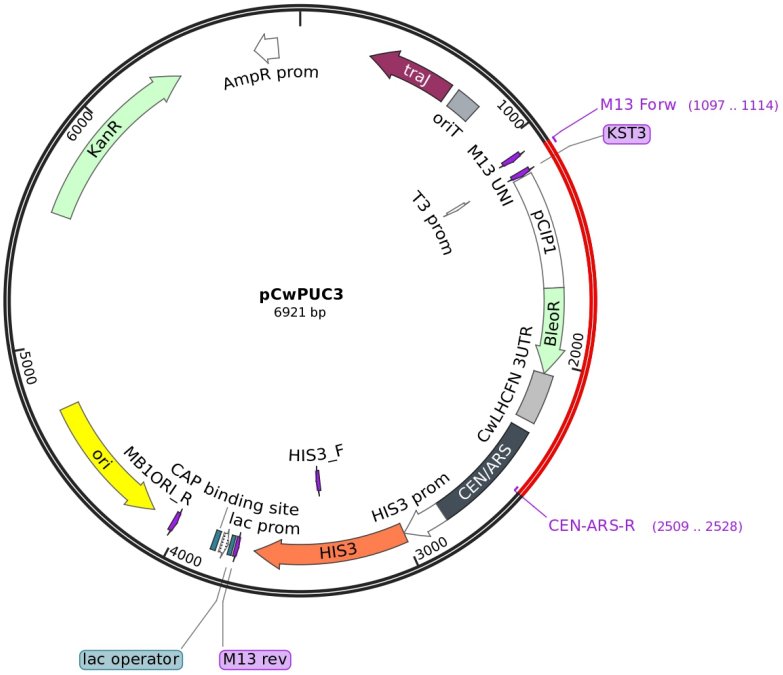

Figure 2.3: Plasmid pCwPUC3 with the amplified area for the colony PCR screen marked in red. Created with SnapGene™.

MiniPrep Protocol

The Thermo Scientific GeneJET Plasmid Miniprep Kit contains three solutions and multiple column tubes. From each overnight culture, 1 mL was placed in an Eppendorf and then placed in a micro-centrifuge at 6800 x g for two minutes. The pellet was resuspended using 250 µL of Resuspension Solution, 250 µL of Lysis Solution and 350 µL of Neutralization Solution, the tube was mixed in between the addition of each solution, and the mix was centrifuged at 12 000 g for five minutes. The supernatant was transferred to a Miniprep Column tube to be centrifuged for one minute at 12 000 x g, the fluid in the tube was discarded. The content of the column was washed two times with 0.5 mL of Wash Solution and centrifuged at 12.000 g for 60 seconds. The column was dried by doing another centrifuge step for 1 minute with the same amount of g. Finally, the column was moved to a new Eppendorf tube, 50 µL of Elution Buffer was added and then spun at 12.000 x g for 2 minutes. The quantity of DNA was measured using a NanoDrop and the purified plasmid were kept in a freezer at -18 °C.

Fragment Sequencing

To be certain that the isolated plasmids are the expected one, The isolated fragments were verified by Sanger sequencing, performed externally (Eurofins). The sequencing method is Sanger sequencing. Each potential colonies were sent to the contractor in Eppendorf tube with a barcode with the solution found in the Table 2.4.The results were retrievable online few days after.

Table 2.4: Composition of sample send to Eurofins for sequencing

Component	Volume [µL]
Plasmid MiniPreped	5
Primer	2.5
Deionized water	2.5

Transformation of Verified Plasmid in *E.coli*

Transformation in DH10B The purified episomes were transformed into the strain of bacteria DH10B following the same process as the transformation of DH5 described in Section 2.2.4. The transformant were picked the same way as explained in [reference], and the colonies were PCR screened by the same method described in Section **??**.

2.2.5 Establishment of a Conjugation Protocol for *Coscinodiscus wailesii*

Donor Cells Growth

The DH10B cells that were transformed and selected with the plasmid of interest, were stock on agar plate with 50 mg/mL kanamycin were prepared for the conjugation overnight in autoclaved glass tube with 5 mL of SOC medium recipe in appendix Table A.5 (Sambrook and Russell, 2001), plus (50 µg/mL) and 5 µL of gentamycin (20 µg/mL). For each culture, a swap made with a pipette tip was taken from the colonies on the culture plate conserved in a fridge and put in incubation overnight at 37C and agitation at 220 rpm.

Harvesting of *C. wailesii* Cells

As a first step, the number of cells in each culture bottle was estimated using cell counting techniques 2.1.3. Considering that we aimed to conjugate 20,000 cells per Petri dish, the number of bottles needed by experiment was estimated to be 20. The bottles selected for cell harvesting were kept static until all the cells settled to the bottom of the flasks, as the bottles were kept static with daily manual mixing the cells were already on the bottom of the bottles at the beginning of each experiment. One hundred milliliter of a mix of cells and medium was siphoned from the bottom of each bottle using a serological pipette of 50 mL, as shown in Figure 2.4. The content of each serological pipette was poured in a 50 mL Falcon tube. Then the falcon tubes were put in a centrifuge at 100 g for four minutes. The tube was then maintained standing in tube racks before conjugation.

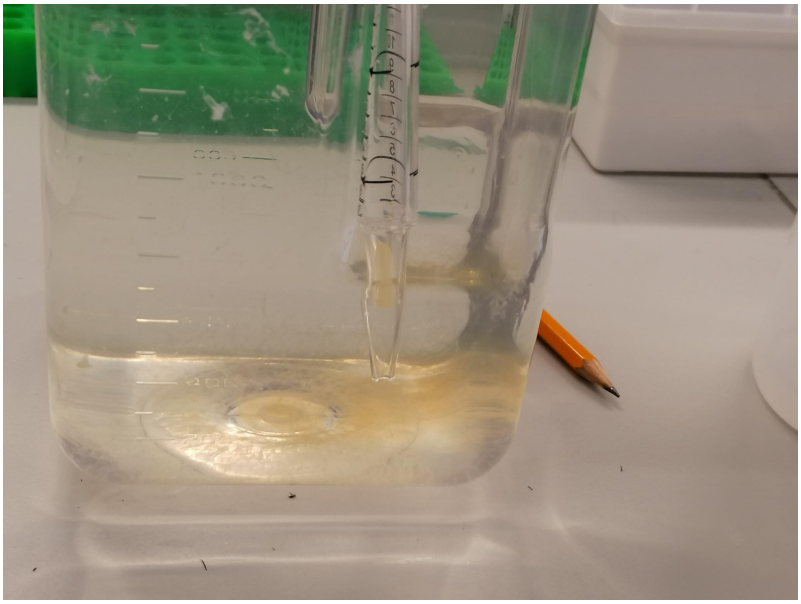

Figure 2.4: Harvest of *C. wailesii* cells with a serological pipette 50 mL. Only the bottom, where the cell naturally settled was pipetted to concentrate the cells.

Conjugation Protocol

Prior to the co-incubation, culture plates were prepared with 49% L1 medium, 45% MQ water, 1% agar, and 5% LB medium (Bertani, 1951), and maintained refrigerated at 4 °C (Karas et al., 2015). Before the beginning of the conjugation, the co-incubation plates were kept open in a sterile cabinet at room temperature to dry.

When the plates were ready, for each incubation, 200 µL of concentrated *C. wailesii* cells were transferred to an Eppendorf tube using a cut pipette tip and 200 µL of the corresponding transformed DH10B *E. coli* at the wanted concentration was added. The tubes were flipped four or five times to ensure a complete mixing and the solution was plated on the co-incubation plate under the sterile hood and left to dry for ten minutes. Once dry, the plates were placed in the dark in an incubator at 30 C for 90 minutes. After the incubation, the plates were moved to an algae cabinet with a temperature of 18 °C in presence of light for four hours to allow the cells to recover. Once the recovery period finished, 1 mL of L1 medium was poured on the plate to rehydrate and free the cells from the gel with the help of a plate scraper. The liquid phase was collected in an Eppendorf and 200 µL was plated in the selective in-agar plates containing 50 mg/mL zeocin prepared in the same condition as described in 2.1.6 and let for growth for four days.

For each experiment positive controls and negative controls were made. The positive controls were made of *C. wailesii* cells that did not undergo conjugation experiment in antibiotic free in-agar medium and with diatom cells that underwent conjugation in antibiotic free in-agar medium. The negative control plates were made of non conjugated *C. wailesii* in-agar plates containing 50 mg/mL zeocin. All the controls as well as the experimental plates as described in 2.1.6.

