TRANSCRIPTION OF THE LARVICIDAL *cry4Ba* GENE FROM *Bacillus thuringiensis* IN THE GREEN ALGAL *Chlamydomonas reinhardtii* CHLOROPLAST

THANATE JUNTADECH

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…………………………………………
Mr. Thanate Juntadech
Candidate

………………………………………....
Assoc. Prof. Chanan Angsuthanasombat, Ph.D.
Major-advisor

..………………………………………
Asst. Prof. Kittisak Yokthongwattana, Ph.D.
Co-advisor

……………………………………
Lect. Yun-kiam Yap, Ph.D.
Co-advisor

…………………………………………
Mr. Sithichoke Tangphatsornruang, Ph.D.
Co-advisor

..……………………………………..
Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

..………………………………………..
Assoc. Prof. Apinunt Udomkit, Ph.D.
Program Director
Doctor of Philosophy Program in
Molecular Genetics and Genetic Engineering
Institute of Molecular Biosciences
Mahidol University

Thesis
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TRANSCRIPTION OF THE LARVICIDAL *cry4Ba* GENE FROM *Bacillus thuringiensis* IN THE GREEN ALGAL *Chlamydomonas reinhardtii* CHLOROPLAST

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for the degree of Doctor of Philosophy
(Molecular Genetics and Genetic Engineering)
on
November 26, 2013

……………………………………..…
Mr. Thanate Juntadech
Candidate

..……………………………………….....
Assoc. Prof. Apinunt Udomkit, Ph.D.
Chair

………………………………………...
Assoc. Prof. Chanan Angsuthanasombat, Ph.D.
Member

..…………………………………..……
Asst. Prof. Kittisak Yokthongwattana, Ph.D.
Member

..…………………………………
Lect. Thanyanan Wannathong, Ph.D.
Member

..………………………………..............
Mr. Sithichoke Tangphatsornruang, Ph.D.
Member

..…………………………………
Prof. Banchong Mahaisavariya,
M.D., Dip Thai Board of Orthopedics
Dean
Faculty of Graduate Studies
Mahidol University

..……………………………………..…
Prof. Prasert Auewarakul, M.D., Dr. med.
Director
Institute of Molecular Biosciences
Mahidol University

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Thanate Juntadech

TRANSCRIPTION OF THE LARVICIDAL *cry4Ba* GENE FROM *Bacillus thuringiensis* IN THE GREEN ALGAL *Chlamydomonas reinhardtii* CHLOROPLAST

THANATE JUNTADECH 4837260 MBMG/D

Ph.D. (MOLECULAR GENETICS AND GENETIC ENGINEERING)

THESIS ADVISORY COMMITTEE: CHANAN ANGSUTHANASOMBAT, Ph.D., KITTISAK YOKTHONGWATTANA, Ph.D., SITHICHOKE TANGPHATSORNRUANG, Ph.D., YUN-KIAM YAP, Ph.D.

ABSTRACT

The well-known single cell green alga *Chlamydomonas reinhardtii* has been recognized as a potential platform for expression of recombinant proteins due to its potential for large scale production as well as low maintenance costs. In this study, transcription of the 3.4-kb mosquito-larvicidal *cry4Ba* gene from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) in transgenic chloroplasts of *C. reinhardtii* under control of the promoter and 5′-UTR of photosynthetic *psbA* gene was achieved. Inverted repeats in a chloroplast genome of the host strain with deleted photosynthetic *psbA* genes were selected as recombination targets. Three transformant lines showing stable and site-specific integration of intact *cry4Ba* and *psbA* genes into *C. reinhardtii* chloroplast genomes were obtained by means of dual-phenotypic screening *via* exhibition of resistance to spectinomycin and restoration of the photosynthetic activity. Achievement in co-transcription of recombinant *cry4Ba* and *psbA* transgenes in all stable lines revealed by RT-PCR and Northern blot analyses demonstrates the sufficiency of this system's transcription machinery, offering further innovation for *Bt*-insecticidal protein production.

KEY WORDS: *Chlamydomonas*/ CHLOROPLAST TRANSFORMATION/ INVERTED REPEAT/ *Bti-cry4Ba* TRANSCRIPT/ *psbA* PROMOTER

125 pages

การถอดรหัสของยีนโปรตีนสารพิษ *cry4Ba* จากแบคทีเรีย *Bacillus thuringiensis* ในคลอโรพลาสต์ของสาหร่ายสีเขียว *Chlamydomonas reinhardtii*

TRANSCRIPTION OF THE LARVICIDAL *cry4Ba* GENE FROM *Bacillus thuringiensis* IN THE GREEN ALGAL *Chlamydomonas reinhardtii* CHLOROPLAST

ธเนศ จันทเดช 4837260 MBMG/D

ปร.ด. (อณูพันธุศาสตร์และพันธุวิศวกรรมศาสตร์)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: ชนันท์ อังศุธนสมบัติ, Ph.D., กิตติศักดิ์ หยกทองวัฒนา, Ph.D., สิทธิโชค ตั้งภัสสรเรือง, Ph.D., Yun-Kiam Yap, Ph.D.

บทคัดย่อ

Chlamydomonas reinhardtii เป็นสาหร่ายสีเขียวเซลล์เดียว ที่ได้รับการยอมรับว่าเป็นต้นแบบที่มีศักยภาพสำหรับการแสดงออกของโปรตีนลูกผสม (recombinant proteins) เนื่องด้วยเป็นเซลล์ที่มีประสิทธิภาพในการผลิตโปรตีนได้ปริมาณสูงและมีค่าใช้จ่ายในการบำรุงรักษาต่ำ ในการศึกษานี้ได้ประสบความสำเร็จในการถอดรหัสหรือการสังเคราะห์อาร์เอ็นเอของยีนที่สร้างโปรตีนฆ่าลูกน้ำยุง *cry4Ba* ที่มีขนาด 3.4 กิโลเบส จากแบคทีเรีย *Bacillus thuringiensis* สายพันธุ์ *israelensis* ในคลอโรพลาสต์ของสาหร่าย *C. reinhardtii* ภายใต้การควบคุมของโปรโมเตอร์และ 5'UTR (บริเวณที่ไม่มีการสร้างโปรตีน) ที่ได้จากยีนสังเคราะห์แสง *psbA* ในการนี้ตำแหน่งที่บรรจุยีน (recombination targets) ได้ถูกเลือกให้อยู่บริเวณที่เป็น inverted repeats ภายในคลอโรพลาสต์ของสาหร่ายสายพันธุ์ซึ่งไม่มียีน *psbA* จากการศึกษาพบว่าได้สาหร่ายลูกผสม 3 ตัวอย่างซึ่งสามารถแสดงการมีอยู่อย่างจำเพาะและถาวรของยีน *cry4Ba* และ *psbA* ภายในจีโนมคลอโรพลาสต์ของ *C. reinhardtii* โดยได้มาจากการคัดเลือกแบบลักษณะของการแสดงออกทั้ง 2 วิธี อันได้แก่การแสดงออกของความต้านทานยาปฏิชีวนะ spectinomycin และการคืนความสามารถของกระบวนการสังเคราะห์แสง โดยที่ความสำเร็จในการสังเคราะห์อาร์เอ็นเอของยีนลูกผสม *cry4Ba* และ *psbA* ในทุกตัวอย่างของสาหร่ายลูกผสมนั้นได้ผลมาจากการวิเคราะห์ด้วย RT-PCR และ Northern blot ซึ่งได้แสดงถึงความเพียงพอของทรัพยากรที่ใช้ในการถอดรหัสดีเอ็นเอของระบบนี้นำพาไปสู่นวัตกรรมก้าวหน้าสำหรับการผลิตโปรตีนสารพิษจากแบคทีเรีย *Bt* ในอนาคตต่อไป

125 หน้า

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LIST OF ABBREVIATIONS

% (v/v)	Percent volume by volume
% (w/v)	Percent weight by volume
% (w/w)	Percent weight by weight
°C	Degree Celsius
Amp	Ampicillin
APN	Aminopeptidase N
ATA	Aurintricarboxylic acid
bp	Base pair (s)
Bt	*Bacillus thuringiensis*
Bti	*Bacillus thuringiensis* subsp. *israelensis*
cDNA	Complementary deoxyribonucleic acid
CIAP	Calf intestinal alkaline phosphatase
cm	Centimeter (s)
Cry	Crystal protein
CTAB	Cetyltrimethylammonium bromide
Cyt	Cytolytic protein
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	dATP, dGTP, dTTP, dCTP
DTT	1,4-Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
et al	And other
EtBr	Ethidium bromide
FdUrd	5-fluorodeoxyuridine
g	Gram (s)

LIST OF ABBREVIATIONS (cont.)

GRAS	Generally regarded as safe
HS	High salt
hr	Hour (s)
ICP	Insecticidal crystal protein (s)
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Inverted repeat (s)
kb	Kilobase (s)
kDa	Kilodalton (s)
L	Liter (s)
LB	Luria-Bertani medium
LF	Left recombination fragment
M	Molar (s)
mt	Mating type
mg	Milligram (s)
min	Minute (s)
ml	Milliliter (s)
mM	Milimolar (s)
mRNA	Messenger ribonucleic acid
MW	Molecular weight
ng	Nanogram (s)
nm	Nanometer (s)
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
psi	Pound per square inch
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

LIST OF ABBREVIATIONS (cont.)

rpm	Revolutions per minute
RF	Right recombination fragment
RT	Reverse transcription
SDS	Sodium dodecyl sulphate
sec	Second (s)
supsp.	Subspecies
TAP	Tris acetate phosphate
TBP	Tris bicarbonate phosphate
Ti	Tumor-inducing
Tris	Tri (hydroxymethyl)-aminothane
U	Unit (s)
UTR	Untranslated region
UV	Ultraviolet
μg	Microgram (s)
μl	Microliter (s)
μm	Micrometer (s)
μM	Micromolar (s)

CHAPTER I
INTRODUCTION

Thus far the concept to employ other organisms as a host for the purpose of better yield and quality of the required expression product has been available as the advance in recombinant technology as well as the engineering technique. A number of protein expression systems such as bacteria, yeast, animal cell lines and plants are available, each of which offers different characteristics in terms of protein yield, ease of manipulation and cost of operation [1]. Bacterial and yeast systems are still the popular choice in many applications as their short duplication time and mass-production performance. However, these systems are not suitable for some eukaryotic proteins that require post-translational modifications as they show inability to produce the properly folded functional molecules, and poor yields of complex proteins [2]. Protein production in animal or human cell lines offers the better folding and assembling of complex molecules however they require high capital costs [3]. The plant system, on the other hand, is economical for mass production and low risk of toxin contamination but it still has several shortcoming including the lengthly production time consume and gene flow by pollen to the native species [4].

Owning to the achievement in delivering transgenes into plant chloroplast genomes [5], the field of chloroplast genetic engineering has developed rapidly. In general, expression of foreign genes in the chloroplast genome provides several advantages over that of the nuclear genome. The position effect as well as gene silencing, the common phenomena in the nuclear transgenic, can be avoided as the transgenes are inserted specifically *via* homologous recombination process and it has been no report of gene silencing in the chloroplast system [6]. In addition, chloroplasts have the ability to express polycistrons or multiple genes in a single transformation event. Moreover, in the perspective of biosafety, the chloroplast expression system is an environmentally friendly approach based on the fact that the chloroplast DNA is

eliminated during pollen maturation and is not transmitted to the next generation [7]. These benefits make the chloroplast system to be a promising bioreactor.

Cry4Ba, one of the four major insecticidal proteins produced from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*), is specifically toxic against mosquito larvae such as *Aedes* and *Anopheles* species that are the vectors of widespread deadly contagious diseases, including dengue-viral hemorrhagic fevers, viral encephalitis and malaria [8-9]. The achievement of over-expression of the recombinant *Bti*-Cry4Ba protein was reported in *Escherichia coli* [10]. A further effort was made until that time to manipulate this toxin into the cyanobacterial host, which exists in an environment akin to that of the target larvae [11]. Although the bacterial system holds some potential for producing this insecticidal protein, the expensive cost of industrial-scale production, particularly with regard to bacterial growth media, still hinders wide-scale usage of *Bti* toxins.

Unicellular photosynthetic micro-algae *Chlamydomonas reinhardtii*, on the other hand, offers a compromise between the bacterial and plant systems in terms of production cost and timescale based on the fact that they use natural resources of light and CO_2 for photosynthetic growth and propagate by cell division in approximately every eight h [12]. Moreover, the time spent from initial transformation to large-scale production is surprisingly short, approximately six months [13]. These attractive characteristics as well as benefits of the chloroplast system of *C. reinhardtii* should facilitate for expressing the mosquito-larvicidal *Bti-cry4Ba* gene.

In order to construct the transformation vector harboring *cry4Ba* gene to be expressed in the chloroplast of *C. reinhardtii*, controlling sequences of an algal strong promoter have to be first considered. It is interesting to note that the translation of many chloroplast genes can be stimulated by light as the highest level of light induction has been reported for the photosynthetic *psbA* gene-encoded D1, a core protein of photosystem II [14]. Together with the fact that, promoter/5 UTR plays an important role in defining the levels of accumulation of recombinant proteins, whereas the identity of the 3 UTR has little or no effect [15]. Therefore the light-enhanced promoter in combination with its analogous 5'UTR sequence was selected to investigate the pliability of *C. reinhardtii* chloroplasts as biological factories capable

of producing the biological control agent of prokaryotic *cry4Ba* gene product. Hence, objectives of this study were :

1. Construction of the chloroplast transformation vector containing the *Bti-cry4Ba* transgene under control of promotor/5'UTR and terminator of the photosynthetic *psbA* gene
2. Transformation of *C. reinhardtii* chloroplasts with chloroplast transformation vector coated Gold particles using the bombardment technique
3. Transcription and translation analyses of the *Bti-cry4Ba* transgene in the transgenic chloroplast of *C. reinhardtii*

CHAPTER II
LITERATURE REVIEW

2.1 General background of *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii is a single-celled green alga about 10 µm in diameter. This alga is the member of *chlamydomonadaceae* family which belongs to *Chlamyomonas* genus. For ages, scientists have identified species of *Chlamydomonas* based solely on morphological criteria which generally has two anterior flagellae, one or more pyrenoids within a basal chloroplast and a distinct cell wall [12] (**Figure 2.1**).

A single cup-shaped chloroplast occupies the basal two thirds of the *C. reinhardtii* cell and contains a distinctive body of pyrenoid where the CO_2 fixation and the dark reaction of photosynthesis occur. The 203-kb circular structure of approximately 100 copy numbers of the *C. reinhardtii* chloroplast genome possesses two copies of a 22-kb inverted repeat (IR) sequence separated by two nearly equally sized unique regions of 80 and 78 kb [16]. Wild-type *C. reinhardtii* is easily grown in defined liquid or agar media at neutral pH without the requirement of supplementary vitamins or other co-factors. The average doubling time is approximately 6-8 h under the light intensity of 50-200 µmol photons m^{-2} s^{-1} and culture temperature of 25 °C. The sexual cycle of *C. reinhardtii* occurs through the fusion of two different haploid mating types, designated plus (mt^+) and minus (mt^-), into a diploid zygote which can normally sustain drastic conditions, especially nitrogen deprivation [17] whereas in the plentitude condition, the diploid zygote would reverse to four haploid vegetative cells by meiosis (**Figure 2.2**). Unlike the nuclear genome, cell division *via* meiosis of chloroplast DNA is regularly transmitted to progeny only from mt^+. This non-Mendelian behavior was initially described as maternal inheritance [18].

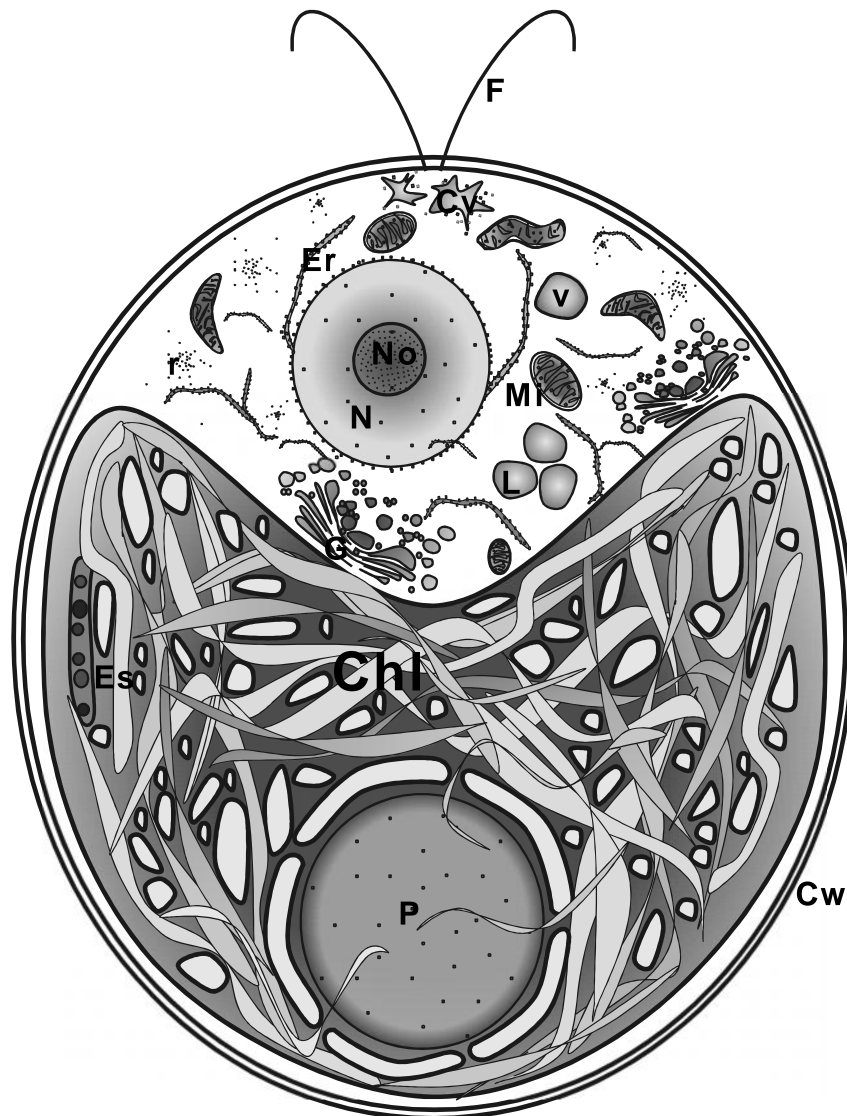


Figure 2.1 An interphase *Chlamydomonas* cell (modified from [12])

Cell length, 10 µm; Chl, chloroplast; Cv, contractile vacuole; Cw, cell wall; Er, endoplasmic reticulum; Es, eyespot; F, flagella; G, Golgi apparatus; L, lipid body; Mi, mitochondria; N, nucleus; No, nucleolus; P, pyrenoid; r, ribosomes; S, starch grain; v, vacuole.

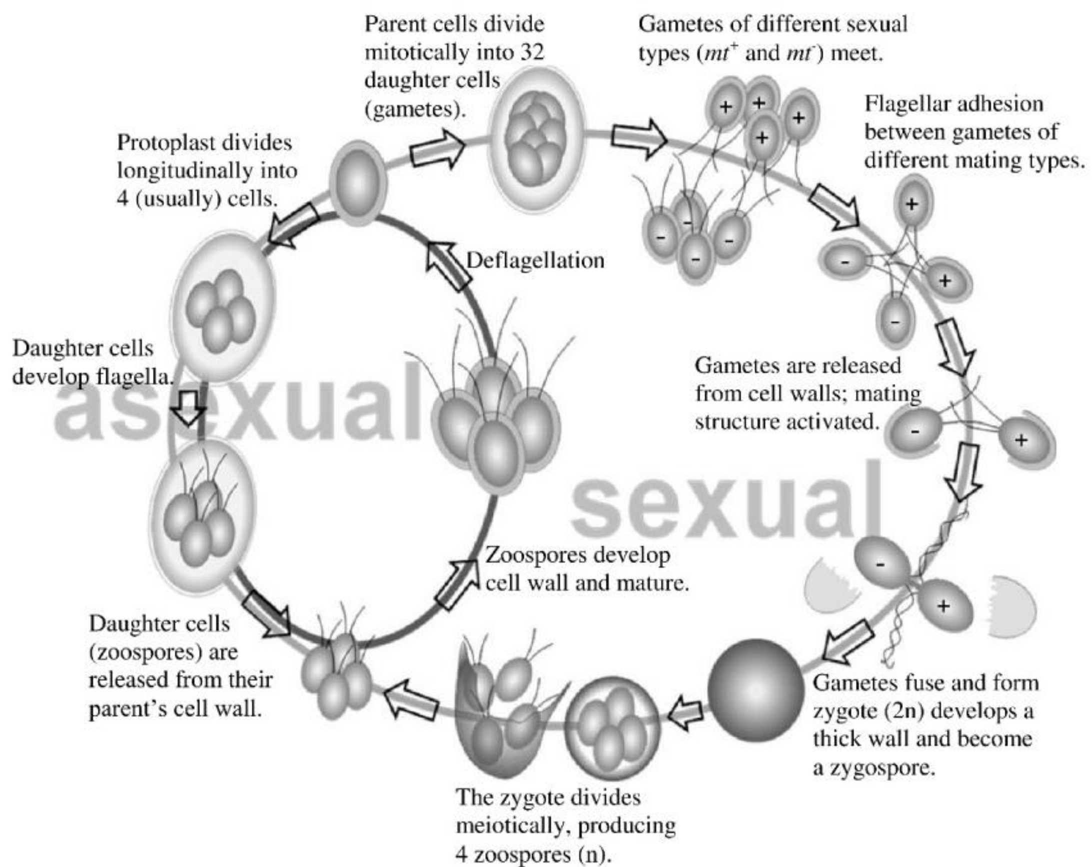


Figure 2.2 ***Chlamydomonas*** **life cycle** (modified from [12] and http://www.metamicrobe.com/chlamy)

In the plentitude condition, the vegetative haploid cells reproduce asexually by fission which the protoplast divides to form 4-8 zoospores similar to the parent. Under conditions of nitrogen starvation, vegetative cells of each mating type, mt^+ and mt^-, develop into gametes before they are attracted to each other. Cell walls are lysed and the mating structure is generated. Fusion of sex specific structure between fertilization tubule of mt^+ and the mating structure of mt^- begins at the anterior ends of the cells and continues laterally from anterior to posterior. The newly formed diploid zygote is not flagellated and serves as a dormant form of the species in the soil. Once the environment was suitable to grow, zygote undergoes meiosis to form 4 haploid zoospores.

C. reinhardtii offers attractive characteristics that make it the most widely used as the laboratory research organism such as: (i) The ability to grow phototrophically or heterotrophically, utilizing acetate as a carbon source. (ii) The ability to induce gametogenesis and carry out genetic crosses between haploid cells of opposite mating types. (iii) There is a variety of selectable markers for nuclear and chloroplast transgenic lines that could be easily observed as colonies on selectable solid medium. (iv) Both nuclear and chloroplast genomes have been fully sequenced, providing a wealth of information for transformation of non-native genes as well as manipulation of endogenous genes [19]. Thus far, *C. reinhardtii* has been used to elucidate aspects of photosynthesis, phototaxis, flagella assembly, cell wall biogenesis, gametogenesis, cell cycle events, mating process and bioreactor for transgene expression [20, 21].

