Norwegian University of
Science and Technology

Developing tools to genetically engineer the microalga Nannochloropsis

Vy Nguyen

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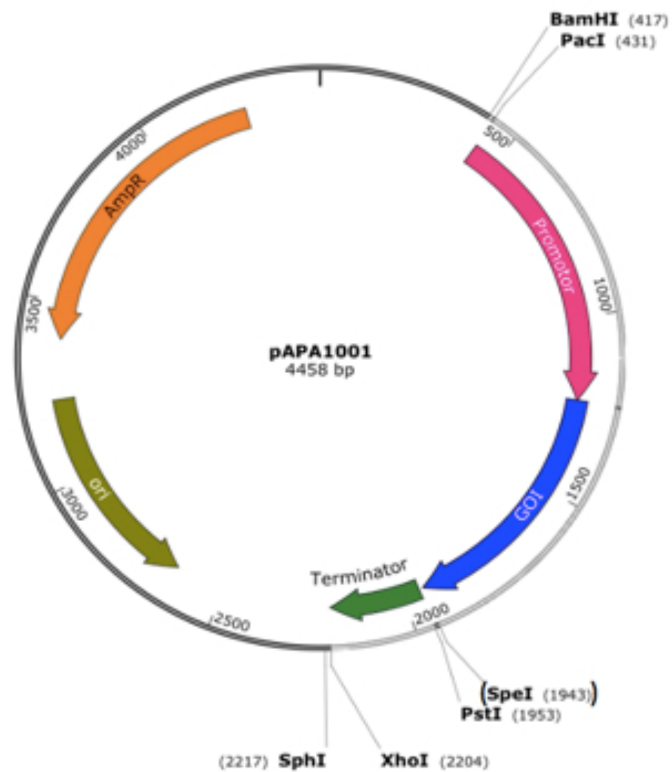
Supervisor: Martin Frank Hohmann-Marriott, IBT

Norwegian University of Science and Technology
Department of Biotechnology

Developing tools to genetically engineer the microalga *Nannochloropsis*

VY THUY NGYEN



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ABSRACT

Nannochloropsis species have been investigated as promising candidate for biofuel and nutraceuticals production in algae. These eukaryotic microalgae belongs to a diverse group of brown algae containing haploid genome enabling the opportunity for metabolic engineering. This project describes the development of an efficient vector family pAPA1001 for transformation by electroporation in *Nannochloropsis oceanica* CCMP1779 by a modular BioBrick assembly strategy. The endogenous promoters with their associate terminators ensure constitutively strong expression of zeocin, hygromycin B or paromomycin in transformed eukaryotic algae cell. Sequence of the designed transformation plasmids revealed one point mutation in the promoter and one in the terminator region that were evaluated to be not problematic for the gene regulation. Successful transformation by electroporation was shown with the plasmids pAPA_0169_Z and pAPA_0602_Z, revealing expression of the zeocin resistant gene in *N. oceanica* CCMP1779. The transformation efficiency based on colony PCR verified algal clones was 6.5×10^{-8} and 1.2×10^{-8} colonies/ cell/ µg for electroporation using pAPA_0169_Z and pAPA_0602_Z, respectively. Transformation with the other four plasmids pAPA_0169_H, pAPA_0602_H, pAPA_0169_P and pAPA_0602_P was not successful due to technical issues. Electroporation transformation using the plasmid pAPA_0169_Z resulted in very low number of ¨false positive¨ resistant clones compared to the previously used pSELECT100 plasmid, providing a useful tool for simpler and more effective transformant selection of the oleaginous algae *N. oceanica.*

¨Don´t give up learning and improving.

That´s why we have a life to grow.

So never take things as criticism, only as constructive comments.¨

ꕤ- Alice Mühlroth - ꕤ

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ABBREVIATIONS

TAG	triacylglycerol
LC-PUFAs	long chain polyunsaturated *n*-3 fatty acids
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
GOI	gene of interest
HR	homologous recombination
bp	base pair
EDTA	ethylene diamine tetra acetic
TUB	β-tubulin
HSP	heat shock protein
UEP	ubiquitin extension protein
VCP	violaxanthin/chlorophyll α-binding protein
LDSP	lipid droplet surface protein
MSC	multiple cloning site
UTR	untranslated region
PCR	Polymerase Chain Reaction
LB	lysogeny broth
OD	optical density
TD	Touch Down
HF	High-Fidelity
IPTG	isopropyl β-D-1-thiogalactopyranoside

Part I
Nannocloropsis oceanica
and GENETIC ENGINEERING

Part I. *Nannochloropsis oceanica* and GENETIC ENGINEERING

1. Biofuel production

There are growing concerns about the diminishing world oil reserves, the environmental deterioration and biotic health problems related to fossil fuel consumption. Currently, about 90% of global energy use is provided by petroleum based fuels (Yen et al. 2013). The increasing energy demand shows evidences that the conventional oil reserves that can be commercially exploited will be vanished after 2050 (Campbell and Laherrère, 1998; Ho et al. 2011). This has led to vast interest in searching for highly sustainable and renewable energy sources.

Biofuels, which are produced from photosynthetic biomass, are considered one of the most feasible energy alternatives to reduce our high dependency on fossil fuels. The first generation of biofuels are produced directly from food crops that could be lipid-based (biodiesel; rapeseed, palm, etc.) or sugar-based (bioethanol; sugarcane, corn, etc.) feedstock (Brennan and Owende, 2010; Chen et al. 2011). Those plants transform the solar energy into the carbon storage products, which then can be transformed into biodiesel and bioethanol (Maity et al. 2014). However, they threaten the food chain due to the need of an excessively large area of arable land for producing a sufficient amount of crop-based fuel. This leads to increased pressure on farm land needed for food production to feed the growing population which is expected to reach 7.2 billion in 2050. One more problem could be intense growth requirements that might affect ecosystem balance and biodiversity (Parmar et al. 2011). Although various non-food feedstock including food crops that have already fulfilled their food purpose (for instance waste vegetable oil) have been successfully converted into second generation of biofuels. However, there is still shortage in meeting overall global energy demands (Naik et al. 2010).

The disadvantages related to the first and second generation of biofuels can be overcome by microalgae which is also known as the third generation biofuel source. Many eukaryotic microalgae have the ability to store significant amounts of energy-rich compounds while consuming CO_2. Such compounds like triacylglycerol (TAG) and starch can be utilized for biofuels production, including biodiesel and ethanol (Chen et al. 2011). Studies showed that many algae can accumulate more than half their dry weight biomass as lipids (Hu et al. 2008). According to a recent study, the theoretically estimated annual oil production from algae can reach a maximum of 40,700 – 53,200 L ha^{-1} $year^{-1}$, which is 5 to 6 times higher than that from palm oil (Weyer et al. 2009). Annual oil production of algae has also been reported to be 100-

fold greater than that of soybeans, a major feedstock currently being used for biodiesel in the USA (Christi, 2007). In addition, the biggest advantage in water usage is that microalgae are able to grow in brackish and saline water as well as water produced from oil and gas extraction (Quinn et al. 2012), hence it does not compete with farm land and there is no competition to the food supply compared to the first and second generation biofuels. Microalgae also have some other advantages such as a high degree of environmental tolerance and conversion of microalgal biomass to biofuel. These could help in mitigating the environmental problems related to greenhouse gas and waste water. Hence, the benefits of using microalgae as feedstock for biofuel production is enhanced (Maity et al. 2014).

The production of biofuels using microalgae is a very promising to replace conventional biofuel production approaches. Despite of laboratory-scale research on involved process, the selection of organism and successful cost effective industrial application of biofuel production remains an upstream challenge, due to our limited knowledge of their metabolic systems (Weeks, 2011). The lipid yields obtained from algal mass culture efforts performed to date fall short of the theoretical maximum and have made algal oil production technology prohibitively expensive (Sheehan et al. 1998; Hu et al. 2006). Among 3,000 different microalgae species that are potential to produce biofuel have been analyzed by the U.S. Department of Energy´s Aquatic Species Program includes *Chlorella vulgaris, Chlorella emersoni, Neochloris oleoabundans, Phaeodactylum tricornutum* and *Nannochloropsis* sp. Most of them have drawbacks that have been preventing the emergence of an economically viable algal biofuel industry (Sheehan et al. 1998). There are still several technical barriers that are needed to be overcome before microalgae can be used as an economically viable biofuel feedstock. This includes production management, harvesting, fuel extraction, refining and residual biomass utilization (Scott et al. 2010; Pienkos et al. 2009). Many of the engineering processes related to increase of both growth and lipid content must be developed and optimized considering the key parameter of efficient strain selection.

2. Essential fatty acid for dietary supplement

Humans and other animals can synthesize most of the fats they need from their diet (Groff et al. 1995). However, there are essential fatty acids that cannot be synthesized by the body in a sufficient amount for the maintenance of mental and cardiovascular health (Plourde et al. 2007; Khozin-Goldberg et al. 2011). Among those essential fatty acids, long chain polyunsaturated *n*-3 fatty acids (LC-PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and

docosahexaenoic acid (DHA, 22:6n-3) are of increasing interest, due to their many positive effects for human health and their use as feed for fish farming (Mühlroth et al. 2013). The beneficial health effects of *n*-3 fatty acids have been extensively studied to have positive effects include anti-viral, anti-bacterial and anti-fungal (Das, 1999; Ormarsson et al. 2012). It was also experimentally proved that *n*-3 fatty acids are important for human brain development during both the fetal and postnatal period and dietary DHA is needed for the optimum functional maturation of the retina and visual cortex (Chang et al. 2009). In fish, LC-PUFAs play crucial roles in osmoregulation (Tocher, 2010) and are also required for normal growth, immunity and stress tolerance, especially at early stage of development (Bell et al. 2003).

The main source of *n*-3 LC-PUFAs for human consumption is currently marine fish and in particular the fish oils from fat fish. However, as the natural source is at peak harvesting, alternative sustainable sources for *n*-3 LC-PUFAs for both marine aquaculture and human consumption should be developed soon. The aquaculture industry has started to replace fish oil and fish meal with plant seed meals and vegetable oils in order to secure growth in the production (Nasopoulou, 2012). Currently, the most common vegetable oils used for fish feed production have been soybean, linseed, rapeseed, sunflower, palm oil and olive oil.

Studies have shown that microalgae contain large quantities of high-quality EPA and DHA, and thus they are considered a good potential source of these valuable fatty acids (Spolaore et al. 2006; Vazhappilly and Chen, 1998). Compared to terrestrial crop plants, microalgae present a few advantages as *n*-3 LC-PUFA sources, such as commonly occurring genes for the biosynthesis of these nutrients, simpler fatty acid profiles and higher growth rates (Martins et al. 2013). Phototrophic species including eustigmatophyte *Nannochloropsis* have long been used by the aquaculture industry as a source of *n*-3 LC-PUFAs for larval fish through the enrichment of live feeds (e.g., rotifers). However, the production is remained low due to low biomass density, making photosynthetic production of *n*-3 LC-PUFAs still profitably challenging at the present. Hence, there is need in isolation and genetically improvement of strains that are potential for *n*-3 LC-PUFAs production.

3. Introduction to *Nannochloropsis oceanica*

Planktonic *Nannochloropsis* is a genus of unicellular eukaryotic microalgae and is classified under the class Eustigmatophyceae of the Heterokontophyta, a diverse algal group that includes brown algae and diatoms (Van Den Hook et al. 1995). All eustigmatophyceae are unicellular and have coccoid shape. There are six recognized species in the *Nannocloropsis* genus including five marine ones (*N. gaditana, N. granulata, N. oceanica, N. oculata* and *N. salina*) and one freshwater specie (*N. limnetica*) (Li et at., 2014). Species of this genus are 2-5 μm in size and are morphologically similar, which makes their identification through traditional observation difficult (Li et al. 2011). Therefore, sequences for 18S rRNA is usually used to identify different species of *Nannochloropsis* (Karlson et al. 1996). Moreover, microalgae *Nannocloropsis* possess haploid genome (Killian et al. 2011).

Many species of *Nannochloropsis* have been investigated by studies for their biomass production, lipid composition and content under different growth conditions (Rodolfi, 2009; Hu and Gao, 2006, Tonon et al. 2002). It is a robust industrial algal specie that has been extensively grown in outdoor ponds and photo-bioreactors for aquaculture as fine rotifer feed (Sukenik et al. 2009; Rodolfi et al. 2009). Some species of *Nannochloropsis* such as *N. oculata* (Renaud et al. 1991) and *N. gaditana* (Ferreira et al. 2009) are used in marine aquaculture as an important source of eicosapentaenoic acid and is economically important. Moreover, *Nannochloropsis* is considered appealing feedstock for biodiesel production due to their high growth rate and ability to accumulate high amounts of lipids (up to 47.5% of biomass) (Kang et al. 2015). Under nitrogen depletion condition, *Nannochloropsis* can accumulate lipids exceeding 60% of its biomass, making it an excellent candidate for industrial biofuel production (Radakovits et al. 2012). In this regard, many different species of *Nannochloropsis* have been studied including *N. gaditana* (Simionato et al. 2011, 2013), *N. oculata* (Vooren et al. 2012), *N. salina* (Sforza et al. 2012) and *N. oceanica* (Dong et al. 2013; Pal et al. 2013; Solovchenko et al. 2014).

The specie of interest in this project is *N. oceanica* which has been chosen due to its high biomass production. It has been reported that *N. oceanica* was able to produce 69% lipid (dry weight) in a 23-d batch culture (Xiao et al., 2013), which is higher than that (60% of dry weight) of several other *Nannochloropsis* spp. reported by Rodolfi et al. (2009). Therefore, *N. oceanica* is a good candidate for development into a model organism for algal biofuel and nutraceuticals production. However, as unimproved algae are unlikely to possess all of the traits necessary for

economic production of biofuels and nutraceuticals (Wijffels, 2010), there is significant need for strain optimization (Radakovits et al. 2010).

It has been shown that there are two general approaches to improve lipid production in microalgae *N. oceanica* (1) To increase the desired lipids content per unit of biomass and (2) To increase the biomass density to maximize biomass per culture volume or area (Mühlroth et al. 2013). However, optimization of growth conditions that increase desired lipids is challenging. The rational for this is that many microalgae do not produce large amounts of storage lipids during exponential phase of growth. Instead, TAGs only accumulate under abiotic stress such as lack of nitrogen. Under depletion, microalgal cells slow down their proliferation and start producing energy storage products, which then in turn decreases the biomass yield. Currently, there is increasing effort for improving the *Nannochloropsis* strains by long selection processes or genetic engineering for industrial applications (Yen et al. 2013). It stands to reason which approach selection or genetic engineering is more beneficial to achieve an industrial strain.

4. Genetic engineering

Genetic engineering is the direct manipulation of an organism´s genome using a set of technologies to change the genetic makeup of an organism to produce improved or novel organism (Alberts et al. 2008). Firstly, exogenous DNA is prepared by isolating and copying the genetic material of interest using molecular cloning methods or by synthesizing the DNA. Then these DNA sequences are incorporated into the host organism indirectly through a vector system or directly through micro-injection, macro-injection and micro-encapsulation techniques.

The gene to be inserted, or gene of interest (GOI) which are transferred into the host organism must be combined with other regulatory genetic elements including promoter and terminator sequence that are necessary for gene expression, and selectable marker gene as well. The promoter region initiates transcription of the gene (Old et al. 1985). It is the main determinant in modulation of level and timing of gene expression (Lodish et al. 2000). The choice of promoter with suitable strength and features, such as inducible or constitutive, regulates the rate of transcription of the GOI. For inducible promoter, which is usually used in expression vector, the protein synthesis is initiated by environmental changes, which can be artificially controlled by e.g. hormones, biotic or abiotic stresses (Venter et al.2010). Gene expression may also be constitutive (i.e. protein is continually transcribed). Furthermore, studies show that promoter

strength is greatly influenced by its nucleotide sequence (Li et al. 2014). Terminator, which is located downstream of the GOI, defines the end of a gene and initiates the process of releasing the newly synthesized RNA from the transcription machinery (Lodish et al. 2000). Termination is coupled to the process that cleaves and polyadenylates the 3′ end of a transcript. The terminator also plays an important role in RNA processing and contributes to variability in RNA half-life, and ultimately gene-expression. The selectable marker which usually confers antibiotic resistance to the organism is essential to verify transformed cells with integrated foreign gene. All the genetic elements are constructed in the vector (generally a plasmid) by using recombinant DNA techniques including molecular cloning, restriction digestions and ligations (Berg et al. 2010).

In the following step, this vector is transferred into the host genome by transformation. Foreign genes are usually integrated into the host genome by random insertion. However, methods have been developed to insert genes into target position in the host´s genome by homologous recombination (HR). Then, any specific gene can be altered or inactivated by direct gene replacement. Although homologous integration of transfected DNA into the genome was considered to be an extremely rare event in other organisms, techniques for this process have been established for several model systems (Sodeinde et al. 1993; Smithies et al., 1985). In many cases, transformation results in stable expression of transgenes, from either the nucleus or the plastid, but in some cases only transient expression is observed (Quin et al. 2012).

In microalgae, the research into the genetic engineering is still in its fetal phase. However, there are some various methods for genetic modification have been evaluated to increase both the TAG and EPA production of microalgae. In addition to the highly efficient transformation by nuclear electroporation (Chen et al. 2008; Chen et al. 2001), it is also possible to perform genetic manipulation on microalgae by biolistic microparticle bombardment (Apt et al. 1996; Dunahay, 1995; Fischer et al. 1999; Jakobiak et al. 2004). Another methods of transformation have been used to transfer DNA into microalgal cells includes agitation in the presence of glass beads or silicon carbide whiskers (Dunahay et al. 1995; Kindle, 1990) and *Agrobacterium tumefaciens*-mediated gene transfer (Kumar, 2004). Historically, the fresh water algae *Chlamydomonas reinhardtii* has been the focus of most molecular and genetic phycological research. This specie is the model system for diverse biotechnological research areas with the availability of complete genome sequencing (Leon-Banares et al. 2004). However, low biomass production rates in most of these strains have kept them from becoming industrially relevant.

Transformation methods have also been developed for the model diatom *Phaedactylum tricornutum* but there is limitation in available molecular genetic tools for this species (Apt et al. 1996; Zaslavskaia et al.2000).

Currently, the *Nannochloropsis* genus has awaken large interest and therefore motivation in the field of transgenic microalgae. Genomic information and high-efficient nuclear transformation using electroporation of some *Nannochloropsis* species has been made available. This includes *N. gadinata* (Radakovits et al. 2012), and *N. oceanica* (Vieler et al. 2012). Various genetic modification tools have been released recently such as homologous recombination and overexpression of target genes (Kaye et al. 2015; Kilian et al. 2011; Li et al. 2014) which had permitted the genetic and metabolic engineering of *Nannochloropsis* spp. Optimizing the lipid enrichment and the lipid composition by genetic manipulation is easier and more stable in haploid *N. oceanica* than in diploid genome of model diatom *Phaedactylum tricornutum*. Moreover, due to limitation in available tools in molecular analysis in the current model *C. reinhardtii*, the achievement of homologous gene replaces in *N. oceanica* (Kilian et al. 2011) may open up potential opportunities to develop it into an alternate model organism representing marine, oleaginous microalgae.

5. General aims of the project

This project was to establish novel plasmids for *N. oceanica* transformation by electroporation with the aim of strain improvement using genetic engineering approach. These plasmids carry different promoter-terminator segments along with different selectable marker genes. Molecular cloning strategies used to construct these plasmids were validated based on transformation efficiency, with the intention to optimize transformants selection and target homologous recombination in *N. oceanica*.

Part II
MOLECULAR CLONING

Part II. MOLECULAR CLONING

1. Introduction

1.1 Molecular cloning

Molecular cloning or gene cloning is a set of experimental methods used to insert the gene-of-interest (GOI) into an expression cloning vector being introduced into a target cell. For expressing and synthesizing the encoded protein from the GOI, plasmids contain regulatory elements including promoter and termination sequence.