Alternatively, a part of the collected cells were transferred to a liquid culture bottle with L1 medium and 50 mg/mL zeocin to observe if the recovery is more efficient in liquid phase, as it was proposed by Diner et al. (2016). The negative controls were made of non conjugated *C. wailesii* in L1 medium and 50 mg/mL zeocin. The positive controls were made of *C. wailesii* cells that did not underwent a conjugation experiment in antibiotic free L1 and with diatom cells that underwent conjugation in antibiotic free L1.

Chapter 3

Results

3.1 Cultivation of *Coscinodiscus wailesii*

The species *coscinodiscus wailesii* was chosen for this study as its culture specificity is relatively welldocumented and its transcriptome has been sequenced as part of The Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Keeling et al., 2014).

3.1.1 Culture Optimization

Optimization of Culture Bottles

To learn how to produce in the most efficient way the biggest quantity of *C. wailesii* cells, we looked at the importance of constant shaking and on the extent on what the bottom surface matters.

Cells were grown under four conditions:

1 C1 : standing up no shaking
2 C2 : laying down no shaking
3 C3 : standing up shaking
4 C4 : laying down shaking

The evolution of the growth in each condition was estimated by counting using a Sedgwick-Rafter cell counting chamber (Chandler, 1899). The results are represented in the Table 3.1.

During the experiment, it was possible to see that the group G3 and G4 show an immediate decay from day one. All the culture in a bottle laying down on the shaking table (G4) had no living cells

Table 3.1: Cell density (cells mL-1) of *C. wailesii* in different culture conditions of a 16 days period. The four groups on the Y axis refer to different growth condition. The group G1 act as a control and represent the group where the culture bottle stood up and were still, the group G2 represent the bottles that were laid down on the side and maintained still, the group G3 represent the bottles that stood on a shaking table at a speed of 60 RPM, and the group G4 includes bottles that laid down on a shaking table at a speed of 60 RPM. The cultures were simultaneous and at normal growth conditions.

	Cells per mL			
	C1	C2	C3	C4
Day 1	253	380	380	380
Day 3	300	436	320	33
Day 7	535	268	200	
Day 9	598	670	190	
Day 11	413	455	225	
Day 16	185	190	83	

with-in the first week of the experiments. The group G3, composed of the bottle standing up on the shaking table decade in a slower pattern then. The difference of cell concentration over time in the groups G1 and G2 were not significantly different from one another and both show growth until day 9. Based on these results, the doubling time of the *C. wailesii* was of 8 days.

3.2 Selective Medium for Conjugation

In-agar Growth of *Coscinodiscus wailesii*

The establishment of a conjugation protocol for *C. wailesii* required good cell growth in agar media for selection. Following the protocol detailed in Material and method, six Petri dishes were plated with different agars to observe the different growth efficiency, results are listed in Table 3.2. The different agar used for the experiment were: the Super Clean Agar is the agar purified following the protocol of Turnsek (2017), protocol in section **??**. The Clean Agar is the medium made from untreated the Bacterial agar (A5306) from Sigma-Aldrich directly and the classic agar results from classical bacterial agar.

From the approximately 150 cells added in each plate, the observations of development of the diatoms in the vessel are reported in the table x. Only colonies having four cells or more were counted as the cells might undergo a non sexual reproduction in the culture before being added to the plate.

Table 3.2: Reported observable colonies of *C. wailesii* by using a different type of agar.

Agar medium	**Number of after 2 days**	**Mean**
Super Clean Agar 1	85	71
Super Clean Agar 2	57	-
Clean Agar 1	30	27
Clean Agar 2	24	-
Classic Agar 1	41	47.5
Classic Agar 2	54	-

In that experiment, the Super Clean Agar offered the most efficient growth seen as the formation of cell chains, as shown in Figure 3.1.

Picking Colonies

Four colonies were taken from the control plate (0 µg/mL) who lead to noticeable growth. In four cases, the cells ultimately divided normally within three days.

Antibiotic Resistance of *C. wailesii* in in-agar 25%

Following the experimentation mentioned in 2.1.6. The resistance of *C. wailesii* to the antibiotic zeocin in an in-agar culture partially made with salt water was tested. The cells were incubated for four days. Example of the results of the three concentrations tested can be found below in the Figure 3.2, 3.3, and 3.4.

The growth of cells seemed normal for the control (0 µg/mL zeocin). In contrast, the plates with an in-agar medium with a concentration of 50 µg and 100 µg per milliliter had no colonies.

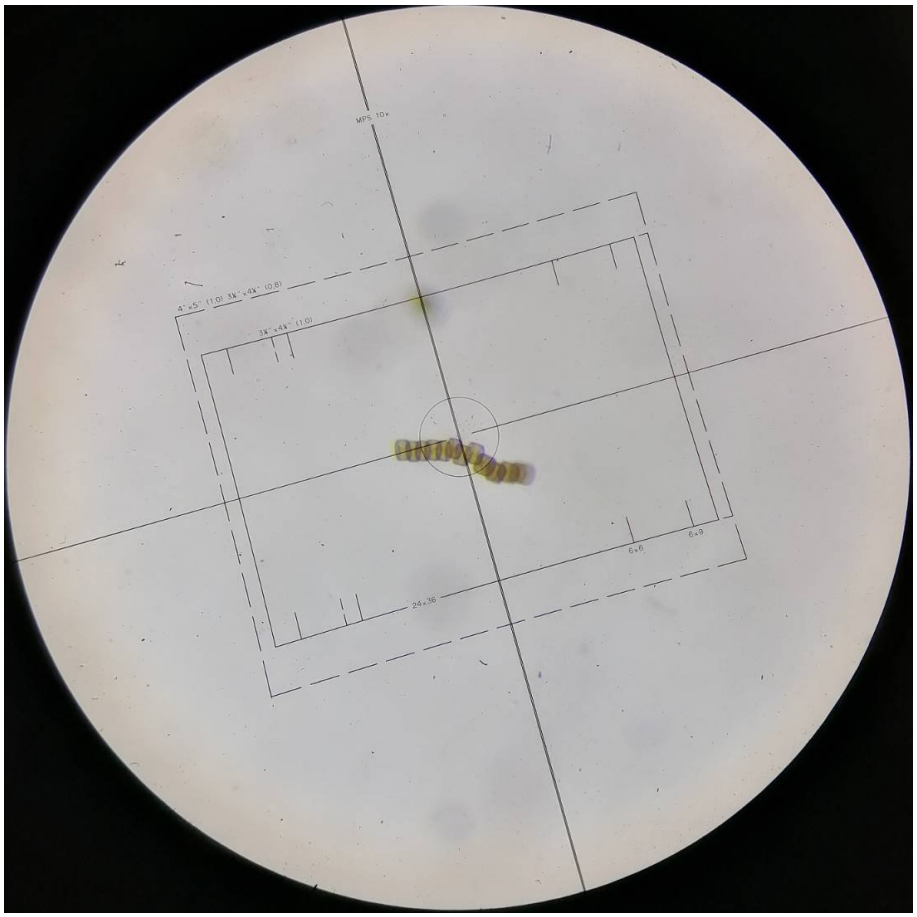

Figure 3.1: A *C. wailesii* colony on Super Clean Agar after five days. Microscope magnification x40.

3.3 Results of Molecular Biology Experiments

3.3.1 Fragment Amplification Results

As listed in section 1.4.2], the experiment requires amplifying different DNA fragments. Those portions of DNA correspond to parts of episomes that had been used for the conjugation experiments. The templates used for the amplification were of different origins, the linearized backbones were amplified from pPtPuc3, the promoters and terminators from *C. wailesii* genomic DNA, and the zeocine resistance gene (BleoR) from pPtPuc3.

The basis of the construction of the molecular tools lays on the production of the DNA fragments composing the plasmids : pCwPUC1, PCwPUC2a, and PCwPUC3. As displayes in Table 3.3, 3.4, and 3.5, all fragments were produced in sufficient amount except the LHCF4a term. If more than one amplification was realized, only the one with the highest concentration of DNA was used and then reported in the tables.

Table 3.3: Results from amplification of fragment composing the plasmid pCwPUC1.