2.2 The genetic transformation of *C. reinhardtii*

The nuclear transformation has become a routine technology after the tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* capable of delivering foreign genes into the plant nucleus was achieved [22]. Although nuclear transformation by *Agrobacterium* Ti-plasmid has been demonstrated to work with *C. reinhardtii* [23], using vortex-mixing technique with DNA-coated glass beads or silicon carbide whiskers is preferable as it requires simple equipments and inexpensive supplies [24]. The highest frequency of glass bead transformation could be obtained by using the cell wall deficient strain or the wild-type strain whose cell walls were enzymatically removed by autolysin or the gamete lytic enzyme [24, 25]. In *C. reinhardtii*, the transformation efficiency of nuclear transformation *via* glass bead procedure could yield up to 10^3 transformants per 1 μg of DNA however the higher yield could be obtained by using the electroporation procedure that showed the maximum yield of 2×10^5 transformants per 1 μg of DNA [26]. Unfortunately, the nuclear transformation had a serious drawback by low consistency of transgene expression level due to the position effect as well as epigenetic gene silencing [7].

The chloroplast transformation was first achieved in the *Chlamydomonas* by employing the Tungsten or Gold particles as carriers coded with the plasmid DNA.

The DNA-coated particles are delivered into the target organelles *via* high pressure helium gas using bombardment mechine [5]. It is interesting to note that chloroplast transformation frequency could be maximized by growing cells for several generations in medium containing 5-fluorodeoxyuridine (FdUrd) prior to perform a bombardment. Given that FdUrd-treated cells would decrease the chloroplast DNA copy number, the lower of genetic materials might therefore increase the proportion of transgenic chloroplast genomes [27]. Integration of transgene specific to the chloroplast genome provides additional attractions over targeting into the nucleus genome including the precision transgene integration through homologous recombination process, the ability to perform polycistronic transcripts and the absence of silencing mechanisms as well as from glycosylated post-translation modification [28]. The early attempts to express foreign genes in the *C. reinhardtii* chloroplast involved the use of reporter genes and antibiotic resistance genes for transformant selection. The further studies were focused on expression of pharmaceutical and therapeutic-important proteins including antibody and vaccine. **Table 2.1** showed examples of recombinant proteins in each application field producing in the *Chlamydomonas* chloroplast.

Table 2.1 Recombinant proteins produced in the *C. reinhardtii* chloroplast (modified from [29, 21])

Gene expressed	Application/Comment
Aminoglycoside adenine transferase [30]	Reporter, confers spectinomycin and streptomycin resistance
-Glucuronidase [31]	Reporter, catalyzes the conversion of substrates to colored products
Renilla luciferase [32]	Reporter protein, luminescent
Aminoglycoside phosphotransferase [33]	Reporter, confers kanamycin and amikacin resistance
Green fluorescent protein [34]	Reporter protein, fluorescent
HSV8-lsc [35]	Pharmaceutical, first mammalian protein expressed
83K7C IgG1 [36]	a full-length human IgG1 monoclonal antibody, directed against anthrax protective antigen 83
Cholera toxin B subunit fused to foot and mouth disease VP1 [37]	Pharmaceutical, vaccine
White spot syndrome virus protein 28 [38]	Vaccine
Bovine mammary associated serum amyloid [39]	Therapeutics, oral delivery
Blood sugar level-regulating hormone, type I diabetes treatment [40]	Therapeutics, proinsulin

2.3 Selectable markers for transformation of the *C. reinhardtii* chloroplast

Marker genes used in the transformation processes aim to give a selective advantage to the transformed cells and to eliminate the non-transformed cells. The initial strategy of marker genes for the chloroplast transformation was based on the rescue of chloroplast deletion mutants using wild-type DNA fragment. Photosynthetic *atpB* gene encoded the chloroplast ATP synthase was knockouted and the wild type *atpB* gene was used to restore the defect under a photoautotrophic condition [5]. Mutant lines could be viable by growing in enrich TAP medium containing acetate as a carbon source. Positive lines with recovered photosynthetic activity could be simply screened by growing the transformed algae in the minimum HS medium without any carbon source [41].

The requirement of photosynthetic mutant host for the previous scheme was overcome using the antibiotic resistant scheme. This scheme employed mutations in 16s RNA (*rrnS*) which provided resistance to spectinomycin and streptomycin, or in the gene for 23s RNA (*rrnL*) which provided resistance to erythromycin [42]. Nevertheless the rDNA mutation for the antibiotic resistance had an important drawback by its spontaneous mutation that appeared with a frequency similar to the genuine transformants [43]. Another limitation of this strategy was due to the integration site of genes of interest that had to be located close to the locus of the rDNA marker if they were harbored by the same transformation vector [44].

Further improvement based on the transgenic expression of the antibiotic resistant genes controlled by the chloroplast-specific expression cassette was developed. The first chloroplast-specific antibiotic resistance marker originated from bacterial aminoglycoside adenine transferase (*aadA*) gene, conferring resistance to a variety of antibiotics including spectinomycin and streptomycin [30]. The AadA protein catalyzes the covalent transfer of an AMP residue from ATP to spectinomycin consequently converting the antibiotic into an inactive form of adenylylspectinomycin that no longer inhibits protein biosynthesis specific on 30S subunit of 70S ribosomes as present in the chloroplast [30, 45]. In the experiment by Minagawa and Crofts [46], another photosynthetic gene, *psbA*-encoded D1, a core protein of photosystem II [14], was employed together with the spectinomycin resistant *aadA* gene for selection of

Chlamydomonas chloroplast transformants. It is interesting to note that the secondary screening *via* photosynthetic complementation offered an important helpful in ruling out the spontaneous mutation possibly induced by frequent drug screening [47].

Differences in various properties such as dominance, cell-autonomy and portability could be used to categorize marker genes. Each property conferred the different advantages and drawbacks. Some markers are dominant including *aadA* gene which provides the resistance against spectinomycin and streptomycin by directly inactivating antibiotics, while others are recessive such as the point mutation of *rrnS* or *rrnL* that provides resistance against various antibiotics by relieving the binding sensitivity of antibiotics on individual rRNA [44]. It is interesting to note that dominant markers give the higher transformation frequency than recessive ones based on the fact that the presence of dominant markers in a minority of chloroplast genomes is adequate to confer an effect at early stages of selection. On the other hand, the recessive markers confer the resistance only when the random segregation of chloroplast genomes harbor enough copies of transformed plastid for the selectable phenotype to emerge [44]. Another property is the cell-autonomous, for example the antibiotic resistance *rrnS* or *rrnL* genes, which confer the selectable phenotype only to the cell in which they occupy. In contrast, inactivation of antibiotics by the translation product of genes such as *aadA* gene offers protection not only to the cells they reside but also to neighboring cells by diminishing the effective concentration of antibiotics [44]. The *rrnS* and *rrnL* genes are also classified as markers that have to integrate in a specific locus of the chloroplast genome, while others are portable and can be inserted in any locus of the chloroplast genome such as the *aadA* gene driven by chloroplast expression signals (promoter, 5'UTR and 3'UTR) [44].

2.4 Regulation of gene expression in the *C. reinhardtii* chloroplast

In plant and algae, chloroplast protein accumulation was generally regulated by transcription [48], RNA processing [49], mRNA stability [50], protein turnover [51], protein toxicity [38] and by the translation step which has been reported as the key rate-limiting step [52, 53]. To enhance the chloroplast transgene expression, many reports have shown the importance of *cis*-acting elements such as promoters and

5'UTRs which drive efficient transcription and translation [15, 31]. On the other hand, 3'UTRs had only a minor influence on transgene expression, but they appeared to be required for transcription termination and mRNA stabilization [15, 54]. In *C. reinhardtii*, the promoter/5'UTRs generally used for chloroplast transgene expression were derived from the endogenous *atpA*, *rbcL*, *psbA* and *psbD* genes [55] as well as from the exogenous *lac* operon system from *E. coli* [56]. The 5'UTRs of photosynthetic genes including *psbA* and *psbD* offered a particular attraction over the others as they could enhance the transgene expression level by light [15]. Unlike eukaryotic system, in the *C. reinhardtii* chloroplast, the role of conjugated polyadenylation or poly(A) tails was only recognized as an instability determinant resulting in degradation of RNA and subsequent failure in transcript accumulation [54]. Interestingly, it has been reported that the higher expression level of recombinant genes could be achieved if they were fused with the coding sequences including the ATG start codon of the endogenous gene [57]. However that utility was not generalized and remained to be clarified owing to an adverse effect as reported by Michelet and co-workers [58].

It is interesting to note that the anterograde signaling, the nucleus encoded protein that is specifically targeted to the individual chloroplast gene or small subsets of genes, also plays an important role in controlling the chloroplast gene expression [59, 60]. In *C. reinhardtii*, the nucleus-encoded Nac2 chloroplast protein specifically required for the stable accumulation of chloroplast *psbD* mRNA encoding the D2 reaction center protein of PSII is of particular interest. Application of Nac2 was performed by conjugating with the inducible promoter which acted as a switch therefore the activation of PSII could be transiently turned off, and as a consequence generated anaerobic condition suitable for hydrogen production [61]. In addition, this scheme has been utilized if the transgene expression product was toxic to the host cell which was difficult to recover [38]. Another example demonstrates the application way to enhance the *C. reinhardtii* chloroplast transgene expression by mutating the anterograde signaling that exhibits the negative feedback regulation in the chloroplast [58]. This application based on the fact that the chloroplast encoded *psaA* gene, composed of three exons which were transcribed independently as precursors, was required the nuclear encoded factors for assembling *psaA* mRNA during splicing. The

nuclear-mutant strains defective in splicing of these separated precursors of chloroplast *psaA* mRNA showed over-accumulation of the *psaA*-exon1 precursor [62, 63]. The splicing deficient mutant therefore could apply to increase in the translation product of recombinant gene expressed under the promoter/5'UTR of *psaA*-exon1 [58].

2.5 General background of *Bacillus thuringiensis*

Thus far, the agricultural insect pests and the insect vectors of important human diseases have been mainly controlled by using chemical insecticides. However, the excessive usage of these synthetic insecticides has led to several problems, including effects on agroecosystem and environment, *i.e.*, beneficial or non-target species and on the adverse effects in human health, *i.e.*, cancer and several immune system disorders [64]. The bioinsecticides on the other hand provide high specificity, efficiency, environmental friendliness and lack of harmful side-effects [65].

Bacillus thuringiensis (Bt), an entomopathogenic Gram-positive bacterium has been used successfully as a biopesticide for many years. Spores of *Bt* can be isolated from a wide variety of environments including fresh water, soil, the rhizosphere, the phylloplane, grain dusts and insects, crustaceans, annelids and insectivorous mammals [66, 67]. *Bt* had been first discoverd in 1901 by the Japanese biologist, Shigetane Ishiwata, who isolated and identified this bacterium as the causal agent for a silkworm disease before Berliner discovered the insecticidal activity in 1911 [68]. Many years later, commercial productions and the use of *Bt* as a spray formulation for insect control had established in France, and since 1958, the first commercial *Bt* formulations were available for field testing in the USA. Until the 1970s, it was generally agreed that the insect targets of *Bt* were only order *Lepidoptera* (moths and butterflies) [69]. In 1995, the market volume of *Bt* formulae was at an estimated 90 million US dollars and 67 formulas were registered worldwide. Bioinsecticides received more and more attention from many research groups especially those from *Bt* as shown tendency to rise up 90% of all biopesticides [70] and 2% of the total insecticidal market [71].

During sporulation stage, the lysis of *Bt* cells would release spores and inclusion bodies which act as poisons [9]. When *Bt* cells were engulfed and digested by susceptible insect larvae, toxins from the lysis cells will damage epithelial cells of the insect gut. It is generally believed that these toxins act by creating pores in the cell membrane [72]. The toxin proteins accumulated as crystal inclusions were specific toxic to many types of insect larvae [74]. Toxicity of all *Bt* strains covers a wide range of insect host larvae including the orders *Diptera* (mosquitoes and files), *Lepidoptera* (moths and butterflies), *Coleoptera* (beetles and weevils) and *Hymenoptera* (wasps and bees). [65, 74]. Some of them are also toxic against *Homoptera*, *Orthoptera*, *Mallophaga* and *Nematodes* [75].

2.6 Classification and nomenclature of *Bt* -endotoxins

Bt synthesizes insecticidal crystal proteins (ICPs) in the cytoplasm. These crystal proteins comprise of two gene families, Cry (crystal -endotoxins) and Cyt (cytolysins) distinguished by amino acid sequences [65]. The classification of Cry proteins was originally based on the range of specific target larvae [76]. However due to the increase in the number and variety of Cry proteins, this classification was abandoned for the current system based on amino acid sequence similarity [65]. The better illustration for the large number of expected new Cry species was done by changing the original Roman to Arabic numerals in the primary rank [65]. For example, the 130-kDa CryIVB toxin, which is produced by *Bt* subsp. *israelensis* and specifically toxic to the *Aedes* and *Anopheles* mosquito-larvae was reclassified as Cry4Ba [77]. The available of evolution relationship between each *cry* gene and their phylogenetic interaction provides interesting insights into relationship between the sequence divergence of active toxins and the extensive range of target specificity [78]. To date, more than 500 different sequences of *cry* genes have been classified into 67 groups (Cry1-Cry67) [79].

2.7 Mode of action of *Bti*-Cry toxins

Bt subsp. *israelensis* (*Bti*) has been used worldwide for controlling mosquitoes. Crystal inclusions of *Bti* toxins are principally formed by Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa providing the specific toxicity against many species of mosquito larvae, *i.e.*, *Aedes* species (vector of dengue and yellow fever), *Culex* species (vector of West Nile virus and filariasis) and *Anopheles* species (vector of malaria) [8, 9, 80]. Interestingly, these four *Bti* toxins have a synergistic effect or the phenomena that the toxicity of the whole *Bti* crystal inclusion is much higher than that of each of the Cry and Cyt proteins in this crystal [81]. After *Bti* crystal inclusions are ingested by susceptible mosquito larvae, they are dissolved in the alkaline environment of larval gut prior to release the soluble proteins. In the case of the Cry4Ba, N-terminal, C-terminal and intra-molecular portions of protoxin were cleavage resulting in an active toxin comprised of 18- and 46-kDa protein fragments which remain associated and retain toxicity [82].

The insecticidal activities of the *Bt*-Cry toxins are proposed to involve several steps that begin with the solubilization of the crystalline inclusions in the midgut of susceptible larvae, followed respectively by proteolytic processing of the protoxin by midgut proteases, binding of the active toxins to the midgut receptors, a conformational change, insertion of the toxin into the apical membrane and oligomerization of the inserted toxin to form lytic pores. Three different models of mode of action of *Bt*-Cry were proposed. The first model was proposed by Knowles and Ellar [83] in 1980s, explained that after binding with the receptor, Cry toxins inserted into membrane or perturbed the plasma membrane to generate small pores. Generation of these pores led to the influx of ion and water following by cell swelling and cell lysis (colloidal-osmotic lysis), respectively. This model was furthered modified by Bravo and co-workers [84] focusing on receptor binding steps in which the toxin molecules were bound the epithelial membrane *via* a cadherin-like protein. After binding with the receptor, conformational change in the toxin molecules was occurred leading to toxin activation *via* N-terminal removal and formation of the oligomeric toxin structure. Subsequently, toxin oligomers bound to APN (aminopeptidase N) prior to concentrate in lipid rafts, where toxins inserted their structure into the membrane to generate pores. Recently, it has been reported that APN

might possible act as a Cry4Ba receptor identified by the physiological effect of RNA interference knockdown of APN transcripts in *Aedes aegypti* larvae [85]. In contrast to the first model, Zhang and co-workers proposed that that after binding with the receptor, cell lysis caused by Cry toxins is occurred due to triggering of adenylyl cyclase/protein kinase A cascades prior to destabilize the cytoskeleton and ion channels [86]. The last model proposed by Jurat-Fuentes [87] would be the combination between the first and the second models that toxicity of Cry toxins were mediated *via* both colloidal osmotic lysis and signal transduction cascades after binding of the Cry toxin to its receptor.

Up to now, the precise structural feature of a Cry toxin-induced pore is not clearly elucidated. Thus far, the most well-known description for the membrane-insertion and pore-formation stage of Cry toxin has been known as the "umbrella-like" model [88, 89]. In this model, only 4 and 5 inserted into the lipid membrane while the remain helices spread over the membrane surface like the opening of an umbrella [90]. This mechanistic model has been supported by substantial evidence involving the role of both 4 and 5 during pore formation. For instance, 4 plays a part in ion conduction which generally forms as the layer on the inside surface of the pore lumen [91, 92]. On the other hand, 5 is hydrophobic which could take part in toxin-pore oligomerization [93, 94].

Until very recently, the update evidence about the fuctional significance of 4- 5 loop particularly at the residue Asn166 located within Cry4Ba pore forming domain have been elucidated (**Appendix 1**). Important factor required for the larvicidal and biochemical characteristics function of Cry4Ba residue 166 was the polarity but not charge *per se* which had been previously reported by Kanintronkul Y. and co worker [95]. It was clearly signified that the specific substitutions of Asn166 with polar-uncharged or charged residue such as Gln, Cys, Asp or Arg could retain high toxicity against the mosquito larvae, whereas replacement with the hydrophobic amino acid, Ala and Ile for example, almost totally abolished the larvicidal activity [95]. Substantial evidence has preferentially supported the vital of hydrophobicity of residue 166 within Cry4Ba by the study of ion channel characteristics using planar lipid bilayer. The assay clearly indicated that the ion permeation was found to be reduced in N166A, and N166I mutants, where as in N166D mutant could retain the

property near the WT level [96]. Additionally, observation of the molecular dynamic of toxin pore opening using the VMD software provided the supported evidences showing that the trimeric pores of the mutants which were substituted by hydroprobic residues of Ala and Ile did not open up, while that of N166D opened more but still less than that of the wild type [96].

2.8 Application of *Bt*-Cry toxins in agriculture and forestry

Thus far, applications of *Bt* toxin have been focused directly on controlling the agricultural and forest pests offering an important breakthrough in decreasing the exploitation of the chemical insecticide [27]. Most of *Bt* products are based on spore-crystal preparation of a few wild-type strains. Important strains for the direct application to control leaf-feeding *lepidopteran* crop pests or forest defoliators were *Bt* subsp. *kurstaki* (*Btk*) HD1 expressed Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa proteins or *Btk* HD73 that expressed the Cry1Ac protein, *Bt* subsp. *aizawai* HD137 that expressed slightly different Cry toxins such as Cry1Aa, Cry1B, Cry1Ca and Cry1Da proteins, *Bt* subsp. *san diego* and *Bt* subsp. *tenebrionis* that expressed the Cry3Aa protein [97-98].

The study fields of genetic engineering were developed rapidly after a new technology of transgenic plants had been achieved, offering the ability to produce insecticidal toxins continuously in *Bt*-plants or *Bt*-crops and also protected toxins from UV degradation [99]. Presently, more than 40 million hectares of *Bt*-crops including the important economic plants such as soya, corn, cotton and canola were grown world-wide resulting in a significant reduction on the use of chemical insecticides [100, 101]. However, it is not to say there have not been problems with the technology. The initial transgenic plants, delivered by the recombinant toxin genes into the nuclear genome, produced low levels of Cry proteins. This problem however was solved by changing the nucleotide sequences of *cry* genes to optimize their expression with codon usage of plants [102]. Attempts were also made to engineering *Bt-cry* gene into plant chloroplast genomes due to the growing public concern of genetic pollution *via* pollination. The first achievement was demonstrated by the Cry1Ac insecticidal protein with much higher expression levels than nuclear

transgenic plants [103]. In the meantime, several *Bt*-Cry toxins have been expressed in plant chloroplasts with a similar high production level as shown more than 10% in tobacco [104] and cabbage [105]. The protein accumulation could be up to 45% of the total soluble protein and remained stable even in senescing leaves as shown in tobacco chloroplast expressing *Bt-cry2Aa2* with a putative chaperonin in the same operone [106].

2.9 Application of *Bti*-Cry4Ba toxin in control of human-disease carrying mosquitoes

Owning to an advancement in *Bt*'s molecular biology particularly of the complete sequence of the *cry4Ba* gene (Genbank accession number of X07423 [107]), this tools were used not only to overcome the low efficacy and short half-life in nature of *Bti* but also to improve the expression system of Cry4Ba toxin *via* engineered microorganisms [88]. For controlling human-disease carrying mosquitoes, *i.e.*, the *Aedes*, *Culex* and *Anopheles* species, toxin products were based on *Bti* such as VectoBac® and Teknar® which were mainly composed of Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa toxins [108].

As a suitable candidate, *E. coli* had been shown to be the hyper-expression system of the recombinant *Bti*-Cry4Ba toxin that was first reported by Angsuthanasombat and co-workers [10]. An effort was made until that time to manipulate this toxin into the cyanobacterial host which existed in an environment akin to that of the target larvae nevertheless its utility has been limited due to low levels of protein expression [111]. Studies of new strains with improved range of insecticidal activity through combination of genes that did not occur naturally were further investigated. For example, the combined mosquitocidal proteins of *Bti* and *Bacillus sphaericus* (*Bs*) produced in a novel *Bti* strain showed 20-fold more toxic than either of the parental strains to larvae of *Culex* species [109].

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

Ampicillin	Sigma
Cetyltrimethylammonium bromide (CTAB)	Sigma
Coomassie brilliant blue R-250	Sigma
Urea	Merck
Chloroform	Merck
Phenol	Merck
Isopropanol	Merck
Deoxyribonucleotide triphosphates (dNTPs)	Promega
100-bp ladder DNA marker	Sib*Enzyme*
Lamda DNA-*Bst*EII digested	Biolabs
Lamda DNA-*Hin*dIII digested	Biolabs
Standard protein marker	Bio-RAD
Prestained protein marker	Bio-RAD
Spectinomycin	PhytoTechnology Laboratories
DIG probe	Roche
AntiDIG	Roche
CDP-Star	Roche

All other unlisted chemicals and reagents were analytical grade purchased from various suppliers.

3.1.2 Enzymes and accessory buffers

Restriction endonucleases	Biolabs, Promega
RQ1 RNase-free DNase	Promega

Calf intestinal alkaline phosphatase (CIAP)	Promega
T4 DNA ligase	Promega
T4 DNA polymerase	Biolabs
Phusion® high-fidelity DNA polymerase	Biolabs
RNaseOUT™	Invitrogen
SuperScript™ II RT	Invitrogen
Ribonuclease A	Sigma
Lysozyme	Sigma

All restriction endonuclease enzymes and their buffers were commercially provided by specified companies.

3.1.3 Recombinant plasmids

pMU388 [10] contains gene of interest, mosquito-larvicidal *cry4Ba*, from *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) (Genbank accession number: X07423) (**Figure 3.1**).

Plasmid ChT1-2 was constructed for expression of Cry4Ba in the *C. reinhardtii* chloroplast. The vector is designed to offer high-level expression in the chloroplast system by using the promoter/5'UTR of photosynthetic *psbA* gene. Plasmid ChT1-2 contains single fragment of the recombination target, the expression cassettes of *cry4Ba* and anti-drug *aadA* genes (**Figure 3.1**).