In *Nannochloropsis* species several cloning vectors with different integrated promoters have been identified as usable. There have been reports of successful genetic transformation of *N. oculata* (Chen, 2008, Li 2009) using foreign promoters. However, only 0.8% of the transformants contains the transferred fragment after 1.5 months of cultivation, indicating that the transferred DNA fragment was unstable in the transformed microalgae and the promoters are probably not ideal for stable expression. Recently, gene transfer by electroporation in *N. gadinata* was performed successfully using for the first time endogenous promoters identified through preliminary sequencing of the species genome. Three selected promoters are from the genes encoding β-tubulin (TUB, Nga00092), heat shock protein 70 (HSP, Nga07210) and the ubiquitin extension protein (UEP), all of them driving the bleomycin resistant gene (Radakovits et al. 2012). It has been shown that the efficiency of the transformations was strongly affected by the promoter used, and the most efficient transformation was achieved using the TUB promoter. Another nuclear transformation in *Nannochloropsis* species used endogenous promoter from violaxanthin/chlorophyll α-binding protein (VCP) genes, VCP1 and VCP2. The promoter regulates the expression of Sh *ble* selectable marker gene, which confers resistance to zeocin, which was patented (Kilian et al. 2011). The VCP1 promoter is a unidirectional promoter while the VCP2 promoter is bidirectional. They are light-inducible promoters, which drive gene-expression in different light intensities. Moreover, a nuclear transformation method has been established by Vieler et al. (2014) employing the pSELECT100 vector with the promoter sequence of the stress-inducible endogenous lipid droplet surface protein (LDSP). This promoter regulates the expression of the hygromycin B resistant gene and resulted in robust expression under nitrogen starvation conditions. The drawback of the pSELECT100 is that it contains many restriction enzyme cutting sites in the promoter region and that it was not designed for homologous recombination.

In previous studies (Chernyavskaya, 2014; Anley; 2015) plasmid pSELECT100 was used for biolistic transformation and electroporation in *N. oceanica*, with low transformation efficiency and high amount of false positive mutants. Growth of untransformed clones on antibiotic-selective medium have made it complicated to identify pSELECT100 positive cells. Hence in order to optimize the efficient selection of transformants, there is a need to develop an expression cassette that has a strong and stable expression of the selection marker and therewith a good promoter. In this manner, a high expression level of selected reporter genes can be achieved, which in turn simplifies the screening for transformed algal clones. In addition to the expression of a selectable marker gene, a high expression of genetically introduced genes of interest is also essential for industrial application. If successful transformation and stable expression of the desired gene are established, genes encoding important proteins of lipid metabolism pathway can be inserted into the genome of *N. oceanica*. This would allow optimization of oil production in *N. oceanica,* which presents possibilities for the future of third generation biofuels. A novel plasmid with a strong, stable promoter driving gene expression under different conditions such as nutrition starvation and in the stationary phase can also be applied later for creating gene knockouts by HR.

Different vectors with integrated promoters were previously engineered for different applications in *Nannochlropsis.* However, due to disadvantages and irrelevance of those plasmids, Kutschera (2015) developed a cloning strategy to achieve a novel plasmid family pAPA1001 for transformation of *N. oceanica* that is expected to have constant expression in all phases of the cell cycle and in different growth conditions, including nutrition depletion. The aims of these plasmids are to optimize transformation selection and accommodate future homologous recombination approaches to achieve gene overexpression or gene knockouts.

1.2 Vector construction

The plasmids pAPA1001 was created based on the pUC19 backbone with progressive insertion of promoter - terminator and reporter gene sequence. The vector construction was based on simple exchange of these sequences to establish six different vector constructs. The overall vector construction strategy is illustrated in Figure 1.

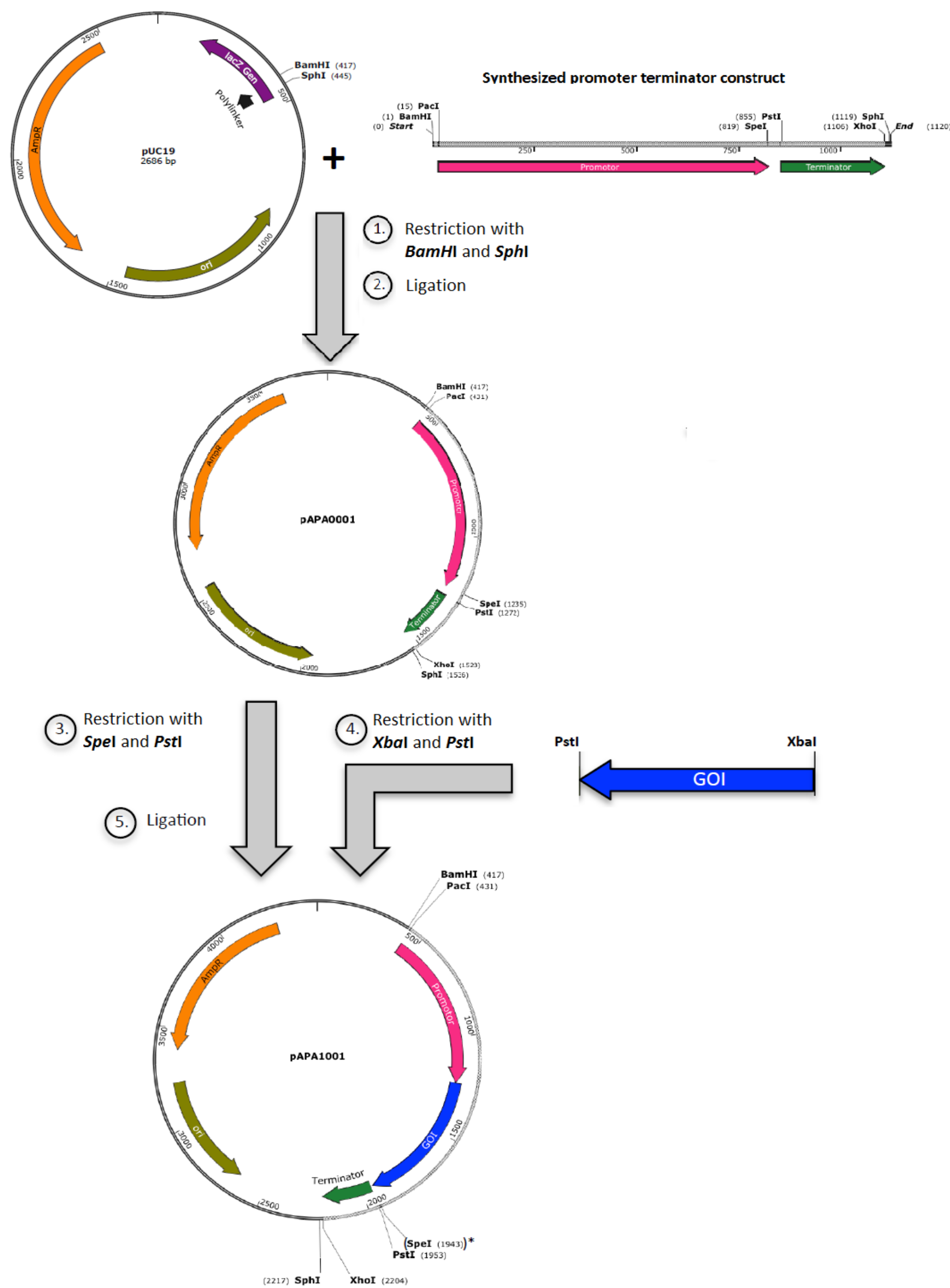

Figure 1: Scheme of the cloning strategy. The plasmid pUC19 and the synthesized promoter-terminator sequences were enzyme-digested with the enzymes *Bam*HI and *Sph*I (1) and subsequently ligated together (2). To insert the GOIs or reporter genes, the formed plasmid was cut with *Spe*I and *Pst*I (3). The PCR amplified GOI was restricted with the restriction enzymes *Xba*I and *Pst*I(4). The *Spe*I and *Xba*I overhang are compatible and ligation with the GOI with the plasmid (5) results in the final expression construct. (Adapted from Kutschera, 2015). The partial construct pAPA0001 after step 2 is the general form of pAPA_0169 and pAPA_0602. The final construct pAPA1001 after step 5 is the general form of pAPA_0169_X or pAPA_0602_X with integrated promoter terminator sequence and reported (X = Zeocin resistance gene, Paromomycin resistance gene or Hygromycin B resistance gene, Z= Zeocin resistance gene, P= Paromomycin resistance gene, H = Hygromycin B resistance gene).

Vector backbone

A compact high copy number plasmid pUC19 (2,686 base pair (bp)) was used as the foundation vector. This vector contains an *AmpR* gene encoding resistance against ampicillin along with a segment of the β-galactosidase gene (*lacZ'*) of the lactose operon of *E. coli* under the control of the regulatory *lac* promoter; a short DNA sequence of 54 bp with a multiple cloning site (MCS); and pMB1 origin of DNA replication. For a pUC19 cloning experiment, DNA from a source organism is cut with one of the restriction endonucleases for which there is a recognition site in the multiple cloning site. This source DNA is mixed with pUC19 plasmid DNA that has been treated with the same restriction endonuclease. After ligation with T4 DNA ligase, this cloning vector-insert construct is usually amplified by transformation with a laboratory strain (for instant *E. coli* DH5α) under ampicillin selection (Glick et al.2010). In this project, plasmid pUC19 was used as backbone for the vector construction of pAPA1001.

Promoter and terminator sequences

Promoter and terminator sequences were determined by previous study (Kutschera, 2015) to be chosen from the genes *nanno_169* (from NannoCCMP1779_3874) and *nanno_602* (from NannoCCMP1779_11694) from the genome sequenced by RNAseq (Vieler et al. 2014). Those two genes showed a high and constant expression rate through microarray assessments of *N. oceanica* expression studies under different conditions (Mühlroth, 2013).

As constant gene expression is desired, genes encoding enzymes of lipid metabolism and photosynthesis pathway were excluded in the microarray assessment of all candidate promoters due to their possible interference with the expression system during cultivation. Sequences of the genes of interest were selected and copied from the corrected gene model of *N. oceanica* CCMP1779 genome (Winge, 2014) and compared to the total genome of the strain to find the start codon of the genes therewith identifying the promoter region (Pan et al. 2011, Vieler et al. 2012). Subsequently, potential genes were either elucidated by comparing with the RNA gene sequence extended from the correct gene model or with the protein sequences for finding possible introns close to the promoter region. Introns close to the promoter region are undesirable because they would indicate binding of additional transcription factors at the region. To further narrow the promoter region down, the gene upstream of the selected gene was identified and the promoter direction was determined. If the upstream gene has the same transcriptional orientation as the featured gene, the promoter sequence is located between two genes at a distance of approximately 300-1200 bp (see Figure 2). In this way, the potential

promoter sequences were selected narrowing them down to a length of less than 1000 bp. If the upstream, featured gene has opposite orientation, the promoter might be bidirectional and utilizing these promoter sites may interfere with cell metabolism. The same approach as for the promoter sequences mentioned above was used to determine terminator sequence (Winge, 2014). The terminator containing 3' untranslated region (UTR) and were used with the associated promoter in the expression constructs. The screening of suitable promoter and terminator involved 15 gene sequences in total of which only two showed to be promising candidate sequences because the *Nannochloropsis* genome contains many introns.

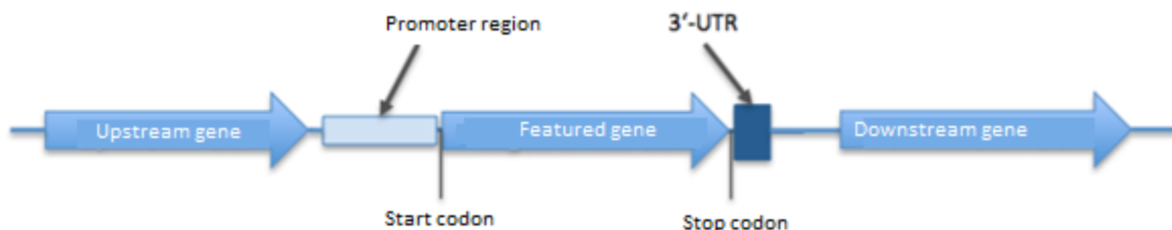

Figure 2: Scheme for the identification of the promoter and terminator sequences of a selected gene (Adapted from Kutschera, 2015). The promoter region is limited by the end of the 3'-UTR of the upstream gene and the start codon of the selected gene. The 3'-UTR of the selected gene was determined by sequence comparisons with a cDNA database. This contains the terminator sequence.

The gene *nanno_602* encodes a general substrate transporter with a major facilitator - superfamily domain. It had the third highest expression of all *N. oceanica* genes by microarray expression analysis. The gene consists of a single exon and the expression is very similar (91% sequence identity) to the homolog protein in *N. gaditana.* The gene *nanno_169* encodes a CLP Protease as a single exon. Furthermore, the expression of the selected gene by the promoters is about twice as high as that of the LDSPs promoter used in the plasmid pSELECT100.

The promoter and terminator fragments of *nanno_169* (NannoCCMP1779_3874) and *nanno_602* (from NannoCCMP1779_11694) were then synthesized by Eurofins Genomics (Eurofins Medigenomix, Germany) and resulted in the *construct_0169* (1089 bp) and *construct_0602* (1220bp), respectively (see Appendix A1.1). The plasmids pEX-K4-No:3874 and pEX-K4-No:11694 contain these two promoter - terminator synthetic constructs, respectively and both possess kanamycin resistance gene as a selectable marker. Both of the synthesized constructs begin with a *Bam*HI and end with a *Sph*I recognition site.

In addition, *construct_0169* and *construct_0602* were also designed to facilitate insertion of any genes instead of the resistant marker genes. While *Spe*I and *Pst*I restriction site sequences mediate BioBrick compatibility of the report genes, there are other interfaces including *Pac*I and *Xho*I that could be used in the future for including native *N. oceanica* sequences that will enable homologous recombination. To sum up, both constructs contain a *Bam*HI, *Pac*I, *Spe*I, *Pst*I, *Xho*I and *Sph*I restriction site (see Figure 1-Synthesized Promoter Terminator construct and Appendix A1.1).

Partial construct pAPA0001

Plasmid pAPA0001 is the general form of pAPA and fully compatible to all reporter gene insertion approaches. They are derived by insertion of the synthesized *construct_0169* and *construct_0602* into the pUC19 backbone by *Bam*HI and *Sph*I restriction and ligation, resulting in pAPA_0169 and pAPA_0602 respectively.

Reporter genes

Efficient isolation of transformants is greatly facilitated by the use of selection markers, including antibiotic resistance and/or fluorescent/ biochemical markers. For transformation selection in microalgae, various antibiotic resistant genes have been used including bleomycin, paromomycin, hygromycin, chloramphenicol and others (Radakovist et al. 2010). The selection of suitable reporter genes was established in previous study (Kutschera, 2014) based on already published transformation and expression systems for *N. oceanica*. Different antibiotic resistant genes and their lethal dose were studied and have already been successfully used as reporter genes (Vick et al. 2012; Anley, 2015). Based on these data, the zeocin, hygromycin B and paromomycin resistance genes were selected for inclusion in the expression constructs. Sequence of reporter genes were presented in Appendix A1.2. The expression “Reporter gene” and “gene of interest (GOI)” are used interchangeably in this project.

Final vector construct

Each final vector construct is composed of a synthesized promoter – terminator sequence separated by an inserted reporter gene. Base on BioBrick assemble approach (Knight, 2003), the restriction sites *Spe*I and *Pst*I are used to add another sequence to a target sequence. Each BioBrick part consists of a circular, double-stranded vector with precisely defined flanking sequences called prefix and suffix located upstream and downstream of a gene, respectively. The upstream sequence consists of *EcoR*I and *Xba*I restriction sites, whereas the downstream

sequence consists of *Spe*I and *Pst*I restriction sites. In partial construct pAPA0001, the *Spe*I site at the 5 'end and the *Pst*I site at the 3' end are present in the DNA sequence between promoter and terminator. Hence, it was decided to amplify reporter genes with designed primers adding *Xba*I (5'-end) and *Pst*I site (3'-end). Reporters gene sequence with introduced *Xba*I and *Pst*I are shown in Appendix A1.3. Due to compatibility of *Spe*I and *Xba*I-generated sticky ends, GOIs are inserted into the partial constructs pAPA0001 to form final vector construction of pAPA1001. However, during the ligation of these sticky ends, the restriction site is lost and a *Spe*I/*Xba*I scar is formed in the final constructs, which cannot be opened again (Figure 3). In this way, six plasmids differing in the promoter-terminator sequence and reporter gene combination can be created with a few steps. Their general form is pAPA1001 while naming system for each plasmid is pAPA_0169_X or pAPA_0602_X depending on promoter-terminator sequence and reporter gene used for construction (X = Zeocin resistance, Parm resistance or Hygromycin B resistance, Z= Zeocin resistance, P= Paromomycin resistance, H = Hygromycin B resistance).

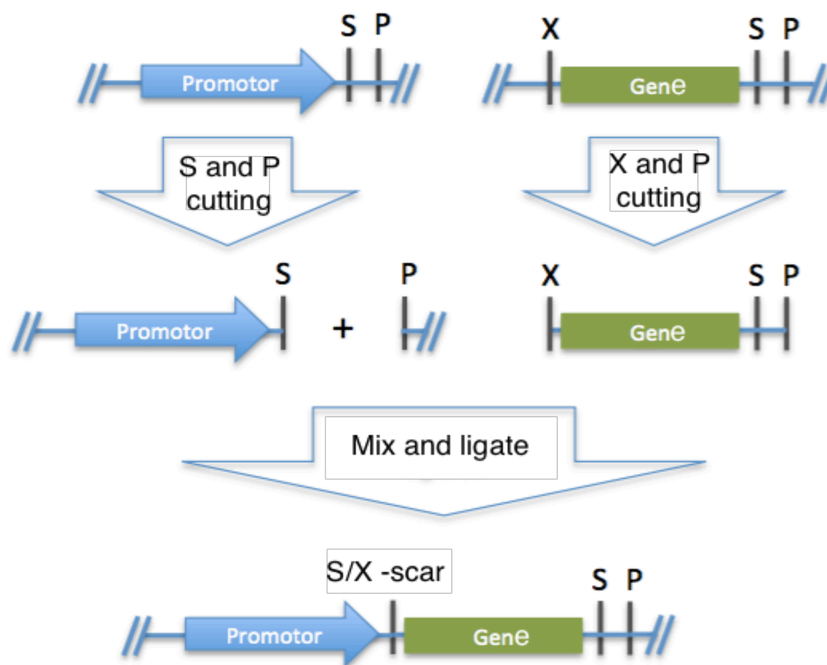

Figure 3: Schematic of BioBrick-based cloning of GOI into partial construct pAPA0001. The overhangs of S and X interfaces are compatible. The ligation product of S/X compatible ends results in the loss of the S/X restriction site and a "scar" (S/X-scar). The BioBrick compatibility is maintained by the additional S restriction site in the added sequence. In this way, several sequences can be added after the building block principle sequentially (adapted from Kutschera, 2015). Restriction enzymes: S: *Spe*I, P: *Pst*I, X: *Xba*I.

1.3 Objectives of the study

The aim of this study is to generate a novel family of plasmid for efficient heterologous expression of genes including antibiotic-based selection markers in *N. oceanica.* The use of a modular BioBrick assembly strategy allows to modify plasmids easily to include native DNA sequences that facilitate targeted homologous recombination.