Fragment	**Successful Amplification**	**DNA Concentration in ng/mL**
LHCFN prom	Yes	4.5
BleoR	Yes	28.7
LHCFN term	Yes	52.5
Vector	Yes	11.7

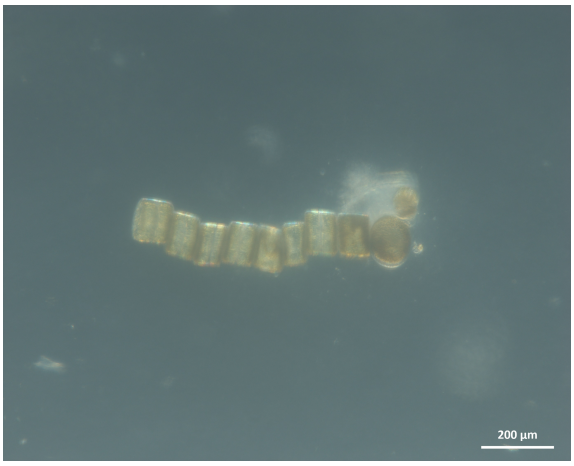

Figure 3.2: *C. wailesii* colony grown in agar without zeocin for four days. The cells grew normally by forming colonies in chains.

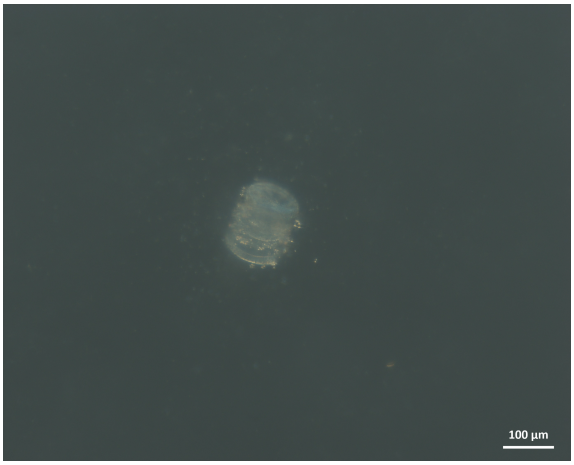

Figure 3.3: Two adjacent cells of *C. wailesii* maintained in agar with 50 µg/mL zeocin for four days in normal growth conditions. The cells showed sign of advanced decay.

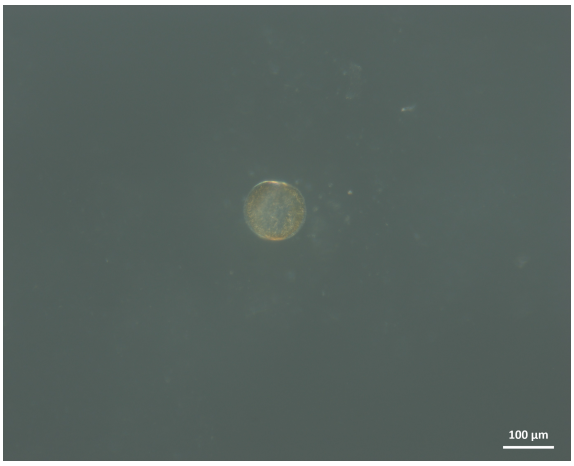

Figure 3.4: *C. wailesii* maintained in agar with 100 µg/mL zeocin for four days in normal growth conditions. The cells showed sign of decay.

Table 3.4: Results from amplification of fragment composing the plasmid pCwPUC-2a. As the fragment LHCF4a term was never successfully amplified, the DNA concentration of the other fragments composing pCwPUC-2a were not measured.

Fragment	**Sucessfull Amplification**	**DNA concentration in ng/mL**
LHCF4a prom	Yes	-
BleoR	Yes	-
LHCF4a term	No	-
Vector	Yes	-

Table 3.5: Results from amplification of fragment composing the plasmid pCwPUC3.

Fragment	**Successful Amplification**	**DNA concentration in ng/mL**
CIP1 viral prom	Yes	7.9
BleoR	Yes	19.6
LHCFN term	Yes	52.5
Vector	Yes	50.9

3.3.2 Gibson Assembly and Transformation Results

Because the fragments were assembled using a Gibson Assembly (Gibson et al., 2009), the fragment would be assembled with compatible ends coming from the primers used for the amplification. Every assembly lead to the growth of many colonies on the selective media with kanamycin. Because of the possibility to have vectors ligating on themselves, making transformant which got replicable plasmids with a resistance to kanamycin only the rate of false positive was high.

Selection of Potential Transformant

The amplified fragment would have an expected length of 1432 base pairs. To discriminate the true positives over the false positives the smallest colonies present on the Petri dishes were collected and grown on a new Petri dish. An example of the secondary culture can be seen on Figure 3.5.

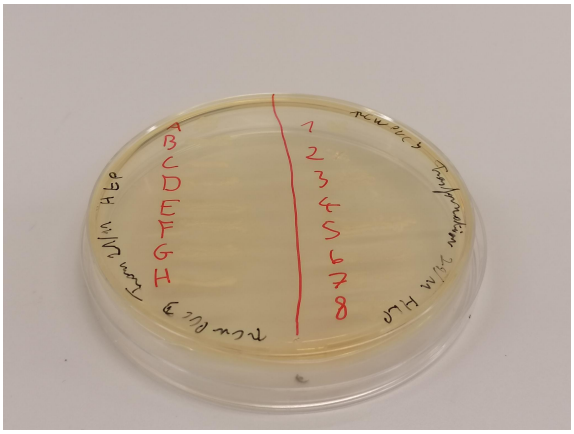

Figure 3.5: Petri dish with agar and kanamycin to maintain transformant that may or may not have the full plasmid. Colonies on the left side of the plat (indicated by letters) comes from the tranfoirmation of pCwPUC1. Colonies on the right side of the plate (indicated by numbers) are from the transformation of pCwPUC3.

The picked colonies were amplified during a PCR screen using “M13 Forw” as forward primer and the reverse primer “CEN-ARS-R” before to be compared by gel electrophoresis.

Sequencing Results

The four clones that gave a match on the PCR screen were sent to sequencing with the same primers as for the PCR screen ("M13 Forw" as forward primer and the reverse primer "CEN-ARS-R").

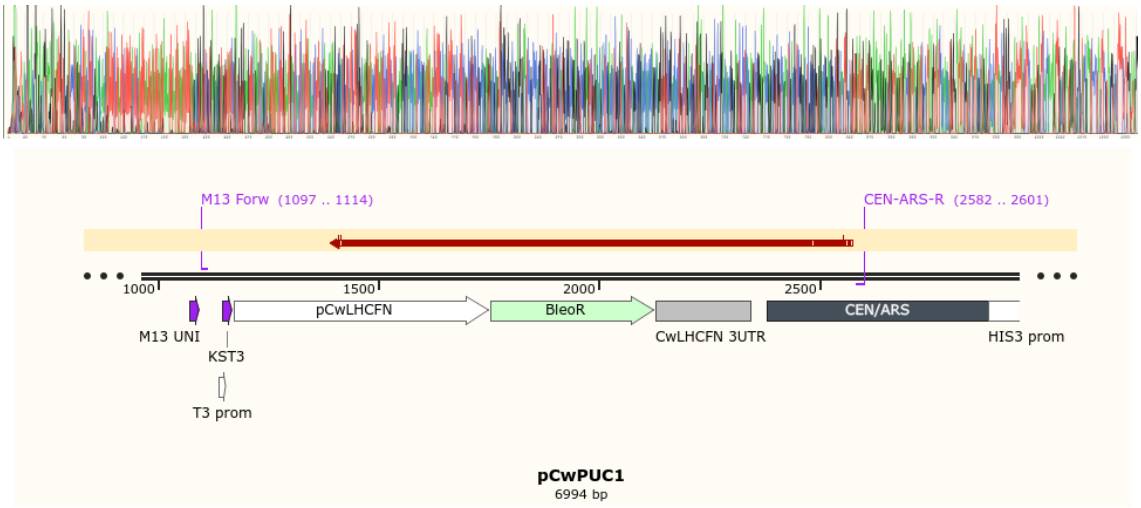

Figure 3.6: Sequencing of clone A after an attempt of transformation with the plasmid pCwPUC1. With 1188 base pairs aligned, it has two mismatchs and four gaps. Screenshot taken from Snapgene™.