Plasmid Ch2T-3 harbors double fragments of recombination targets (left and right recombination fragment) flanking 3 expression cassettes of recombinant photosynthetic *psbA*, *cry4Ba*, and anti-drug *aadA* genes (**Figure 3.1**).

3.1.4 Bacterial and algal strains

E. coli, JM109 strain [*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17*(r_k^-, m_k^+), *relA1*, *supE44*, *(lac-pro AB)*, (F' *traD36 proAB* $lacI^q$ (lacZ)M15)], was ordered from Promega.

C. reinhardtii, wild-type CC400 and photosynthetic *psbA* mutant CC744 strains, were ordered from the Chlamydomonas Resource Center; http://www.chlamycollection.org.

E. coli was used for general DNA manipulation whereas the wild-type and mutant algal strains were respectively used as the experimental control and the expression host for the recombinant genes.

3.1.5 Synthetic oligonucleotide primers

All synthetic oligonucleotides served as primers were purchased from Sigma-Aldrich (www.sigmaaldrich.com). Primers for site-directed mutagenesis and those for construction and verification are shown in **Table 3.1** and **Table 3.2**, respectively.

3.1.6 Luria-Bertani (LB) medium

One liter of LB broth contains 10 g peptone, 5 g yeast extract and 10 g NaCl. LB agar was prepared by adding 15 g bacto-agar in one litre of LB broth. The medium was sterilized *via* autoclaving at 121°C, 15 psi for 15 min. For selective medium, ampicillin was aseptically added to 100 μg/ml final concentration respectively at 45-50°C.

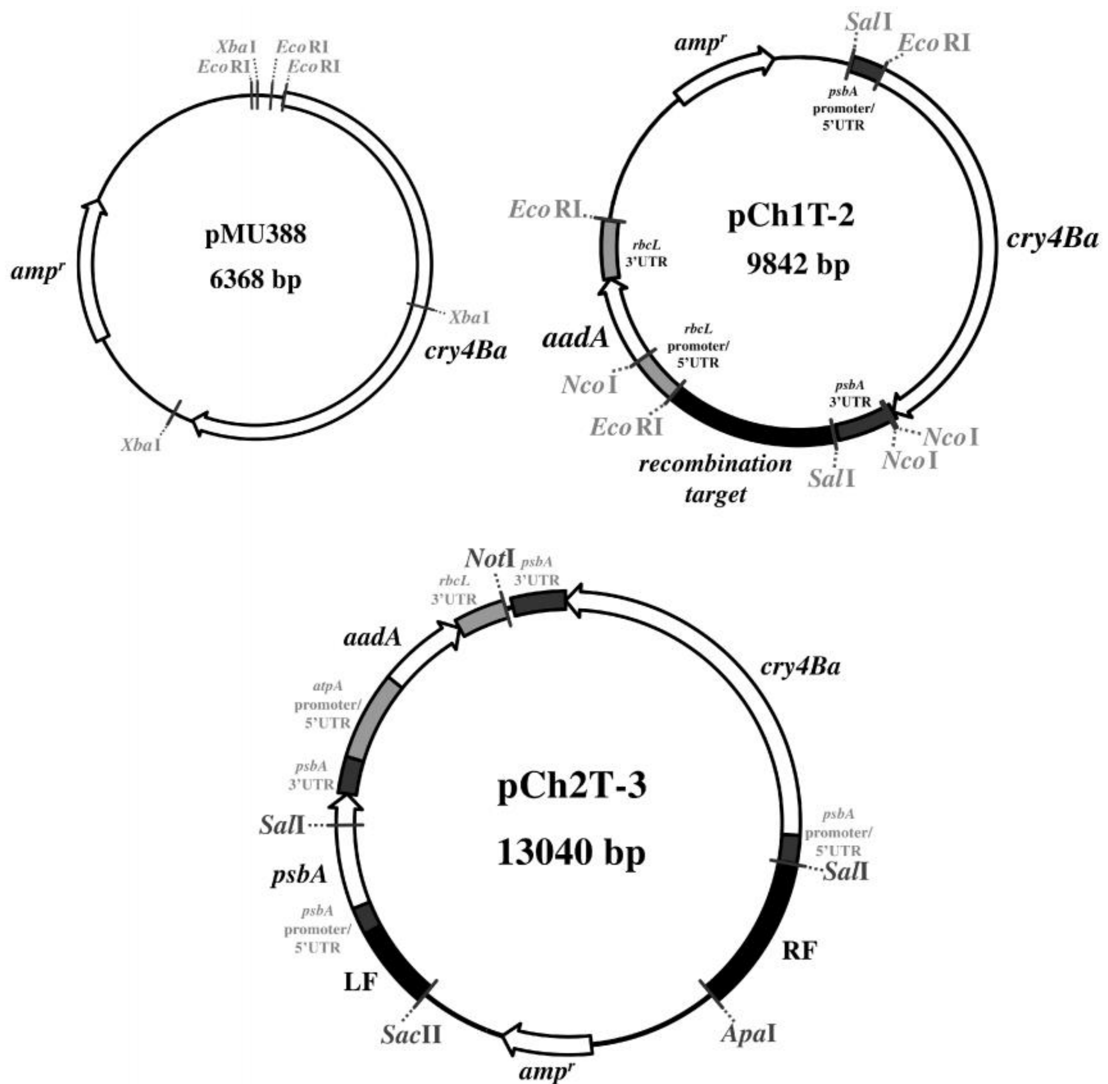


Figure 3.1 Physical maps of recombination plasmids

Schematic diagrams represent the recombination plasmid pMU388, pCh1T-2 and pCh2T-3. The *aadA* and *psbA* genes are selectable markers and *cry4Ba* is the gene of interest. The orientation of each gene is indicated by arrows. The independent homologous regions, left recombination fragment (LF) and right recombination fragment (RF), are indicated by black bars. The ampicillin resistance gene is indicated by *amp*^{r}. Relevant restriction enzymes introduced in this study are shown.

Table 3.1 Mutagenesis primers [a]

Primer	Original	Sequence
4BΔ*Xba*I-f	*T*	5'-GAGTTTCCAGAGGAACAAC-3'
4BΔ*Xba*I-r		3'-GTTCCTCAAAGGTCTCCTT-5'
PUC18ds-f	*AATT*	5'-GAGCTCGTCGACGTAATCATGG-3'
PUC18ds-r		3'-GGCTCGAGCAGCTGCATTAG-5'

Table 3.2 Construction and verification primers [b]

Primer	Site	Sequence
RF-f	*Sal*I	5'-GGCCGTCGACGCTAGCAATATCTGATGGTAC-3'
RF-r	*Apa*I	5'-GGCCGGGCCCGAATCTCAGTTCTAGTGCTAG-3'
LF-f	*Sac*II	5'-GCCCCGCGGACGTTAGTCGATATTTATACAC-3'
rbcL-3'UTR-r	*Sac*II *Not*I	5'-CCCCGCGGTGGCGGCCGCTC-3'
L1		5'-CTAATGTCAAATCAATCTGTAAATGC-3'
L2		5'-GCTCTAGATTAGTTGTTTGAGCTAGAG-3'
R1		5'-TCACTCGTTCATGCAAATTAATTCAATGC-3'
R2		5'-CTTGGTGCTCTTTGCTCAGTTGGAC-3'
aadA-f		5'-AAGTCACCATTGTTGTGCACG-3'
aadA-r		5'-GACGGGCTGATACTGGGC-3'
DII-f		5'-CGGAATTCGGAGGAATAAATATGTACCCTGCGGACAAAATAGATAATACG-3'
DII-r		5'-TTGATTATTGGGTCGACTTATTAATGTGTCCAAGCAAATGAAAC-3'
4B946		5'-GAACATCTCGTGTAAGTGCTGC-3'
4B-ORF		5'-ATGAATTCAGGCTATCCGTTAGCG-3'
psbA-f		5'-ATGACAGCAATTTTAGAACG-3'
psbA-r		5'-TTAGTTGTTTGAGCTAGAGT-3'

[a] Highlighted letters indicate mutated nucleotides from the original ones written above.

[b] Relevant restriction endonuclease sites are underlined and designated above

3.1.7 High-salt (HS), tris-bicarbonate-phosphate (TBP) and tris-acetate-phosphate (TAP) media

HS and TBP media were used as a minimal medium whereas TAP medium was used as an enriched medium, respectively. To make each medium, the following stock solutions have to be prepared:

- **Beijerinck's solution** (1 L)

NH_4Cl (MW. 53.49) 10 g

$MgSO_4 \cdot 7H_2O$ (MW. 246.47) 0.4 g

$CaCl_2 \cdot 2H_2O$ (MW. 147.02) 0.2 g

Autoclaving

- **Phosphate solution**, pH 7.0 (1 L)

KH_2PO_4 (MW. 136.09) 29.6 g

K_2HPO_4 (MW. 174.18) 57.6 g

Without adjusting, the pH should be 7.

Autoclaving

- **TAP salts** (1 L)

NH_4Cl (MW. 53.49) 15 g

$MgSO_4 \cdot 7H_2O^*$(MW. 246.47) 4 g

$CaCl_2 \cdot 2H_2O$ (MW. 147.01) 2 g

Autoclaving

*Tap salts for TBP medium is replace $MgSO_4$ with $MgCl_2$

- **Hutner's micronutrient** (1 L)

The 50 g EDTA was dissolved in about 800 ml distilled water prior to adjusting the pH with KOH to 6.5-6.8. The following trace minerals were added one by one (let each one dissolved completely before adding another one)

H_3BO_3 (MW. 61.83) 11.4 g

$ZnSO_4 \cdot 7H_2O$ (MW. 287.58) 22 g

$MnCl_2 \cdot 4H_2O$ (MW. 197.91) 5.1 g

$FeSO_4 \cdot 7H_2O$ (MW. 278.01) 5 g

$CoCl_2 \cdot 6H_2O$ (MW. 237.93)	1.6 g
$CuSO_4 \cdot 5H_2O$ (MW. 249.69)	1.6 g
$Na_2MoO_4 \cdot 2H_2O$ (MW. 241.95)	0.7 g

Autoclaving (The solution should be pale green and turn purple upon storage.)

To prepare HS medium, one liter of medium contains 50 ml Beijerinck's solution, 25 ml phosphate solution and 1 ml Hutner's micronutrient. The pH can be adjusted to 6.8 by 1N HCl before autoclaving.

To prepare TBP medium, one liter of medium contains 40 ml of 2M Tris-HCl (pH 7.5), 25 ml TAP salts, 1 ml phosphate solution and 1 ml Hutner's micronutrient. The volume was brought to 950 ml and autoclaved. Finally, 50 ml of 0.2-µm filter sterilized 0.5M $NaHCO_3$ was added when the medium cool down.

To prepare TAP medium, 1 liter of medium contains 2.42 g Tris base, 1 ml glacial acetic acid, 25 ml TAP salts, 1 ml phosphate solution and 1 ml Hutner's micronutrient. For TAP agar plate, add 20 g of agar to the medium before volume adjustment and autoclaving.

3.1.8 Plasmid DNA extraction solutions

- **0.5 M EDTA**, pH 8.0 (100 ml)

EDTA·2Na, (MW. 372.24)	18.61 g

The pH can be adjusted to 8.0 by NaOH before autoclaving

- **1 M Tris-HCl**, pH 8.0 (100 ml)

Tris (MW. 121.14)	12.11 g

The pH can be adjusted to 8.0 by HCl before autoclaving

- **5 mg/ml RNaseA** (1 ml)

RNase A	5 mg

The Rnase A solution can be made free of Dnase by boiling for 15 min before storage at -20°C.

- **STET buffer** (50 ml)

1 M Tris-HCl, pH 8.0	2.5 ml
0.5 M EDTA, pH 8.0	1.25 ml
Triton-X100	50 µl
Sucrose	0.4 g

- **10 mg/ml Lysozyme solution** (1 ml)

1 M Tris-HCl, pH 8.0	10 µl
Lysozyme	0.01 g

- **5% (w/v) CTAB** (100 ml)

CTAB (MW. 364.45)	5 g
Autoclaving	

- **1.2 M NaCl** (50 ml)

NaCl (MW. 58.44)	3.5 g
Autoclaving	

3.1.9 Algae total genomic DNA extraction solutions

- **2× Lysis solution** (50 ml)

1 M Tris-HCl, pH 8.0	10 ml
0.5 M EDTA, pH 8.0	0.2 ml
SDS (MW. 288.38)	3 g

- **20 mg/ml Proteinase K** (0.5 ml)

Proteinase K	10 mg

- **5 M NaCl** (50 ml)

NaCl (MW. 58.44)	14.6 g
Autoclaving	

- **CTAB/NaCl solution** (50 ml)

NaCl (MW. 58.44)	2.05 g
CTAB (MW. 364.45)	5 g
Autoclaving	

3.1.10 Algal total genomic RNA extraction solutions

- **ATA solution** (50 ml)

1 M Tris-HCl, pH 8.0	2.5 ml
0.5 M EDTA, pH 8.0	0.5 ml
5 M NaCl	3 ml
ATA (MW. 422.35)	42.24 mg
SDS (MW. 288.38)	1 g

Autoclaving

- **3 M KCl** (50 ml)

KCl (MW. 74.55)	11.18 g

Autoclaving

- **8 M LiCl** (100 ml)

LiCl (MW. 42.40)	33.92 g

Autoclaving

3.1.11 Agarose gel electrophoresis solutions

- **10Î TAE buffer**, pH 8.3 (1L)

Tris (MW. 121.14)	4.85 g
EDTA (MW. 292.24)	930.6 mg
Acetic acid (MW. 60.05)	2.29 ml

The pH can be adjusted to 8.3 by NaOH.

- **Gel-loading dye** (1 ml)

Bromophenol blue	0.01 g
Ficoll 400	1.5 g
0.5 M EDTA	100 µl

- **20× MOPS**, pH 7.0 (1L)

MOPS (MW. 209.3)	83.72 g
Sodium acetate (MW. 82.03)	1.64 g

1 M EDTA 20 ml

The pH can be adjusted to 7.0 by NaOH before autoclaving.

- **RNA loading buffer**, pH 8.0 (1ml)

87% Glycerol 575 µl

1 M EDTA, pH 8.0 1 µl

Autoclaving

- **0.5 M NaOH solution** (1L)

NaOH (MW. 40.01) 20.01 g

3.1.12 *E. coli* competent cell preparation solutions

- **0.1 M $CaCl_2$** (100 ml)

$CaCl_2 \cdot 2H_2O$ (MW 147.02) 1.47 g

Autoclaving

- **0.1 M $MgCl_2$** (100 ml)

$MgCl_2 \cdot 6H_2O$ (MW. 203.30) 20.33 g

Autoclaving

- **SOB medium**, pH 7.0 (1L)

Tryptone 20 g

Yeast extract 5 g

NaCl (MW. 58.44) 500 mg

KCl (MW. 74.55) 186.38 mg

The volume of solution is adjusted to 990 ml before autoclaving

An autoclaved solution is further added by a sterilized 1 M $MgCl_2$ 10 ml

- **TB buffer** (100 ml)

PIPES (MW. 302.40) 302.4 mg

$MnCl_2 \cdot 4H_2O$ (MW. 197.90) 1.09 g

KCl (MW. 74.55) 1.86 g

Autoclaving

3.1.13 Rapid plasmid size screening solution

- **Lysis buffer** (50 ml)

1 M EDTA	250 µl
Sucrose (MW. 342.30)	5 g
SDS (MW. 288.38)	125 mg
1 M NaOH	5 ml
1 M KCl	3 ml
Bromophenol blue	25 mg

3.1.14 Northern blot solutions

- **20× SSC**, pH 7.0 (1L)

NaCl (MW. 58.44)	175.3 g
Sodium citrate, dihydrate (MW. 294.10)	88.2 g

The pH can be adjusted to 7.0 by HCl before autoclaving.

- **Transfer buffer** (1L)

1 M NaOH	10 ml
NaCl (MW. 58.44)	175.32 g

Autoclaving

- **1 M Na-phosphate buffer**, pH 7.2 (100 ml)

1 M NaH_2PO_4 (MW. 137.95)	15 ml
1 M Na_2HPO_4 (MW. 141.96)	70 ml

Autoclaving

- **Modified Church and Gilbert hybridization buffer** (100 ml)

1 M Na-phosphate buffer	50 ml
SDS (MW. 288.38)	7 g
1 M EDTA	1 ml

Autoclaving

- **Hybridization washing buffer I** (1L)

20× SSC	100 ml
SDS (MW. 288.38)	1 g

Autoclaving

- **Hybridization washing buffer II** (1L)

20×SSC	10 ml
SDS (MW. 288.38)	1 g

Autoclaving

- **Maleic acid buffer**, pH 7.5 (2L)

Maleic acid (MW.116.07)	23.22 g
NaCl (MW. 58.44)	17.54 g

The pH can be adjusted to 7.5 by NaOH before autoclaving.

- **Washing buffer** (1L)

Tween 20	3 ml
Maleic acid buffer, pH 7.5	997 ml

Autoclaving

- **Blocking solution** (40 ml)

Skimmilk	4 g
Maleic acid buffer, pH 7.5	40 ml

- **Detection buffer**, pH 9.5 (1L)

Tris (MW. 121.14)	12.11 g
NaCl (MW. 58.44)	5.84 g

The pH can be adjusted to 9.5 by HCl before autoclaving.

3.1.15 Protein isolation and SDS-PAGE solutions

- **2× Lysis buffer** (50 ml)

1 M Tris-HCl, pH 6.8	12.5 ml
SDS (MW. 288.38)	3.5 g
Urea (MW. 60.06)	6 g
99.5 % Glycerol	10 ml

- **10× SDS-PAGE running buffer** (1L)

Tris (MW. 121.14)	30.29 g
Glycine (MW. 75.07)	144.13 g
SDS (MW. 288.38)	10 g

- **Staining solution for acrylamide gel** (1L)

Coomassie Brilliant blue R-250	2 g
Methanol	500 ml
Acetic acid	100 ml

- **Destaining solution** (1L)

Methanol	100 ml
Acetic acid	200 ml

3.1.16 Western blot solutions

- **Wet-blot transfer buffer** (1L)

Glycine	69.06 g
Tris (MW. 121.14)	3.03 g
SDS	400 mg

The volume of solution is adjusted to 800 ml by distilled water. The solution is further added by 200 ml methanol before use.

- **10× PBS**, pH 7.4 (1L)

NaCl	80.0 g
KCl	2.0 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

The pH can be adjusted to 7.4 by NaOH before autoclaving.

- **PBS-T buffer** (500 ml)

Tween 20	0.5 ml
20× PBS	25 ml

3.1.17 Miscellaneous

24-well titration plate	Costar
MJ Mini™ Personal Thermal Cycler	Bio-RAD
Geldoc® system	Bio-RAD
PDF-1000/He particle bombardment system	Bio-RAD
Trans-blot SD	Bio-RAD
RNeasy plant mini kit	QIAGEN
GENECLEAN® II kit	Qbiogene
UV 1601 spectrophotometer	Shimadzu
Blood counting chambers	BOECO
Medical X-Ray Processor 102	KODAK
Amersham Hybond™-N+	GE Healthcare
PROTRAN® nitrocellulose transfer membrane	Schleicher & Schuell
Hybridisation tube and oven	Thermo Hybaid

3.2 Methods

3.2.1 Plasmid DNA extraction from *E. coli* by CTAB method [110]

A single colony of *E. coli* was inoculated into 3 ml LB medium containing 100 µg/ml ampicillin then grown at 37˚C with 200 rpm shaking overnight. Bacterial cell pellet was harvested in the 1.5-ml microcentrifuge tube by centrifugation at 5,000×*g* for 1 min. The pellet was resuspended in 200 µl of STET buffer (8% sucrose, 0.1% Triton X-100, 50 mM Tris (pH 8.0) and 50 mM EDTA) and 5 µl of lysozyme solution (10 mg/ml). The mixture was incubated at room temperature of 5-10 min then boiled for 45 sec and centrifuged at 10,000×*g* for 15 min. After the pellet was removed with sterile toothpick, the plasmid DNA and low molecular weight RNA were recovered from the supernatant *via* addition of 5% CTAB into supernatant fraction to give 1/10 (v/v) of CTAB. The solution was mixed by vortex prior to centrifugation at 10,000×*g* for 5 min. The pellet was resuspended in 300 µl of 1.2 M NaCl and 3 µl of RNaseA *via* vigorous vortex prior to incubating at 37˚C for 30 min. Proteins and plasmid DNA were separated by adding an equal volume of chloroform, mixing and packing *via* centrifugation at 10,000×*g* for 5 min. The aqueous phase was transferred to a new microcentrifuge tube. Plasmid DNA was precipitated *via* adding two volumes of absolute ethanol and kept at -20˚C for 30 min. The mixture was centrifuged at 10,000×*g* for 15 min at 25˚C to pellet the DNA before the DNA pellet was washed with 70% ethanol, air dried and dissolved in 20 µl of sterile distilled water.

3.2.2 Total genomic DNA extraction from *C. reinhardtii* cells

A single colony of *C. reinhardtii* was inoculated into 50 ml TAP medium then grown on a constant-illumination rotary shaker (approximately, 100 µmol photons/m^2·s of light intensity and 100 rpm of rotation frequency) at 25˚C until reach the log-phase state with a density of 2-4×10^6 cells/ml (5-7 days). Cell pellet was harvested in a 15-ml microcentrifuge tube by centrifugation at 5,000×*g* for 1 min then resuspended in 2.5 ml of sterile distilled water and 2.5 ml of 2× lysis solution (200 mM Tris-HCl pH 8.0, 2 mM EDTA and 6% SDS). The solution was added by 30 µl of

20 mg/ml proteinase K and incubated at 25°C for 25 min. Then the solution was added by 1 ml of 5 M NaCl and mixed gently prior to adding 800 µl of CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) and incubating at 65°C for 10 min. Proteins and DNA were separated by adding an equal volume of phenol:chloroform:isoamylalcohol (25:24:1), mixed and centrifuged at 4°C, 10,000×*g* for 5 min. The aqueous phase was kept and purified twice with an equal volume of chloroform as above. DNA dissolved in the final aqueous phase was precipitated by adding two volumes of isopropanol and kept at -20°C for 30 min. The mixture was centrifuged at 10,000×*g* for 15 min at 4°C to pellet the DNA before the DNA pellet was washed with 70% ethanol, air dried and dissolved in 0.5 ml of sterile distilled water.