2. Materials and Methods

2.1 Experimental procedures

General protocols used throughout this experiment

Gel electrophoresis - Electrophoresis is used to verify PCR reaction, restriction digests and separate desired DNA fragments for later purification. Agarose gels (0.8% agarose (w/v)) added with GelRed™ Nucleic Acid Gel Stain with the concentration 0.04 µL /ml (Biotum, USA) in 1xTAE buffer (a pH 8 tris-acetate buffer containing EDTA) was used. Samples were added with 10X loading dye (containing 0.25% bromophenol blue and 0.25% xylene cyanol FF). GeneRuler™ 1kb DNA ladder Plus (Thermo Scientific) was used as DNA fragment size indicator. The gel electrophoresis was performed at 90 Volts for 60 minutes. The DNA fragments were visualized under UV light (λ = 312 nm) on a ChemiDOc XRS+ Imaging System (Bio-Rad, USA). For downstream gel extraction, Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA) was used.

Gel and PCR products purification - For purification of PCR products and post-gel electrophoresis DNA, Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA) was used.

Preparing competent cells – Heat shock-competent *E. coli* DH5α cells were used in this project. Media and antibiotic concentration used for growing *E. coli* and detailed reagents used for making competent *E. coli* are shown in Appendix A1.4 and A1.5 respectively. Competent *E. coli* cells were made by inoculating 1 µL of thawed DH5α cells in 20 mL of SOC Medium overnight at 37°C on shaker. A volume of 3 mL of this culture was transferred to 300 mL pre-warmed yB medium and grown at 37˚C in shaking condition until the optical density (OD) at 600 nm reaches 0.3-0.4. The culture is then incubated on ices for 5 minutes, before being transferred into six chilled 50 mL falcon tubes and centrifuged at 4000 rpm at 4˚C in IEC Table

Top centrifuge. The supernatant was discarded, and the pellet was resuspended in 15 mL chilled TfBI medium before being centrifuged for 10 minutes at 4000 rpm at 4˚C. Again, the supernatant was discarded, and the pellet was carefully resuspended in 1 mL chilled TfBII medium by using a 1000 µL pipette with a cut-off tip. The solution was transferred into sterile 1.5 mL Eppendorf tubes in an aliquot of 100 µL and snap-frozen using liquid nitrogen. The competent cells were then stored at -80˚C.

Plasmid isolation – The Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) was used for isolation of plasmids. *E. coli* overnight culture of 5-10 mL of LB media added with ampicillin (100 µg/mL) was centrifuged for pellet obtaining, following the manual.

2.2 Vector construction

Here, molecular cloning was performed in order to construct vectors containing promoter and terminator sequences along with reporter genes, based on established protocol in previous study (Kutschera, 2015)

Amplification of reporter genes

Touch Down (TD) PCR is a PCR reaction with gradually decreasing annealing temperature. This approach ensures that the primers in the reaction binds properly to the DNA samples. Touch Down PCR is applied to avoid non-specific binding of primers that have different annealing temperature and thereby increases amplification of desired DNA sequences. In the first cycles, binding of the primers is very specific, while tthe last cycles achieve a maximal sequence amplification. Phusion® High-Fidelity (HF) DNA Polymerase (New England Biolabs ® Inc., USA) known for its inherent proofreading capacity by removing non-complementary sequences was used along with 5X Phusion® HF Buffer (GC rich). Phusion polymerase has shorter extension time, more robust and high yield amplification, that is relevant for PCR reactions requiring high accuracy and long amplicons. The zeocin resistant gene was amplified from the pPtPUC3, hygromycin B resistant gene from the pSELECT100 and paromomycin resistant gene was amplified from the pRS426- ble aphAVIII-gfp plasmid. Site directed mutagenesis with primers Hygro_mut fwd: AACTGCCCGCTGTTCTCCAGCC and Hygro_mut rev: CCTCCGCGACCGGCTGGAG were used to introduce mutation that delete a *Pst*I site in the middle of hygromycin B resistance gene. This aimed to prevent hygromycin B resistant gene from being truncated at unwanted *PstI* cutting site while being treated with *Pst*I

restriction enzyme. PCR thermal cycle to introduce mutation from CTGCAG to CTCCAG is presented in Appendix A.6. Summary of primers designed in previous study (that were purchased from Sigma-Adrich® Oslo, Norway) to amplify reporter genes is given in Appendix A.7. Reaction mixtures can be seen in Table 1 and gradient thermal cycles are shown in Table 2. For reporter gene amplification, DNA templates were added with an amount of 50-100 ng. Annealing temperatures were estimated using NEB´s T_m calculator, but were optimized using an annealing temperature gradient when necessary to increase specific binding of primers. The thermal-cycler used was a C1000 Touch™ thermal cycler (Bio-Rad Laboratories, USA).

Table 1: Reaction mixtures for Touch Down PCR.

Component	**Volume (µl)**
DNA Template (Plasmid)	~50-100 ng
5X Polymerase Buffer (GC rich)	6
10 mM dNTPs	1.5
10 µM Forward primer	2.5
10 µM Reverse primer	2.5
2U / µL Fusion Polymerase	0.5
ddH_20, autoclaved	Fill up to 30

Table 2: Thermal cycler protocol for reporter gene amplification.

Step	Temperature	Time	Cycles
1. Initiate Denaturation	95°C	1 min	1
2. Denaturation	98°C	20 sec	10
3. Annealing Touchdown	66°C to 56 °C	30 sec	
4. Elongation Normal cycles	72°C	1 min	
5. Denaturation	98°C	20 sec	15
6. Annealing	62 °C	30 sec	
7. Elongation	72°C	1 min	
8. Final Elongation	72°C	5 min	
9. Holding	4°C	-	

Digested enzyme restriction, ligation and transformation

Following the strategy presented in part 2.2 (Final vector construct), enzymatic protocol (New England Biolabs® Inc.) were utilized for molecular cloning. An amount of 1 µg of substrate DNA was applied to 1 unit of restriction enzyme to complete digestion in a 50 µL reaction in 60 minutes.

DNA fragments were subsequently ligated together. DNA ligation involves creating a phosphodiester bond between the 3'hydroxyl end of one nucleotide and the 5'phosphate of another utilizing the activity of T4 DNA ligase from the T4 bacteriophage. This enzyme ligates DNA fragments having overhanging "sticky ends" together. A ligation with molar ratio of 1:3 vector to insert was applied. Ligation was performed using T4 DNA ligase in 1X T4 DNA Ligase Reaction Buffer (New England Biolabs® Inc.), following NEB´s protocol and set up overnight at 16°C. Summary of enzymatic restriction and ligation setup was showed in Appendixes A1.8 and A1.9.

Transformation used to transfer plasmids DNA into competent *E. coli* cells were performed as followed. An aliquot of 100 µL competent cells was thawed on ice for 5 minutes before DNA was added. A ligation volume of 20 µL was used for transformation. The tubes were then gently mixed and incubated on ice for 30 minutes. Further, the cells were heat shocked at either 37˚C for 2 minutes or 42°C for 45 seconds, then held on ice for 3 minutes. A volume of 1mL lysogeny broth (LB) was added for each Eppendorf tube and then the cell cultures were incubated at 37˚C on the shaker (150rpm) for 90 minutes. 100 µL of transformed cells were plated directly out onto LB-plate containing antibiotic and incubated overnight at 37˚C. In this project, transformation into *E. coli* cells were employed to amplified target plasmids for downstream electroporation.

Overnight ligation mixture was spread on LB agar plate containing ampicillin 100 µg/mL. *E. coli* colonies that were appeared on the plates would be later sub-cultured onto LB agar plate containing ampicillin along with X-gal and IPTG to screen for possible successful transformants before genomic DNA of confirmed white clones were extracted for colony PCR.

Blue/ white screening

Blue/ white screening is an efficient technique for the identification of recombinant DNA clone. It bases on the activity of β-galactosidase, an enzyme occurring in *E. coli* coded by *lacZ* operon, which cleaves lactose into glucose and galactose. The activity of operon produces β-galactosidase that metabolizes the lactose in cultured environment of bacteria. This β-galactosidase activity of bacterial cells can be governed by adding X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and IPTG (Isopropyl β-D-1-thiogalactopyranoside). If β-galactosidase is produced, it hydrolyzed X-gal to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by normal cells that harbor *lacZ* operon, therefore appear blue in colour.

In this project, the plasmid vectors used in cloning were manipulated in a way that alters this *lacZ* operon in the plasmid backbone pUC19. An MCS is present within the *lacZ* sequence. This sequence can be nicked by various restriction enzymes to insert the foreign DNA. When *construct_0169* and *construct_0602* were successfully inserted into pUC19 in MCS fragment, they deleted a residue in the *lacZ* gene, leading to its dysfunction, and β-galactosidase was prevented from expression. Without β-galactosidase, X-gal keeps constant as colourless and hence successful recombinant clones appear as white colonies indicating the integration of exogenous DNA into the backbone at MCS site. If the foreign DNA is not inserted into the vector or if it is inserted at a location other than MCS, the *lacZ* gene in the plasmid vector in the host *E. coli* cell still produces a functional enzyme and bacterial clones turn blue.

This method was used to screen for successful transformants. Colonies appeared on LB plates containing smpicillin 100 µg/mL after transformation were picked up by sterile toothpick and transformed into a master plate containing X-gal and IPTG. For each individual LB agar plates containing about 25mL solidified LB medium, 40 µL of the ready-to-use Thermo Scientific X-Gal Solution (stock concentration 20 mg/mL) and 40 µL of 100 mM ready-to-use Thermo Scientific IPTG Solution was added. The plates were spread evenly with a sterile spatula and let dry for about 30 minutes before using. White colonies appeared on master plates later were taken for further genomic colony PCR for verification of successful transformants.

Colony PCR on *E. coli*

Transformants were screened for DNA sequence mutations in the target region by performing colony PCR. A small amount of cells derived from each transformed *E. coli* were lysed by firstly being dissolved in 50 µL sterilized MQ water. Single colonies were picked up by sterilized toothpicks or pipette tips. The colony pools were then vortexed carefully for at least 30 seconds before they were boiled at 96°C 10 minutes for DNA release. The predicted size of the amplicons was determined by an in silico PCR with the program SnapGene® 2.8.2. Lysate was used as template for the PCR under activity of DyNAzyme II DNA Polymerase (Thermo Scientific). Summary of primers used for colony PCR was shown in Appendix A1.10. After about 2 hours of amplification by PCR, DNA samples of the colonies were separated and visualized through gel electrophoresis. Reaction mixtures and thermo-cycler conditions are shown in Tables 3 and Table 4.

Table 3: Reaction mixtures for 20 µL PCR reaction.

Component	Volume(µl)
DNA template	2.0
ddH_20, autoclaved	13.3
10 X Optimized DyNAzyme Buffer	2.0
10 mM dNTPs	0.5
10 µM forward primer	1.0
10 µM reverse primer	1.0
DyNAzyme II DNA Polymerase	0.2
Total	20.0

Table 4: Thermal cycles of PCR reaction.

Step	Temp and time
1. Initiate Denaturation	94°C for 3 min
2. Denaturation	94°C for 30 sec
3. Annealing	50°C for 30 sec
4. Elongation	72°C for 2 min 15 sec[1]
Cycles	34
5. Final Elongation	72°C for 10 min
6. Holding	4°C for infinite

[1] 15 seconds per kb is sufficient for plasmid DNA, genomic DNA requires roughly twice that time (NEB, 2015a).

DNA sequencing

The DNA sequencing was performed at GATC Biotech Company (www.gatc-biotech.com). For sequencing, ~5 µL of 80-100 ng/µL plasmid DNA (400-500 ng total) were mixed with 5 µL of 5 pmol of primers. The total sample volume of 10 µL of the samples was reached by adding sterilized milliQ water. Primers were designed to be 50-100 bps outside the desired locus that needs to be sequenced, because the first few bp are unlikely to be sequenced properly. Sequence length that are usually obtained is about 1000 bp, which explains why sometimes it needs more than one primer to sequence a DNA sample. The plasmids were sequenced using primers that started in the pUC19 backbone which was summarized in Appendix A1.11. Sequencing result were analyzed using SnapGene® 2.8.2 and online program on Benchling (https://benchling.com).

Freeze stock

The successful bacterial clones containing constructed plasmids were stored by using 80% glycerol as described in the following procedure. Overnight culture of single colony in 5mL LB medium added with selectable antibiotics at 37°C on the shaker (150rpm) was centrifuged at room temperature for 10 minutes at 2000 rpm. Harvested pellet was then resuspended in 0.5mL LB medium containing antibiotic and 0.5mL 80% glycerol before it was transferred into cryotubes and stored at -80°C. The shelf life of the stock lasts for several years, as long as it is handled properly without subsequent freeze and thaw cycles.

3. Results

3.1 Reporter genes amplification

After the outlining a cloning strategy for the plasmids, primers required for amplification of the reporter genes were designed and ordered. The predicted size of the respective amplified products was 1045 bp, 394 bp and 838 bp for the coding region of hygromycin B, zeocin and paromomycin, respectively. Gel electrophoresis image revealed DNA bands of the amplified resistance-conferring genes, confirmed the successful amplification by TD PCR (see Figure 4).

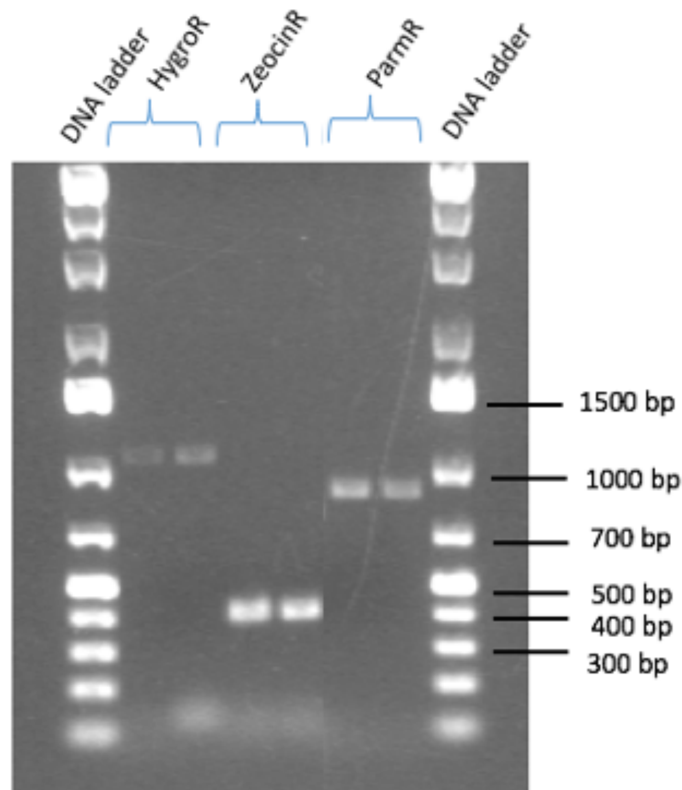

Figure 4: Gel image with DNA bands after separation of the amplified reporter gene sequences. The gel result indicates the expected size of the resistance-conferring genes. From left to right: DNA ladder, hygromycin B resistance gene (1045 bp), zeocin resistance gene (394 bp) and paromomycin resistance gene (838 bp), DNA ladder.

3.2 Creating the partial constructs pAPA0001

In the first step, pAPA0001 was formed by inserting the *construct_0169* and *construct_0602* into the pUC19 backbone by *Bam*HI and *Sph*I restriction and subsequent ligation. After 12-16 hours, colonies appeared on the LB–ampicillin 100 µg/mL, indicating possible successful transformation of plasmids pAPA0001 into *E. coli* cells. For pAPA_0169, colonies sub-plating on LB medium containing ampicillin along with X-gal and IPTG (for blue/white screening) showed 3 of 60 colonies that appeared as white clones (see Figure 5). For the second vector (pAPA_0169), 15 out of 75 clones were white.

Figure 5: Blue/ white screening of ampicilin-selected colonies resulted from transformations of after attempted ligating of *construct_0602* into pAPA0001. The plate showed three white clones, indicating the possible insertion of promoter-terminator sequence in *construct_0602* into pUC19. In these white clones, a residue in the *lacZ* region was deleted and β-galactosidase was prevented from expression to hydrolyze X-gal into blue pigment.

Subsequently, pAPA0001-based constructs containing promoter-terminator sequences was sent for sequencing. The plasmids were sequenced using primers that started at the pUC19 backbone. The forward and reverse primers bind outside the MCS on both sides. Sequencing results for the plasmids pAPA0001 including pAPA_0169 and pAPA_0602 are shown in Figure 6A and 6B, respectively. The arrows indicate the direction of sequencing, where gaps and mismatches are shown as white bars in the arrow. There are some mismatches in the alignment of received sequencing results and plasmid sequences, that are expected after about 1000 bp the sequencing information, as sequencing results become unreliable at longer sequencing length. The alignment of DNA sequencing result of pAPA_0602 and expected DNA template showed in Figure 7A with closer view and more details revealed one point mutation in the promoter sequence at the 890 bp position where T was mutated to C. This mutation was revealed in the context of the whole promoter sequence in Figure 7B. The alignment of DNA sequencing result of pAPA_0169 and expected DNA template (see Figure 8A) revealed one mismatch in the terminator sequence at base pair 1577 (T was mutated to C), which was then shown in the context of the whole terminator sequence in Figure 8B.

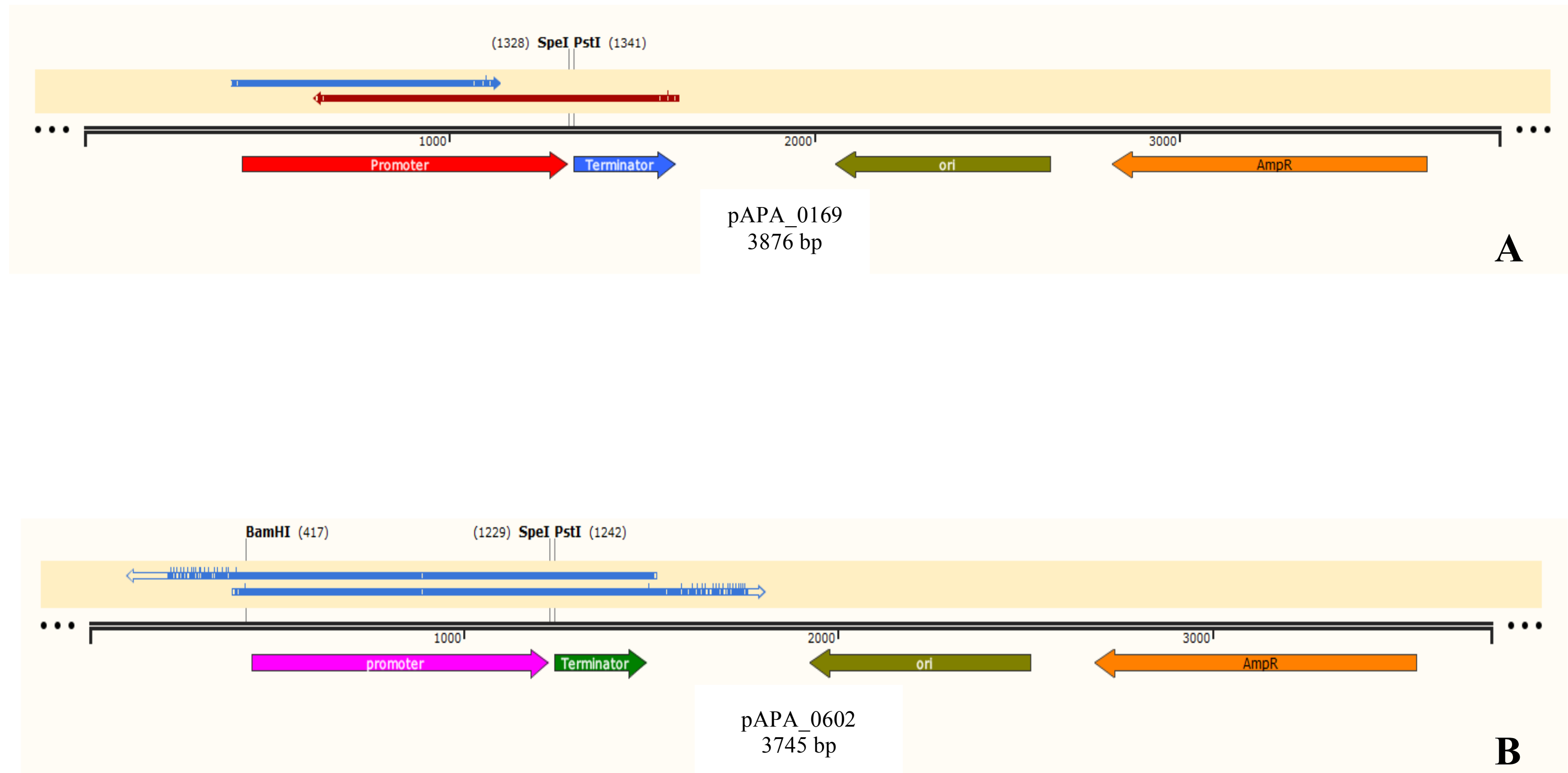

Figure 6: Alignment of DNA sequencing result (pAPA_0169, colony no.9) (**A**) or (pAPA_0602, colony no.7) (**B**) and DNA template. SnapGene® 2.8.2. was used for visualization.