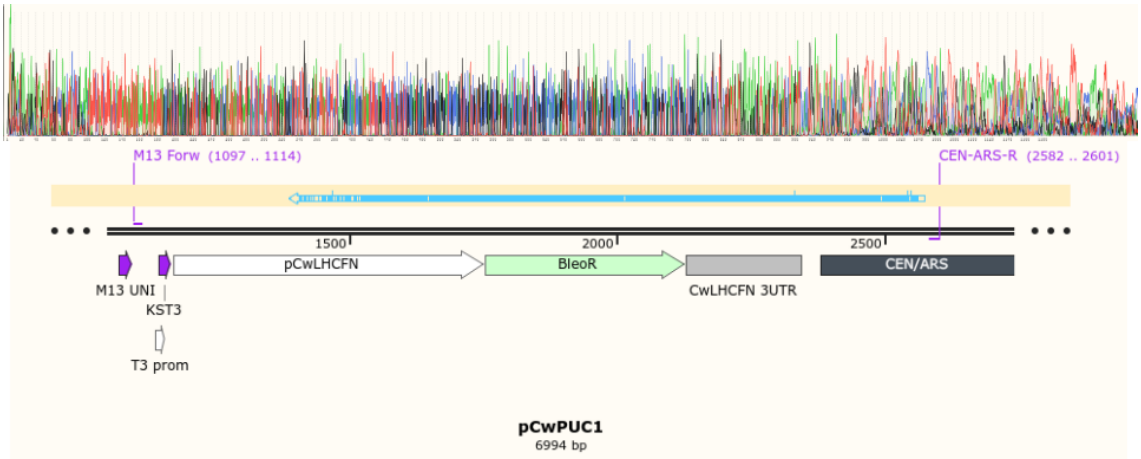

Figure 3.7: Sequencing of clone B after an attempt of transformation with the plasmid pCwPUC1. With 1188 base pairs aligned, it has 28 mismatchs and seven gaps. Screenshot taken from Snapgene™.

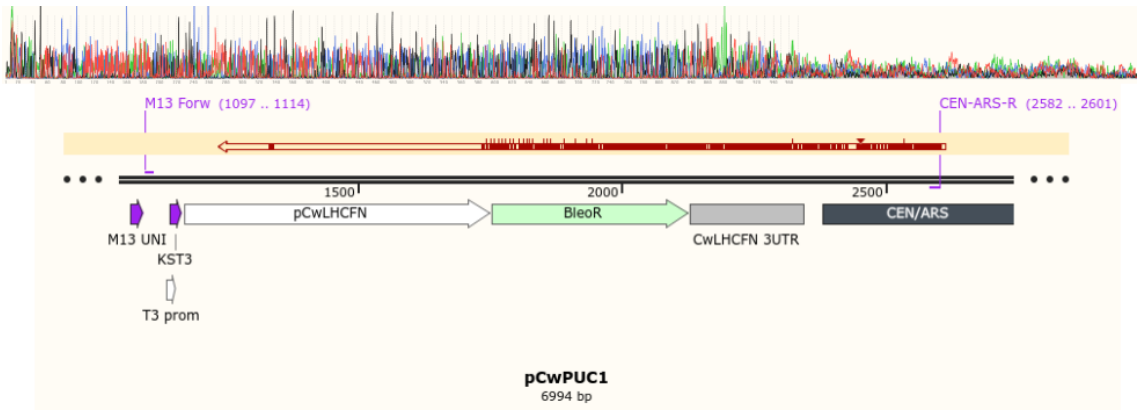

Figure 3.8: Sequencing of clone D after an attempt of transformation with the plasmid pCwPUC1. With 976 base pairs aligned, it has 30 mismatches and 26 gaps (the longest being 1413 base pairs long). Screenshot taken from Snapgene™.

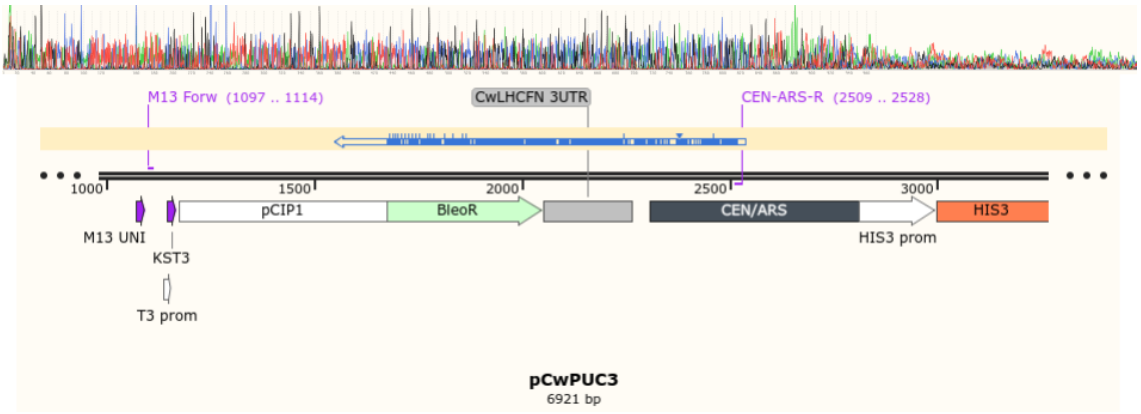

Figure 3.9: Sequencing of clone 2 after an attempt of transformation with the plasmid pCwPUC3. With 976 base pairs aligned, it has 28 mismatches and 24 gaps. The promoter pCIP1 was not detected by the sequencing. Screenshot taken from Snapgene™.

From the results from the sequencing analysis, shown is Figures 3.6, 3.7, 3.8, and 3.9. It seemed that the colonies A and B contains all the expected fragments. On the other hand, the colonies D and 2 seem to have many correspondence with the expected fragments but with uncertainty on the promoter, for the plasmid 2 no correspondence were found.

3.3.3 Axenisation of *Coscinodiscus wailesii* Culture

Axenisation of Diatoms (Mönnich et al., 2020)

The algae culture obtained after the treatment using the method proposed by Mönnich et al. (2020) was successful in leading to axenisation, as no bacterial growth could be observed in the tube with L1 medium with peptone. However, the axenised cells never divided and slowly died over a two weeks period.

Axenisation of *Coscinodiscus wailesii* after Nagai et al., (1998)

Due to the failure on the experiment because a part of the cells went through the sieve two kinds of culture were collected. One with the bigger cells that went through all the Petri dishes and another one with the cells that stayed for 12 hours in the Petri dish 2 with a 1/20 dilution. Both groups developed visual growth of microorganisms in the test tube with L1 + 5% peptone. The growth was less prominent than in the positive control (untreated *C. wailesii*).The tubes can be seen in Figure 3.10.

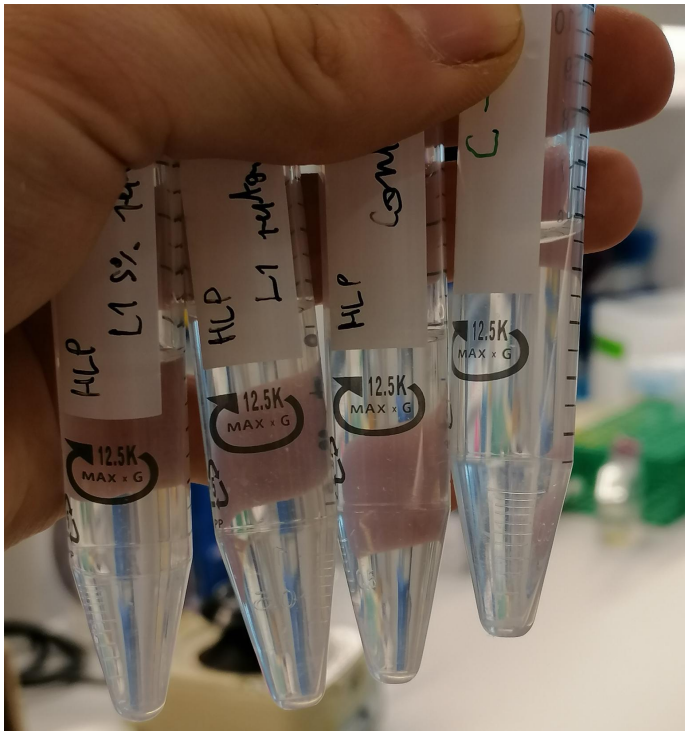

Figure 3.10: Results of the test of axenity of L1 with 5% peptone. From right to left Tube with treated algae, tube with algae that stayed in the Petri dish 2 for twelve hours, tube with control positive, and tube with control negative.