3.2.3 Chloroplast RNA extraction from *C. reinhardtii* cells

The procedure for RNA extraction was based on the manufacturer's instructions of the RNeasy plant mini kit. The pellet from 50 ml log-phase cell was harvested *via* centrifugation at 5,000×*g* for 1 min. The sample was immediately placed in liquid nitrogen and grinded thoroughly with the mortar and pestle. Cell powder and liquid nitrogen were decanted into the RNase-free 1.5 ml microcentrifuge tube following by evaporating liquid nitrogen. To lyse the sample, 450 µl RLC buffer was added and the sample was mixed vigorously by vortex. The lysate was transferred to a QIAshredder spin column placed in a 2-ml collection tube prior to centrifuge for 2 min at full speed. The supernatant of the flow-through was carefully transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube. The cleared lysate was added by 0.5 volume of absolute ethanol and mixed by pipetting. The sample was then transferred to an RNeasy spin column placed in a 2 ml collection tube prior to centrifuge at 8000×*g* for 15 sec prior to discard the flow-through. To wash the bound RNA, 700 µl of RW1 buffer was added and the column was centrifuged at 8000×g for 15 sec. After discarding the flow-through, the column membrane was washed twice by adding 500 µl RPE buffer prior to centrifuge at 8000×g for 2 sec. The column was placed in a new 1.5-ml microcentrifuge tube and the 30 µl RNase-free water was added to the center of the column membrane. The column was centrifuged at 8000×*g* for 1 min in order to elute the bound RNA.

3.2.4 Total RNA extraction by ATA (Aurintricarboxylic acid)

The pellet from 50 ml log-phase cell was harvested by centrifugation at 3,000×*g* for 1 min. The sample was immediately placed in liquid nitrogen and grinded thoroughly with the mortar and pestle. One drop of -mercaptoethanol and 600 µl of ATA (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 5 mM EDTA pH 8.0, 2 mM ATA and 2% (w/v) SDS) were added to the grinded sample. The melted sample was transferred to a 1.5-ml microcentrifuge tube, added 84 µl of 3 M KCl and mixed by vortex. The sample then was placed on ice for 15 min before cell debris was discarded twice *via* centrifugation at 10000×*g* for 5 min. After the supernatant was transferred to a new 1.5-ml microcentrifuge tube, it was added by 1 volume of 8 M LiCl, mixed by upside down the tube and kept at -20°C for 30 min. The RNA was pelleted *via* centrifugation at 10,000×g, 4°C for 15 min and the genomic DNA was digested with RNase-free-DNaseI RQ1 at 37°C for 10 min prior to adjusting the total volume to 400 microliter. Proteins and RNA were separated twice by adding an equal volume of phenol/chloroform/IAA, mixing and packing by centrifugation at 10,000×*g* for 5 min. The supernatant was added by an equal volume of chloroform and done as above to separate the remaining phenol. RNA from the final supernatant was precipitated by adding 1 volume of 8 M LiCl and kept at -20°C for 30 min. RNA was pelleted *via* centrifugation of the mixture at 10,000×*g* for 15 min at 4°C before the RNA pellet was washed with 75% ethanol, air dried and dissolved in 20 µl of RNase-free sterile distilled water.

3.2.5 Determination of concentrations of DNA and RNA

The concentration and purity of DNA and RNA were determined by using the spectrophotometer at the wavelengths 260 and 280 nm. One absorbance at 260 nm corresponds to a concentration (*) of 50 µg/ml for double-stranded DNA and 40 µg/ml for single-stranded RNA. Concentrations of nucleic acids (µg/ml) were calculated by the following formula: [A_{260} × (dilution factor) × (*)] / 1000. The purity of DNA and RNA determined by the absorbance ratio of A_{260}/A_{280} was respectively represented around 1.8 and 2.

3.2.6 DNA amplification by polymerase chain reaction (PCR)

The MJ Mini^{TM} Personal Thermal Cycler (Bio-RAD) was used to perform PCR. The reaction mixture (25 μl) composed of 50 ng of recombinant plasmid DNA template, 5 pmol of both forward and reverse primers, 1× Phusion GC buffer (contains 7.5 mM $MgCl_2$ in the provided 5× concentration.), 100 μM of dNTPs and 1 unit of Phusion DNA polymerase.

The reaction mixture was carried out with the following conditions as shown in **Table 3.3**. The amplification products were examined by 0.8% agarose gel electrophoresis.

3.2.7 Site-directed mutagenesis

Based on the procedure of Stratagene's QuickChange^{TM} site-directed mutagenesis kit (**Figure 3.4**), the mutagenic primers complemented to opposite strands of DNA templates were amplified during temperature cycling by the activity of Phusion DNA polymerase. Owing to primer incorporation, of mutated plasmids containing staggered nicks were generated. Following the PCR amplification, products were then treated with *DpnI* (target sequence: 5'-G^{m6}ATC-3') in order to digest the parental DNA template. The nicked vectors incorporating the desired mutation were transformed into *E. coli* strain JM109 competent cells followed by screening for mutant clones. The reaction mixture is the same as the simple PCR reaction. PCR profiles for site-directed mutation are shown in **Table 3.4**.

Table 3.3 PCR profiles for extension reactions

Period	Number of cycle	1.4-kb RF fragment	4.5-kb LF-*psbA-aadA* fragment	3.4-kb *cry4Ba*, 0.4-kb *aadA*, 1.0-kb *psbA*	2.3-kb L1L2 fragment	5.2-kb R1R2 fragment
Pre-denaturation	1	98°C/30sec	98°C/30sec	98°C/30sec	98°C/30sec	98°C/30sec
Denaturation	30	98°C/10sec	98°C/10sec	98°C/10sec	98°C/10sec	98°C/10sec
Annealing		58°C/30sec	63°C/30sec	55°C/30sec	55°C/30sec	55°C/30sec
Extension		72°C/1.5min	72°C/5min	72°C/3.5min	72°C/3min	72°C/5.5min
Final extension	1	72°C/3min	72°C/10min	72°C/7min	72°C/6min	72°C/10min

Table 3.4 PCR profiles for site-directed mutagenesis reactions

Period	Number of cycle	pMU388	pUC18
Pre-denaturation	1	98°C/30sec	98°C/30sec
Denaturation	30	98°C/10sec	98°C/10sec
Annealing		50°C/30sec	40°C/30sec
Extension		72°C/6.5min	72°C/3min
Final extension	1	72°C/6.5min	72°C/3min

Plasmid pMU388 was mutagenized by the 4B *Xba*I-f and 4B *Xba*I-r primers. Plasmid pUC18 was mutagenized by the pUC18ds-f and pUC18ds-r primers.

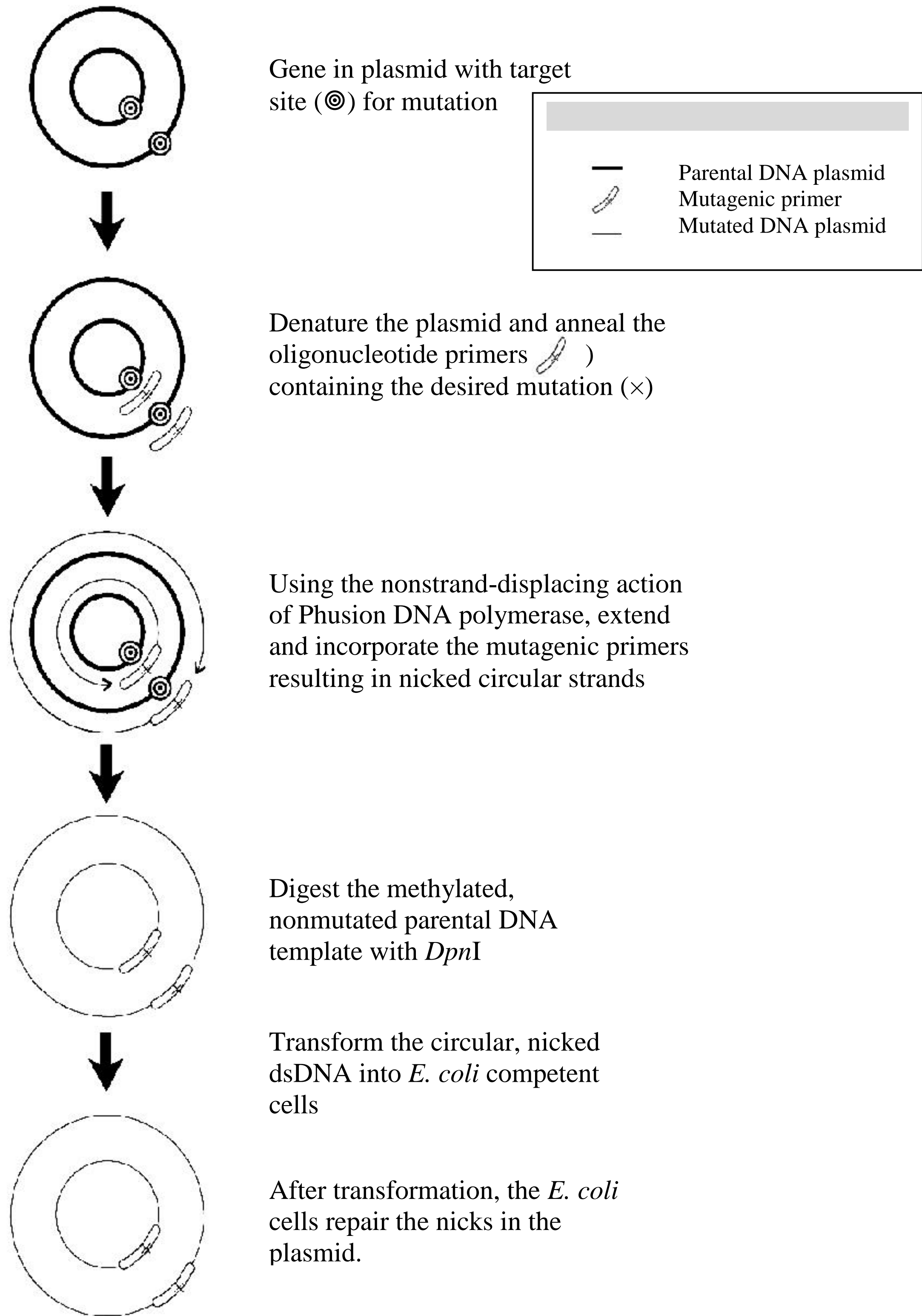


Figure 3.2 An overview of QuickChange site-directed mutagenesis method

(Modified from instruction manual of QuickChange® site-directed mutagenesis kit)

3.2.8 Agarose gel electrophoresis of DNA [111]

An appropriate amount of agarose powder was dissolved in 1×TAE buffer, (40 mM Tris-HCl pH 8.0, 40 mM acetic acid and 2.5 mM EDTA) under boiling temperature to ensure the homogeneity of the gel solution. When the gel mixture was cooled down to about 60°C, the mixture was poured into the mold and allowed to cool and solidify at 25˚C. DNA sample was mixed with gel-loading dye (15% (w/v) ficoll 400, 0.1% bromophenol blue, 5 mM EDTA) at ratio 1:5 and loaded into a well of the gel submerged in TAE buffer pH 8.0 and the electrophoresis was carried out with constant voltage at 100 volts for 1 h. After electrophoresis was completed, the gel was stained in 2 μg/ml ethidium bromide solution for 5 min and destained in water for 10 min. The DNA patterns were visualized under UV light by the Geldoc® system.

3.2.9 Agarose gel electrophoresis of RNA

The electrophoresis chamber, mold and comb were soaked with 0.5 M NaOH solution for at least 3 h in order to eliminate the contamination of RNase. To remove any trace of remaining reagents, all electrophoresis apparatus were rinsed twice with distilled water. The sterile 1×TAE buffer was used for preparing the agarose gel and carring out the electrophoresis.

In general, RNA can form secondary or tertiary structures which are difficult for analysis by native gel electrophoresis. To prevent the secondary structure, 2.7 μl of RNA sample (5-30 μg) was resolved in 7.3 μl of RNA sample buffer [50 μl 20×MOPS (0.4 M MOPS pH 7.0, 100 mM sodium acetate and 20 mM EDTA), 180 μl 37% formaldehyde and 500 μl deionised formamide] which disrupts hydrogen bonds within the molecule. After incubating at 65˚C for 10 min and on ice for 5 min, RNA sample was briefly spun down, added by 3 μl of RNA loading buffer (50% glycerol and 1 mM EDTA pH 8.0). After entire sample was loaded onto the gel, electrophoresis was carried out with constant voltage at 100 volts for 1 h. Subsequently, the gel was stained in 2 μg/ml ethidium bromide solution for 5 min and destained in water for 10 min. The DNA patterns were visualized under UV light by the Geldoc® system.

3.2.10 Denaturing formaldehyde-agarose gel electrophoresis of RNA

All electrophoresis apparatus were eliminated the contamination of RNase and the RNA loading sample were prepared as above.

One gram of agarose was heated in 72 ml of sterile distilled water until dissolved and cooled down to about 60°C. The dissolved agarose was added by 10 ml of 10X MOPS and 18 ml of 37% formaldehyde in a chemical fume hood. The mixture was poured into the mold and allowed to cool and solidify at 25°C about 1 h. The prepared RNA sample was loaded into a well of the denaturing gel submerged in 1X MOPS running buffer. The electrophoresis was carried out with constant voltage at 40 volts for 3 h. After electrophoresis was completed, the denaturing gel was stained in 2 μg/ml ethidium bromide solution for 5 min and destained in water for 30 min. The RNA patterns were visualized under UV light by the Geldoc® system.

3.2.11 Purification of DNA from agarose gel by Geneclean II Kit

The excised gel was placed into a 1.5-ml microcentrifuge tube and weighted to determine the approximate volume of the gel (100 mg ~ 100 μl). Three volumes of 6 M NaI were added and the mixture was incubated at 55°C until the gel slice was completely dissolved. The proper amount of resuspended GLASSMILK was added into the reaction (5 μl of GLASSMILK or silica matrix for DNA sample 5 μg) and the mixture was incubated and gently mixed at room temperature for 15 min to allow binding of the DNA to the bead. The DNA-bound bead was pelleted *via* centrifugation at 13,000 rpm for 5 sec and the supernatant was discarded by pipetting. The silica matrix was washed three times with 500 μl of wash buffer (10 mM Tris-HCl pH 7.4, 0.5 mM EDTA, 50 mM NaCl and 50% ethanol) by vortex, followed by pelleting the DNA-bound bead by spinning as above. After the third wash, buffer was removed by pipetting and the pellet was air dried at room temperature for 10 min. To elute the bound DNA from the bead, 10 μl of TE buffer (100 mM Tris-HCl pH 8.0 and 10 mM EDTA) was added to the bead and mixed prior to incubate at 55°C for 5 min. The mixture was centrifuged at 13,000 rpm for 30 sec and the eluted DNA sample was carefully transferred to a new microcentrifuge tube by pipetting.

3.2.12 Restriction endonuclease digestion of the plasmid DNA [111]

Plasmid DNA was analyzed by restriction endonuclease digestion. The digestion reaction was performed in 20 µl of mixture containing 500 ng of plasmid DNA, 1×restriction endonuclease buffer, 1 U of restriction endonuclease and double distilled water to a final volume of 20 µl. The reaction was carried out at the conditions recommended by the manufacturer. The final DNA digestion patterns were analyzed by agarose gel electrophoresis.

3.2.13 Dephosphorylation of DNA fragment with calf intestinal alkaline phosphatase (CIAP)

Calf intestinal alkaline phosphatase (CIAP) is used to prevent the self-ligation of linear DNA vector by catalysis of 5'-phosphate groups from both 5'-termini. In order to dephosphorylate the overhang ends, the linear DNA was incubated with CIAP, which had been diluted in 1× reaction buffer (50 mM Tris-HCl pH 9.3, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine) to a final concentration of 0.01 unit/µl at 37°C for 30 min. Another equal aliquot of diluted CIAP was added and incubated continuously at 37°C for an addition of 30 min. In case on blunt ends, the reaction was incubated at 37°C for 15 min and again at 56°C for 15 min. For the purpose of DNA recovery, the phenol:chloroform (1:1) extraction and absolute ethanol precipitation were respectively performed before the pellet was rehydrated in sterile distilled water.

3.2.14 Blunt end generation by T4 DNA polymerase

In general, T4 DNA polymerase catalyzes the synthesis of DNA in the 5' to 3' direction and requires the presence of template and primer. This enzyme also has the 3' to 5' exonuclease activity, therefore the blunt end DNA can be generated from both 3' overhang and recessed ends. The reaction was performed in 1×reaction buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$ and 1 mM DTT) supplemented with 50 µg/ml BSA and 100 µM dNTPs. One unit of T4 DNA polymerase was added per microgram of DNA and incubated for 15 min at 12°C. The reaction was stopped by heating to 75°C for 20 min. The phenol:chloroform (1:1) extraction was performed and DNA was precipitated by absoluted ethanol.

3.2.15 DNA ligation

The DNA vector and the inserted DNA fragments were digested with restriction enzymes prior to purify by Geneclean II kit. Digested fragments of DNA inserts and vector were combined in 3:1 molar ratio. The ligation mixture contained 1×ligation buffer, 5U of T4 DNA ligase and sterile distilled water in a final volume of 20 μl. The mixture was then incubated overnight at 16°C. The amount of vector was fixed at 50 ng. The following equation shows the calculation of the appropriate amount of DNA inserts (ng):

$$\frac{\text{amount of vector (ng)} \times \text{insert size (kb)}}{\text{vector size (kb)}} \times \text{(insert:vector mass ratio)}$$

3.2.16 Preparation of *E. coli* competent cells by the $CaCl_2$ method [111]

An overnight culture of a single colony of *E. coli* in 3 ml LB broth was inoculated into new LB broth at 1:100 dilution and incubated at 37°C until $O.D._{600}$ was between 0.3-0.4. The cell culture was chilled on ice for 15 min before 10 ml of culture was transferred into pre-chilled 15-ml tube. The cells were pelleted by centrifugation at 3,000×*g*, 4°C for 10 min. The pellet was resuspended in 5 ml of ice-cold 0.1 M $CaCl_2$, placed on ice for 20 min and then centrifuged at 3,000×*g*, 4°C for 10 min. The cells pellet was resuspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 1 h. Glycerol was added to the cell suspension to 20% (v/v) final concentration. The cell suspension was kept at -80°C.

3.2.17 Preparation of *E. coli* competent cells by the simple and efficient method (SEM)

Ten colonies of *E. coli* were inoculated into 25 ml of SOB medium (pH 7.0) (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl and 2.5 mM KCl). After sterilization, a sterile $MgCl_2$ solution is added to 10 mM final concentration just before use and cultured at 18°C with vigorous shaking. After 24-h incubation, a starter culture was inoculated into 250 ml of SOB medium in a 2-liter flask and incubated in the same condition until the $O.D._{600}$ reached 0.5-0.6. The bacterial culture was chilled on ice for 10 min before cells were harvested by

centrifugation at 2500×*g*, 4°C for 10 min. The pellet was gently resuspended in 80 ml of cold TB buffer (10 mM pipes, 55 mM $MnCl_2$ and 250 mM KCl), incubated on ice for 10 min and centrifuged at 2,500×*g*, 4°C for 10 min. Cells were gently resuspended in 20 ml of cold TB buffer again, and DMSO was slowly added to give a 7% final concentration. The suspension was mixed by swirling and incubated in an ice bath for another 10 min. The 200 µl aliquots of the competent cells were swiftly dispensed to the 1.5-ml microcentrifuge tube and immediately frozen in liquid N_2. These competent cells were kept at -80°C.

3.2.18 Transformation of plasmid DNA into *E. coli* by the heat-shock method

An aliquot of 100 µl *E. coli* competent cells was mixed with 50 ng of ligated products. Cells were placed on ice for 30 min, incubated at 42°C for 90 sec and immediately placed on ice for 5 min. The transformed cells were mixed with 900 µl of SOC or LB medium, incubated at 37°C for 1 h with shaking at 200 rpm and centrifuged at 3,000×*g* for 1 min. A 400 µl aliquot of the supernatant was discarded. An aliquot of 250 µl of transformed culture was spread on the LB agar plates containing ampicillin (100 µg/ml) and incubated overnight at 37°C.

3.2.19 Selection of recombinant *E. coli* clones by rapid plasmid size-screening method

A single colony of transformant was transferred into 30 µl of pre-warmed lysis buffer (5 mM EDTA, 10% (w/v) sucrose, 0.25% (w/v) SDS, 100 mM NaOH, 60 mM KCl and 0.05% (w/v) bromophenol blue). The mixture was incubated at 37°C for 5 minutes then placed at 4°C for 5 min and centrifuged at 20,800×*g* for 5 minutes. 20 µl of supernatant was loaded onto an agarose gel and the electrophoresis was performed. The colony containing only vector was used as a negative control.

3.2.20 Verification of insertion fragments in recombinant plasmid by PCR and restriction digestion analysis

A gene amplification system of MJ Mini^{TM} Personal Thermal Cycler (Bio-RAD) was employed as a thermocycler for the PCR amplification with primers specific to the inserted genes and the appropriated temperature profile. The recombinant plasmid was verified again by analyzing the pattern of restriction digested products. Recombinant plasmids were digested by the appropriated restriction enzymes recognizing the specific sequences of inserted gene. The pattern of digested products indicated the correct orientation of inserted genes.

3.2.21 DNA sequencing

Double stranded DNA templates for sequencing were prepared from the selected clones by mini-scale DNA preparation (CTAB method). The DNA sequences of selected clones were determined by Macrogen DNA sequencing service (www.macrogen.com).

3.2.22 Autolysin preparation [24]

Both mating types of *C. reinhardtii*, designated plus (mt^+) and minus (mt^-), were separately cultivated up to about 10^7 cells/ml in 250 ml of TAP medium using the 1 liter flask. Cells of each mating type were pelleted by centrifugation at 1,000×*g* for 5 min and resuspended by 1 liter of TAP-N medium (NH_4Cl was replaced with the same concentration of KCl) with shaking at 50 rpm of rotation frequency for 24 h under light in the 2 liter flask. The differentiation of both mating types into sexually competent gametes was induced by the nitrogen deprivation of TAP-N medium. Both mating types were mixed in the 2 liter flask and let stand under light for 1-2 h to allow mating. The autolysin released from the mating cells was harvested from the cell suspension culture *via* centrifugation at 6,000×*g* for 5 min. The separate supernatant containing autolysin was filtered *via* 0.45-µm membrane unit prior to keep in -80°C until use.

3.2.23 Transformation of recombinant plasmids into the *C. reinhardtii* chloroplast by the glass bead method [24]

Glass beads, 0.45 mm in diameter, were washed with concentrated sulfuric acid, rinsed thoroughly with distilled water, dried and autoclaved. The microcentrifuge tube containing 400 µl of host cells was added by 300 mg of sterile glass beads, 5 µg of the recombinant plasmid and the mixture was agitated by vortex with variety of time periods (5, 10, 15, 20, 25 and 30 sec). The beads were allowed to settle and cells were spread on selective agar plates containing 100 µg/ml spectinomycin. The plates were allowed to dry and then sealed with Parafilm prior to incubate in the dark for 24 h and under 100 µmol photons/m^2·s of light intensity. The transformant colonies were visible after 6-8 days of growth. Control plates contained the mixture of agitated cells without the recombinant plasmid.

3.2.24 Transformation of recombinant plasmids into the *C. reinhardtii* chloroplast by the bombardment method

To begin with, 1 µm gold particles (Bio-Rad) were prepared. To prepare particles for up to 60 shots, 30 mg of gold particles were weighed into a sterile 1.5-ml microcentrifuge tube, added 500 µl of absolute ethanol, mixed by vortex for 1 min and let stand for 15 min. Particles were centrifuged for 1 min at 10,000Î *g* and the supernatant was removed. Particles were then washed for 3 times by adding 500 µl of the sterile distilled water and mixing by vortex for 1 min. After let stand the particle solution for 1 min, the supernatant was removed prior to finally resuspend by 500 µl of sterile 50% glycerol. The particles were left on ice until used, or could store at 4˚C as gold stock.