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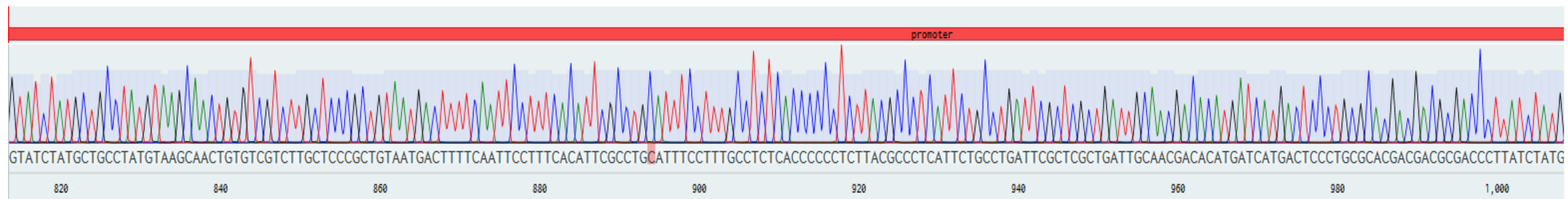
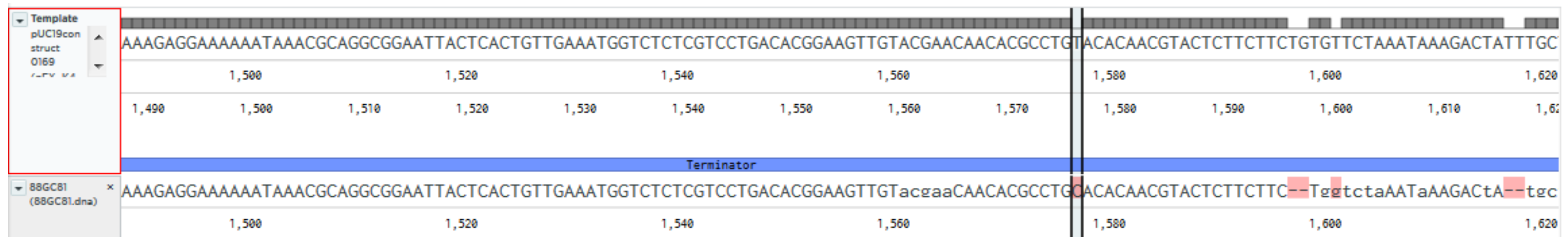


A

```
 434 GGGTTCCTTG AGCTTTCATC CTTCAGAGCC CGTGTTGGTG ACAGTGGGAT
 484 ACGATGGAGT GGGATGTTGT TGGTTTCGGG ATTTGTGAGG AGGATGAGCG
 534 GGGGGGGTTT GACGGATGTA AATAGAGCGA CACGAGCGGT GGCGCATGTG
 584 AATCAGCAAG CGTATTTATG GGAACAATAA TGAACGAAAG ATTTAACGAA
 634 CCAATAAAAG GTATACGTAC ACATAAGATG CAGGCTTTCT TTAATCCCTG
 684 CCCTCTGTAC AGCAAAGTAG CCGAGCAAAG TAGCCGCTCA GCAGGGCAAA
 734 GGTCGGCCCA TGGTTCTTTC CTTTCCTCTT CTCTCGCATG ACAAAAGGAC
 784 GAGCTTTAGG GATTCTGCCT AGACTGTATC TATGCTGCCT ATGTAAGCAA
 834 CTGTGTCGTC TTGCTCCCGC TGTAATGACT TTTCAATTCC TTTCACATTC
 884 GCCTGCATTT CCTTTGCCTC TCACCCCCCT CTTACGCCCT CATTCTGCCT
 934 GATTCGCTCG CTGATTGCAA CGACACATGA TCATGACTCC CTGCGCACGA
 984 CGACGCGACC CTTATCTATG CGTCTGAGCC ACCACACAAA GTGTAAACCT
1034 CACACAGACC TTCACTCTGC GCCTTTCATT AGCCCAAGCT CAAGCGCACG
1084 TGCGTCAGCG GGGTACGACG TAAGACCTCT CGCCTACGAC CTCCCCAAAT
1134 CAAATCATTT ACGACTTCTT TCTGTCGAAA CCTACCGCAT CTCACTCCCT
1184 CCGTCAACAT TCTCGCCTTC CCGCACTGGC TCTATACCCT ACAGC
```

B

Figure 7: Sequencing result of pAPA_0602 (colony no.7) shows one mismatch in the promoter at base pair 890 (T was mutated to C) (**A**). Online program on Benchling (https://benchling.com) was used for visualization. This point mutation was revealed in the context of the whole promoter sequence in (**B**).
Color code: mismatch




```
1343 AGTGAGGGGG AGGGGGAGGG TGGACTTGAG AGTGTGGAAG TGAGTGAGGG
1393 TAGAAGATAG AGTAAGGATG GCAGACAAGA TGACGATAAA GAAGACGTGG
1443 AAGAGAGGGA AATACACGTA CAAAGGAAGA AAACAAGAAA AGGAAAAAGA
1493 GGAAAAAATA AACGCAGGCG GAATTACTCA CTGTTGAAAT GGTCTCTCGT
1543 CCTGACACGG AAGTTGTACG AACAACACGC CTGCACACAA CGTACTCTTC
1593 TTCTGTGTTC TAAATAAAGA CTATTTGCT
```

B

Figure 8: Sequencing result of pAPA_0169 (colony no.9) shows one mismatch in the terminator at position of base pair 1577 (T was mutated to C) (**A**). Online program on Benchling (https://benchling.com) was used for visualization. This point mutation was revealed in the context of the whole terminator sequence in (**B**).

Color code: T rich region, mismatch, poly(A)site

3.3 Construction of resistance-conferring plasmids pAPA1001

The next series of cloning steps described here were used to generate final constructs pAPA1001 by integration of antibiotic selectable marker genes into partial constructs pAPA0001. The vectors pAPA_0602 and pAPA_0169 were restricted with *Spe*I and *Pst*I followed by the insertion of PCR amplified resistance-conferring genes digested with *Xba*I and *Pst*I. Figure 9 shows gel image of PCR product after screening for transformants of pAPA_0169 and pAPA_0602 carrying the zeocin or paromomycin resistant gene. The clones carrying the plasmid pAPA_0169_Z, pAPA_0602_Z, pAPA_0169_P, pAPA_0602_P have expected size of 1336 bp, 1237 bp, 1779 bp and 1680 bp, respectively.

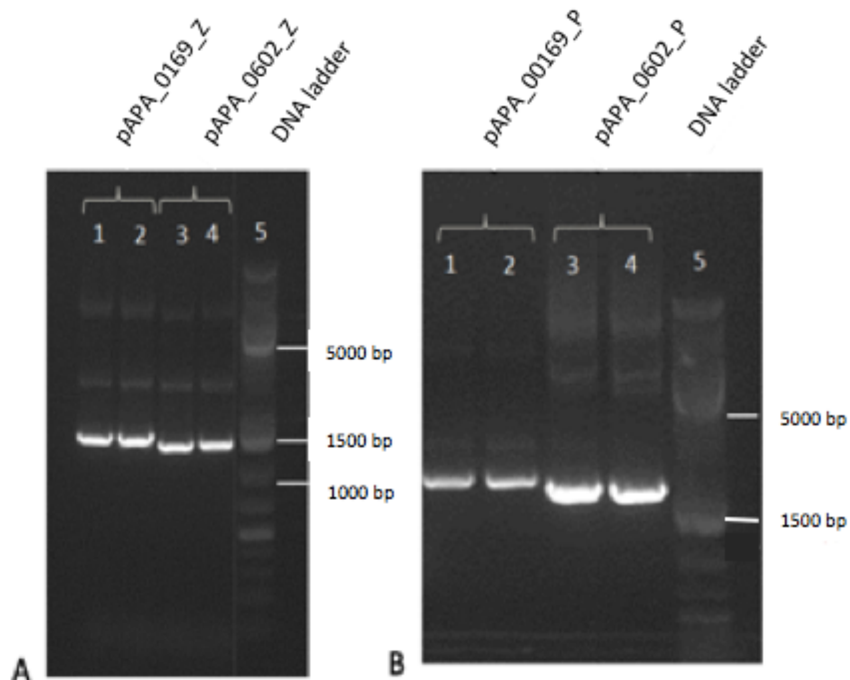


Figure 9: Gel image of colony PCR amplicons. The insert of GOI was amplified using PCR, with primers specific for each resistance gene. From left to right are gel bands of pAPA_0169_Z (Lane 1&2A 1336bp), pAPA_0602_Z (Lane 3&4A 1237bp), pAPA_0169_P (Lane 1&2B 1779 bp) and pAPA_0602_P (Lane 3&4B 1680 bp).

These clones were then sequenced to confirm identity. Due to large size of the DNA fragments multiple sequencing steps were required to span the constructed sequences. For this primers HygroRfwd, ZeocinRfwd and ParmRfwd were used to bind outside the reporter genes in addition to forward and reverse primers that bind to the pUC19 backbone at either promoter or terminator site. Figure 10 shows DNA sequencing result of 2 out of 6 construct candidates for pAPA_0169_Z and pAPA_0602_Z. The DNA sequences are identical, except the ´inherited´ mistmatches from previous cloning steps (mutation from T to C in the terminator of pAPA_0169, mutation from T to C in the promoter of pAPA_0602).

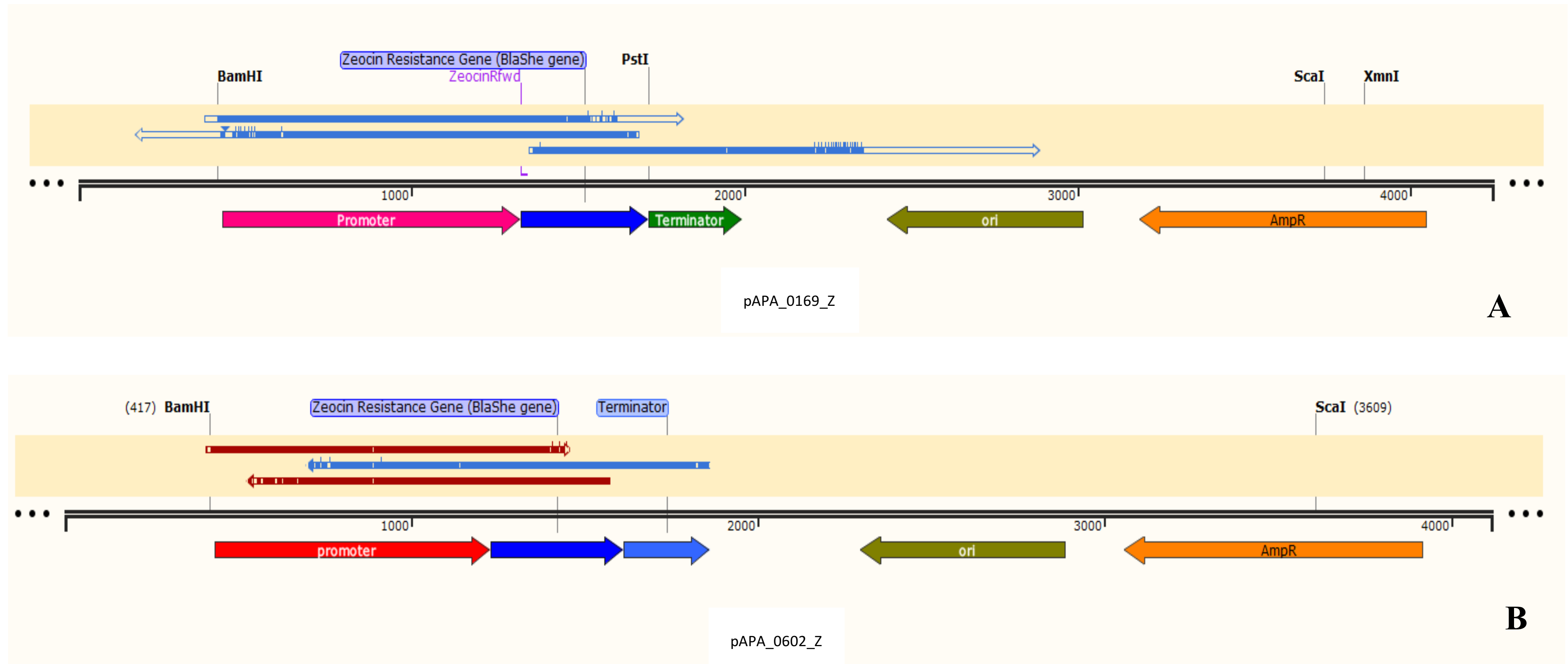


Figure 10: Visual representation of DNA sequencing result of pAPA_0169_Z (colony no. 3) (**A**) and pAPA_0602_Z (colony no.7) (**B**). Given the large size of the insert, about 1.4 kpb, multiple primers were used to allow for overlapping coverage: M13_INSERT_short_fw, M13_INSERT_short_rv and ZeocinRfwd. The sequencing results revealed successful integration of zeocin resistant gene into the middle site of promoter-terminator fragment.

4. Discussion

Molecular cloning was performed to establish the plasmid family pAPA1001 for transformation in eukaryotic microalgae *N. oceanica*. Generally establishing vectors for eukaryotic organisms meets some certain difficulties due to the complexity of the genome in chloroplast and nucleus (Lodish et al. 2000). Challenges such as exon-intron and post transcriptional processes have to be taken in account during plasmid construction.

For the plasmid constructions of pAPA1001, the amplification of reporter genes was confirmed to be successful, resulting in created overhangs for ligation with the promoter-terminator containing constructs. Blue/ white screening was used to confirm successful *E. coli* transformants of ligated constructs. During the screening, some clones appeared in white color while others are in blue. The blue clones are probably either self-ligated clones with no desired insert. This leaves functional *lacZ* operon that is capable of transforming X-gal to blue pigment 5,5'-dibromo-4,4'-dichloro-indigo.

Progressive sequencing results of plasmid constructs showed that insertion of the promoter-terminator sequences and GOIs into the pUC19 backbone was successful with the expected sequences except one mismatch in the pAPA_0602-based plasmid found in promoter region and one mismatch in the pAPA_0169-based plasmid in terminator region. In both case T was mutated to C.

The promoter is a regulatory region of DNA located upstream of a gene and plays an important role in transcription regulation (Lodish et al.2000). The eukaryotic promoter is a unique stretch of DNA sequence consisting of a core, proximal and distal region. The core promoter usually refers to the region that comprises distinct elements, such as the TATA-box or GC-rich sequences. For pAPA_0602, T was mutated to C at the 890 bp position in the promoter sequence (see Figure 7B). Since this point mutation occurred in the middle of the promoter sequence not belonging to the core or proximal promoter region, it is probably not affecting the gene expression of a GOI.

The terminator is a section of nucleic acid sequence that marks the end of a gene transcription and consists of T rich area, polyadenylation site, a cleavage signal and GU-rich sequence in eukaryotes (Proudfoot, 2011). The AATAAA sequence – poly(A) site is located 10–30

nucleotides upstream of the cleavage site, and was shown to be absolutely required for mRNA 3´-end polyadenylation. Moreover, other sequences including upstream sequence element and downstream sequence element also play important roles in enhancing 3´-end formation. The actual nucleotides of cleavage site can also influence the efficiency of this process. The mismatch is in the terminator sequence at base pair 1577 in pAPA_0169. However, this exchange of T for C in plasmid pAPA_0169 occurred at a site that is in short distance away from the T-rich area (see Figure 8B), and is probably not significant for 3´- formation.

Ligation of promoter-terminator backbone and the different resistant genes encoding zeocin, paromomycin or hygromycin B showed expected band-sizes for vectors carrying the resistance-conferring genes. This indicates the successful insertion of the selection marker into the promotor-terminator vector backnones (pAPA_0602 and pAPA_0169). The alignment of DNA sequences to theoretical vector template in SnapGene® 2.8.2 showed high similarity; except the ´inherited´ mismatches from previous cloning steps (mutation from T to C in the terminator of pAPA_0169, mutation from T to C in the promoter of pAPA_0602), which was evaluated not an essential factor in gene expression regulation. The strategy of Biobrick approach was successful allowing later changes of the vector in a fast and easy manner.

In conclusion, six plasmids containing two different promoter sequences and three different antibiotic resistance-conferring genes were constructed:
+ pAPA_0169_Z (4247 bp)
+ pAPA_0602_Z (4116 bp)
+ pAPA_0169_H (4898 bp)
+ pAPA_0602_H (4767 bp)
+ pAPA_0169_P (4691 bp)
+ pPAPA_0602_P (4560 bp)
These plasmids will be used for transformation of *N. oceanica* by electroporation (see Section III).

Part III
ELECTROPORATION

Part III. ELECTROPORATION

1. Introduction

Transformation by electroporation is one of the methods to transfer exogenous DNA into a host organism. This method is based on the application of pulses of electric current through the cells, resulting in the formation of transient holes in the cell membrane (León et al. 2007). During the time that the pores are open, the exogenous genetic material (i.e. transformation vector) is then transferred into the cells. In eukaryotic cells, based on the vector construction, the foreign DNA remains either as plasmid or can become incorporated into the nuclear or plastid genome. This incorporation occurs randomly, or by homologous recombination (HR).

Gene transfer by electroporation has been applied in bacterial, animal and plant systems for over 30 years (Neuman et al. 1982; Zimmermann et al. 1975). The method has various advantages, as it is simple, low in cost and producing transformants with high efficiency without the need of large DNA amount. At present, transformation by electroporation was established in many marine organisms from prokaryotic cells to eukaryotic red algae, green algae and diatoms (Qin et al. 2012). Gene transfer by electroporation was reported as a powerful tool to transform *Nannocloropsis* sp., which is currently considered as the ¨star¨ marine alga for potential biofuel production (Kilian et al. 2011; Vieler et al. 2012; Radakovits et el. 2012). Moreover, several genes involved in the nitrogen metabolism were knocked out by the HR method (Kilian et al.2011), demonstrating that targeted genetic manipulation in this organism is possible. This technology therefore appears promising to advance further genomic research and optimize the lipid production capabilities (Pan et al. 2011).

Various factors, such as electric voltage, resistance, amount of the inserted genetic material or cell amount resulting in different transformation efficiencies were determined experimentally in these studies (Kilian et al. 2011; Vieler et al. 2012; Radakovits et al. 2012). In addition, it has been shown that for *Nannochloropsis* species, the insertion of linearized plasmid DNA, with free ends, by electroporation is more efficient than transformation with circular plasmid DNA (Kilian et al., 2011, Zhang et al. 2013) Generated transformants can be identified by the positive cell selection on the selective medium, followed by the colony PCR for confirmation of plasmid DNA-insertion into the host genome.