It is important to notice that during the experiment, most of the cells went through the sieve in the first Petri dish. Those cells were collected after spending 12 hours in a Petri dish with antibiotics and later the axenity was tested using peptone 5%. In both case the axenisations of our culture of *C. wailesii* were unsuccessful.

3.4 Establishment of Conjugation Protocol

3.4.1 Harvesting of *C. wailesii* Cells Results

Harvesting the cells appeared to be challenging, as it was difficult to use all the isolated and concentrated cells because of the volume of the *C. wailesii* cells. Sometimes the cells stayed up to one hour in small Eppendorf tubes. Because of the small absolute number of cells and difference in cell concentration from bottle to bottle, it was difficult to be certain of the number of *Coscinodiscus wailesii* cells that were actually plated.

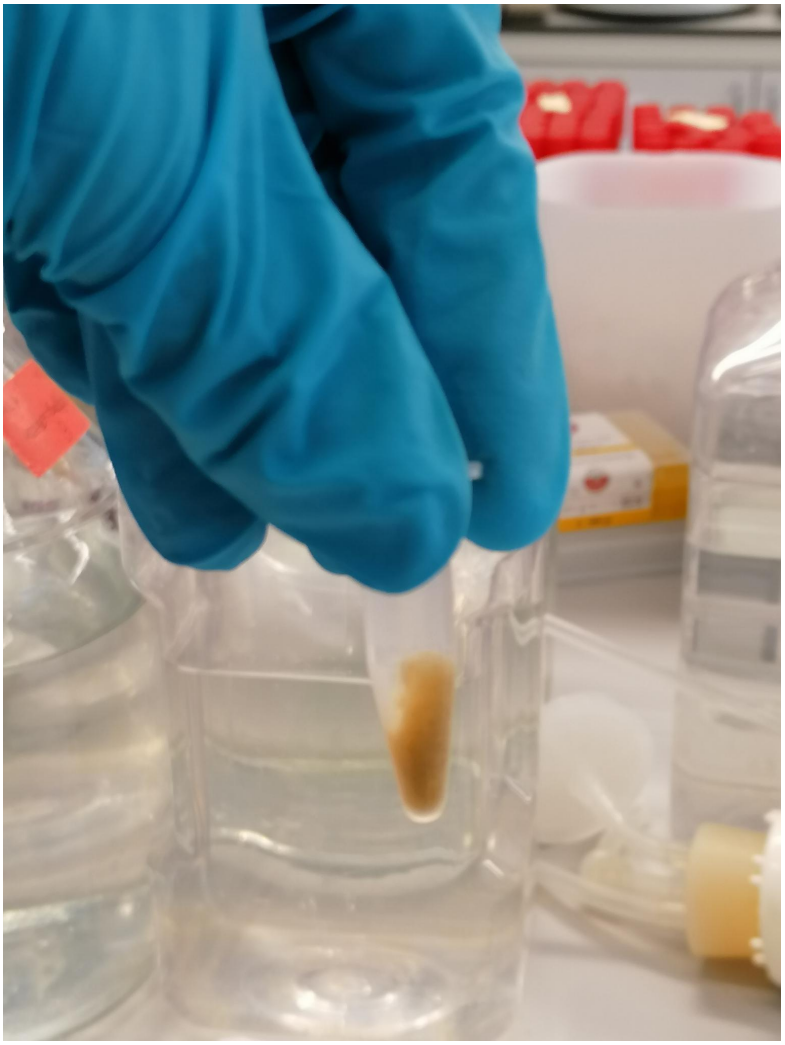

Figure 3.11: An example of a concentration of about 25.000 cells of *Coscinodiscus wailesii*. The accumulated cells formed a thick slurry that was difficult to pipette.

3.4.2 Results of Conjugation Plates Before Growth

Every time the experience has been intended, the in-agar pouring was achieved in the respect of the temperature limits described in Section 2.1.6, which should have not killed the *C. wailesii* cells. The agar seemed to be consistent with no to little irregularity in texture. With the proper gentle mixing, the cells did not accumulate at the bottom of the plates.

3.4.3 Results of Conjugation Plates After Growth

After four days of growth, all the positive control plates had observable growth. Almost all single cells turned into colonies. No growth was observed on the negative control plates. With the experimental plates, at the fourth day of growth it was possible to observe two cells touching another (in one case three cells were bound in the agar), those group of two to three cells were referred as protocolonies, an example of what was called a protocolony can be fine in Figure 3.12.

The number of those may be late colonies were marked and their number were from 0 to 4 protocolonies per plate with at least a protocolony. Even after one extra week of growth, no protocolony was observed to have more cells, and they slowly perished.

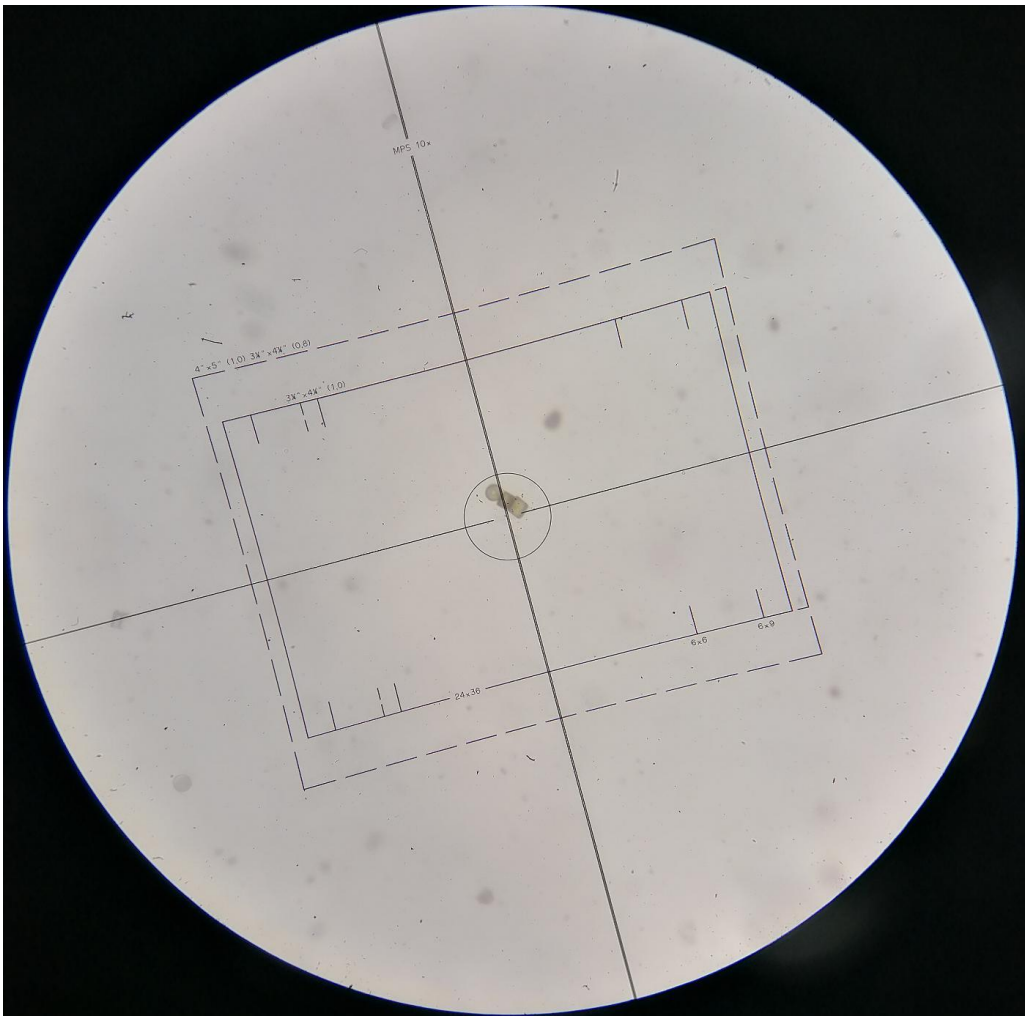

Figure 3.12: A protocolony of *C. wailesii* in agar with 50 mg/mL zeocin. Three cells really close to one another that could had been a colony that grew slowly.

3.4.4 Results of Liquid Selective Medium

The cells that underwent conjugation and were put L1 medium and 50 mg/mL zeocin did not show any growth and the cells rapidly lost colors by the fourth day. The positive controls showed growth in both cases and no growth was observed in the negative control. As a whole, none of the three attempts to transfer an antibiotic resistance gene to *C. wailesii* by conjugation seemed to be successful as the cells never survived or at least grew in medium with 50 mg/mL zeocin.