Five ml of the log-phase state with a density of 10^7 cells/ml of *C. reinhardtii* were spread onto a TAP agar plate containing a final concentration of 100 µg/ml spectinomycin (Sigma) and briefly air-dry under sterile conditions. Concurrently, the prepared gold particles were coated with plasmid DNA by adding in a particular order into a 1.5-ml microcentrifuge tube. The microcentrifuge tube was continuously vortexed during addition of each solution: For up to 6 shots, 50 µl of the gold particle suspension (~ 3 mg in 50% glycerol), 5 µl of 1 µg/µl plasmid DNA, 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine free base. After each addition, the

suspension was vortexed for 3 min and let stand on ice for 1 min. The DNA-coated gold particles were then pelleted *via* centrifugation at 10,000×*g* for 2 seconds. After removing the supernatant, the DNA-coated gold particles were washed with 140 µl of 70 % ethanol and again with 140 µl of absolute ethanol without disturbing the pellet. After removing the supernatant, the pellet was resuspended gently in 48 µl of absolute ethanol by tapping the side of the microcentrifuge tube several times. Six µl of DNA-coated gold particles (microcarrier suspendsion) were pipetted onto each macrocarrier disc for bombardment in the PDS-1000/He instrument. Each target plate was bombarded under 25 inches Hg chamber vacuum, 9 cm target distance and 900 psi Helium pressure. Bombarded plates were kept in the dark at 25°C for 24 h before incubated under 100 µmol photons m^{-2} s^{-1} of light intensity. Control plates contained wild-type cells which were not bombarded.

3.2.25 Selection of the chloroplast transformants by drug screening and recovery of photosynthetic activity

The bombarded *C. reinhardtii* cells spread on the TAP agar containing spectinomycin drug were screened for the transformants containing the recombinant antibiotic *aadA* gene. About 2 weeks, the drug resistant colonies were collected as well as subcultured in the HS liquid minimum medium. About 1 week, HS medium of the reproducible strain that restored the activity of photosynthetic *psbA* gene should be changed from clear to greenish colors.

3.2.26 A PCR dioxigenin (DIG) probe synthesis

PCR amplification parameters (cycling conditions, template concentration, primer sequence, and primer concentration) were optimized for each template and primer set in the absence of DIG. Then an optimized profile was employed in order to generate probe *via* incorporating DIG into the PCR reaction. To synthesize the DIG probe, a 1:6 ratio of DIG-dUTP:dTTP with the non-proof reading DNA polymerase were used. To evaluate the efficiency of probe labeling, PCR products were visualized *via* agarose gel electrophoresis and ethidium bromide staining. The incorporation of DIG into the probe should be migrated through the agarose gel at a slower rate than an unlabelled PCR product.

3.2.27 Northern blot analysis

RNA in denaturing formaldehyde-agarose gel was transferred onto a positively charged nylon membrane (GE Healthcare) by the downward capillary transfer method. One sheet of nylon membrane, 7 sheets of absorbant paper (Whatman 3 MM) and a stack of paper towels several centimeters in height were cut to the size of the gel. The membrane and gel were cut off a corner to mark for orientation prior to wetting. Membrane was respectively immersed in sterile deionized water until completely wet and 10×SSC (1×SSC pH 7.0: 150 mM NaCl and 15 mN sodium citrate) for at least 5 min. Four sheets of absorbant paper were immersed in the sterile transfer buffer (0.01 N NaOH and 3 M NaCl). Gel was rinsed with sterile distilled water and immersed in the transfer buffer. To assemble the transfer system, three centimeters in height of a paper towel stack, 3 sheets of dry absorbant paper, 2 sheets of wet absorbant paper, a membrane, gel and 2 sheets of wet absorbant paper were respectively placed into the glass tray. The side of the gel corresponding to the bottom of the wells should be against the membrane therefore it minimizes the distance of RNA migration from gel to membrane. After transferring for overnight, the gel was stained with ethidium bromide to verify the transfer. The membrane was immersed in 6×SSC prior to use in the next step.

Membrane with RNA side up was rolled and inserted into the hybridization tube (Hybaid) containing 10 ml of the sterile hybridization buffer (modified Church and Gilbert buffer: 0.5 volume of 1M Na-phosphate buffer pH 7.2 and 7% (w/v) SDS) and sample was incubated at 55°C for 30 min with continuously rolling in the hybridization oven (Hybaid). After the incubation was finished, the 100 ng of the 95°C preheated DIG-probe was added prior to incubation at 55°C for overnight.

To wash off the unbound probe, membrane was immersed twice in the hybridization washing buffer I (2×SSC and 0.1% SDS) for 5 min each, twice in the 55°C preheated hybridization washing buffer II (0.2×SSC and 0.1% SDS) for 15 min each and one time in the washing buffer (0.3% (v/v) tween in maleic acid buffer pH 7.5) for 5 min. The washed membrane was incubated with blocking solution (10% skimmilk in maleic acid buffer) for 30 min in order to prevent non-specific binding of primary antibody to the membrane. Blocked membrane was subjected to incubate with

antibody solution (a 1:10,000 dilution of antiDIG (Roche) conjugated with the alkaline phosphatase in fresh blocking solution) for 30 min. After incubation, the excess antibody was washed out of the membrane twice by the washing buffer for 5 min each. The membrane was then immersed in the detection buffer pH 9.5 (0.1 M Tris-HCl and 0.1 M NaCl) for 5 min prior to use in the detection step.

The detection reagent, CDP-Star (Roche), was pipetted on to the washed membrane which was placed RNA side up on the sheet of Saran Wrap^{TM}. The membrane was incubated with the reagent for 5 min and the excess reagent was drained. Then the membrane was wrapped up carefully without any air bubbles and placed on an X-ray film cassette. A sheet of autoradiography film (Super RX, Fuji Medical X-Ray Film) was placed on top of the membrane. The exposed film was developed using CURIX 60, a film processor machine (Agfa).

3.2.28 Reverse transcription and polymerase chain reactions (RT-PCR)

Reverse transcription reaction was performed to synthesize the first-strand cDNA in preparation for later PCR amplification. The 10 μM of specific reverse primer was added to 2 μl of extracted RNA (250 ng). The mixture was heated at 65˚C for 5 min and chilled on ice. A mixture composed of 10 mM dNTPs, 0.1 mM DTT, 2 μl of First-Strand buffer, 40 units of RNaseOUT^{TM} (Invitrogen) and 50 units of SuperScript^{TM} II RT (Invitrogen) was added to the reaction before the final volume was adjusted to 20 μl with RNase-free water. The final reaction was incubated at 55˚C for 1 h, followed by deactivation at 70˚C for 10 min. The RT product was used as the template for the next step.

The PCR reaction was performed in the MJ Mini^{TM} Personal Thermal Cycler. The reaction mixtures of RT and PCR were carried out with the following conditions as shown in **Table 4.5**. The amplification products were examined by 0.8% agarose gel electrophoresis.

Table 3.5 RT-PCR profiles and reaction mixtures

RT reaction	
Total RNA (250 ng)	1-2 µl
Specific reverse primer (10 pmol)	2 µl
• 65°C, 5 min	
• On ice, at least 1 min	
• Add the mixture;	
5× First-Strand buffer	2 µl
dNTPs (10 mM)	0.5 µl
0.1 M DTT	0.5 µl
RnaseOUT	0.5 µl
SuperScript II, RT	0.5 µl
Sterile distilled water	2 µl
Total	10 µl
• 55°C, 60 min	
• 70°C, 15 min	

PCR reaction	
First-strand cDNA	1 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
5×Phusion GC buffer	5 µl
dNTPs (10mM)	0.5 µl
Phusion DNA polymerase	0.5 µl
Sterile distilled water	17 µl
Total	25 µl

Period		***cry4Ba***	***psbA***
Pre-denaturation		98°C/30sec	98°C/30min
30 cycles	Denaturing	98°C/10sec	98°C/10sec
	Annealing	55°C/30sec	55°C/30sec
	Extension	72°C/3.5min	72°C/1min
Final extension		72°C/6min	72°C/2min

3.2.29 Calculation the amount of total chlorophylls

C. reinhardtii harbouring recombinant genes was inoculated in the 50 ml TAP medium with 100 µg/ml spectinomycin and incubated at 37°C with shaking at 100 rpm for 5 days. The pellet from 10 ml of the 5-day culture was harvested by centrifugation and was resuspended with 500 µl of 2× lysis buffer (250 mM Tris pH 6.8, 2 M urea, 20% (v/v) glycerol and 7% SDS). The 50 µl of the resuspended pellet was mixed by 950 µl of 80% acetone prior to measure the absorption and the 80% acetone was used as a blank. The concentration of non-chlorophyll, chlorophyll a and chlorophyll b were respectively measured by using the spectrophotometer at the wavelengths 720, 663 and 645 nm which were subsequently used to determine the concentration of the total chlorophyll (nmol/µl) according to the modified Arnon equation [112]:

$$\textbf{Chlorophyll a (nmol/ml)} = \frac{(\text{dilution factor}) \times \{[12.7(O.D._{663}-O.D._{720})]-[2.69(O.D._{645}-O.D._{720})]\}}{0.893491}$$

$$\textbf{Chlorophyll b (nmol/ml)} = \frac{(\text{dilution factor}) \times \{[22.9(O.D._{645}-O.D._{720})]-[4.68(O.D._{663}-O.D._{720})]\}}{0.9075014}$$

Total chlorophyll (nmol/ml) = chlorophyll a + chlorophyll b

The sample containing the 3 nmol of the total chlorophylls was collected to analyze protein profiles on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.2.30 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The 10 ml of 5-day log-phase *C. reinhardtii* cells was collected and pelleted by centrifugation. The cell pellet was resuspended with 500 µl of lysis buffer (250 mM Tris-HCl pH 6.8, 2 M urea, 20% (v/v) glycerol, 7% SDS) before cell solution was added by 100 mM dithiothreitol (DTT). Cell solution was homogenized by a paintbrush and centrifuged at 10,000×*g* for 5 min to precipitate any insoluble materials. Supernatant equivalent to 3 nmol of the total chlorophylls was loaded into each well of polyacrylamide gel for SDS-PAGE analysis.

SDS-PAGE was performed using the Bio-RAD Mini-Protein II system. Polyacrylamide gel for SDS-PAGE is composed of separating and stacking gels as described in **Table 3.6**. After the gel was polymerized, the comb was removed and wells were flushed with distilled water. The electrophoresis was performed in Tris-glycine buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine and 0.1% SDS). The protein samples and 8 µl of Broad range protein marker (Bio-RAD) were loaded into wells and the electrophoresis was performed with constant voltage of 100 volts/gel until the dye front reached the bottom edge of the gel. The electrophoresis equipments were disassembled and the gel was stained with shaking in Coomassie staining solution (0.1% Coomassie brilliant blue R250, 50% methanol and 10% glacial acetic acid) for 2 h. The gel was then destained with shaking in destaining solution (20% methanol and 10% glacial acetic acid) until the background was clears.

Table 3.6 Preparation of SDS-PAGE gel (2 gels)

Solution	5% Stacking gel	10% or 12% Separating gel
40% Acrylamide solution	0.5 ml	2.5 ml or 3 ml
1.5 M Tris-HCl, pH 8.8	-	2.5 ml
1.0 M Tris-HCl, pH 6.8	0.5 ml	-
Distilled water	2.92 ml	4.8 ml or 4.3 ml
10% SDS	40 µl	100 µl
TEMED	4 µl	4 µl
10% (w/v) Ammonium persulphate (APS)	40 µl	100 µl

3.2.31 Western blot analysis

The gels containing protein samples separated on 10% SDS-PAGE were equilibrated in the transfer buffer (192 mM glycine, 25 mM Tris-HCl pH 8.3, 0.04% SDS, 20% methanol) for 10 minutes. The nitrocellulose membrane and 4 pieces of filter paper were cut to the same size as the gel prior to immerse in transfer buffer for 10 min. The blotting apparatus (Bio-RAD) was assembled by putting 2 pieces of soaked filter paper over the buffer-soak sponge on the clear side of the cassette following by placing the nitrocellulose membrane, the polyacrylamide gel, the other 2 pieces of soaked filter paper and another sponge, respectively. The assembled cassette was inserted into the container as the black side of the cassette was turned to the same color of the container. Electroblotting was carried out at 80 volts for 1 hour and 120 volts for another 1 hour in the cold room.

After the blotting process, the nitrocellulose membrane was briefly stained with Ponceau S (2% Ponceau S and 3% Trichloroacetic acid) and washed with distilled water to remove the excess staining solution. Subsequently, the standard protein marker bands were marked by pencil. Then the membrane was pre-blocked by immersing in blocking solution which the 5% (w/v) skimmilk was dissolved in 1×PBS

(25 mM Tris-HCl pH 8.3, 192mM glycine and 20% (v/v) methanol) for 2 h or 4°C overnight. To detect the Cry4Ba toxin, the membrane was incubated with a new blocking solution containing 1:50 dilution of primary antibody (2F-1H2 mouse antibody [112]) specific to domain III of the Cry4Ba protein for 1 h at 25°C with shaking. The membrane was washed 3 times with PBS-T buffer (1×PBS containing 0.1% (v/v) Tween20) for 5 min each. Subsequently, the membrane was incubated with the blocking solution containing 1:20,000 dilution of secondary antibody (alkaline phosphatase conjugated with anti-mouse IgG) for 1 h at 25°C with shaking. The membrane was washed 3 times with PBS-T buffer for 5 min each and twice with PBS buffer for 5 min each. Signal development and detection steps were done as Northern blot.

3.2.32 Mosquito larvae feeding

Two-day-old *Aedes aegypti* larvae were hatched from eggs (supplied by the mosquito-rearing facility of the Institute of Molecular Biosciences, Mahidol University, Thailand) and reared at room temperature, 25°C. Feeding assays were performed in 1 ml *C. reinhardtii* cell suspension (10^7cells of *C. reinhardtii* suspended in distilled water) in a 24-well titration plate (Costar, USA), with 5 larvae per well and a total of 25 larvae. An equal number of starving larvae were used as negative control. Phenotypic changes were observed after a 5-day incubation period.

CHAPTER IV
RESULTS

In this work, two transformation vectors were constructed and two transformation strategies, glass bead and bombardment methods, were used to deliver the constructs into *C. reinhardtii* chloroplast genomes. The first construct, pCh1T-2, contains a target fragment for the homologous recombination process and two separated expression cassettes of *cry4Ba* and *aadA* genes (**Figure 4.1**). However, upon transformation by the first construct using the bombardment method, only the expression cassette of *aadA* gene can be detected by PCR screening in the recovered spectinomycin resistant putative transformants. Therefore, a second construct, pCh2T-3, that contained three separated expression cassettes of *cry4Ba*, *aadA* and *psbA* genes which are located between the two target fragments for the homologous recombination process (RF; right recombination fragment and LF; left recombination fragment) was constructed. Upon transformation by the second construct using the bombardment method three transformants were obtained and all recombinant genes could be detected by PCR screening**.** Flowcharts illustrating the construction strategies of pCh1T-2 and pCh2T-3 were shown in **Appendix 2** and **Appendix 3**, respectively.

The non-photosynthetic *psbA*-deleted (CC744) and wild-type (CC400) strains were used as hosts for insertion of pCh1T-2 and pCh2T-3, respectively. Plasmid pCh1T-2 contains a single 0.7-kb recombination fragment which was identical to the non-coding region between *psbA* and *rrn5* genes lying on the inverted repeats (IR) of *C. reinhardtii* chloroplast genomes. On the other hand, pCh2T-3 contains a 0.7-kb left recombination fragment (LF) and a 1.4-kb right recombination fragment (RF) which are identical to the non-coding regions between *chlN* and *rrn5* genes lying on the IR of *C. reinhardtii* chloroplast genomes.

After transformant lines were screened *via* spectinomycin resistance and restoration of photosynthesis, Northern blot and Western blot analysis were

subsequently performed to check the transcription and translation products of the *cry4Ba* transgene.

4.1 Construction of p4B harboring the *cry4Ba* expression cassette

The *cry4Ba* gene from pMU388 was mutagenized to silence the *Xba*I restriction site (NCBI reference X07423, bases 1837 to 1842) by 4B *Xba*I-f and 4B *Xba*I-r primers (**Table 3.1**) prior to modifying the promoter and terminator sequences. In order to express the *cry4Ba* gene in the chloroplast system, the original 5'- and 3'-controlling sequences were substituted with the 5 UTR in combination with promoter and the 3 -UTR of *psbA* gene providing by p546 vector (**Appendix 4**). In this process, a 3.5-kb fragment of blunt-*Eco*RI/*Xba*I of *cry4Ba* gene from pMU388 was ligated with a 3.5-kb fragment of blunt-*Nco*I/*Xba*I of linearized p546 generating p4B plasmid containing the *cry4Ba* expression cassette (*psbA-cry4Ba*) (**Figure 4.1**). DNA sequencing analysis of part of p4B plasmid indicated that the *Eco*RI site (which overlapped with the ATG start codon of *cry4Ba* gene) was reconstituted and confirmed that the *cry4Ba* gene open reading frame was intact (**Figure 4.2**).

After the ligated product of p4B was delivered to *E. coli* grown on LB agar containing ampicillin, colonies of transformants were primarily screened by rapid size screening (**Figure 4.3A**). Comparing with the DNA pattern of p546, five clones showing the same pattern of DNA shifted band were selected to be analyzed by digesting with *Eco*RI. It revealed that the expected DNA pattern was observed only from clone number 1 (**Figure 4.3B-C**).

4.2 Construction of p4Bds harboring the *cry4Ba* expression cassette flanked by the *Sal*I restriction site

In order to further facilitate ligation of the product *psbA-cry4Ba* expression cassette into the chloroplast transformation vector, the flanking restriction sites were changed to *Sal*I *via* subcloning into pUC18ds which contained the mutagenized *Eco*RI to *Sal*I site within the multiple cloning sites of pUC18. In this process, a 4.2-kb

fragment of blunt-*Kpn*I/*Sac*I of the *psbA-cry4Ba* expression cassette from p4B was ligated with a 2.6-kb fragment of *Sma*I/*Sac*I of pUC18ds to generate p4Bds (**Figure 4.4**).

After the ligated product of p4Bds was delivered to *E. coli* grown on LB agar containing ampicillin, colonies of transformants were primarily screened by rapid size screening (**Figure 4.5A**). Comparing with the DNA pattern of pUC18ds, two clones with the different pattern of DNA shifted band were selected to be analyzed by digesting with *Eco*RI. The results showed that the expected DNA pattern was observed only from clone number 2 (**Figure 4.5B-C**).

4.3 Construction of pCh1T-1 harboring the *aadA* expression cassette and a target of homologous recombination

Plasmid p242 (*Chlamydomonas* stock center, Duke university, Durham, NC, USA; http://www.chlamy.org.), harboring 1.4-kb fragment of the recombination target, identical to the non-coding region between the *psbA* and *5S rRNA* genes of the *C. smithii* chloroplast genome, was linearized by *Eco*RI. Plasmid p463de, harboring the antibiotic *aadA* cassette, was cut by *Eco*RI and a 1.6-kb *Eco*RI-flanked *aadA* product was subcloned into a 4.0-kb *Eco*RI-linearized p242 generating pCh1T-1 (**Figure 4.6**).

After the ligated product of recombinant plasmid pCh1T-1 was delivered into *E. coli* grown on LB agar containing ampicillin, colonies of transformants were primarily selected by the rapid size screening method (**Figure 4.7A**). Comparing with the DNA pattern of p242, four clones with two patterns of DNA shifted band were selected to be analyzed by digesting with *Sal*I. The results showed that the expected DNA pattern was observed only from clone numbers 1-2 (**Figure 4.7B-C**).

4.4 Construction of pCh1T-2 harboring expression cassettes of *aadA* and *psbA-cry4Ba* and a recombination target

Finally, p4Bds, harboring the *psbA-cry4Ba* cassette, was cut by *Sal*I and a 4.2-kb *Sal*I-flank *psbA-cry4Ba* product was subcloned into the corresponding cleavage site of 5.7-kb pCh1T-1 generating pCh1T-2 (**Figure 4.8**).

After the recombinant plasmid pCh1T-2 was delivered into *E. coli* grown on LB agar containing ampicillin, colonies of transformants were primarily screened by rapid size screening (**Figure 4.9A**). Comparing with the DNA pattern of pCh1T-1, four clones with the different pattern of DNA shifted band were selected to be analyzed *via* digesting with *Eco*RI, *Sal*I and *Sma*I restriction enzymes. The results indicated that the expected DNA pattern was observed only from clone number 4 (**Figure 4.9B-C**).