The selection of genetic elements is crucial for determining the stability and frequency of exogenous DNA expression in algal expression systems (Qin et al. 2012). Gene expression is an important parameter for the functioning of antibiotic-resistance conferring genes as a positive selective marker. In electroporation established in *N. gaditana* (Radakovits et al.2012), transformation efficiency of 12.5 x 10^{-7} colonies/ cell/ μg was achieved using vector with β-tubulin promoter. Vieler et al. (2012) reported the use of vector pSELECT100 with endogenous LDSP promoter (lipid droplet surface protein) in *N. oceanica* CCMP1779. This obtained 125 resistant colonies per μg of linearized pSELECT100 transformation plasmid from 5 x 10^{8} cells, which gives transformation efficiency of 2.5 x 10^{-7} colonies/ cell/ μg. In another study, Kilian et al (2011) obtained 2500 transformants using 3 μg transformation vector containing VCP promoter (the amount of algal cells underwent transformation is 10^{9}). The transformation efficiency in this studied is 8.3 x 10^{-7} colonies/ cell/ μg.

Objectives of the study

In this Master thesis, a novel family of plasmid pAPA1001 (Section II) was established. These plasmids, with endogenous promoters driving expression under various conditions, may increase the transformation efficiency by electroporation in *N. oceanica* CCMP1779. Additionally, by choosing different selection markers encoding antibiotic resistant genes such as zeocin, hygromycin B or paromomycin, attempt to optimize the transformant selection after electroporation will be taken. The expression of the GOIs, in this case the various selection markers driven by endogenous promoters were assessed based on molecular analysis by colony PCR of the genomic DNA of the algal clones.

2. Materials and Methods

2.1 Strain and growth condition

N. oceanica CCMP1779 obtained from National Center for Marine Algae and Microbiota (NCMA, former CCMP,) was used as the host strain. The cells were grown in 50% seawater that was filtered and autoclaved afterwards. The seawater was then enriched with Cell-Hi NC medium (obtained commercially from Varicon Aqua Solutions), referred to as "Miracle Mix" with complex medium components (1mL for 1L of 50% seawater). Moreover, as ampicillin up to concentration of 200 µg/mL was not toxic to the *N. oceanica* specie (Anley, 2015), hence ampicillin 50 µg/mL was used to prevent possible contamination of the algal cultures. The cells were grown under photoautotrophic condition at 23°C under a constant light intensity of 100 µE m^{-2} s^{-1} and continuous rotational shaking using a Heidolph® Rotamax 120 Orbital Shaker.

2.2 Plasmids for transformation

The family of plasmid pAPA1001 achieved from part II was utilized for electroporation in *N. oceanica* CCMP1779 (see Figure 11). The vector constructs pAPA1001 contains six plasmids that were created based on the pUC19 backbone with progressive insertion of the two native constitutive promoters and reporter gene sequences.

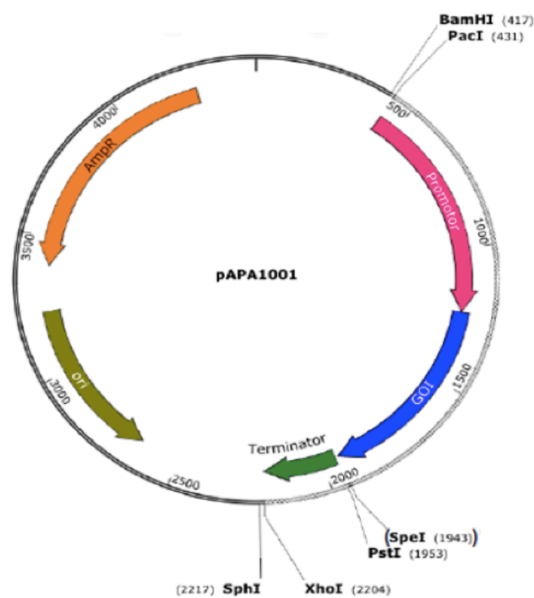


Figure 11: The map of general form of plasmid family pAPA1001. These plasmids were used in the transformation of *N. oceanica* CCMP1779 cells. The regions of the plasmids were annotated including the promoter (pink), selectable marker gene or GOI (blue), terminator (green), ampicillin resistance cassette (orange) and pMB1 origin of DNA replication (light green).

The promoter and terminator mediate the expression of the antibiotic resistant gene zeocin, hygromycin B or paromomycin. In addition, the plasmid family pAPA1001 includes an ampicillin resistance cassette and pMB1 origin of DNA replication due to the pUC19 backbone.

In addition, plasmid pSELECT100 that was used successfully as transformation plasmid in previous study (Vieler et al. 2012; Chernyavskaya, 2014; Anley; 2015) was employed as positive control to evaluate effect of different transformation parameters on transformation efficiency. The plasmid contains hygromycin B resistance gene.

Plasmid amplification

Heat-shock transformation of *E. coli* cells was used to amplify the transformation plasmids. Extraction of the amplified plasmid with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) was used for isolation of plasmids.

Plasmid verification

Verification by restriction enzyme is an essential step to confirm the amplified plasmids in previous step. Double enzyme digestion with *Sma*I and *Pst*I was set up for identification of plasmids pAPA1001 based on NEB's online tools, Double Digest Finder. A volume of 2 µL of pAPA1001 was added with 5 µL CutSmart® Buffer (Biolabs), 1 µL *Sma*I and filled up with sterilized water to 50 µL. The mixture was incubated at 25°C for 5-15 minutes before addition of 1 µL *Pst*I-HF® and continuous incubation at 37°C for 5-15 minutes. The cut plasmid parts were visualized by gel electrophoresis.

To identify the amplified pSELECT100 plasmid, the DNA was cut with *Sma*I restriction enzyme. Three different fragment sizes were predicted to be obtained after restriction because the plasmid pSELECT100 has three cutting sites for the *Sma*I restriction enzyme. Resulting fragments were then visualized by gel electrophoresis.

Plasmid linearization

The four plasmids pAPA_0169_Z, pAPA_0602_Z, pAPA_0169_P and pAPA_0602_P were linearized by either *Sca*I or *Bam*HI and visualized by gel electrophoresis. The two plasmids pAPA_0169_H and pAPA_0169_H were cut with *Ssp*I as they have more than one *Sca*I or

*Bam*HI cutting site. pSELECT100 was linearize by *Bam*HI restriction enzyme. Enzymatic restriction mixture was set up based on New England Biolabs® Inc. protocol. An amount of 1 µg of substrate DNA was applied to 1 unit of restriction enzyme to complete digestion in a 50 µL reaction in 60 minutes.

Plasmid purification and concentration

For purification of plasmids after linearization, Wizard® SV Gel and PCR Clean-Up System Kit (Promega, USA) was used. To concentrate plasmid DNA, Amicon Ultra-0.5 Centrifugal Filter Unit (Merck Millipore, Germany) was used.

2.3 Electroporation protocol

The electroporation protocol performed in this project was based on previously reported work (Killian et al. 2011; Vieler et al. 2012) with some adjustments of parameters. The workflow for electroporation is summarized in the diagram (see Figure 12). The agal cells were harvested by centrifugation at 4000xg 4°C for 10-15 minutes when they reached an exponential growth phase at a density of 2-7 x 10^6 cells/ mL. Further, the collected cells were washed 3 times with ice cold 375 mM sorbitol solution and resuspended in a final volume of 0.2 mL to a transformation concentration of 5 x 10^8 to 5 x 10^9 cells/ mL. Cell concentration was determined by means of $OD750_{nm}$ measurement using a predetermined regression based on direct cell counts (Chernyavskaya, 2014). As the electroporation system applies electric pulses, the presence of salt from the growth medium, which is a good electrical conductor, can cause flow of electricity that destroys the cells. Therefore, it is essential to avoid any rests of growth medium by repetitive washing the cells. An aliquot of 100 µL of the final mixture comprising algal cells and 3 µg enzymatic linearized plasmids were distributed into aliquots in 2mm chilled electroporation cuvettes. The amount of algal cells utilized for each transformation is 5 x 10^8. All the washing steps, concentrating and distribution of the cells were conducted on ice. Keeping cells at 0°C often improves cell viability and thus results in higher effective transfection frequency, especially at high power which can lead to heating (Potter et al. 1984).

Electroporation was performed in sterile condition using Bio-Rad (http://) Gene Pulser II Electroporation System set to 600 Ohm shunt resistance, 50 µF capacitance and field strength of voltage at 2200V. After the pulse the cells were immediately collected from the electroporation cuvettes and resuspended into 10 mL 50% sterile sea water added Cell-Hi NC Medium solution and allowed to recover for overnight in continuous light with shaking condition. After that all the algal cells were collected by centrifugation at 4000xg 4°C for 10-15 minutes before they were spread on selection agar plates containing antibiotic according to the selection marker of the plasmid, which prevented growth of *N. oceanica* wild type.

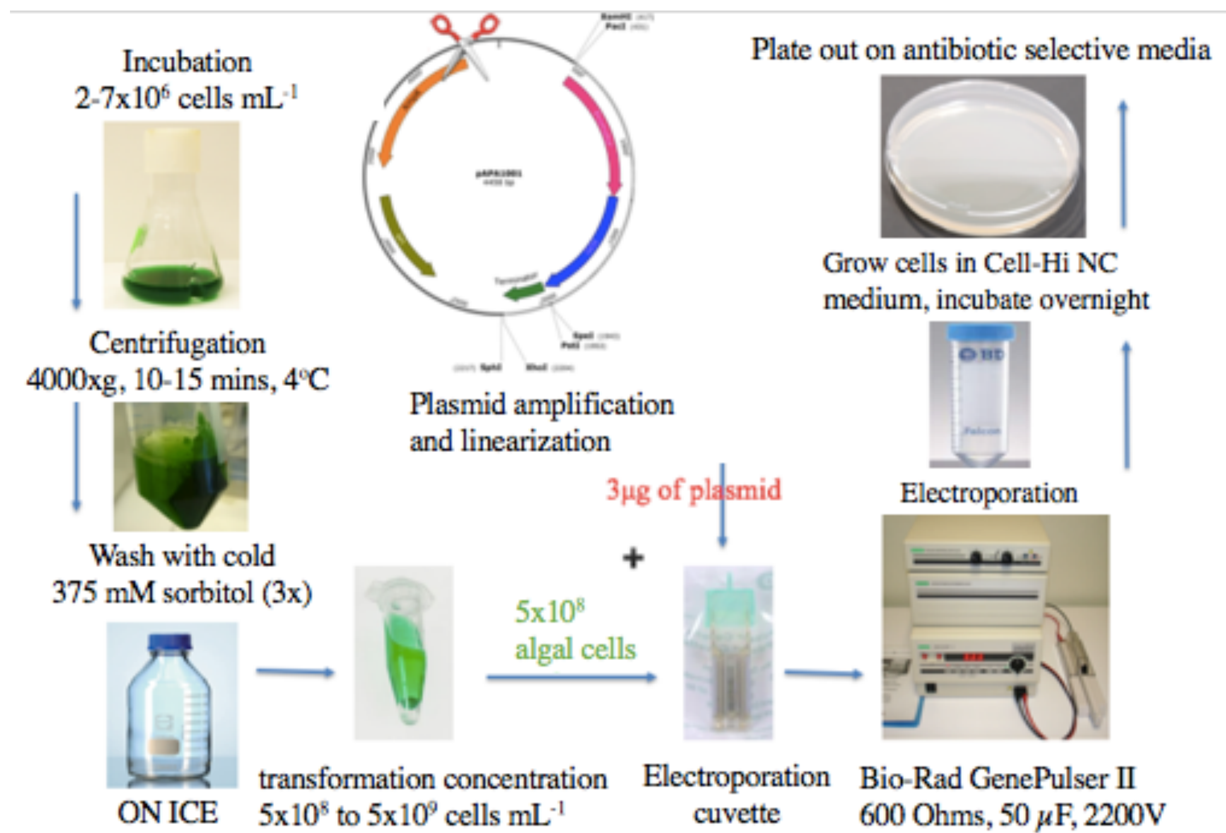


Figure 12: The workflow for transformation by electroporation of *N. oceanica*. Algal cells at density of 2-7 x 10^6 cells/ mL were harvested, washed with sorbitol and concentrated. These steps should be performed on ice. The washed and concentrated cells are transferred into the 2mm electroporation cuvette followed by addition of linearized plasmid pAPA1001. The cells are further exposed for electrical pulse by Bio-Rad Gene Pulser II Electroporation System with specific parameter. The cells are then recovered in Cell-Hi NC medium overnight before being spread on antibiotic selective media.

2.4 Transformant screening

Selection of transformants was conducted after approximately 14 days of incubation on antibiotic selective medium. The concentration of 2 µg/mL or 5 µg/mL for zeocin, 150 µg/mL for hygromycin B and 40 µg/ mL for paronomycin were chosen for transformants selection because of the consistent inhibition of algal growth at low concentrations by these antibiotics (Vieler et al., 2012). Summary of stock solution concentration and storage condition of used antibiotics is presented in Appendix 2.1. The number of colonies appeared on selective plates were counted and colonies were transferred onto ¨master plates¨ with the same selective medium concentration. Colonies on these master plates were transferred to new master places with selective medium every month. Transformation efficiencies were determined using a standard formula:

$$Transformation\ efficiency = \frac{(Number of colonies\ verified\ by\ colony\ PCR)/(Number\ of\ cells\ plate\ out)}{Amount\ of\ plasmid(\mu g)}$$

To verify the presence of transformation construct within the cells (and thus that the transformation experiment was a success), genomic colony PCR of *N. oceanica* CCMP1779 cells, which grew on antibiotic selective medium, was performed using an efficient colony PCR procedure (Daboussi et al. 2014). Cell lysates from resistant colonies were prepared by dissociation of colonies in 20 ml of lysis buffer (1% TritonX-100, 20 mM Tris–HCl pH 8, 2 mM EDTA) in an Eppendorf tube. The tubes were vortexed for at least 30 seconds and then kept on ice for 15 min. After heating for 10 min at 85°C, tubes were cooled down at room temperature. After a 1:5 dilution in water and a brief centrifugation to pellet cell debris, supernatants were used immediately or stocked at 4°C. Five microliters of cell lysates were used for the PCR amplification of the genomic targets with specific primers binding to integrated sequence. Sequences of primers used and components of reaction mixture are present in Tables 5 and 6, respectively. The PCR products were then analysed by electrophoresis migration on agarose gel.

Table 5: Primers for genomic colony PCR in *N. oceanica* CCMP1779.

Transformation plasmid	Primer name	Primer sequence (5'-3')
pAPA_0169_Z	3874_Fwd	TTGGATTGCAGCAAGGCGGTGT
	ZeocinRrev	atatCTGCAGTCAGTCCTGCTCCTC
pAPA_0602_Z	11694 _Fwd	GAGCCCGTGTTGGTGACAGTGG
	ZeocinRrev	atatCTGCAGTCAGTCCTGCTCCTC
pAPA_0169_H	3874_Fwd	TTGGATTGCAGCAAGGCGGTGT
	HygroRrev	atatCTGCAGCTATTCCTTTGCCCTC
pAPA_0602_H	11694 _Fwd	GAGCCCGTGTTGGTGACAGTGG
	HygroRrev	atatCTGCAGCTATTCCTTTGCCCTC
pAPA_0169_P	3874_Fwd	TTGGATTGCAGCAAGGCGGTGT
	ParmRrev	atatCTGCAGACACCATCAGGTCCC
pAPA_0602_P	11694 _Fwd	GAGCCCGTGTTGGTGACAGTGG
	ParmRrev	atatCTGCAGACACCATCAGGTCCC

Table 6: Reaction mixtures for genomic colony PCR in *N. oceanica* CCMP1779.

Component	Volume(µl)
DNA template	5.0
ddH_20, autoclaved	15.9
10 X Optimized DyNAzyme Buffer	2.5
10 mM dNTPs	0.5
10 µM forward primer	0.5
10 µM reverse primer	0.5
DyNAzyme II DNA Polymerase	0.1
Total	25.0

To evaluate the effect of DNA extraction buffers and incubation times in colony PCR, the primers for detection of the endogenous 18S rRNA gene was used as internal control:

Forward primer 18SF1: 5´- CAGAGGTGAAATTCTTGGA -3´

Reverse primer 18SR: 5´- TCACCTACGGAAACCTTGTTACGAC -3´

Thermal cycler conditions to amplify integrated DNA sequence in *N. oceanica* nuclear genome and to perform internal control using 18S rRNA primers are shown in Table 7.

Table 7: Thermal cycles of PCR reaction using either primers to amplify target DNA sequence or 18S RNA primers.

Step	Target primers	18S rRNA primers	Cycles
1. Initiate Denaturation	94^oC for 2 min	94^oC for 2 min	
2. Denaturation	94^oC for 30 sec	94^oC for 30 sec	30
3. Annealing Touchdown	**63^oC** for 30 sec	**50^oC** for 30 sec	
4. Elongation	72^oC for **1 min**	72^oC for **1 min 12 sec**	
5. Final Elongation	72^oC for 5 min	72^oC for 5 min	
6. Holding	4^oC for infinite	4^oC for infinite	-

3. Results

3.1 Plasmid amplification

Plasmid were amplified through cloning in *E. coli* cells and subsequent purified with the Wizard® *Plus* SV Minipreps DNA Purification System (Promega). The product yield was 300 - 500 ng/µL measured with NanoDrop1000 spectrophotometer (Thermo Scientific). This yield was satisfactory for later transformation by electroporation of *N. oceanica* CCMP1779.

3.2 Plasmid verification and linearization

To confirm the identity of amplified plasmids pAPA1001 in Section 3.1, the plasmid was cut with both restriction enzymes *Sma*I and *Pst*I. Either of the restriction enzyme *Sca*I, *Bam*HI or *Ssp*I was used to linearize plasmids. A complete digest should result in fragmented with different lengths. A summary of fragments resulted after enzyme restriction is presented in Table 8.

Table 8: Full length of plasmids pAPA1001

and resulted fragment size after double enzyme digestion by *Sma*I and *Pst*I.

	Full length (bp)	Fragment length being digested by *Sma*I and *Pst*I (bp)		
pAPA_0169_Z	4247	2949	1009	289
pAPA_0602_Z	4116	2917	910	289
pAPA_0169_H	4898	2949	1949	
pAPA_0602_H	4767	2917	1850	
pAPA_0169_P	4691	2949	1742	
pAPA_0602_P	4560	2917	1643	

Enzymes *Sma*I and *Pst*I-HF® cut pAPA_0169_Z and pAPA_0602_Z at two and one sites, respectively. Gel electrophoresis result (see Figure 13) revealed fragment distribution of the partially digested plasmid. The bands of 289 bpwere not visualized for both plasmid, indicating unsuccessful enzymatic digestion at *Pst*I cutting site. However, the band of 1009 bp (for pAPA_0169_Z) and band of 910 bp (for pAPA_0602_Z) indicated these two plasmids were cut by *Sma*I. Those two thick bands on the top of both lines have predicted size of 3237 bp (for pAPA_0169_Z) and 3206 bp(for pAPA_0602_Z), which are the "backbone" of the plasmids after being cut by *Sma*I. Even though the gel image did not show three expected bands of the plasmid being cut by double digestion using *Sma*I and *Pst*I, it was consistent with the plasmids map and therefore correct assembly of the pAPA_0169_Z and pAPA_0602_Z was assumed. The other four plasmids pAPA_0169_H, pAPA_0602_H, pAPA_0169_P, pAPA_0602_P were checked for correct assembly using the same approach.