Chapter 4

Discussion

4.1 Cultivation of *Coscinodiscus wailesii*

4.1.1 Culture Optimization

The culture of C. wailesii is challenging because of its cell size, besides other factors. The size of the cell results in a low absolute concentration of cells in the culture. This is challenging, as the conjugation success rate depends more on the number of cells than on the biomass. To increase the chance to get exconjugants, we tried to be as loyal as possible to the original publication (Karas et al., 2015), this implied that a big volume of culture would be necessary. The classical use of a bottle with aeration gave poor results. As there is little publication describing optimal cultivation technique for the diatom of the genre *Coscinodiscus* and that the cell seemed to not support continues air agitation, we tried to inquire the usefulness of a shaking table to speed up the growth (Svenning, 2014).

To ensure to produce a sufficient amount of *C. wailesii* in the most efficient way, a growth experiment testing the importance of the bottom surface of the cell culture bottle and of constant shaking was carried out. As the *C. wailesii* settles easily, we made the hypothesis that a greater surface by volume could lead to a higher growth rate. Surprisingly, the best growth were obtained using still bottles. It was thought that agitation would improve nutrient uptake. After deeper literature review, one article published in 1999 observed slower growth in still *Coscinodiscus granii* cultures than on agitated one. The study found a correlation between the slower growth with constant agitation and a diatom parasite *Pirsonia diadema*, even if the studied could not determine if the enhance parasitic contamination were a cause or a consequence of the slow growth it seem that the *C. granii* cells multiply faster when not agitated (Kühn and Hofmann, 1999).

It did not seem that the surface of the bottom of the bottles has an influence on the growth and maximum concentration of *C. wailesii*.

As the optimization of the culture of *C. wailesii* was not the primary purpose of that study, only one experiment has been carried out. The conclusions made with the available information were that the optimal way to cultivate *C. wailesii* with classical day-night cycle and light intensity for diatom, using a standard L1 medium and letting the cells sediment on the bottom of the bottles. Regular manual agitation showed to be efficient to break the mucilage that could block the diffusion of the nutrient

4.2 Selective Medium for Conjugation

To conjugate *C. wailesii*, it is necessary to cultivate the exconjugants for around 14 days in a selective medium. This medium also facilitates the picking of the forming colonies. Previous experimentation showed that *C. wailesii* grow poorly on agar like *P. tricornutum* (Karas et al., 2015). The same problem has been experienced when cultivating the extraconjugant cells of *T. pseudonana*. To ensure a proper development of the antibiotic resistant exconjugant *C. wailesii*,

the protocol for pour plating of *T. pseudonana* by Turnsek (2017) was adapted. The production of Super Clean Agar was easy to carry out and even if a loss of 50% in mass with the raw agar can be observed, the quantity produce following the Turnsek protocol gives enough agar to make plate for any project. The growth efficiencies of *C. wailesii* in the different types of agar were compared in an experiment. Even if the original goal was to see how fast the colonies would develop in the Super Clean Agar, it was also interesting to see if the use of an expensive agar is necessary. As it can be seen in the Table 3.2 even the classical agar led to growth of colonies of *C. wailesii*, although it was not as efficient as the Super Clean Agar. The experiment was performed only once. Its robustness is not so strong, but the super clean agar was used in the conjugation experiments.

Zeocin was chosen as the selective factor of selective medium to ultimately test the success of the conjugation experiment, as it was the antibiotic used in the first publication of the first conjugative DNA transfer of diatom (Karas et al., 2015). As Zeocin is expensive (NOK 281.5 per milliliter (ZeocinTM Selection Reagent, ThermoFisher Scientific), the concentration of 50 µg/mL was favored over 100 µg/mL.

Picking the colonies of *C. wailesii* in 0.25% agar using an uncut pipette tip was not possible. We would recommend to cut the tips to get an opening of approximately a millimeter of diameter to be precise without damaging the cell.

4.3 Molecular Biology

4.3.1 Episome Designs

The operators designed three plasmids to achieve a conjugative DNA transfer to *C. wailesii*, pCwPUC1, PCwPUC2a, and PCwPUC3. Those plasmids use different promoters and terminators and are detailed in Section 1.4.2. As the promoter of the plasmid PCwPUC2a was never successfully amplified, it was not possible to test it. The strategy of testing more than one plasmid is common within molecular biology (Karas et al., 2015; Diner et al., 2016).

4.3.2 Fragment Amplification

The amplification of the fragments was challenging, and some had to be repeated many times before to find the right annealing temperature and time to obtain enough copies.

4.3.3 Gibson Assembly and Transformation

The Gibson assembly of the plasmids, pCwPUC1 and PCwPUC3, were followed by transformation into competent *E. coli* DH5.

Gibson assembly is a standard method for cloning of two or more fragments in one reaction. It relies on different enzymes and on specific compatible sticky ends for the ligation to take place. The compatible sticky ends were designed using the Snapgene software and were part of the amplification primers of the fragments. This is supposed to prevent that the fragment assembled to another randomly. The process is described in the figure x . Gibson assembly is efficient cost wise in the lab, but requires planning and is more expensive than the digestion ligation.

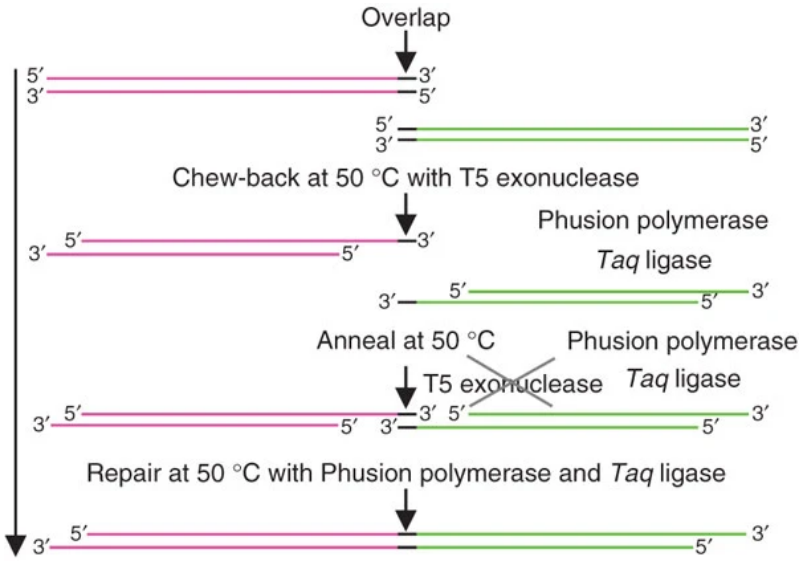

Figure 4.1: Scheme of the process happening to the strand during a Gibson Assembly. Compatible ends are ligated to one another before that at polymerase fills the gap. Retrieved from (Gibson et al., 2009)

The results from the Petri dish from the transformation of the assembled pCwPUC1 and PCwPUC3 plasmids led to an important number of false positive when using kanamycin to select the E. coli transformant. It seems that the backbones, which are the longest fragment of the episomes, had a tendency to ligate on themselves. The selective gene for *E. coli* KanR (kanamycin resitance gene) is located on the vector backbone. The backbone that were self-ligated and transformed in *E. coli* would give a resistance to the colony to the antibiotic without being the expected plasmids. Those false positives forced the operators to carry colony PCR screen to identify the potential colony that got the right genetic material.

Analysis of Transformant

A well-operated transformation can give many transformants. As the Gibson Assembly products are not analyzed before transformation, it is highly possible that incomplete plasmids with the gene resistance to kanamycin got transformed into *E. coli*. Gibson Assembly products can sometimes form plasmid but lacking one or more of the multiple fragments. To ensure that the colonies that would be selected to carry on the experiment have the expected plasmids transformed, we made two-level verifications. At first, the colonies were analysed by colony PCR screening using a specific primer that would amplify the promoter, the resistance gene BleoR, the terminator, and a sub-sequence of backbone from pPtPuc3. The author was suggested to pick the smallest colonies; the idea was that colonies that have the full plasmid would spend more energy replicating that plasmid than the colonies that only have a smaller part of it (**?**). The informal tip to prefer smaller colonies, colonies that have the full plasmid would spend more energy replicating that plasmid than the colonies that only have a smaller part of it (**?**). This subjective approach helped to find colonies where the PCR screen with the primer M13 Forw and CEN-ARS-R were successful. The results from the PCR screen were later confirmed by sequencing.