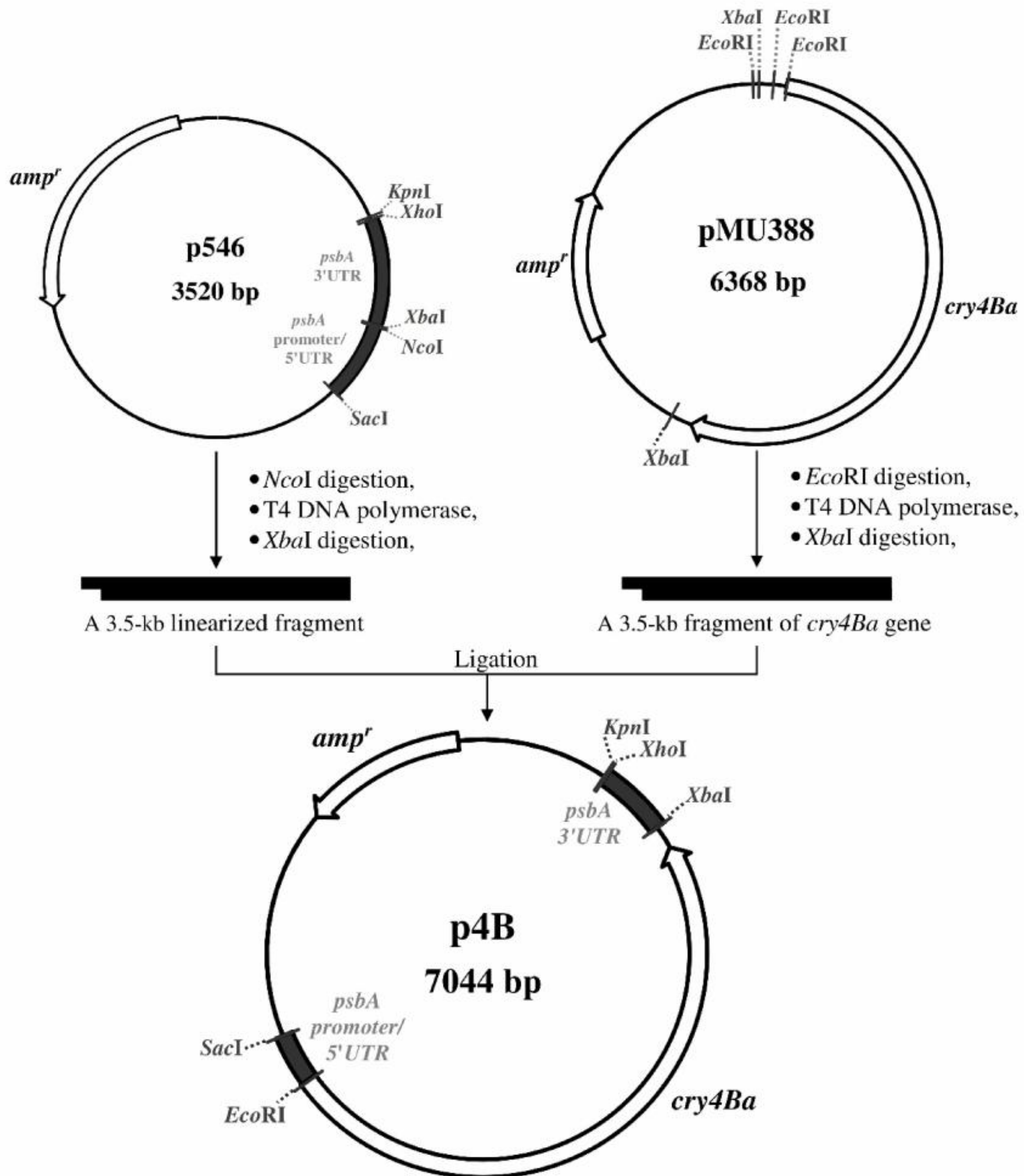
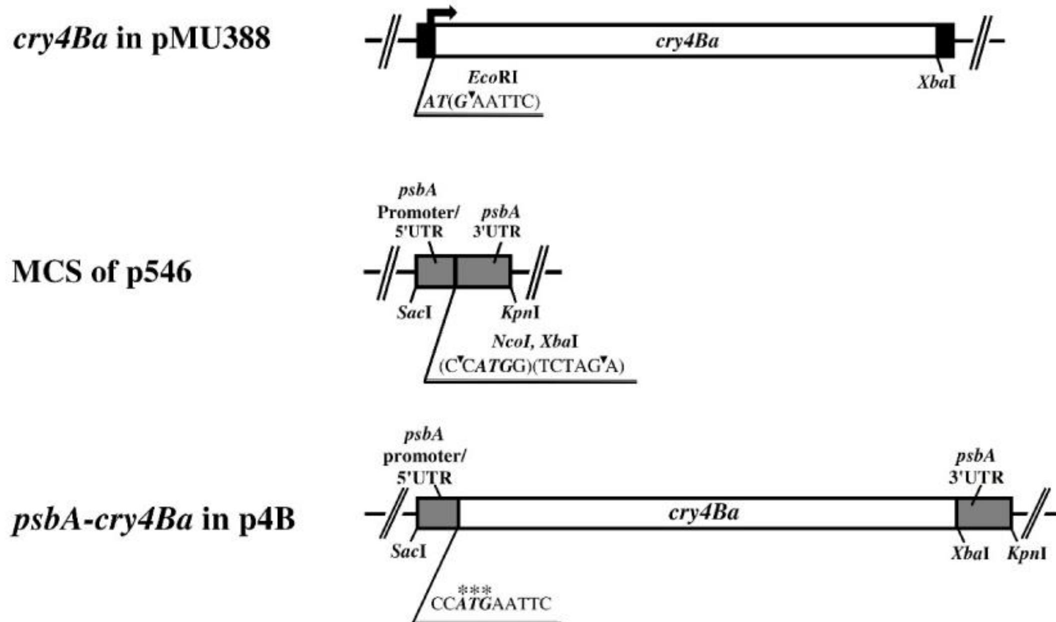


Figure 4.1 Schematic diagram of the p4B construction

A 3.5-kb fragment of the *cry4Ba* gene was excised from mutated pMU388 by *Eco*RI digestion, T4 DNA polymerase treatment and *Xba*I digestion, respectively. The cut product was then ligated with a 3.5-kb fragment of p546 that was obtained by *Nco*I digestion, T4 DNA polymerase treatment and followed by *Xba*I digestion.

(A)



(B)

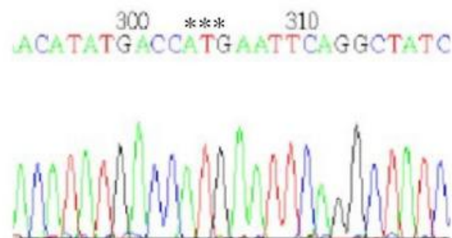


Figure 4.2 Recovery of the start codon (ATG) of *psbA-cry4Ba* in p4B

(A) The start codon of *cry4Ba* from pMU388 (overlapping with *Eco*RI site) was recovered by sequences of *Nco*I from p546. Nucleotide sequences of some restriction sites are shown in brackets with italics indicating the location of the start codon.

(B) The recovery of *cry4Ba* start site (asterisk) was determined by DNA sequencing to ensure that the *cry4Ba* open reading frame is correct.

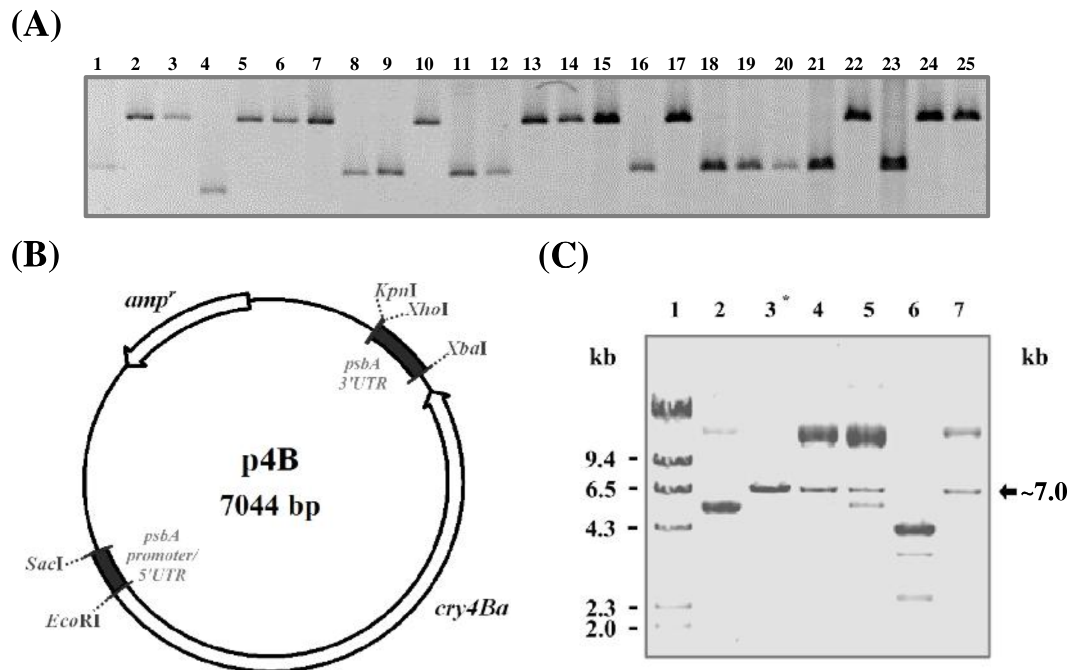


Figure 4.3 Screening and verification of p4B

(A) The figure demonstrates 1% agarose gel from rapid size screening of p4B (a ligation product between the full-length *cry4Ba* gene and p546 harboring the promoter and terminator of *psbA* gene).

Lane 1: Plasmid p546

Lanes 2-25: Selected recombinant clones

Samples showing the same pattern of DNA shifted band (lanes 10, 13, 14, 15 and 17) are respectively designated as clones 1 to 5 for further verification by the restriction endonuclease analysis.

(B) Schematic diagram shows a map of p4B with the position of restriction sites relevant to the construction. The expected product of p4B digested by the *Eco*RI restriction enzyme is ~7.0-kb fragment.

(C) The 1% agarose gel illustrates the restriction endonuclease analysis by *Eco*RI digestion. Only the size of a restriction product from the positive clone (asterisked) is indicated on the right.

Lane 1: /*Hin*dIII DNA marker

Lane 2: Undigested clone1

Lanes 3-7: Digested clone1-5, respectively

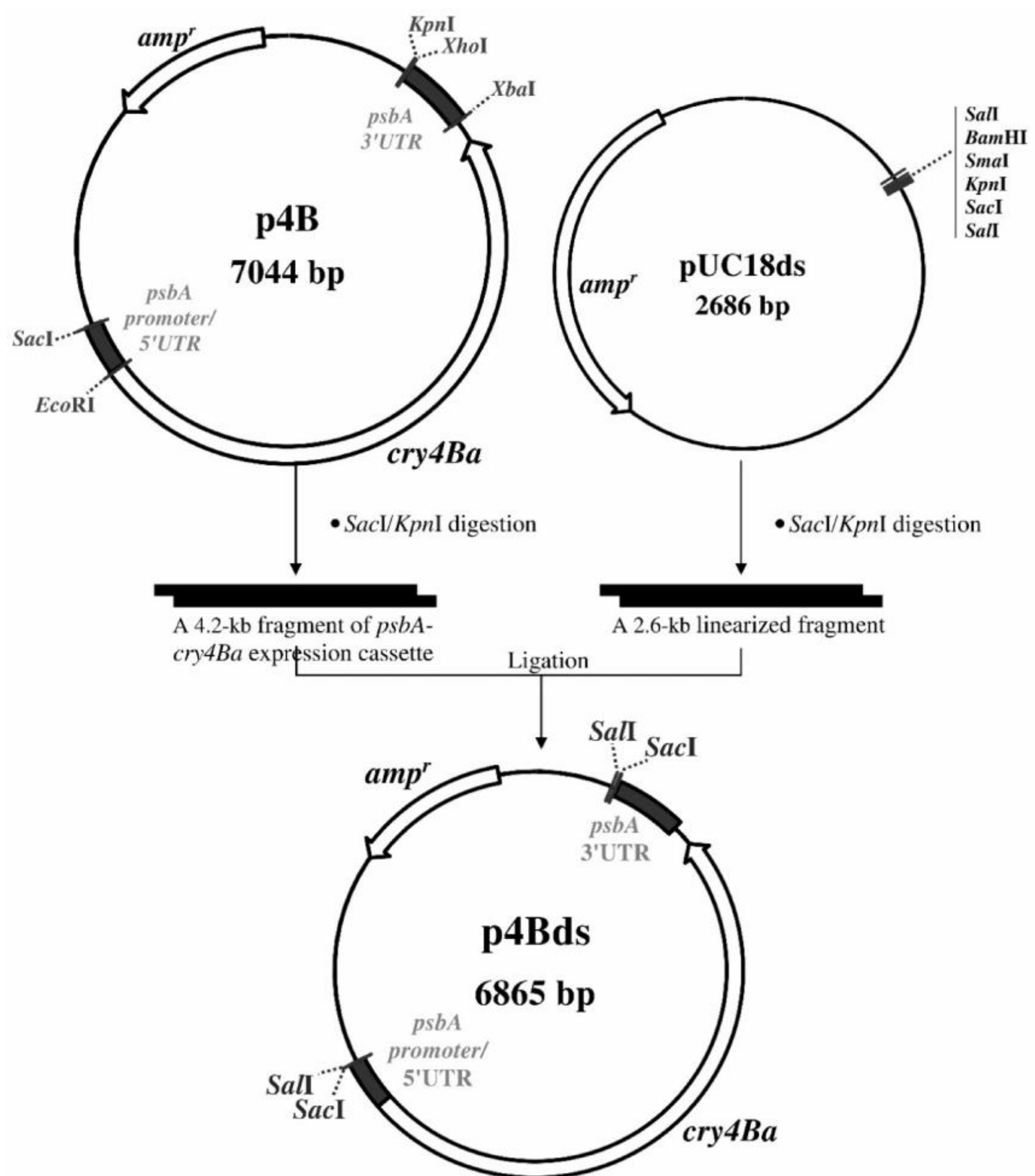


Figure 4.4 Schematic diagram of the p4B construction

A 4.2-kb fragment of *psbA-cry4Ba* was isolated from p4B by *Sac*I/*Kpn*I digestions. The cut product was ligated with the 2.6-kb fragment of pUC18ds digested by *Sac*I/*Kpn*I restriction enzymes.

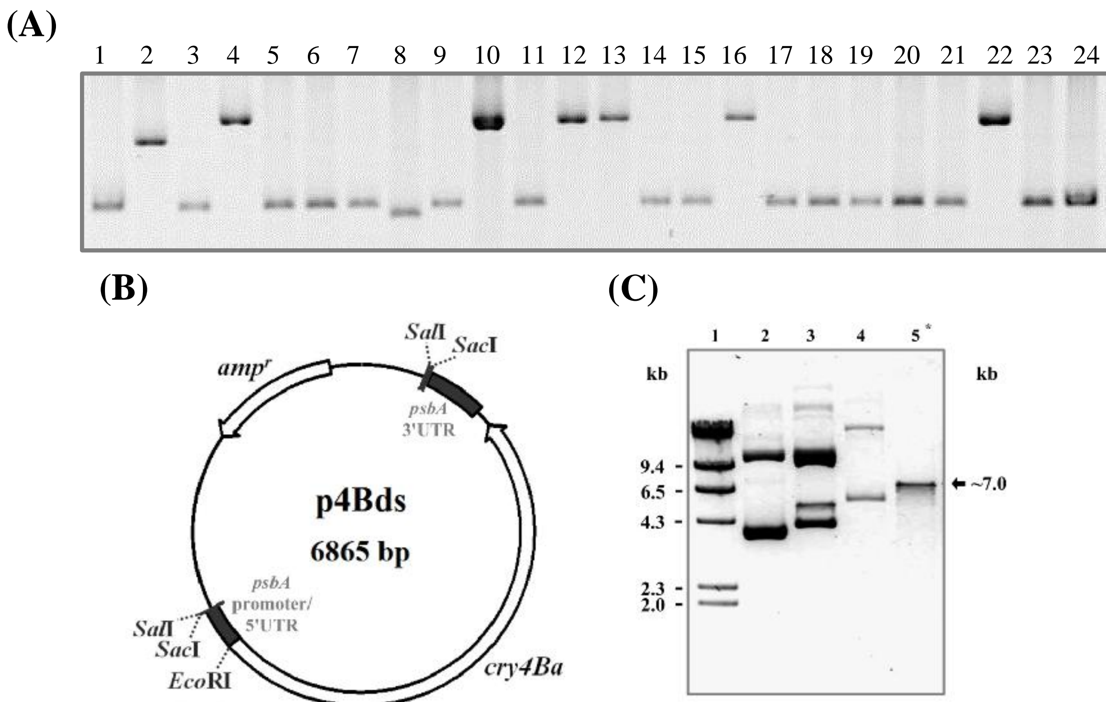


Figure 4.5 Screening and verification of p4Bds

(A) The figure demonstrates 1% agarose gel from rapid size screening of p4Bds (a ligation product between the *psbA-cry4Ba* cassette and pUC18ds).

Lane 1: Plasmid pUC18ds

Lanes 2-24: The selected recombinant clones

Samples showing the different pattern of DNA shifted band (lanes 2 and 4) are respectively designated as clone 1 and 2 for further verification by the restriction endonuclease analysis

(B) Schematic diagram shows a map of p4Bds with the position of restriction sites relevant to the construction. The expected product of p4B digested by the *Eco*RI restriction enzyme is ~7.0-kb fragment.

(C) The 1% agarose gel illustrates the restriction endonuclease analysis by *Eco*RI digestion. Only the size of the restriction product from the positive clone (asterisked) is indicated on the right.

Lane 1: /*Hin*dIII DNA marker

Lanes 2-3: Undigested and digested clone 1, respectively

Lanes 4-5: Undigested and digested clone 2, respectively

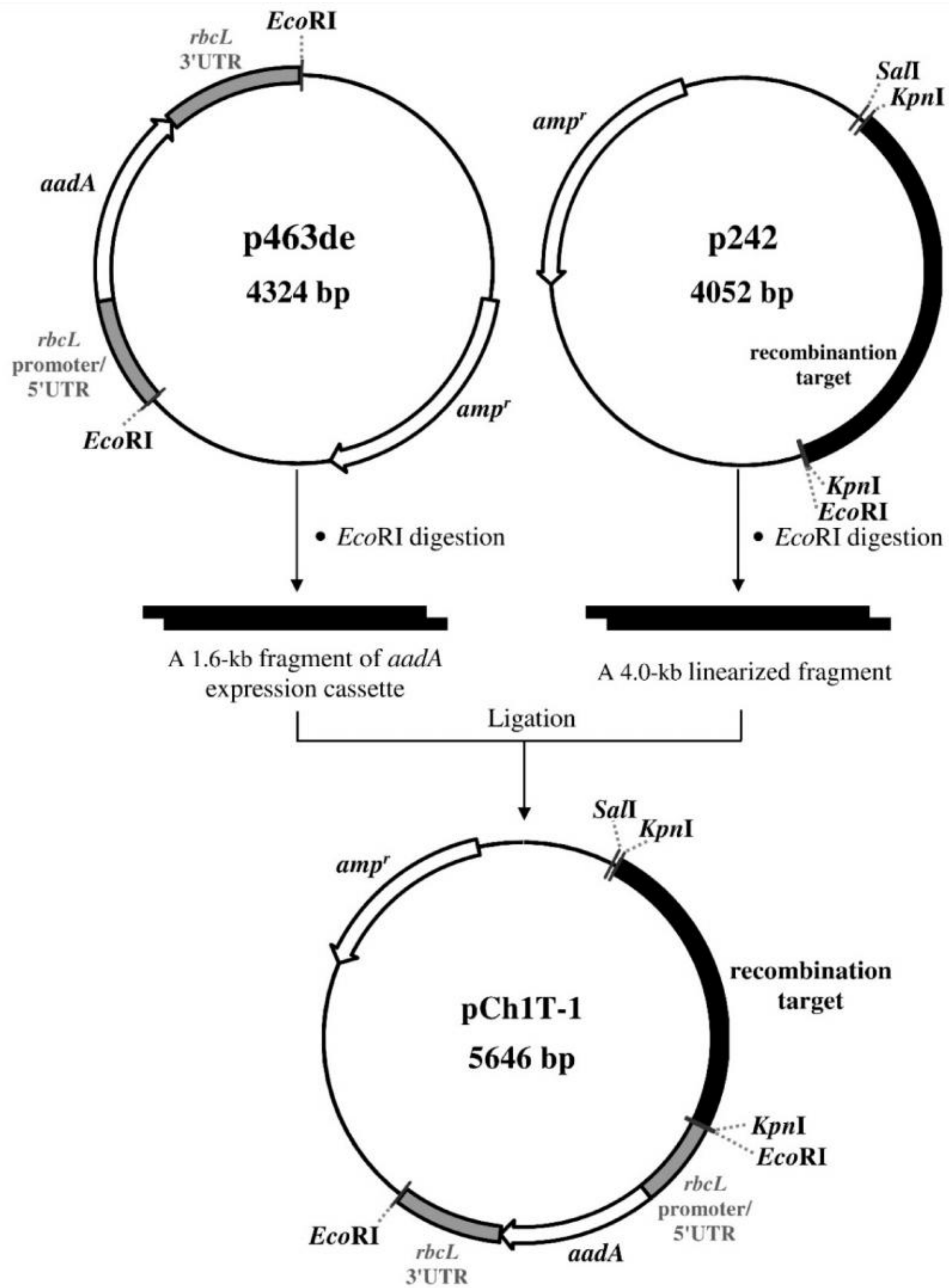


Figure 4.6 Schematic diagram of the pCh1T-1 construction

A 1.6-kb fragment of *aadA* expression cassette was excised from p463de by *Eco*RI digestion. The product was then ligated to the 4.0-kb linearized fragment of p242 digested by *Eco*RI.

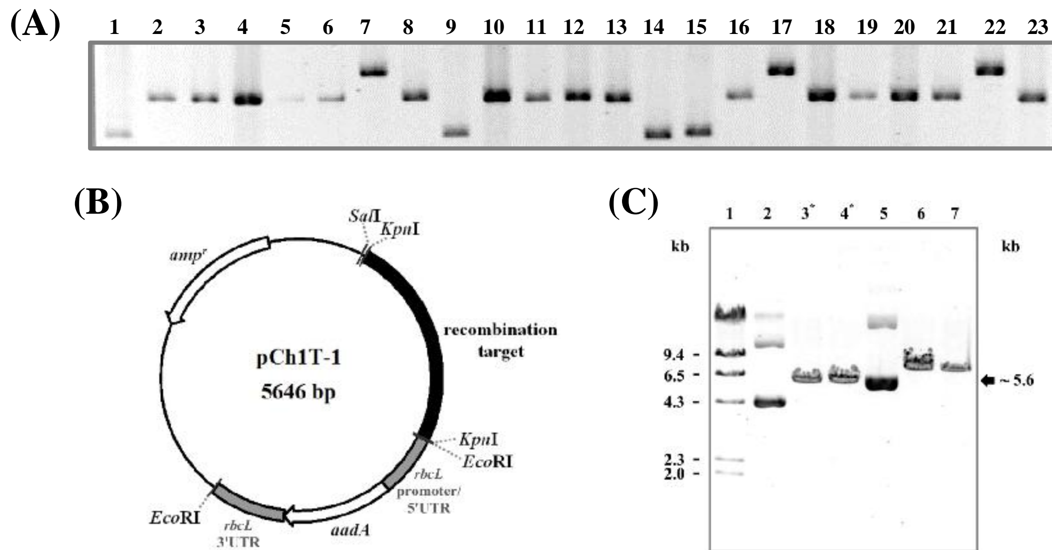


Figure 4.7 Screening and verification of pCh1T-1

(A) The figure demonstrates 1% agarose gel from rapid size screening of pCh1T-1 (a ligation product between the *aadA* expression cassette and p242 harboring a fragment of recombination target).

Lane 1: Plasmid p242

Lanes 2-23: Selected recombinant clones

Samples showing the different pattern of DNA shifted band (lanes 4 and 10 for the first pattern and lanes 7 and 17 for the second pattern) are respectively designated as clone 1 to 4 for further verification by the restriction endonuclease analysis

(B) Schematic diagram shows a map of pCh1T-1 with the position of restriction sites relevant to the construction. The expected product of pCh1T-1 digested by the *Sal*I restriction enzyme is ~5.6-kb fragment.

(C) The 1% agarose gel illustrates the restriction endonuclease analysis by *Sal*I digestion. Only the size of a restriction product from the positive clone (asterisked) is on the right.