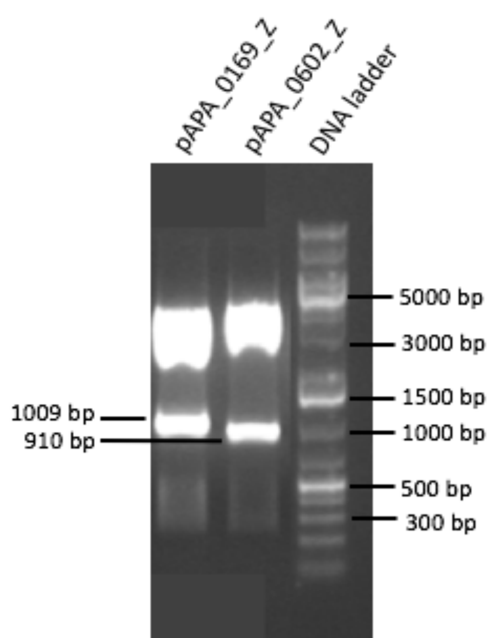


Figure 13: The gel image after digesting pAPA_0169_Z and pAPA_0602_Z with both *Sma*I and *Pst*I-HF® restriction enzymes. The thick bands at the top of both lanes probably have predicted size of 3000 bp. The band at 1009 bp (pAPA_0169_Z) and 910 bp (pPAPA_0602_Z) confirmed that these two plasmids were cut by *Sma*I.

Figure 14 demonstrates complete digestion by either enzyme *Bam*HI or *Sca*I in plasmid pAPA_0169_Z (4247 bp), pAPA_0602_Z (4116 bp) (A), pAPA_0169_P (4691 bp), pAPA_0602_P (4560 bp) (B) that can be used for electroporation. It is worth noting that based on the DNA ladder, the results showed linearized plasmid size bigger then predicted, likely due to the large size of the digested plasmid and electrophoresis/ gel conditions.

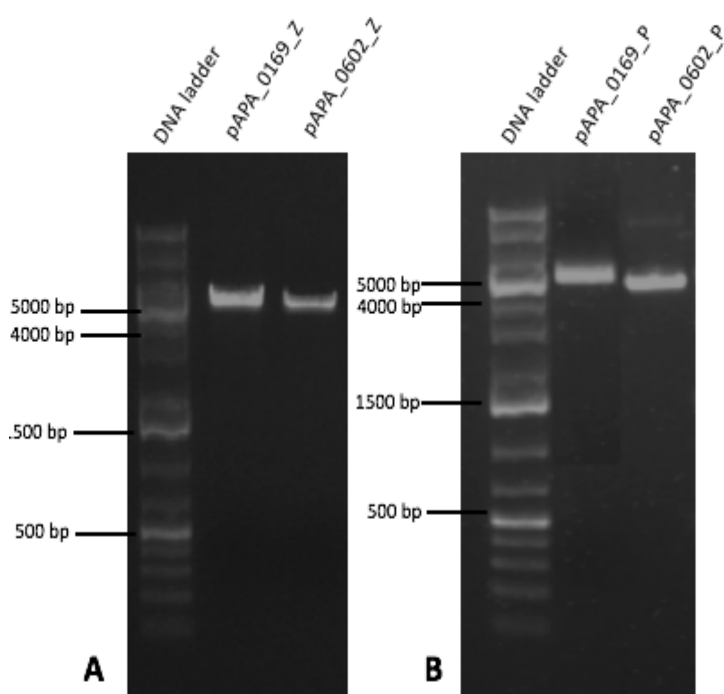


Figure 14: The gel image with DNA bands after separation of the enzymatically digested plasmid using either *Bam*HI or *Sca*I. (A) The band length of 4247-bp and 4116-bp are the result of *Bam*HI digestion, indicating successful linearization of pAPA_0169_Z and pAPA_0602_Z, respectively. (B) The band length of 4691-bp and 4560-bp are the result of *Sca*I digestion, indicating successful linearization of pAPA_0169_P and pAPA_0602_P, respectively.

Endonuclease digestion of control plasmid pSELECT100 by *Bam*HI and *Sma*I resulted in one fragment and three fragments, respectively. The gel image (Figure 15) revealed that the plasmid which was digested by *Bam*HI has one cutting site and resulted in a linearized plasmid with the size of 4031 bp. On the other hand, *Sma*I cut pSELECT100 at three sites approximately at 2650 bp, 1333 bp and 113 bp. Length of fragments obtained after the *Sma*I and *Bam*HI digestion provided confirmation that the plasmid was pSELECT100 and that linearized plasmid was ready to be used for transformation.

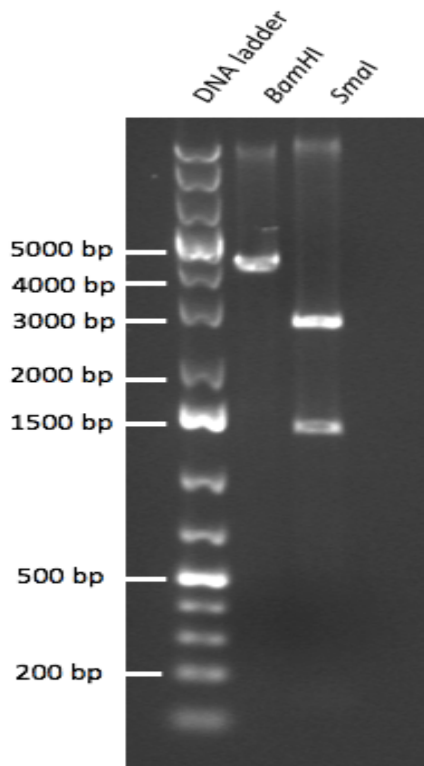


Figure 15: The gel image after digesting pSELECT100 plasmid with *Bam*HI and *Sma*I restriction enzymes. The band length of 4031-bp in the second lane is the result of *Bam*HI digestion, indicating successful linearization of pSELECT100. The two bands in the last lane at 2650-bp and 1333-bp revealed digested fragments after restriction by *Sma*I. The third band at 133-bp was not visualized on the gel image as it might go further down the gel.

3.3 Electroporation of *N. oceanica* CCMP1779

Electroporation of *N. oceanica* CCMP1779 using different plasmid constructs was performed with the Bio-Rad Gene Pulser II Electroporation System. Two replicates of electroporation were performed for each transformation plasmid. For transformation of each plasmid in the pAPA1001 family, transformation with the pSELECT1000 was performed as a positive control. The summary of all transformations performed are presented in Table 9, where each performed electroporation abbreviated with EL_´number

Table 9: The summary of all transformations of *N. oceanica* CCMP1779 performed by electroporation during the master project. Various conditions of plasmid preparation and algal growth, as well as time required for appearance of the colonies, are indicated for all of the transformation experiments. The transformation is given the names abbreviated EL_´number´ for the better distinguishing of performed experiment and their conditions.

Transformation criteria	EL_1	EL_2	EL_3	EL_4	EL_5	EL_6	EL_7	EL_8
Transformation plasmid	pAPA_0169_Z	pAPA_0169_Z	pAPA_0169_H	pAPA_0169_Z	pAPA_0169_H	pAPA_0169_Z	pAPA_0169_Z	pAPA_0169_P
	pAPA_0602_Z	pAPA_0602_Z	pAPA_0602_H	pAPA_0602_Z	pAPA_0602_H	pAPA_0602_Z	pAPA_0602_Z	pAPA_0602_P
Enzyme used for plasmid linearization	*Sca*I	*Bam*HI	*Ssp*I	*Bam*HI	*Ssp*I	*Sca*I	*Sca*I	*Sca*I
Plasmid concentration (µg/ mL)	500	500	260	420	190	500	670	530
Plasmid volume (µL)	6	6	11.5	7.1	15.8	6	4.5	5.7
Axenic growth	No	Yes	Yes	Yes	Yes	Yes	No	No
Harvesting concentration (cells/ mL)	1.32×10^7	1.96×10^7	2.24×10^7	9.98×10^6	9.98×10^6	9.98×10^6	2.01×10^7	2.01×10^7
Cell concentration for transformation (cells/ mL)	9.79×10^8	5.39×10^8	1.02×10^9	7.39×10^8	7.39×10^8	6.59×10^8	5.79×10^8	5.79×10^8
Transformed cell amount (cells)	5×10^8	5×10^8	5×10^8	5×10^8	5×10^8	5×10^8	5×10^8	5×10^8
Visible colonies (weeks)	2-3 weeks	No	No	No	No	2-3 weeks	No	No

3.4 Transformant selection and verification

After 2-3 weeks of incubation of algal cells on agar plates containing either zeocin (2 or 5 µg/mL), hygromycin B 150 µg/mL or paronomycin 40 µg/ mL as a selective marker, the resistant colonies were obtained, as summarized in Table 10. These colonies were subcultured onto ¨master plates¨ containing the same antibiotic concentration. Number of resistant colonies in Table 10 is presented as the ration of clones on ¨master plate¨/ the clones on original selective plates after transformation.

In the electroporation experiment EL_1, the cell culture was contaminated so that it was decided to use 50 µg/mL ampicillin to reduce and prevent contamination in the future. In EL_1 there were approximately 3000 resistant clones counted directly on selective plates containing zeocin 2 µg/mL. It is worth noting that only around half of this amount was observed on ¨master plates¨ where zeocin-resistant clones were sub-cultured (see Table 10). The colonies of size as small as ¼ of the 0.5-10 µL pipette tip was problematic for counting. Moreover, this algal colony size of EL_1 was noticed significantly smaller than that of EL_6. To test if these colonies do indeed represent transformants, 15 random colonies were selected for verification of transformation. Colony PCR analysis of genomic DNA isolated directly from these colonies, which was transformed with pAPA_0602_Z, confirmed the presence of the zeocin resistant gene in 11 out of 15 clones (Figure 16A). In successful transformed clones, a 1156 bp fragment was amplified, whereas in wild-type cells, no amplification product was obtained (lane WT). However, intensity of amplicons greatly differs from clones to clones. Amplification of 18S rRNA served as an internal control for successful DNA extraction and showed bands at size of 870 bp (Figure 16B).

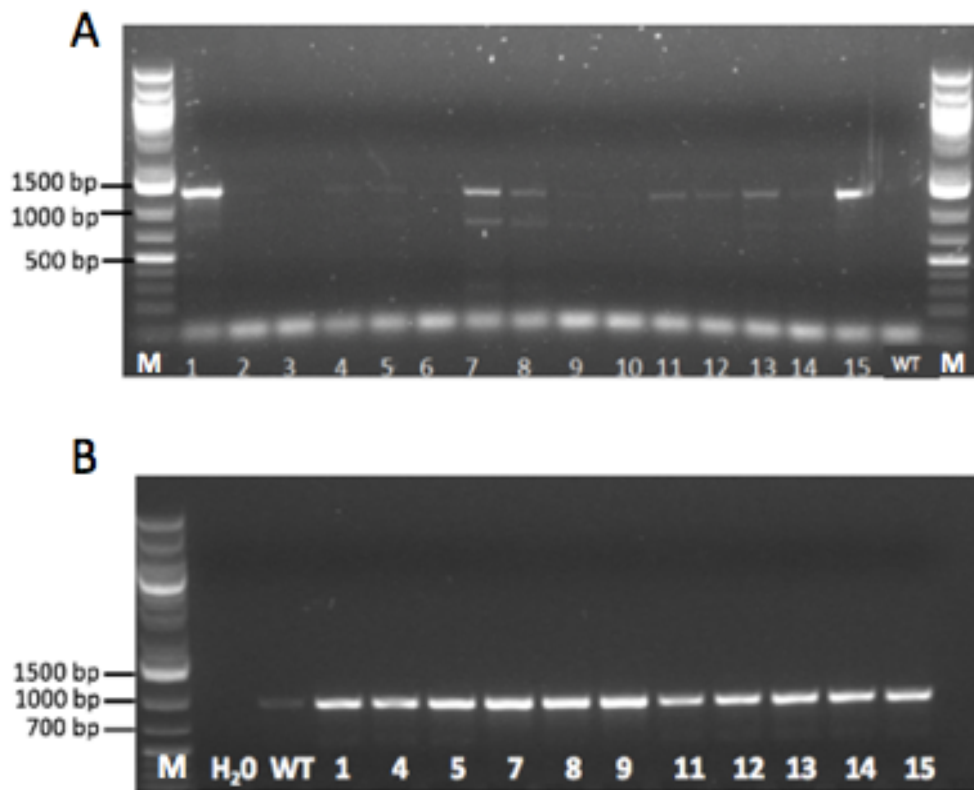


Figure 16: Gel image of colony PCR of the transformed *N. oceanica* CCMP1779 cells in EL_1. Algal cells were transformed with pAPA_0602_Z linearized by *Sca*I. After 2 weeks of incubation on antibiotic selective media, genomic DNA was extracted from colonies and analysed by colony PCR. Lane M: Molecular marker; lane WT: wild type as control (no plasmid); lane H_20: distilled water (negative control); lane with numbers: random single colony of *N. oceanica* mutants. Target primers amplified zeocin resistance gene resulted in 1156 bp PCR product (**A**) in successful transformed cells, while primers amplified 18S rRNA gene resulted in 870-bp PCR product (**B**).

Table 10: Overview of all electroporation experiments. Number of resistant colonies achieved by electroporation of *N. oceanica* CCMP1779 using pAPA1001 as transformation vector and number of verified transformants by colony PCR and transformation efficiency calculated is presented.

	Transformation plasmid	Replicate	Antibiotic & Concentration	No. of resistant colonies	No. of verified transformants	Transformati efficiency
EL_1	pAPA_0169_Z	1	Zeocin 2 µg/mL	Contaminated		
		2		Contaminated		
	pAPA_0602_Z	1		Contaminated		
		2		~1500/3000	11 out of 15	
EL_2	pAPA_0169_Z	1	Zeocin 5 µg/mL	0		
		2				
	pAPA_0602_Z	1				
		2				
EL_3	pAPA_0169_H	1	Hygromycin B 150 µg/mL	0		
		2				
	pAPA_0602_H	1				
		2				
EL_4	pAPA_0602_Z	1	Zeocin 5 µg/mL	0		
		2				
	pAPA_0169_Z	1				
		2				
EL_5	pAPA_0169_H	1	Hygromycin B 150 µg/mL	0		
		2				
	pAPA_0602_H	1				
		2				
EL_6	pAPA_0169_Z	1	Zeocin 5 µg/mL	118/ 159	13 out of 20	6.89×10^{-8}
		2		89/ 90	20 out of 20	6.00×10^{-8}
	pAPA_0602_Z	1		34/ 42	11 out 20	1.54×10^{-8}
		2		32/ 33	8 out of 20	8.80×10^{-9}
EL_7	pAPA_0169_Z	1	Zeocin 5 µg/mL	0		
		2				
	pAPA_0602_Z	1				
		2				
EL_8	pAPA_0169_P	1	Paronomycin 40 µg/ mL	0		
		2				
	pAPA_0602_P	1				
		2				

It was decided not to further perform molecular analysis on electroporation experiment EL_1 because the algal culture was contaminated and this could affect the level of confidence in the repeatability of the transformation procedure.

There were no colonies observed on antibiotic selective plates of electroporation experiment EL_2, EL_3, EL_4, EL_5, EL_7, EL_8. Concurrent transformation of pSELECT100 as positive control for these electroporation experiment did not show resistant colonies either. This indicated unsuccessful transformation in these experiments or failed culturing conditions.

In the electroporation experiment EL_6, *N. oceanica* CCMP1779 was treated with ampicillin 50 µg/mL to prevent possible contamination. Following electroporation, colonies was formed on selective plates containing zeocin 5 µg/mL (Table 10). To determine whether these colonies contained DNA sequences derived from either plasmid pAPA_0169_Z or pAPA_0602_Z, colony PCR was performed on these colonies following the analysis outlined for EL_1. A number of 20 random colonies was selected for each vector transformation replicate. For algal clones transformed successfully with plasmid pAPA_0169_Z and pAPA_0602_Z, a 1199 bp fragment and a 1156 bp fragment was amplified, respectively (Figure 17). These results implied that the algal cells of *N. oceanica* was successfully introduced with the designed plasmid and the zeocin resistance gene was integrated into the nuclear genome. Transformation efficiency was calculated using the standard formula described in Section 2.4, which is lower than that of previous studies reported in *N. oceanica.* In general, electroporation with pAPA_0169_Z resulted in higher transformation efficiency than that with pAPA_0602_Z (6.5×10^{-8} compared to 1.2×10^{-8} colonies/ cell/ µg)

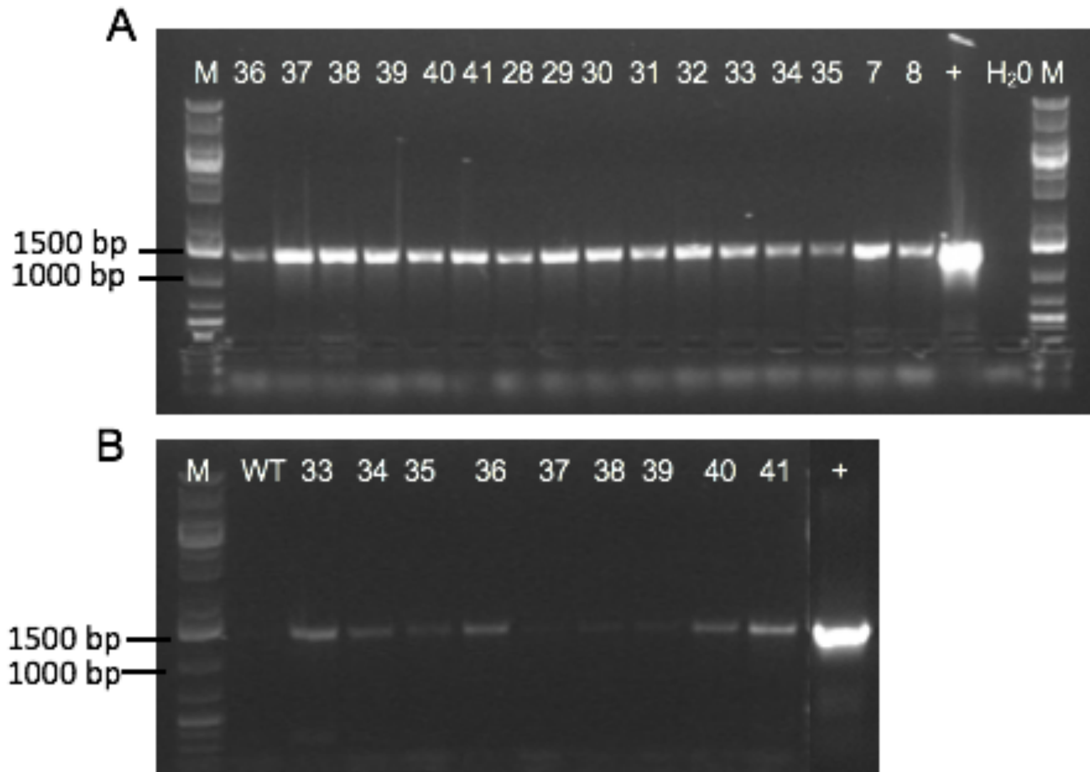


Figure 17: Gel image of colony PCR of the transformed *N. oceanica* CCMP1779 cells in EL_6. Algal cells were transformed with pAPA_0602_Z (**A**) or pAPA_0169_Z (**B**) linearized by *Sca*I. Lane M: Molecular marker; lane WT: wild type as control (no plasmid); lane H_20: distilled water (negative control); lane (+): plasmid (positive control), lane with numbers: random single colony of *N. oceanica.* Target primers amplified zeocin resistance gene resulted in 1156 bp and 1199 bp and PCR product for cells transformed with pAPA_0602_Z and pAPA_0169_Z, respectively.

4. Discussion

In this project electroporation was used to introduce DNA fragments containing vector constructs into *N. oceanica* CCMP1779 genome.