The sequencing results of the colony A (pCwPUC1) and colony B (pCwPUC1) confirmed the PCR results, as the sequencing matches with the expected sequence through the four fragments of the plasmids. This is not the case for the colony D (pCwPUC1) and colony 2 (pCwPUC3), where the promoter appeared no to be inserted, as the sequencing got little to no match in the zone of the promoters. Plasmid with no promoter would prevent the expression of the selective marker, therefore making a potential success of the conjugation impossible.

The results regarding the colony 2 (pCIP promoter) came a short time before that the culture of *C. wailesii* was ready for a conjugation experiment. The plasmids from that colony were none the less isolated and transformed into E. coli DH10B in spite potentially missing as it was not possible to try to obtain more colonies for sequencing before that the culture of diatom degenerate. The plasmid from the colony 2 (pCIP promoter) were then used in the conjugation experiment as well as the one from the colonies : A(pCwLHCFN), B(pCwLHCFN), and D(pCwLHCFN).

4.4 Axenisation of Coscinodiscus wailesii Culture

The culture of Coscinodiscus wailesii (CCMP2513) supplied by NCMA was delivered non-axenic. As the conjugation experiment implies exposing the diatoms to a relatively high concentration of transformed *E. coli*, we thought that the microbiota of the algae could disrupt the conjugation, therefore lowering the transformation rate. *C. wailesii*Two experiments were carried out to obtain axenic *C. wailesii* cultures.

The protocol given by Mönnich et al. (2020) indeed led to axenic cultures initially, but the cultures did not survive. It is possible that as the protocol has been thought to be used with *Thalassiosira rotula* and not *C. wailesii*, that the giant diatom simply did not survive the exposure to the antibiotics. Alternatively, *C. wailesii* could not survive without a specific microbiota that was killed by the process. The latter possibility is contested by the results of the experiment of Nagai et al. (1998), where they obtained axenity of *C. wailesii*. Their protocol was also tested, but did not lead to axenity. The concentration in bacteria of the culture was probably lower than the control as the growth was less prominent in the cultures that were treated. It is not possible to say that the protocol cannot work as the experiment did not happen as planned as the cells went through the sieve.

Overall, we did not manage to get an axenic culture of *C. wailesii* and non-axenic culture were used for the conjugation.

4.5 Establishment of Conjugation Protocol

The protocol to attempt the conjugation experiment was directly inspired from publication of Karas et al. (2015). The method was adapted with the available knowledge to be replicated on *C. wailesii*. which has biological differences with the species of the original paper. As the conjugation experiments did not lead to positive results, it is not possible to clearly state if the DNA transfer from bacteria to *C. wailesii* is possible in the first place. We will now try to give a non-exhaustive list of things that may have gone wrong or what should have been done to increase the chance to maybe have an observable DNA transfer by conjugation in *C. wailesii*.

4.5.1 Transformation of Isolated Plasmids in E. coli for Conjugation

The isolated plasmid were transformed into the *E. coli* strain DH10B. This strain of *E. coli* has the mobilization plasmid pTA-Mob. This artificial plasmid improved conjugal transfer (Strand et al., 2014). The pTA-Mob plasmid also has a resistance gene for gentamicin (20 µg/mL) which prevented the culture to degenerate (losing the plasmid) when maintained in medium with gentamicin.

The cultures were normally transformed following the protocol in Section 2.2.4. The obtained colonies were screened by PCR screen to look for the presence of the pCwPUC1 and pCwPUC3 plasmids. The cultures were not sent to sequencing because the plasmid used in the transformation were verified. It was assumed that the presence of positive matches of the expected size during the colony screening by PCR, meaning that the plasmid were successfully transferred with the resistance gene to kanamycin with the sequences to express the resistance gene to zeocin.

The use of SOC medium (Sambrook and Russell, 2001) instead of LB medium (Bertani, 1951) was

thought to give faster growth and endowed a stronger vitality to the bacteria, which could lead to better conjugation rate (**?**). The advantage of using the SOC medium was that the incubation times were reduced. This differs from the original paper of Karas et al. 2015 as they only used SOC medium to resuspend the cells. As it is commonly used without leading to errors by other researchers it was not thought to be disadvantageous.

4.6 Harvesting of *C. wailesii* Cells

Cultivating enough *C. wailesii* cells required to use twenty bottles of 2 L of L1 medium. When it came to the harvesting, the big size of those diatoms became an advantage as they had the tendency to settle easily on the bottom of the bottle ready to be pipetted. But even the 100 mL medium from the bottom of the bottle contained a lot of water compared to the total volume of the cells. It was important to concentrate the cells to an extreme point to make the conjugation process workable in a lab using Petri dishes. The difficulty of the cell concentration lives in the danger to stress the cell by mechanic shock by centrifugation and by starvation, as the cells would take up quickly the available nutrient present in a small amount of medium. The control in-agar culture showed satisfying growth in absence of selective factor which indicates that the cells were able to recover after the centrifugations and the other manipulation that followed. But maybe the stress could prevent the cells to receive the plasmid or if the plasmids were received, it could prevent their replication as the cells would allocate energy to repair itself. We would therefor advice to keep the diatom cells in normal condition as much as possible to increase the chance of success of the conjugative DNA transfer between bacteria and diatom experiments.

4.3.1 Donor Recipient Ratio Because of the difference of achievable cell concentration between *C. wailesii* and *T. pseudonana*, it is possible to have ratios unbalanced towards a higher number of donor cells. Moreover, it requires diluting the *E. coli* cultures Knowing that a greater proportion of cells could lead to higher conjugation rate (Dominguez and O'Sullivan, 2013), we took a different approach than other strategy done for other publications by having a greater concentration of *E. coli* than *C. wailesii* in the co-incubation plate.

Conjugation Protocol

Creating a protocol to transfer DNA from *E. coli* to *C. wailesii* was the ultimate goal of this master thesis project. The conjugation process implies forcing a physical contact between the recipient cells (*C. wailesii*) and the donor cells (DH10B) for a certain amount of time to maximize the chance that the natural process of conjugation occurs. The composition of the medium of the co-incubation plates was inspired by Karas et al. (2015), they suggested having 5% LB medium in the gel's composition. From the work of Knider (2018) suggested that the use of 5% LB medium would not be insufficient to lead to bacterial growth, which is a sign that the bacteria are thriving. The best alternative found in that study was to use pure algae culture medium with 1% of peptone. It is important to point out that the specie that study was working on was not a diatom. Only one co-incubation time was tried, maybe that a longer incubation could let more time for the cells to make a physical connection for the conjugation to occur. More incubation time could have been tested, but the lacked of time to continue the master thesis project made it impossible.

Possible Errors

It was not possible to demonstrate that conjugation took place during the experiment of that project. In spite that it is not known if the conjugation with *C. wailesii* is possible, it is also possible that not enough cells were used to statistically observe a conjugation. In the experiment of Karas et al. (2015) the rate of transfer per diatom cells was of 4*10, because the real number of *C. wailesii* cells use in the experiment was not exactly known because of the difficulty to concentrate, split, and spread that diatom cells jam. It is possible that the total number of diatom was lower than the aimed 20,000 per plate. To find out if the experiment did not show positive results because of statistic limitation we would have needed to reproduce the same experiment many times to come to that conclusion. Unfortunately, not enough time was allocated to the project to carry out that possibility. One error of the project was that the plasmids pCwPUC1 and pCwPUC3 were not transformed into *C. wailesii* using other techniques than conjugation. It would have been possible to try to electroporate (Karas et al., 2015) or bombard (Apt et al., 1996)the plasmids in *C. wailesii* cells with the plasmids to observe if they would express the resistance gene. Without those experiments, it is difficult to conclude if the conjugation did not take place or if the plasmid were indeed transferred into the *Coscinodiscus* cells but could be expressed. Maybe that the plasmid pCwPUC3 from the colony 2 was indeed incomplete as its sequencing led to uncertain results.

Maybe the experience would have been successful if more plasmids were tried using different promoters, but the number of potential highly expressed natural promoters was limited and it is uncertain that the affinity of the promoters with the polymerase causes the absence of observation of transformed *C. wailesii*.