Lane 1: /*Hin*dIII DNA marker

Lane 2: Undigested clone 1

Lane 3-4: Digested clones 1-2, respectively

Lane 5: Undigested clone 3

Lane 6-7: Digested clones 3-4, respectively

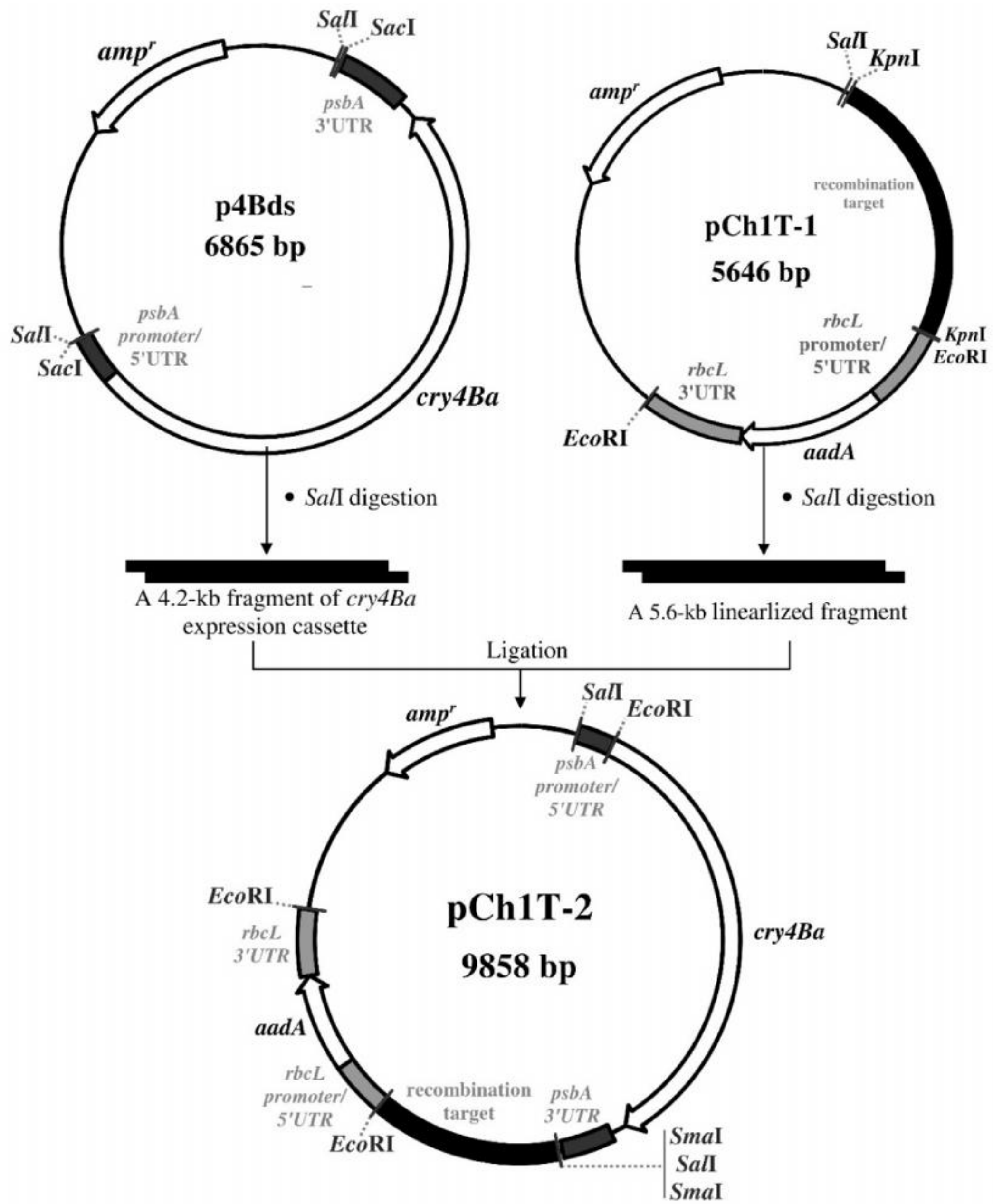


Figure 4.8 Schematic diagram of the pCh1T-2 construction

A 4.2-kb fragment of *cry4Ba* expression cassette was excised from p4Bds by *Sal*I digestion. The product was then ligated to the 5.6-kb linearized fragment of pCh1T-1 digested by the *Sal*I restriction enzyme.

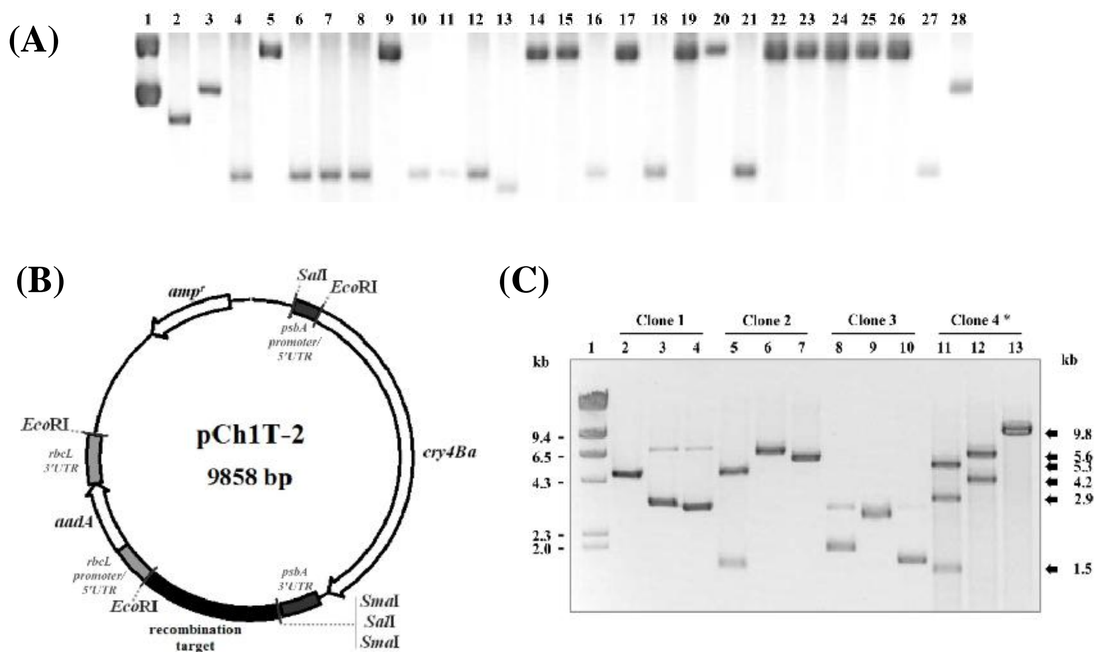


Figure 4.9 Screening and verification of pCh1T-2

(A) The figure demonstrates 1% agarose gel from rapid size screening test of the pCh1T-2 (a ligation product between the *cry4Ba* expression cassette and linearized pCh1T-1).

Lane 1: Plasmid Ch1T-1

Lane 2-28: Selected recombinant clones

Samples showing the different pattern of DNA shifted band (lanes 2, 3, 4 and 5) are respectively designated as clone 1 to 4 for further verification by the restriction endonuclease analyses.

(B) Schematic diagram shows a map of pCh1T-2 with the position of restriction sites relevant to the construction. The expected products of pCh1T-2 digested by *Eco*RI are ~ 5.3-, ~ 2.9- and ~ 1.5-kb fragments, digested by *Sal*I are ~ 5.6- and ~ 4.2-kb fragments and digested by *Sma*I are ~ 5.6-kb fragment.

(C) The 1% agarose gel illustrates the restriction endonuclease analysis by *Eco*RI (lane 2, 5, 8, 11), *Sal*I (lane 3, 6, 9, 12) and *Sma*I (4, 7, 10, 13) digestions. Only the sizes of restriction products from the positive clone (asterisked) are indicated on the right.

4.5 Construction of pCh2T-1 harboring the right recombination fragment (RF)

A 1.4-kb right recombination fragment (RF) (NCBI reference NC_005353, bases 146986 to 148500) was amplified from the *C. reinhardtii* chloroplast genome by RF-f and RF-r primers (**Table 4.2**). The amplified product was cut by *Sal*I and *Apa*I prior to subcloning the product into the MCS of pBluescriptII KS+ at corresponding cleavage sites generating pCh2T-1 (**Figure 4.10**).

After the ligated product of pCh2T-1 was delivered into *E. coli* cells grown on LB agar containing ampicillin, colonies of transformants were primarily screened by the rapid size screening method (**Figure 4.11A**). Comparing with the DNA pattern of pBluescriptII KS+, five clones with two patterns of DNA shifted band were selected to be analyzed *via* double digestion with *Sal*I/*Apa*I restriction enzymes. All clones showed the correct DNA pattern as expected (**Figure 4.11B-C**).

4.6 Construction of pCh2T-2 harboring the RF and the *cry4Ba* expression cassette

Plasmid pCh1T-2 was digested with *Sal*I and *Xho*I restriction enzymes, before a 4.1-kb digested product containing the *psbA-cry4Ba* expression cassette was subcloned into pCh2T-1 at the *Sal*I restriction site generating pCh2T-2 (**Figure 4.12**). It should be noted that *Sal*I and *Xho*I are isoschizomers.

After the ligated product of pCh2T-2 was delivered into *E. coli* cells grown on LB agar containing ampicillin, colonies of transformants were primarily screened by the rapid size screening method (**Figure 4.13A**). Comparing with the DNA pattern of pCh2T-1, two clones with different patterns of the DNA shifted band were selected to be analyzed by digestion with *Apa*I and *Eco*RI restriction enzymes. Only the clone number 1 displayed the expected DNA pattern (**Figure 4.13B-C**).

4.7 Construction of pCh2T-3 harboring expression cassettes of *aadA, psbA* and *psbA-cry4Ba* and two fragments of the homologous recombination targets

Finally, a fragment of the left recombination target or LF (NCBI reference NC_005353, bases 137761 to 138969) in combination with the expression cassettes of *psbA* and *aadA* genes was amplified from pBA157 [46] by LF-f and *rbcL*-3'UTR-r primers (**Table 4.2**). The amplified product digested by *Sac*II and *Not*I restriction enzymes was subcloned into corresponding cleavage sites of pCh2T-2 generating pCh2T-3 (**Figure 4.14**).

After the recombinant plasmid Ch2T-3 was delivered into *E. coli* cells grown on LB agar containing ampicillin, colonies of transformants were primarily screened by the rapid size screening method (**Figure 4.15A**). Three positive clones with the same size of DNA shifted band were selected to be analyzed *via* digesting with *Cla*I, *Eco*RI, *Apa*I and *Sac*II restriction enzymes. All positive clones showed the correct DNA pattern as expected (**Figure 4.15B-C**).

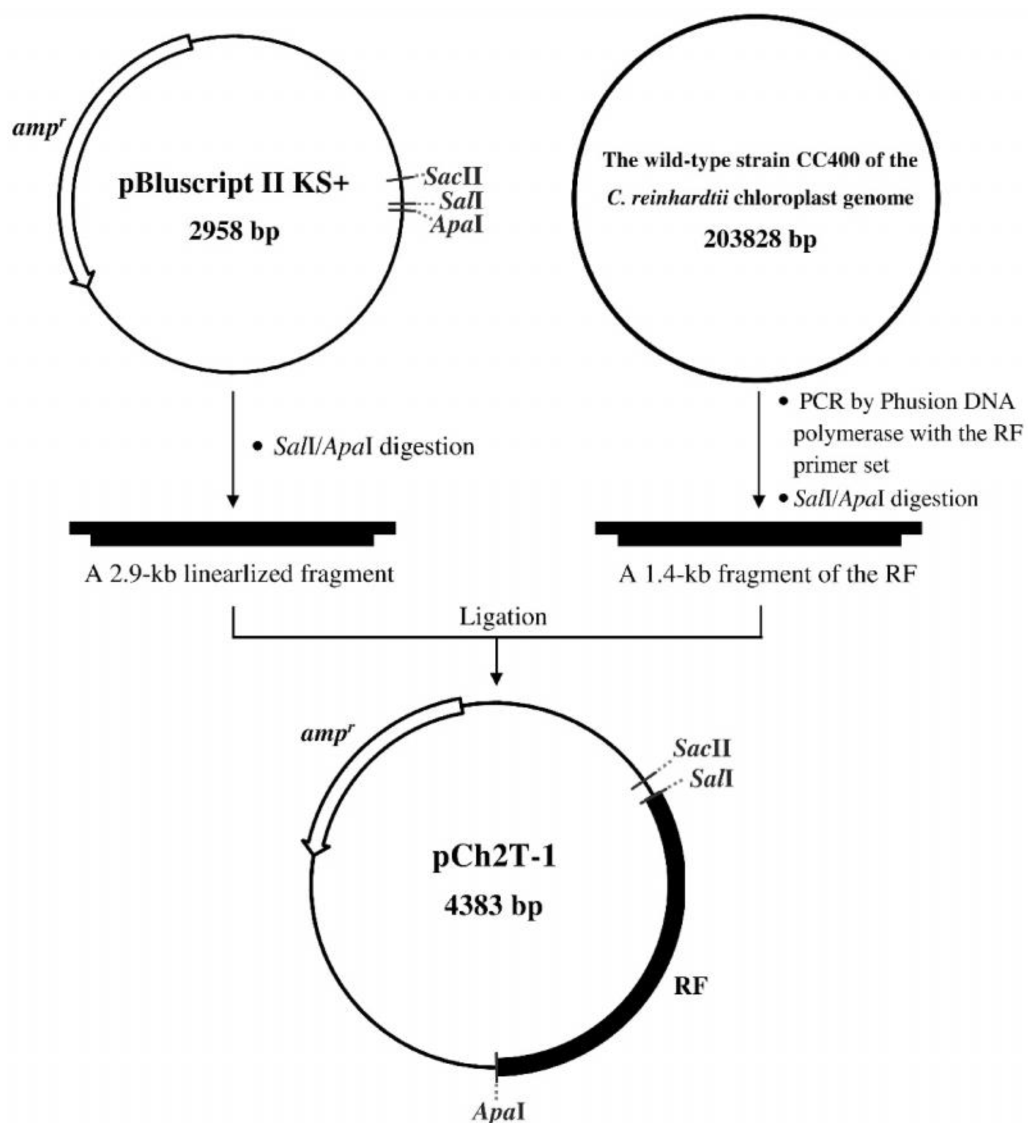


Figure 4.10 Schematic diagram of the pCh2T-1 construction

A 1.4-kb fragment of the RF was amplified from chloroplast genomes of the wild-type CC400 *C. reinhardtii* strain. The amplified product was digested by *Sal*I and *Apa*I restriction enzymes prior to ligate with the 2.9-kb linearized fragment of pBluescriptII KS+ digested with the corresponding restriction enzymes.

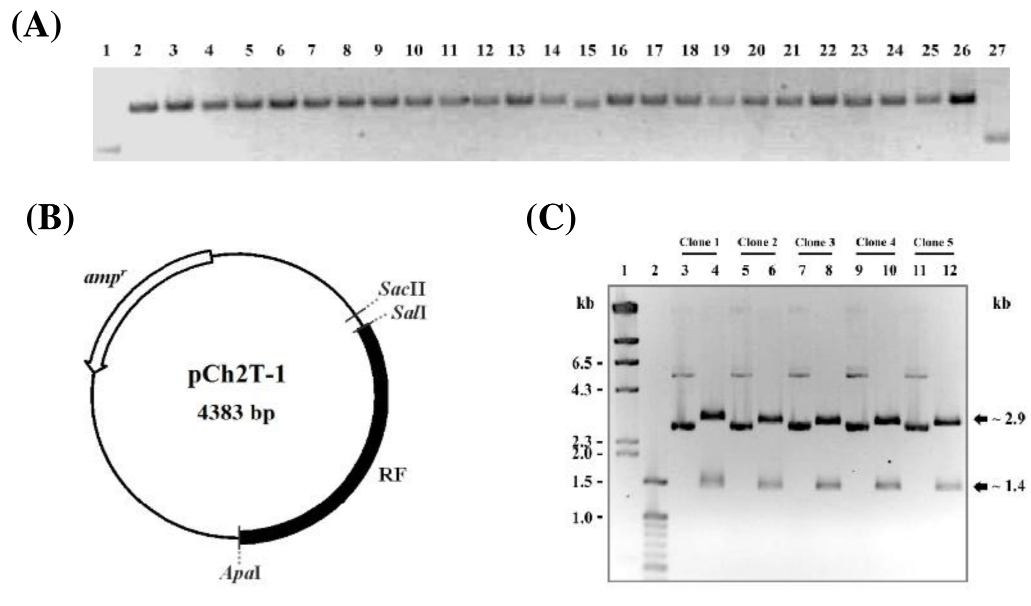


Figure 4.11 Screening and verification of pCh2T-1

(A) The figure demonstrates 1% agarose gel from rapid size screening test of the pCh2T-1 (a ligation product between pBluscript KS+ and the RF).

Lane 1: Plasmid pCh2T-1

Lanes 2-28: Selected recombinant clones

Samples showing the same pattern of DNA shifted band (lanes 2, 3, 4, 5 and 6) are respectively designated as clone 1 to 5 for further verification by the restriction endonuclease analysis.

(B) Schematic diagram shows a map of pCh2T-1 with the position of restriction sites relevant to the construction. The expected products of pCh2T-1 digested by *Sal*I/*Apa*I restriction enzymes are ~ 1.4- and ~ 2.9-kb fragments.

(C) The 1% agarose gel illustrates the restriction endonuclease analysis by *Sal*I/*Apa*I digestions. Only the sizes of restriction products from the positive clone (asterisked) are indicated on the right.

Lane 1-2: /*Hin*dIII and 100 bp DNA ladder DNA markers, respectively

Lane 3-4: Undigested and digested clone1, respectively

Lane 5-6: Undigested and digested clone2, respectively

Lane 7-8: Undigested and digested clone3, respectively

Lane 9-10: Undigested and digested clone4, respectively

Lane 11-12: Undigested and digested clone5, respectively

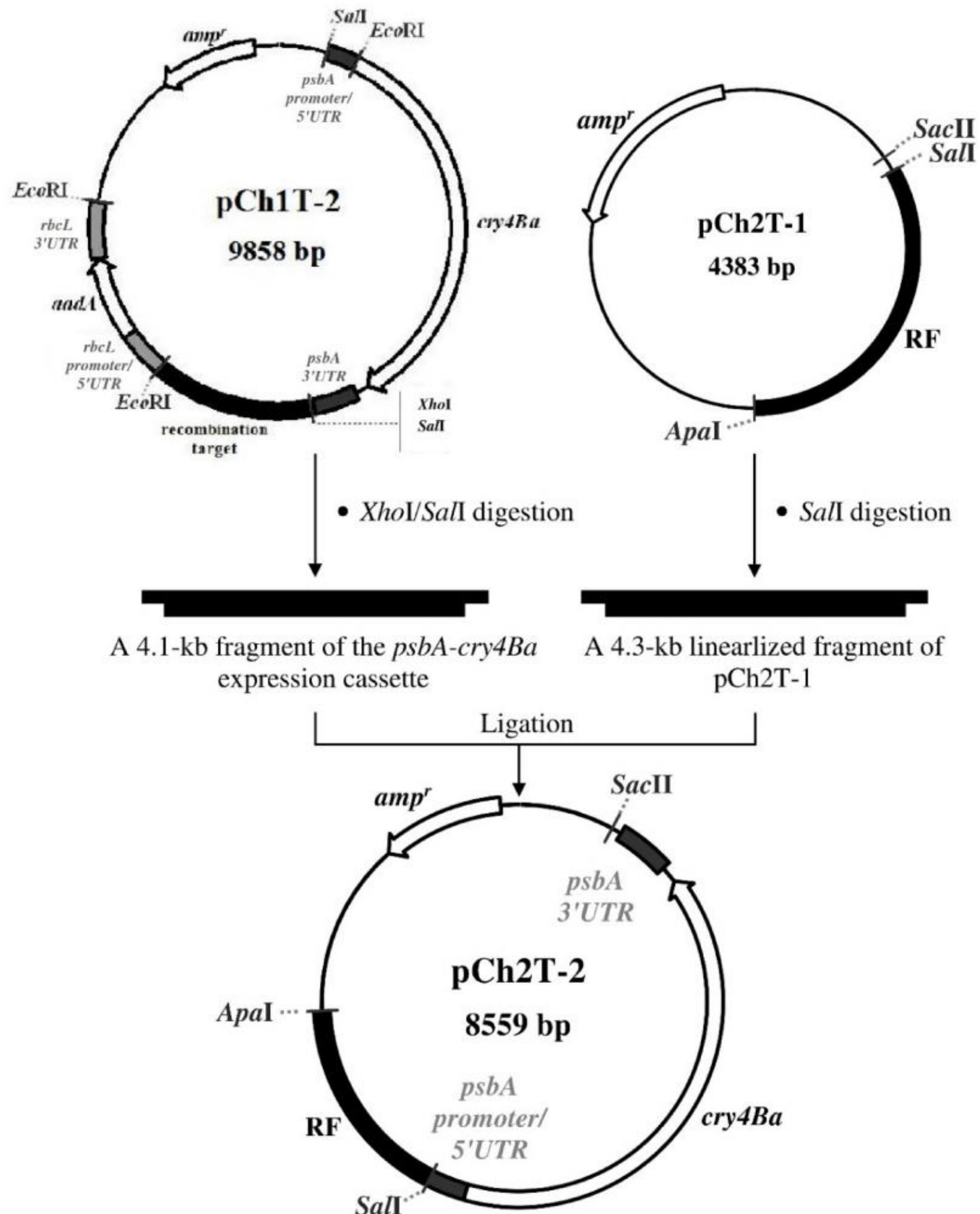


Figure 4.12 Schematic diagram of the pCh2T-2 construction

A 4.1-kb fragment of the *psbA-cry4Ba* expression cassette was isolated from p4Bds by *Xho*I and *Sal*I digestions. The digested product was ligated with the 4.3-kb linearized fragment of p4Bds digested by the *Sal*I restriction enzyme. *Xho*I (CTCGAG) and *Sal*I (GTCGAC) are isoschizomers however the ligated product (GTCGAG) is not cleaved by either enzyme.

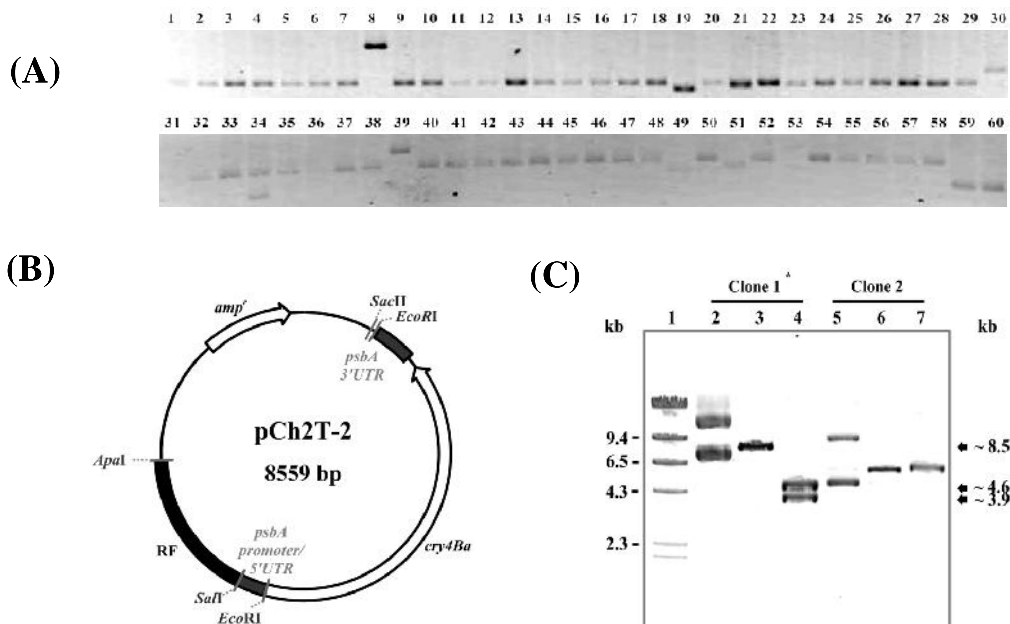


Figure 4.13 Restriction digestion analyses of pCh2T-2

(A) The figure demonstrates 1% agarose gel from rapid size screening test of pCh2T-2 (a ligation product between the *psbA-cry4Ba* expression cassette and pCh2T-1).