Vector construction

A family of vectors was constructed (pAPA1001) containing specific GOI, which confers resistance to either zeocin, hygromycin B or paromomycin. For the transformation, these vector constructs were amplified and then verified by double enzymatic restriction with *Sma*I and *Pst*I-HF® before being utilized further for transformation. Restriction-enzymatic characterization of the vectors pAPA_0602_Z and pAPA_0169_Z (Section 3.2) succeeded for *Sma*I but not for *Pst*I. This could probably be the consequences of technical issues related to the enzyme quality or experimental errors in performing the enzymatic setup. The increase of incubation time for *Pst*I-HF® were considered to optimize the enzymatic activity for digesting other four plasmids (pAPA_0602_H, pAPA_0169_H, pPAPA_0602_P, pPAPA_0169_P) and showed successful digestion by achieving with expected band size.

Transformation and Evaluation of selection specificity

After two to three weeks of incubation of the possible transformed cells, growth on the antibiotic selective medium was observed in experiment EL_1 and EL_6. However, there was bacterial contamination during the cultivation of algae in EL_1, which was not easily recognize by naked eyes, leading to contamination of most of the selective plates after transformation. This bacterial contamination was able to survive under antibiotic activity of zeocin 2 μg/ mL even though it has been reported that zeocin shows strong toxicity against bacteria (Invitrogen, 2012). The bacterial growth may compete with the algae growth, which probably reduced the amount of algae growing on the selection plates thereby reducing the apparent transformation efficiency. In addition, the number of zeocin-resistance algal clones counted on the ¨master-plates¨ was only half the number of algal clones observed on the selective plates right after the transformation. These false positive clones may be observed due to poorly prepared plates (such as insufficient mixing of the antibiotic) which led to decreased activity of zeocin against both, bacteria and wild type *N. oceanica* growth. Furthermore, zeocin is very unstable and may loose activity by prolonged exposure to light, or temperatures of more than 55°C (according to Thermo Fisher Scientific). Also due to the dense growth of bacterial contamination and algae on the same plates, satellite colonies may have formed. Such colonies are not exposed to the

actual antibiotic in the agar because they grow on the top of old cells. In addition, competition for nutrition on agar plates between bacterial and algal cells could be the reason for significantly smaller size of algal colonies on selective plate of EL_1 compared to that of EL_6. Another, rather unlikely, reason for high number of observed algal clones, is that zeocin at concentration of 2 μg/ mL was not very effective in preventing wild type *N. oceanica* growth. It was reported by Anley (2015) that *N. oceanica* species were highly sensitive to zeocin. The concentration of zeocin 2 μg/ mL was reported to completely inhibit the algal growth. The same concentration of zeocin was used by Killian (2011) for transformant selection after electroporation. In a meanwhile, zeocin at concentration 5 μg/ mL was studied to be more efficient in inhibiting wild type *N. oceanica* growth than zeocin at concentration 2 μg/ mL (Anley, 2015). Therefore an increase in concentration of zeocin from 2 μg/ mL (in EL_1) to 5 μg/ mL (in other experiments EL_2, 4, 6,7) was made. If the zeocin concentration of 5 μg/ mL greatly increased the efficiency of transformation in EL_6 is questionable but it could have a positive effect by eliminating false positive mutants.

For electroporation experiments EL_2, EL_3, EL_4, EL_5, EL_7, EL_8, there was failure in transformation. Concurrent transformation of pSELECT100 as positive control for these electroporation experiments also resulted in no antibiotic-resistant colonies. Problems leading to these unsuccessful transformations could probably be related to either plasmid or algae culture preparation, or errors in protocol procedure. It is possible that cells were not in optimal conditions regarding age and cell wall thickness, even though the medium was refreshed on a regular basis. According to the protocol, the transformed cells should be harvested when it reached when the cells during the exponential growth phase at a density of 2-7 x 10^6 cells/ mL in a 500 mL Erlenmeyer flask. The cells should be in dividing phase on the day of electroporation for having a thin cell wall, which facilitates penetration of DNA fragments to the nucleus. For electroporation of EL_7 & 8, the cells were harvested at the concentration of 2.01 x 10^7 cells/ mL, which is a quite high density. This may have caused stress on the cells due to nutrition and light competition. Due to the lack of components for reproduction, the cells were either more vulnerable and easier to be damaged during electric pulses application, or more resistant due to a thicker cell wall during the cell aging. Moreover, it should be noted that in theses experiments (EL_7 and EL_8), the algal culture were not complemented with ampicillin 50 μg/ mL to prevent contamination as in other experiments (EL_2,3,4,5,6). This might be the reason behind the growth of antibiotic-resistant colonies in E_L6, but not in EL_7

& 8. The principle behind the apparent effect of ampicillin on transformation efficiency is still unclear. It may be that the ampicillin may have an effect on the cells which made the cell wall weaker and it is more vulnerable to the electric pulses to form temporary pores on the cell membrane. Consequently, this gives plasmids higher possibility to be transferred into the cell.

It was reported earlier that transformation with linearized DNA vector is more efficient than with circular DNA (Kilian et al., 2011, Zhang et al. 2013). For the set of plasmids pAPA_0169_Z and pAPA_0602_Z, *Sca*I or *Bam*HI was used for linearization. The *Sca*I cutting site is located in *AmpR* gene encoding region which is around 400 bp away from the promoter while *Bam*HI cutting site is 16 bp distant from the promoter. It is likely that the integration of plasmids digested with *Bam*HI in EL_2 and EL_4 was affected as the cutting site is very close to the 5´ regions of the promoter. In a meanwhile, transformation using the same set of plasmids treated with *Sca*I in EL_1 and EL_6 was successful. Therefore, it is important that the chosen cleavage site is not very close to the 5' or 3' UTR regions of the introduced gene to preserve its integrity (Auchincloss et al. 1999).

Delivery of DNA molecules by electroporation is based on the application of an electric field to cells or a tissue to produce transient pores in the plasma membrane of the cells (Weaver et al. 1996). The exposure of electric field imposed to the cells causing an osmotic imbalance and swelling of the cell. At this moment, the cell membrane molecules that hold by the polar bonds were distracted by the electric field induction, leading to the formation of pores among the cell membrane and reversible electroporation may occur (Joannes et al. 2015). Therefore, volume of DNA solution to be added is a factor that can possibly affect transformation according to NEB´s electroporation tips. The relation between DNA volume and transformation efficiency in microalgae is still unknown. It is possible that high concentration of water molecules in plasmid solutions causes osmotic pressure leading to the explosion of algal cells. Irreversible electroporation may happen, making the cell membrane permanently damaged and unable to recover its original shape. Therefore, it is suggested to keep the volume of DNA plasmid low (less than 10 μL) for transformation.

Plasmid size is one of the factors can affect the transformation efficiency. Electroporation studies in bacteria reported that transformation efficiency declines with increasing plasmid size (Hanahan, 1983; Ohse et al. 1995). This could also be an important factor in microalgae transformation. The plasmids pAPA_0169_H, pAPA_0602_H, pAPA_0169_P and

pAPA_0602_P were around 400 - 800 bp longer than the plasmids pAPA_0169_Z and pPAPA_0602_Z; and the bigger size of these plasmids likely influences the ease of ¨transportation¨ of DNA through the temporary pores on algal cell membrane during electric pulses application. The length of the vectors may in turn influence the integration of the foreign DNA into algal genome of *N. oceanica* CCMP1779.

Electroporation of EL_6, which used plasmids pAPA_0169_Z and pAPA_0602_Z as transformation vectors containing the zeocin resistance gene, was successful. To verify the presence of transformation constructs within the cells (and thus that the transformation experiment was successful), genomic PCR of these possibly transformed clones was performed. The transformation efficiency based on these verified clones was calculated to be 6.5 x 10^{-8} and 1.2 x 10^{-8} colonies/ cell/ µg for electroporation using pAPA_0169_Z and pAPA_0602_Z, respectively. In comparison to other studies, this transformation efficiency was low. Vieler et al. (2012) reported transformation efficiency of 2.5 x 10^{-7} colonies/ cell/ µg using transformation vectors carrying the LDSP promoter. Using the same transformation vector, Chernyavskaya (2014) reported transformation efficiency of 4.08 x 10^{-5} colonies/ cell/ µg. However, in the study of Chernyavskaya (2014), antibiotic resistant colonies were not characterized by colony PCR after transformation and this makes the transformation efficiency result uncertain. Other studies obtained transformation efficiency of 8.3 x 10^{-7} colonies/ cell/ µg (Killian et al. 2011) and 12.5 x 10^{-6} colonies/ cell/ µg (Radakovits et al. 2011), utilizing transformation constructs harboring VCP promoter and TUB promoter, respectively. Even though the transformation efficiency is low and incomparable to other published studies, it is interesting that there was a very low number of ´false-positive´ clones on the selective plates in EL_6. There are more than 75% of algal clones, which were observed on selective plates after transformation, grew well on ¨master plates¨ where resistant clones were sub-cultured for further analysis. There are 16 over 20 (80%) of these zeocin resistant clones were verified by genomic colony PCR to have pAPA_0169_Z actually integrated in algae genome, while for pAPA_0602_Z the ratio is 9 over 20 (47.5%). In a study with pSELECT100 utilized in *N. oceanina* for electroporation, Chernyavskaya (2014) reported that there was high amount of false positive clones, where spontaneous mutations allowed the cells to be antibiotic tolerant. This suggests that pAPA_0169_Z is reliable to be used as a vector to optimize the transformation efficiency, which shows high compatibility between the resistant clones on selective plates and on verified clones by colony PCR.

Moreover, the transformation efficiency of electroporation experiments that utilized pAPA_0169_Z was higher than that of electroporation where pAPA_0602_Z served as transformation vector (6.5×10^{-8} compared to 1.2×10^{-8} colonies/ cell/ µg). This could be due to different expression levels of zeocin resistant gene under control of the two promoters. The promoter of the gene *nanno_169* is probably stronger expressed than that of the gene *nanno_602*. It could be possible that important regulatory structures located in the original gene sequence, which is required for efficient gene expression, were eliminated during promoter sequence selection or were destroyed by the addition of the restriction enzyme cleavage sites during synthesis of promoter. This may lead to lower promoter activity which in turn leads to lower expression of GOI (Graw, 2010).

The number of copies of the introduced DNA varies from one to several depending on the DNA concentration and the transformation technique. In *Chlamydomonas,* most integrations occur with a single copy of marker DNA (Gonzdlez-Ballester, 2005), where low concentration of DNA was used (about 100 ng or less per 10^8 cells). In transformants bearing multiple copies of the marker (1 µg and higher), these are often integrated as concatamers of transforming DNA (Kumar et al. 2004). In *Nannochloropsis* species, Radakovits (2012) reported that multiple insertions of transgene occured in some cases by performing Southern blots. The differences in intensity of amplicons from different clones after colony PCR of EL_1 (Figure 16) and EL_6 (Figure 17) might indicate that integration of different number DNA vector constructs into algal genome occurred. Some clones experienced multiple insertion with more integrated DNA fragments in its genome, leading to higher levels of antibiotic resistant-gene expression compared to the other clones.

In summary, two out of eight sets of electroporation experiments of this project resulted in successful transformation of *N. oceanica* CCMP1779. These two successful experiments are transformation with plasmid set pAPA_0169_Z and pAPA_0602_Z. Many possible reasons for failure of other transformations utilizing other four plasmids pAPA_0169_H, pAPA_0602_H, pAPA_0169_P and pAPA_0602_P, were assumed. These includes technical issues in plasmid or algae culture preparation, and may probably be due to characteristic of synthesized plasmids (e.g. plasmid size). Due to limitation of time, no further attempts were made to achieve successful transformation using the four plasmids pAPA_0169_H, pAPA_0602_H, pAPA_0169_P and pAPA_0602_P. Therefore, transformation efficiency of electroporation using these four plasmids could not be studied. However, pAPA_0169_Z was proved as an

efficient transformation vector with a strong and stable promoter which gives reliable transformant selection with very low number of ¨false positive¨ antibiotic-resistant clones.

Part IV
CONCLUSION

Part IV. CONCLUSION

The plasmid family pAPA1001 was designed by using a modular BioBrick assembly strategy that includes six DNA vector constructs for the expression of reporter genes conferring antibiotic resistance. The resistant genes for the antibiotics zeocin, hygromycin B and paromoycin were driven under endogenous promoters with their associated terminator to ensure stable expression in eukaryotic algae cell. Molecular cloning of the plasmids pPAPA1001 revealed one point mutation in the terminator of the plasmid backbone pAPA_0169 and one mutation in the promoter of the plasmid backbone pAPA_0602 (T exchange to C in both cases). However, these point mutations were evaluated to not be effecting the expression of the resistant gene.

Successful transformation by electroporation was shown with the plasmids pAPA_0169_Z and pAPA_0602_Z, revealing expression of the zeocin resistant gene (Sh *ble*) in *N. oceanica* CCMP1779. The transformation efficiency based on colony PCR verified clones was calculated to be 6.5 x 10^{-8} and 1.2 x 10^{-8} colonies/ cell/ µg for electroporation using pAPA_0169_Z and pAPA_0602_Z, respectively. This is low compared to other studies in *N. oceanica* using pSELECT100 as transformation vector. However, it should be emphasized that transformation using the plasmid pAPA_0169_Z resulted in very low number of ¨false positive¨ and therefore have made screening for transformed mutants simpler and more efficient.

Transformation using the other four plasmids pAPA_0169_H, pAPA_0602_H, pAPA_0169_P and pAPA_0602_P was not successful. Hence, transformation efficiency of electroporation for these plasmids was unable to be assessed. Due to unsuccessful transformation of the latter plasmids there should be more future attempts to transfer these vectors into *N. oceanica* CCMP1779 genome to evaluate expression of antibiotic-resistant genes under driven of the promoters. Only then evaluation in a larger context can be performed to identify the novel plasmids which give highest transformation efficiency and to optimize transformant selection. Suggestions to optimize the transformation protocol including plasmid and algae culture preparation, which were discussed, should be taken into consideration in the future transformation experiments.

The promoters selected to be integrated into the transformation vectors family pAPA1001 are constitutively expressed, and hence are expected to have constant expression in all phases of

the cell cycle and under different growth conditions, including nutrition depletion where the cells accumulate lipids. Studies are necessary to investigate gene expression in response to abiotic stresses for instant nitrogen or phosphate limitation. In these experiments several time points should be taken to show the physiological change of the algal cells, such as TAG accumulation and cell growth. Additionally, samples for expression studies should be harvested at various time points. Expression studies can be performed by isolating the total RNA and analysing the mRNA expression of the reporter gene by quantitative Real-time PCR. For studying the expression of the selection marker under nutrition depletion, a reference culture grown under normal conditions has to be used to analyse the expression variation at different conditions.

Once successful transformation and stable expression of the desired genes are established, genes encoding important proteins in the lipid metabolism pathway can be knocked out by HR in the genome of *N. oceanica* genome. This technique enables to further study the importance of genes in lipid metabolism and facilitate the searching for genes expressing key enzymes in these pathways. Genes that should be studied were suggested in various papers e.g. the BTA gene encoding an enzyme that is required for betaine lipid biosynthesis or PDAT which expresses important enzyme for the TAG accumulation (Mühlroth et al. 2014; Abida et al. 2014). The designed and verified plasmids show potential for further studies of the *N. oceanica* genome in order to achieve optimized strains for the oil production. Studying the lipid metabolism of *N. oceanica* creates greater possibility for this algae specie to be used in the future as a source of the third generation biofuel or in the neutraceuticals production.

Part V
APPENDIX

Part V. APPENDIX

Appendix 1. SUPPLEMENTARY INFORMATION FOR MOLECULAR CLONING OF PLASMID FAMILY pAPA1001

A1. 1. Sequence of synthesized promoter-terminator sequence

The constructs based on the promoter and terminator sequences of the *N. oceanica* gene *nanno_602* or *nanno_169* and each containing a *BamH*I, *Pac*I, *Spe*I, *Pst*I, *Xho*I and *Sph*I restriction site.

Color code: *BamH*I *Pac*I *Spe*I *Pst*I *Xho*I *Sph*I

construct_0169 **(1089 bp)**

```
   1 GGATCCATCT TAATTAAGGG TTCCTTGAGC TTTCATCCTT CAGAGCCCGT
  51 GTTGGTGACA GTGGGATACG ATGGAGTGGG ATGTTGTTGG TTTCGGGATT
 101 TGTGAGGAGG ATGAGCGGGG GGGGTTTGAC GGATGTAAAT AGAGCGACAC
 151 GAGCGGTGGC GCATGTGAAT CAGCAAGCGT ATTTATGGGA ACAATAATGA
 201 ACGAAAGATT TAACGAACCA ATAAAAGGTA TACGTACACA TAAGATGCAG
 251 GCTTTCTTTA ATCCCTGCCC TCTGTACAGC AAAGTAGCCG AGCAAAGTAG
 301 CCGCTCAGCA GGGCAAAGGT CGGCCCATGG TTCTTTCCTT TCCTCTTCTC
 351 TCGCATGACA AAAGGACGAG CTTTAGGGAT TCTGCCTAGA CTGTATCTAT
 401 GCTGCCTATG TAAGCAACTG TGTCGTCTTG CTCCCGCTGT AATGACTTTT
 451 CAATTCCTTT CACATTCGCC TGCATTTCCT TTGCCTCTCA CCCCCCTCTT
 501 ACGCCCTCAT TCTGCCTGAT TCGCTCGCTG ATTGCAACGA CACATGATCA
 551 TGACTCCCTG CGCACGACGA CGCGACCCTT ATCTATGCGT CTGAGCCACC
 601 ACACAAAGTG TAAACCTCAC ACAGACCTTC ACTCTGCGCC TTTCATTAGC
 651 CCAAGCTCAA GCGCACGTGC GTCAGCGGGG TACGACGTAA GACCTCTCGC
 701 CTACGACCTC CCCAAATCAA ATCATTTACG ACTTCTTTCT GTCGAAACCT
 751 ACCGCATCTC ACTCCCTCCG TCAACATTCT CGCCTTCCCG CACTGGCTCT
 801 ATACCCTACA GCACTAGTCG TCTGCAGCGG GATTCCAGGG TGAAGGCCTC
 851 AGGGGAATGA TTGATCTTCG AAATGGTCAA CATATGTGTG TTTGTGTGAT
 901 TTATTTGATT TGTGTCATGC ACGAGAAAAA GTGTAATGCG AGTTCAAGGG
 951 AAGTTGGGGT TTCACAAAAG AAAAAGATGG AGTATTGTTT ATTTGGATTA
1001 CAGATGCCTT GCATTTAACG CCCGTGCCTT GAGTTGGAAC GAGAGTAAAA
1051 TGGGGAAATT CTAAATGAAC AATACTCGAG ATAGCATGC
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construct_0602 **(1220bp)**