Many things could explain why the plasmids are unsuitable for expression. Maybe that *Coscinodiscus* requires different factors than the other diatom and therefore the CEN/ARS-HIS3 sequence did not have the same effect as on *T. pseudomonas* or *P. Tricornutum*. It is also possible that the promoters did not have an affinity with the cell machinery of *Coscinodiscus wailesii*.

Future Work

The scientific interest to develop protocol to extend the possibility molecular biology is a worth pursuing path to extend the knowledge and the impact of that science. *Coscinodiscus wailesii* is a singular diatom species with features that makes it interesting for the study of mechanisms behind the development of the frustule with possible application in promising industry such as the development of photonic crystals. It would interesting to continue the experimentation to with the molecular tools that have already been created during that project with testing their expression in *C. wailesii* by using more classical transfer methods as bombardment or electroporation. If the expression of the plasmids pCwPUC1 and pCwPUC3 in *C. wailesii* can be demonstrated, then more conjugation experiments with different parameter such as longer or shorter co-incubation time, recovery time, different temperatures, different ratio of donors:recipient cells, might show that it is possible to transfer DNA in an easy and non-destructive manner to carry more experiments.

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Appendix

A Culture Media

Table A.1: L1 medium recipe. (Hallegraeff & Anderson, 2003) retrieved from (L1 Medium, 2020)

Component	Stock Solutions	Quantity for 1L	Final Molar Concentration
NaNO3	75.00 g L-1 dH_2O	1.0 mL	8.82 x 10-4 M
NaH2 $\cdot PO4H_2O$	5.00 g L-1 dH_2O	1.0 mL	3.62 x 10-5 M
$Na2Si_O3 \cdot 9H_2O$	30.00 g L-1 dH_2O	1.0 mL	1.06 x 10-4 M
Trace Element	Recipe in Table A.2	1.0 mL	-
Vitamins Mix	Recipe in Table A.3	0.5 mL	-

Table A.2: Trace element solution stock composition for the L1 medium(Hallegraeff and Anderson, 2003).

Chemical	Primary Stock Solution	Amount/Volume for 500 mL
Na_2 EDTA$\cdot 2H_2O$	-	2.18 g
$FeCl_3 \cdot 6H_2O$	-	1.575 g
$CuSO_4 \cdot 5H_2O$	0.0245 g	1.5 mL
$Na_2MoO_4 \cdot 2H_2O$	0.199 g	0.5 mL
$ZnSO_4 \cdot 7H_2O$	0.22 g	0.5 mL
$CoCl_2 \cdot 6H_2O$	0.1 g	0.5 mL
$MnCl_2 \cdot 4H_2O$	1.8 g	0.5 mL
H_2SeO_3	0.026 g	0.5 mL
$NiSO_4 \cdot 6H_2O$	0.027 g	0.5 mL
Na_3VO_4	0.0184 g	0.5 mL
K_2CrO_4	0.0194 g	0.5 mL

Table A.3: Composition of the vitamin stock for the L1 medium (Hallegraeff and Anderson, 2003).

Vitamin	Per Liter
Cyanocobalamin (Vitamin B 12)	0.0005 g
Thiamine HCl (Vitamin B 1)	0.1 g
Biotin	0.0005 g

Table A.4: LB medium/agar recipe (Bertani 1951). Components below were added to MQ (1 L), before to autoclaved for 20 minutes at 120 °C.

Component	Quantity
Tryptone	10.0 g/L
Yeast extract	5.0 g/L
NaCl	5.0 g/L
Bactoagar (only for agar plates)	15.0 ml/L

Table A.5: Composition of SOC medium as described in Sambrook and Russell (2001).

Component	Amount per Liter
Yeast extract	5 g
NaCl	0.584 g
KCl	0.186 g
$MgCl_2$	10 mL
$MgSO_4$	10 mL
1M glucose	20 mL

B PCR Primer

Table B.1: List of primer sequence used in the experiments. The part of the sequences that have a complementary for a Gibson Assembly are bold on the table.

Fragment	Primer name	Sequence (5'-3')
	M13 Forw	TGTAAAACGACGGCCAGT
	CEN-ARS-R	TGTGGTCTTCTACACAGACA
LHCFN prom	PUC-pLHCFN-FN	**AGCTGGTACGGGCGTCA**GATAGAAGACACC
	pLHCFN-BleR	**TCAACTTGGCCATGTTGATTATTGG**TATTGAGGAGAAGAAGAGAG
BleoR pCwPUC1	pLHCFN-Ble-F	**CCAATAATCAACATGGCCAAGTTGA**CCAGTGC
	Ble-tLHCFN-R	**TCCGTCACTTCTCAGTCCTGCT**CCTCGGC
LHCFN term	Ble-tLHCFN-F	**AGCAGGACTGAGAAGTGACGG**AAAAGCCTAGAGAAAAG
	tLHCFN-PUC-RN	**ATTCCTGACTGTGTGGAAAGTTTCT**AGAACTACAGCATAATTATATAATCTTTGT
Vector pCwPUC1	tLHCFN-PUC-FN	**AGAAACTTTCCACACAGTCAGGAAT**AACACTAGCTCG
	PUC-pLHCFN-RN	**CTGACGCCCGTACCAGCT**TTTGTTCCCTTTAGTGAG
LHCF4a prom	PUC-pLHCF4L-F	**AAAAGCTGGTACAATGAGAGGGGTG**AAAAGGCAA
	pLHCF4-BleR	**TCAACTTGGCCATTGTTTCGATTAT**GAGAGAAACACGTAAATTATATGA
BleoR pCwPUC-2a	pLHCF4-Ble-F	**ATAATCGAAACAATGGCCAAGTTGA**CCAGTG
	Ble-tLHCF4-R	**AAACCATAAATATTCAGTCCTGCTC**CTCGGC
LHCF4a term	Ble-tLHCF4-F	**GAGCAGGACTGAATATTTATGGTTT**TTGTGGTTTTTACCCAGATG
	tLHCF4-PUC-R	**ATTCCTGACTGTGGTCATGTCTTAT**CCTAGAAAAAATCACCACTTTTTTAC
Vector pCwPUC-2a	tLHCF4-PUC-F	**ATAAGACATGACCACAGTCAGGAAT**AACACTAGCTCG
	PUC-pLHCF4L-R	**CACCCCTCTCATTGTACCAGCTTTT**GTTCCCTTTAGTGA
CIP1 viral prom	PUC-pCIP1-F2	**AAAAGCTGGTACTACGTAGAATCCT**ACGTAAAATGGACGA
	pCIP1-Ble-R	**CAACTTGGCCATTGCGAATGTACG**TGTTGTAGCTC
BleoR pCwPUC-2a	pCIP1-Ble-F	**CGTACATTCGCAATGGCCAAGTTG**ACCAGTGC
Vector pCwPUC3	PUC-pCIP1-R	**AGGATTCTACGTAGTACCAGCTTTT**GTTCCCTTTAGTGAG

C Reactions

Table C.1: Reaction for Colony PCR Screen using RedTaq 2x Master Mix (VWR)

Component	Volume (µL)	Final Concentration
Red Taq 2x Master Mix	12.5	1x
Nuclease-free water	11.5	-
Forward primer (10 M)	0.5	0.2 µM
Reverse primer (10 M)	0.5	0.2 µM
Template DNA	-	-
Total Volume	25	-

Table C.2: Reaction setup for PCR amplification with Invitrogen PlatinumTM SuperFi PCR Master Mix.

Component	Volume [µM]	Final Concentraton
Nuclease-free water	to 50 L	-
2x Platinum SuperFi PCR Master Mix	25.0	1x
Forward primer (10 M)	2.5	0.5 µM
Reverse primer (10 M)	2.5	0.5 µM
Template DNA	-	-
Total reaction volume	50.0	-

Table C.3: Reaction setup for PCR amplification with Phusion Hot Start II HighFidelity PCR Master Mix.

Component	Volume [µM]	Final Concentraton
Nuclease-free water	to 50 L	-
2x Phusion HS II HF PCR Master Mix	25.0	1x
Forward primer (10 M)	2.5	0.5 µM
Reverse primer (10 M)	2.5	0.5 µM
Template DNA	-	-
Total reaction volume	50.0	-

Table C.4: Reaction set-up for Gibson assembly. The volume of the DNA fragment depends on the concentration of the each PCR product.

Component	Volumes
Total Amount of Fragments	1 pmols*X µL
Gibson Assembly MMaster Mix (2X)	10 µL
Deionized H2O	10-X µL
Total Volume	20 µL