```
   1 GGATCCATCT TAATTAAGAA ATGCATGATG ACATATGTAT GTTGACTTTA
  51 TTTTGATGTG CGGGGATTAA GTGAACGGGG GTAGGATGAT GGGACGAGTT
 101 GGATTGCAGC AAGGCGGTGT GATTGATTTC GGGGAAACGA AACTTGAAAA
 151 AGACGACAAT AAATGTGACA AAAAGAATGA GCGCAACAAC AACGATATCG
 201 TGCGAAACGT TTTTTGTTTC TTGTTCGTTT GCTCTCGCTT CATTTCCGTG
 251 TGGGACGCGG CTTGTAAGAC ACTGATCCGC AGGATAATGC CCCTGCCAAA
 301 CTCACGGGAG ACATTTCAAA AGCCAAGTAG TATAGCTTGC TTAAAATGAG
 351 ATGAGATGGA GGTGGTGGCG GTGGCCTTGG CAAGGAGCGC GCATCGTTTT
 401 GGAAGCCATG TCAGGAAACT CAGGCGTGCG GCGGCGCCAG GACGCTGCTT
 451 CTTCGACACA TGTATCTTCT GAGTCCCTTT GGACAACCAT GCCAGCAACC
 501 ATGACAACCA CACGCAAGCA CATACAAACA CAGGGATGAT GGCTATGTGC
 551 CTGTCTTGAA GGGCATGGCG TTTATAGGCG GGGATTCTTG CTTTTTGGTA
 601 TATCCCTCTG CCCCTCGTCT CCTTGCATCA CGGTGATGTT GCCCTTGCTG
 651 CCGCTGCTGC TTCTTCCCTC TAGGGCATCC ATTCACATAC AAGAAACTCT
 701 CACCTTAACG TCCACCCGAA GGCAGGCTCT CTCCGTTTTG TTAGGGCTTC
 751 TGGCTGGCAG AATGTGCAGG AGACATGCGT TTCCCCATGA TGTGAAAGCC
 801 AACAACGCGT GCCGTCCTCA CACGCTAGTG CTCTGACTGT GTCATCATCA
 851 ACCATCCCCC GCACCGCACT TACACGCACC GAAATCATAC ACATATCTAA
 901 AACGTACAGC GACTAGTCGT CTGCAGAGTG AGGGGGAGGG GGAGGGTGGA
 951 CTTGAGAGTG TGGAAGTGAG TGAGGGTAGA AGATAGAGTA AGGATGGCAG
1001 ACAAGATGAC GATAAAGAAG ACGTGGAAGA GAGGGAAATA CACGTACAAA
1051 GGAAGAAAAC AAGAAAAGGA AAAAGAGGAA AAAATAAACG CAGGCGGAAT
1101 TACTCACTGT TGAAATGGTC TCTCGTCCTG ACACGGAAGT TGTACGAACA
1151 ACACGCCTGC ACACAACGTA CTCTTCTTCT GTGTTCTAAA TAAAGACTAT
1201 TTGCTCTCGA GATAGCATGC
```

A1. 2. Reporter genes

Zeocin resistant gene (375 bp)

```
  1 ATGGCCAAGT TGACCAGTGC CGTTCCGGTG CTCACCGCGC GCGACGTCGC
 51 CGGAGCGGTC GAGTTCTGGA CCGACCGGCT CGGGTTCTCC CGGGACTTCG
101 TGGAGGACGA CTTCGCCGGT GTGGTCCGGG ACGACGTGAC CCTGTTCATC
151 AGCGCGGTCC AGGACCAGGT GGTGCCGGAC AACACCCTGG CCTGGGTGTG
201 GGTGCGCGGC CTGGACGAGC TGTACGCCGA GTGGTCGGAG GTCGTGTCCA
251 CGAACTTCCG GGACGCCTCC GGGCCGGCCA TGACCGAGAT CGGCGAGCAG
301 CCGTGGGGGC GGGAGTTCGC CCTGCGCGAC CCGGCCGGCA ACTGCGTGCA
351 CTTCGTGGCC GAGGAGCAGG ACTGA
```

Hygromycin B resistant gene (1026 bp)

```
   1 ATGAAAAAGC CTGAACTCAC CGCGACGTCT GTCGAGAAGT TTCTGATCGA
  51 AAAGTTCGAC AGCGTCTCCG ACCTGATGCA GCTCTCGGAG GGCGAAGAAT
 101 CTCGTGCTTT CAGCTTCGAT GTAGGAGGGC GTGGATATGT CCTGCGGGTA
 151 AATAGCTGCG CCGATGGTTT CTACAAAGAT CGTTATGTTT ATCGGCACTT
 201 TGCATCGGCC GCGCTCCCGA TTCCGGAAGT GCTTGACATT GGGGAATTCA
 251 GCGAGAGCCT GACCTATTGC ATCTCCCGCC GTGCACAGGG TGTCACGTTG
 301 CAAGACCTGC CTGAAACCGA ACTGCCCGCT GTTCTGCAGC CGGTCGCGGA
 351 GGCCATGGAT GCGATCGCTG CGGCCGATCT TAGCCAGACG AGCGGGTTCG
 401 GCCCATTCGG ACCGCAAGGA ATCGGTCAAT ACACTACATG GCGTGATTTC
 451 ATATGCGCGA TTGCTGATCC CCATGTGTAT CACTGGCAAA CTGTGATGGA
 501 CGACACCGTC AGTGCGTCCG TCGCGCAGGC TCTCGATGAG CTGATGCTTT
 551 GGGCCGAGGA CTGCCCCGAA GTCCGGCACC TCGTGCACGC GGATTTCGGC
 601 TCCAACAATG TCCTGACGGA CAATGGCCGC ATAACAGCGG TCATTGACTG
 651 GAGCGAGGCG ATGTTCGGGG ATTCCCAATA CGAGGTCGCC AACATCTTCT
 701 TCTGGAGGCC GTGGTTGGCT TGTATGGAGC AGCAGACGCG CTACTTCGAG
 751 CGGAGGCATC CGGAGCTTGC AGGATCGCCG CGGCTCCGGG CGTATATGCT
 801 CCGCATTGGT CTTGACCAAC TCTATCAGAG CTTGGTTGAC GGCAATTTCG
 851 ATGATGCAGC TTGGGCGCAG GGTCGATGCG ACGCAATCGT CCGATCCGGA
 901 GCCGGGACTG TCGGGCGTAC ACAAATCGCC CGCAGAAGCG CGGCCGTCTG
 951 GACCGATGGC TGTGTAGAAG TACTCGCCGA TAGTGGAAAC CGACGCCCCA
1001 GCACTCGTCC GAGGGCAAAG GAATAG
```

Paromomycin resistant gene (819 bp)

```
  1 ATGGACGATG CGTTGCGTGC ACTGCGGGGT CGGTATCCCG GTTGTGAGTG
 51 GGTTGTTGTG GAGGATGGGG CCTCGGGGGC TGGTGTTTAT CGGCTTCGGG
101 GTGGTGGGCG GGAGTTGTTT GTCAAGGTGG CAGCTCTGGG GGCCGGGGTG
151 GGCTTGTTGG GTGAGGCTGA GCGGCTGGTG TGGTTGGCGG AGGTGGGGAT
201 TCCCGTACCT CGTGTTGTGG AGGGTGGTGG GGACGAGAGG GTCGCCTGGT
251 TGGTCACCGA AGCGGTTCCG GGGCGTCCGG CCAGTGCGCG GTGGCCGCGG
301 GAGCAGCGGC TGGACGTGGC GGTGGCGCTC GCGGGGCTCG CTCGTTCGCT
351 GCACGCGCTG GACTGGGAGC GGTGTCCGTT CGATCGCAGT CTCGCGGTGA
401 CGGTGCCGCA GGCGGCCCGT GCTGTCGCTG AAGGGAGCGT CGACTTGGAG
451 GATCTGGACG AGGAGCGGAA GGGGTGGTCG GGGGAGCGGC TTCTCGCCGA
501 GCTGGAGCGG ACTCGGCCTG CGGACGAGGA TCTGGCGGTT TGCCACGGTG
551 ACCTGTGCCC GGACAACGTG CTGCTCGACC CTCGTACCTG CGAGGTGACC
601 GGGCTGATCG ACGTGGGGCG GGTCGGCCGT GCGGACCGGC ACTCCGATCT
651 CGCGCTGGTG CTGCGCGAGC TGGCCCACGA GGAGGACCCG TGGTTCGGGC
701 CGGAGTGTTC CGCGGCGTTC CTGCGGGAGT ACGGGCGCGG GTGGGATGGG
751 GCGGTATCGG AGGAAAAGCT GGCGTTTTAC CGGCTGTTGG ACGAGTTCTT
801 CTGAGGGACC TGATGGTGT
```

A1. 3. Reporters gene sequence with introduced *Xba*I and *Pst*I cutting site

Color code: *Xba*I *Pst*I

Zeocin resistant gene (394 bp)

```
   1 actgTCTAGA TGGCCAAGTT GACCAGTGCC GTTCCGGTGC TCACCGCGCG
  51 CGACGTCGCC GGAGCGGTCG AGTTCTGGAC CGACCGGCTC GGGTTCTCCC
 101 GGGACTTCGT GGAGGACGAC TTCGCCGGTG TGGTCCGGGA CGACGTGACC
 151 CTGTTCATCA GCGCGGTCCA GGACCAGGTG GTGCCGGACA ACACCCTGGC
 201 CTGGGTGTGG GTGCGCGGCC TGGACGAGCT GTACGCCGAG TGGTCGGAGG
 251 TCGTGTCCAC GAACTTCCGG GACGCCTCCG GGCCGGCCAT GACCGAGATC
 301 GGCGAGCAGC CGTGGGGGCG GGAGTTCGCC CTGCGCGACC CGGCCGGCAA
 351 CTGCGTGCAC TTCGTGGCCG AGGAGCAGGA CTGACTGCAG ttat
```

Hygromycin B resistant gene (1045 bp)

```
   1 actgTCTAGA TGAAAAAGCC TGAACTCACC GCGACGTCTG TCGAGAAGTT
  51 TCTGATCGAA AAGTTCGACA GCGTCTCCGA CCTGATGCAG CTCTCGGAGG
 101 GCGAAGAATC TCGTGCTTTC AGCTTCGATG TAGGAGGGCG TGGATATGTC
 151 CTGCGGGTAA ATAGCTGCGC CGATGGTTTC TACAAAGATC GTTATGTTTA
 201 TCGGCACTTT GCATCGGCCG CGCTCCCGAT TCCGGAAGTG CTTGACATTG
 251 GGGAATTCAG CGAGAGCCTG ACCTATTGCA TCTCCCGCCG TGCACAGGGT
 301 GTCACGTTGC AAGACCTGCC TGAAACCGAA CTGCCCGCTG TTCTCCAGCC
 351 GGTCGCGGAG GCCATGGATG CGATCGCTGC GGCCGATCTT AGCCAGACGA
 401 GCGGGTTCGG CCCATTCGGA CCGCAAGGAA TCGGTCAATA CACTACATGG
 451 CGTGATTTCA TATGCGCGAT TGCTGATCCC CATGTGTATC ACTGGCAAAC
 501 TGTGATGGAC GACACCGTCA GTGCGTCCGT CGCGCAGGCT CTCGATGAGC
 551 TGATGCTTTG GGCCGAGGAC TGCCCCGAAG TCCGGCACCT CGTGCACGCG
 601 GATTTCGGCT CCAACAATGT CCTGACGGAC AATGGCCGCA TAACAGCGGT
 651 CATTGACTGG AGCGAGGCGA TGTTCGGGGA TTCCCAATAC GAGGTCGCCA
 701 ACATCTTCTT CTGGAGGCCG TGGTTGGCTT GTATGGAGCA GCAGACGCGC
 751 TACTTCGAGC GGAGGCATCC GGAGCTTGCA GGATCGCCGC GGCTCCGGGC
 801 GTATATGCTC CGCATTGGTC TTGACCAACT CTATCAGAGC TTGGTTGACG
 851 GCAATTTCGA TGATGCAGCT TGGGCGCAGG GTCGATGCGA CGCAATCGTC
 901 CGATCCGGAG CCGGGACTGT CGGGCGTACA CAAATCGCCC GCAGAAGCGC
 951 GGCCGTCTGG ACCGATGGCT GTGTAGAAGT ACTCGCCGAT AGTGGAAACC
1001 GACGCCCCAG CACTCGTCCG AGGGCAAAGG AATAGCTGCA Gatat
```

Paromomycin reistant gene (838 bp)

```
  1 actgTCTAGA TGGACGATGC GTTGCGTGCA CTGCGGGGTC GGTATCCCGG
 51 TTGTGAGTGG GTTGTTGTGG AGGATGGGGC CTCGGGGGCT GGTGTTTATC
101 GGCTTCGGGG TGGTGGGCGG GAGTTGTTTG TCAAGGTGGC AGCTCTGGGG
151 GCCGGGGTGG GCTTGTTGGG TGAGGCTGAG CGGCTGGTGT GGTTGGCGGA
201 GGTGGGGATT CCCGTACCTC GTGTTGTGGA GGGTGGTGGG GACGAGAGGG
251 TCGCCTGGTT GGTCACCGAA GCGGTTCCGG GGCGTCCGGC CAGTGCGCGG
301 TGGCCGCGGG AGCAGCGGCT GGACGTGGCG GTGGCGCTCG CGGGGCTCGC
351 TCGTTCGCTG CACGCGCTGG ACTGGGAGCG GTGTCCGTTC GATCGCAGTC
401 TCGCGGTGAC GGTGCCGCAG GCGGCCCGTG CTGTCGCTGA AGGGAGCGTC
451 GACTTGGAGG ATCTGGACGA GGAGCGGAAG GGGTGGTCGG GGGAGCGGCT
501 TCTCGCCGAG CTGGAGCGGA CTCGGCCTGC GGACGAGGAT CTGGCGGTTT
551 GCCACGGTGA CCTGTGCCCG GACAACGTGC TGCTCGACCC TCGTACCTGC
601 GAGGTGACCG GGCTGATCGA CGTGGGGCGG GTCGGCCGTG CGGACCGGCA
651 CTCCGATCTC GCGCTGGTGC TGCGCGAGCT GGCCCACGAG GAGGACCCGT
701 GGTTCGGGCC GGAGTGTTCC GCGGCGTTCC TGCGGGAGTA CGGGCGCGGG
751 TGGGATGGGG CGGTATCGGA GGAAAAGCTG GCGTTTTACC GGCTGTTGGA
801 CGAGTTCTTC TGAGGGACCT GATGGTGTCT GCAGatat
```

A1. 4. Media and antibiotic concentration for growing *E. coli* and selecting transformant

	Component	Concentration	
LB medium		(g/L)	
	Bactotryptone	10	
	Yeast extract	5	
	NaCl	5	
	Agar	15	(for plates only)
	pH at 7.5 adjusted with NaOH		
Antibotics [1]		(µg/mL)	
	Ampicillin	100	
	Kanamycin	50	

[1] Before addition of antibiotics, media was cooled to 55°C

A1. 5. Media and solutions for transforming *E. coli*

	Components	Amount	
yB media (500 mL)			
	Bactotryptone	10.00 g	
	Yeast extract	2.50 g	
	KCl	0.38 g	
	KOH	Until pH reach 7.6	Autoclave then add
	Sterile 1 M $MgSO_4$	17 mL	
TfBI Solution (500 mL)			
	Potassium acetate	1.47 g	
	$MnCl_2$	4.95 g	
	RbCl	6.05 g	
	$CaCl_2$	0.74 g	
	Glycerol	75 mL	
	Acetic acid	pH 5.8	Filter sterilize the solution and store at 4˚C
TfBII Solution (100 mL)			
	100 mM MOPS (pH 7.0)	10 mL	Adjust pH with KOH 0.2 to 1M
	$CaCl_2$	1.10 g	
	RbCl	0.12 g	
	Glycerol	15 mL	Autoclave and store at 4˚C in the dark

A1. 6. PCR thermal cycle to delete a *Pst*I site in the middle of hygromycin B resistance gene

Step	Temperature	Time	Cycles
1. Initiate Denaturation	95°C	1 min	1
2. Denaturation	98°C	20 sec	10
3. Annealing Touchdown	64°C to 56 ^{o}C	30 sec	
4. Elongation	72°C	1 min	
Normal cycles			
5. Denaturation	98°C	20 sec	15
6. Annealing	62 ^{o}C	30 sec	
7. Elongation	62.5^{o}C	1 min	
8. Final Elongation	72°C	5 min	
6. Holding	4°C	-	

A1. 7. Primers for reporter genes amplification

Gene for amplification	Primer name	Primer sequence (5'-3')
Zeocin	ZeocinRfwd	actgTCTAGATGGCCAAGTTGACCAG
	ZeocinRrev	atatCTGCAGTCAGTCCTGCTCCTC
Hygromycin B	HygroRfwd	actgTCTAGATGAAAAAGCCTGAACTCAC
	HygroRrev	atatCTGCAGCTATTCCTTTGCCCTC
Paromomycin	ParmRfwd	actgTCTAGATGGACGATGCGTTGC
	ParmRrev	atatCTGCAGACACCATCAGGTCCC

A1. 8. Enzymatic restriction setup

Enzymatic restriction setup for the plasmids pUC19, pEX-K4-No3874 and pEX-K4-No11694

Components	Volume (µL)
Purified plasmid (from 90 – 140 ng/ µl)	30.0
Each enzyme *BamH*I and *Sph*I	1.0
10X CutSmart® Buffer	3.5
Total	35.5
37°C for one hour	

Enzymatic restriction setup for the partial constructs pPAPA0001

Components	Volume (µL)
Purified plasmid (from 75 ng/ µl)	110.0
Each enzyme *PstI*HF**®** and *Spe*I	2.5
10X CutSmart® Buffer	13.0
Distilled water	2.0
Total	130.0
37°C for one hour	

Enzymatic restriction setup for the GOIs

Components	Volume (µL)
Purified plasmid (from 70 – 120 ng/ µl)	30.0
Each enzyme *BamH*I and *Sph*I	1.0
10X CutSmart® Buffer	3.5
Total	35.5
37°C for one hour	

A1. 9. Enzymatic ligation setup of partial construct pAPA0001 and GOIs

	Hygromycin B resistant gene	Zeocin resistant gene	Paromomycin resistant gene
Vector concentration (ng/µl)	62.2	62.2	62.2
Insert concentration (ng/µl)	2.5	10.6	3.5
10X T4 DNA ligase Buffer (µl)	2.5	2.5	2.5
Vector DNA (µl)	0.8	0.8	0.8
Insert DNA (µl)	15.0	3.5	10.7
Nuclease free water (µl)	5.7	17.2	10
T4 DNA ligase (µl)	1.0	1.0	1.0

A1. 10. Primers used for *E. coli* colony PCR

Plasmid	Primer name	Primer sequence (5’-3’)
pAPA_0001	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
	M13_INSERT_short_rv	CAGGAAACAGCTATGAC
pAPA_0169_H	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
pAPA_0602_H	HygroRfwd	actgTCTAGATGAAAAAGCCTGAACTCAC
pAPA_0169_Z	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
pAPA_0602_Z	ZeocinRfwd	actgTCTAGATGGCCAAGTTGACCAG
pAPA_0169_P	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
pAPA_0602_P	ParmRfwd	actgTCTAGATGGACGATGCGTTGC

A1. 11. Primers used for DNA sequencing

Plasmid	Primer name	Primer sequence (5’-3’)
pAPA_0001	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
	M13_INSERT_short_rv	CAGGAAACAGCTATGAC
pAPA_0169_H pAPA_0602_H	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
	M13_INSERT_short_rv	CAGGAAACAGCTATGAC
	HygroRfwd	actgTCTAGATGAAAAAGCCTGAACTCAC
pAPA_0169_Z pAPA_0602_Z	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
	M13_INSERT_short_rv	CAGGAAACAGCTATGAC
	ZeocinRfwd	actgTCTAGATGGCCAAGTTGACCAG
pAPA_0169_P pAPA_0602_P	M13_INSERT_short_fw	GTTTTCCCAGTCACGAC
	M13_INSERT_short_rv	CAGGAAACAGCTATGAC
	ParmRfwd	actgTCTAGATGGACGATGCGTTGC

Appendix 2. SUPPLEMENTARY INFORMATION FOR ELECTROPORATION USING PLASMID FAMILY pAPA1001

A2. 1. Antibiotics used for transformant selection

Antibiotic	Characteristic	Commercial source	Stock solution	Storage condition
Hygromycin B	water-soluble	Thermo Scientific	50 mg/mL	Stored at 4°C
Zeocin	water-soluble	Thermo Scientific	100 mg/ml	Stored at -20°C, darkness
Paronomycin	water-soluble, powder form	Sigma-Aldrich, Merck	50 mg/mL	Stored at -20°C

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