Lane 1: Plasmid pCh2T-1

Lanes 2-60: Selected recombinant clones

Samples showing the different pattern of DNA shifted band (lanes 8 and 39) are respectively designated as clone 1 and 2 for further verification by the restriction endonuclease analysis.

(B) Schematic diagram shows a map of pCh2T-2 with the position of restriction sites relevant to the construction. The expected products of pCh2T-2 digested by *Apa*I are ~ 8.5-kb fragment and digested by *Eco*RI are ~ 3.9- and ~ 4.6-kb fragments.

(C) The 1% agarose gel illustrates the restriction endonuclease analyses by *Apa*I and *Eco*RI digestions. Only the sizes of restriction products from the positive clone (asterisked) are indicated on the right.

Lane 1: /*Hin*dIII DNA markers

Lane 2: Undigested clone1

Lanes 3-4: Digested clone1 with *Apa*I and *Eco*RI, respectively

Lane 5: Undigested clone2

Lanes 6-7: Digested clone2 with *Apa*I and *Eco*RI, respectively

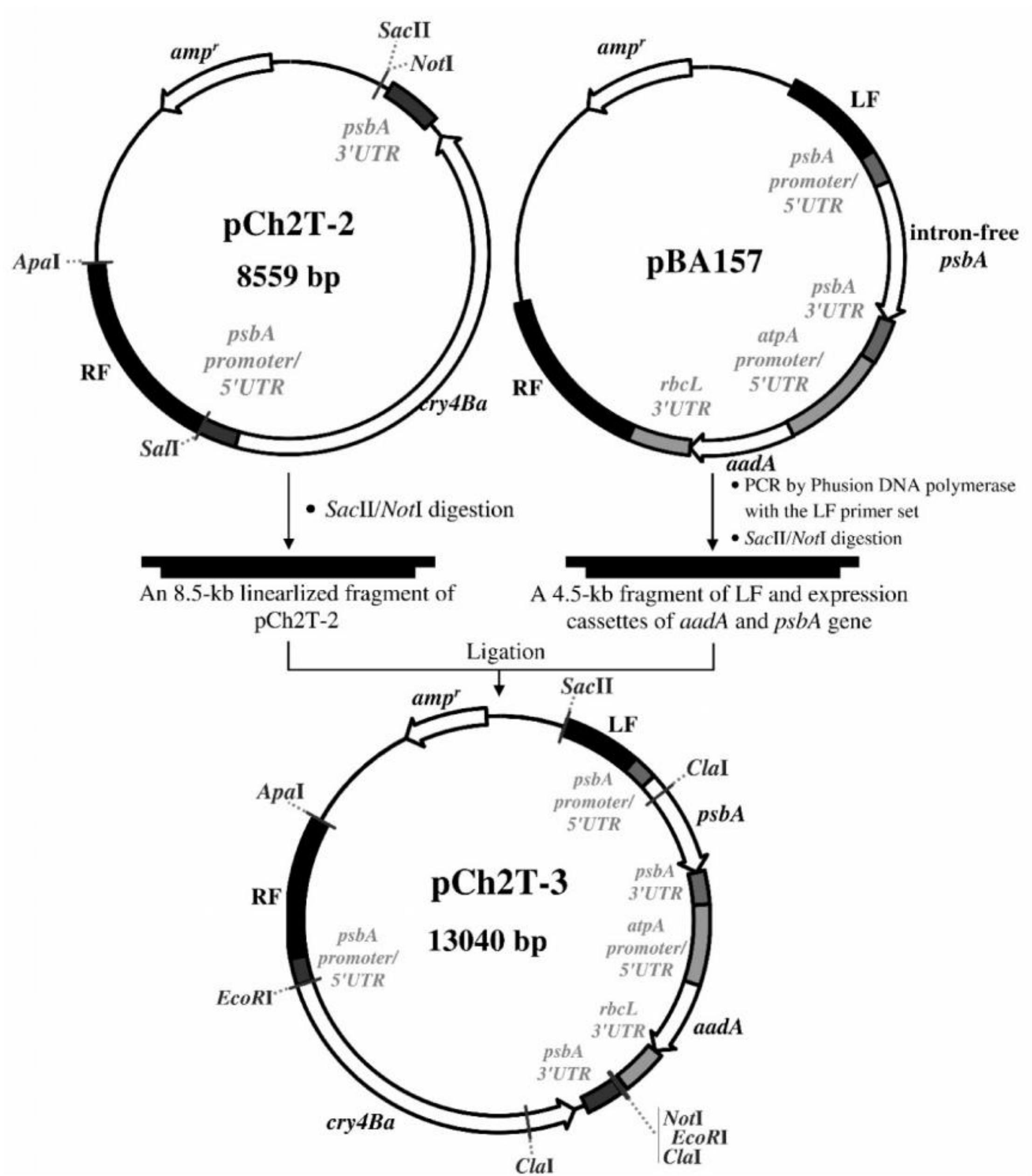


Figure 4.14 Schematic diagram of the pCh2T-3 construction

A 4.5-kb fragment of the LF in combination with expression cassettes of *psbA* and *aadA* genes was amplified from pBA157. The amplified product was digested with *Sac*II and *Not*I restriction enzymes prior to ligation with the 8.5-kb linearized fragment of pCh2T-2 digested with the corresponding enzymes.

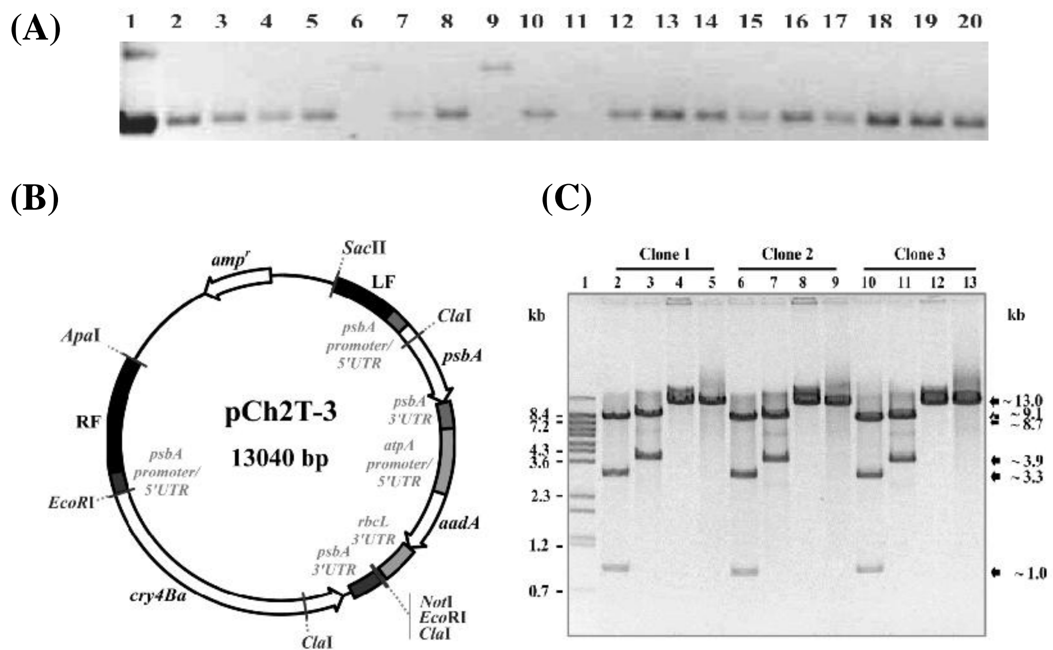


Figure 4.15 Screening and verification of pCh2T-3

(A) The figure demonstrates 1% agarose gel from the rapid size screening test of pCh2T-3 (a ligation product between the LF in combination with expression cassettes of *psbA* and *aadA* genes and the linearized fragment of pCh2T-2).

Lane 1: plasmid pCh2T-2

Lanes 2-20: Selected recombinant clones

Samples showing the same pattern of DNA shifted band (lanes 6, 9 and 11) are respectively designated as clone 1 to 3 for further verification by the restriction endonuclease analyses.

(B) Schematic diagram shows a map of pCh2T-3 with the position of restriction sites relevant to the construction. The expected products of pCh2T-2 digested by *Cla*I are ~8.7, ~3.3 and ~1.0-kb fragments, digested by *Eco*RI are ~9.1- and ~3.9-kb fragments, digested by *Apa*I as well as by *Sac*II are ~13.0-kb fragments.

(C) The 1% agarose gel illustrates the restriction endonuclease analyses by *Cla*I (lane 2, 6, 10), *Eco*RI (3, 7, 11), *Apa*I (4, 8, 12) and *Sac*II (5, 9, 13) digestions. Only the sizes of restriction products from the positive clone (asterisked) are indicated on the right.

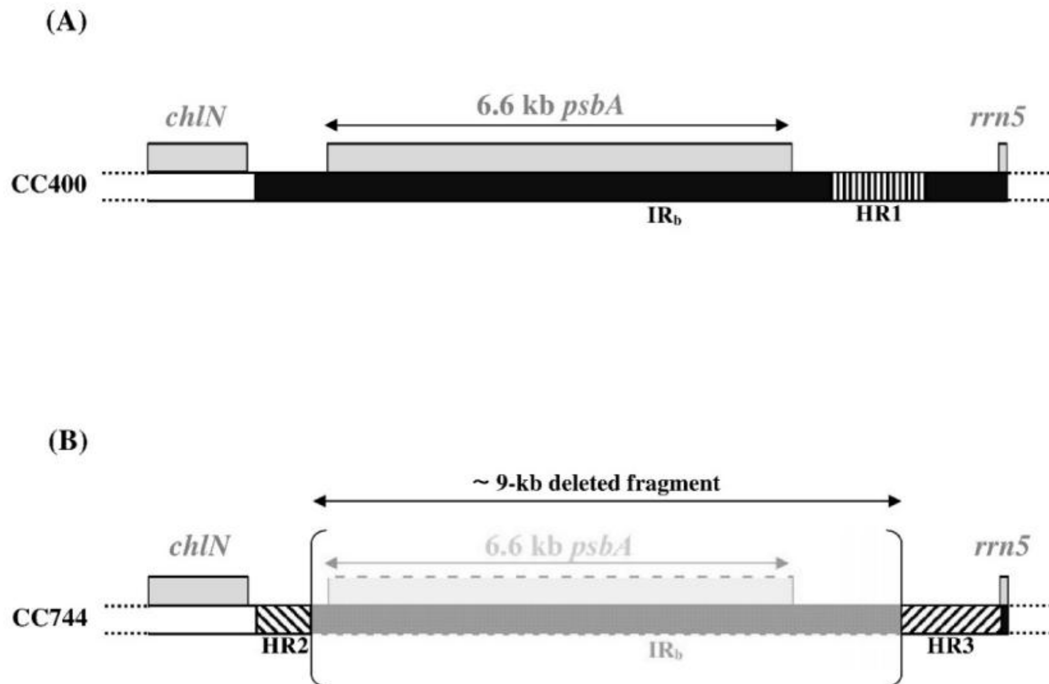


Figure 4.16 Maps of insertion sites on the chloroplast genome of CC400 and CC744 *C. reinhardtii* strains

(A) Map of the insertion site located on IR_b of the CC400 *C. reinhardtii* chloroplast genome. **(B)** Map of the insertion site located on IR_b of the CC744 *C. reinhardtii* chloroplast genome. Approximate 9-kb deleted fragment in CC744 strain is indicated by brackets. The single homologous recombination site used for insertion of pCh1T-2 on the CC400 chloroplast genome is indicated by hatch box HR1. Two homologous recombination sites on the chloroplast genome of CC744 strain used for insertion of pCh2T-3 were indicated by hatch box HR2 and HR3. The location of IR_b on the chloroplast genome was shown as a black bar, the single-copy region as a white bar, *chlN*, *psbA* and *rrn5* genes as grey boxes.

4.8 Optimization of chloroplast transformation conditions of glass beads and biolistic bombardment methods using pCh1T-1 and pCh1T-2

The conditions of chloroplast transformation by glass beads and bombardment methods were optimized by employing the 5.6-kb pCh1T-1 harboring *aadA* drug-resistance marker gene and a fragment of recombinant target.

The 10^7 cells/ml of the log-phase CC400 *C. reinhardtii* strain was prepared and treated with the autolysin prior to transformation with pCh1T-1 using the glass beads method. Among the agitated time period of 5, 10, 15, 20, 25 and 30 sec however there was no colony on the selective agar plate. To solve this problem, the bombardment method, generally offering the higher chloroplast transformation efficiency, was employed.

The optimized pressure and distance for delivering recombinant genes into *C. reinhardtii* chloroplast genomes were determined *via* bombarding cells with pCh1T-1. After bombardment parameters were tried, the condition using the 900-psi pressure and the 9-cm distance was found to be suitable to generate the transformant lines. All putative clones were subjected to verify the existence of *aadA* gene by PCR technique using *aadA*-f and *aadA*-r primers (**Table 4.2**). The 0.4-kb fragment could be amplified from chloroplast genomes of positive clones corresponding to the PCR product of pCh1T-1 used as the positive control (**Figure 4.17**).

Those optimized bombardment conditions were subsequently used to deliver pCh1T-2, harboring a fragment of recombinant target and the expression cassettes of *aadA* and *cry4Ba* genes, into chloroplast genomes of CC400 *C. reinhardtii* cells. However, only the *aadA* amplification product could be observed from all transformant lines (**Figure 4.18**).

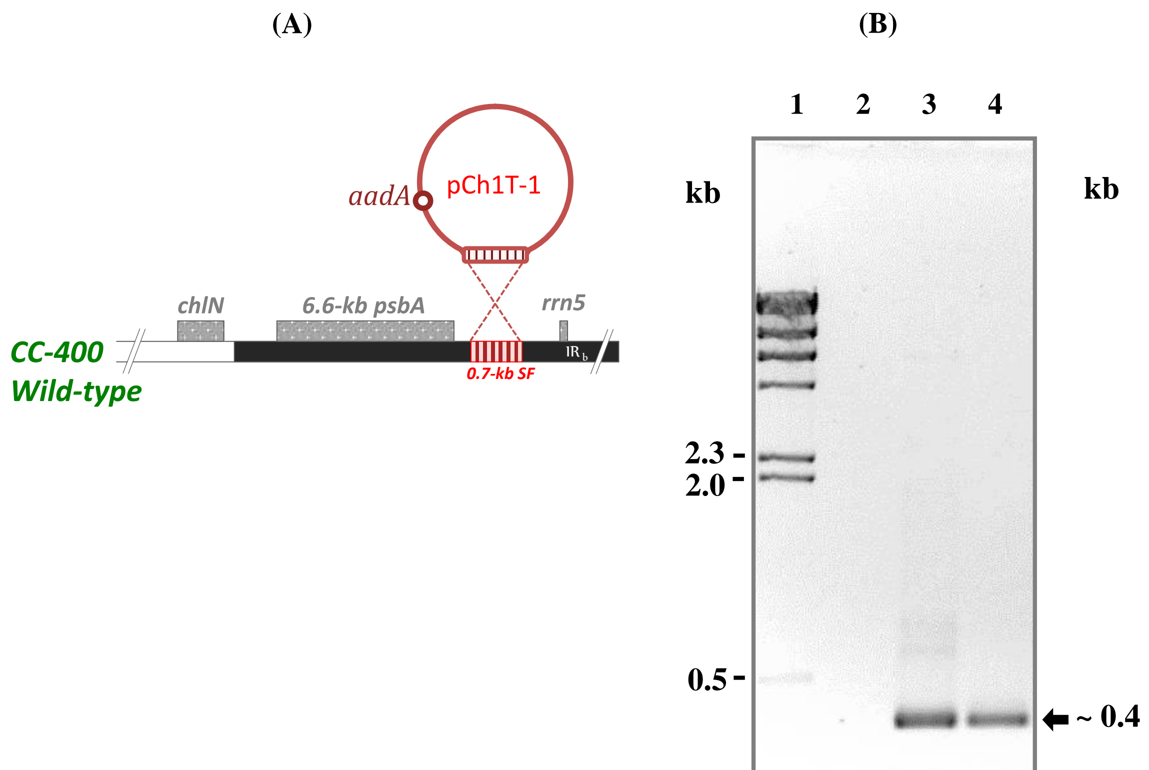


Figure 4.17 Amplification of the *aadA* transgene from CC400 *C. reinhardtii* transformed by pCh1T-1

(A) Integration of pCh1T-1 vector containing the recombination *aadA* gene into a single target of the homologous recombination site of CC400 *C. reinhardtii* chloroplast genomes

(B) The 50 ng of total DNA extracted from non-transformed and transgenic lines was subjected for PCR verification by using *aadA*-f and *aadA*-r primers with the expected product at 413 bp. The amplification product was analyzed by 0.8% agarose gel electrophoresis with ethidium bromide staining

Lane 1: /*Hin*dIII DNA marker

Lane 2: PCR product of total DNA extracted from non-transformed line

Lane 3: PCR product from pCh1T-1

Lane 4: PCR product of total DNA extraction of the transgenic line

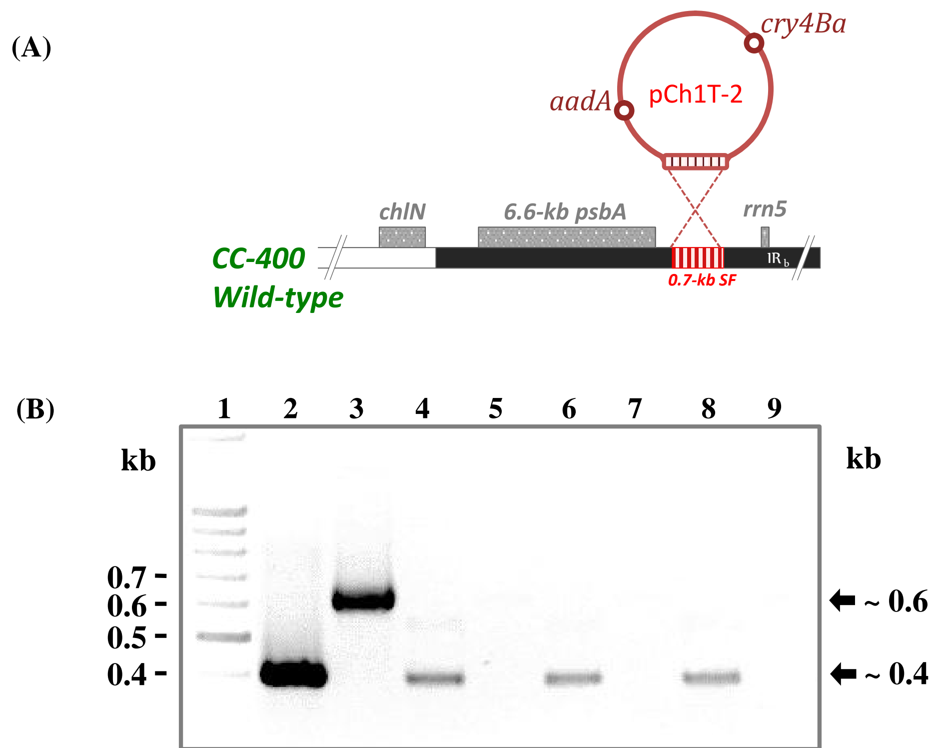


Figure 4.18 Amplification of *aadA* and *cry4Ba* transgenes from CC400 *C. reinhardtii* transformed by pCh1T-2

(A) Integration of pCh1T-2 vector containing the recombination *aadA* and *cry4Ba* genes into a single target of the homologous recombination site of CC400 *C. reinhardtii* chloroplast genomes

(B) The 50 ng of the total DNA extracted from non-transformed and transgenic lines was subjected for PCR verification by using *aadA* and *cry4Ba*-domain II (DII) primer sets with expected products at 413 bp and 633 bp, respectively. The amplification product was analyzed by 0.8% agarose gel electrophoresis with ethidium bromide staining.

Lane 1: 100-bp DNA ladder

Lane 2: PCR product of *aadA* from pCh1T-2

Lane 3: PCR product of *cry4Ba*-DII from pCh1T-2

Lanes 4-5: Respective PCR products of *aadA* and *cry4Ba*-DII from line 1

Lanes 6-7: Respective PCR products of *aadA* and *cry4Ba*-DII from line 2

Lanes 8-9: Respective PCR products of *aadA* and *cry4Ba*-DII from line 3

4.9 Chloroplast transformation with pCh2T-3 by the biolistic bombardment method

Since transgenic lines with pCh1T-2 construct could not be detected the signal of the *cry4Ba* transgene, attempts were made by improving a new transformation construct, pCh2T-3, harboring 2 recombination fragments. The later construct contains recombinant *aadA*, *cry4Ba* and intron-less *psbA* genes which were controlled by the individual expression cassette. Five ml of 10^7 cells/ml log-phase of *psbA*-deleted CC744 *C. reinhardtii* were spread on TAP agar supplemented with 100 μg/ml of spectinomycin prior to bombarding with pCh2T-3 under 900 psi pressure at the distance of 9 cm. After 3 weeks of dual-phenotypic screening *via* exhibition of resistance to spectinomycin and the restoration of the photosynthetic activity, three transformant lines were recovered (**Figure 4.19**).

4.10 Verification of specific integration of the *cry4Ba* transgene in chloroplast genomes by PCR analyses

Three primer pairs specific to recombinant *psbA* (*psbA*-f and *psbA*-r), *aadA* (*aadA*-f and *aadA*-r) and *cry4Ba* genes (4B-ORF and R1) were employed to verify the existence of inserted genes. The templates from pCh2T-3 and from the total genomic DNA of CC744 strain were respectively used as positive and negative controls for the PCR analysis. The result indicated that all lines exhibited majority bands of full-length recombinant 1.0-kb *psbA* (**Figure 4.20**), 0.4-kb *aadA* (**Figure 4.21**) and 3.4-kb *cry4Ba* (**Figure 4.22**) genes, respectively. To further identify whether the transgenes are specifically integrated into the inverted repeats of chloroplast genomes, PCR identification with the other two pairs of specific primers were employed. L1/L2 primer pairs bind to the recombinant *psbA* genes and the endogenous *ChlN* gene, whereas R1/R2 primer pairs bind to the recombinant *cry4Ba* gene and the endogenous *rrn5* gene. Results revealed that a 2.3 kb could be produced from the L1/L2 amplification. On the other hands, 5.2-kb product was amplified from the annealing of R1/R2 primers (**Figure 4.23**). A correct orientation of *cry4Ba* transgene is ascertained *via* restriction analyses of the 5.2-kb R1/R2 product (**Figure 4.24**).

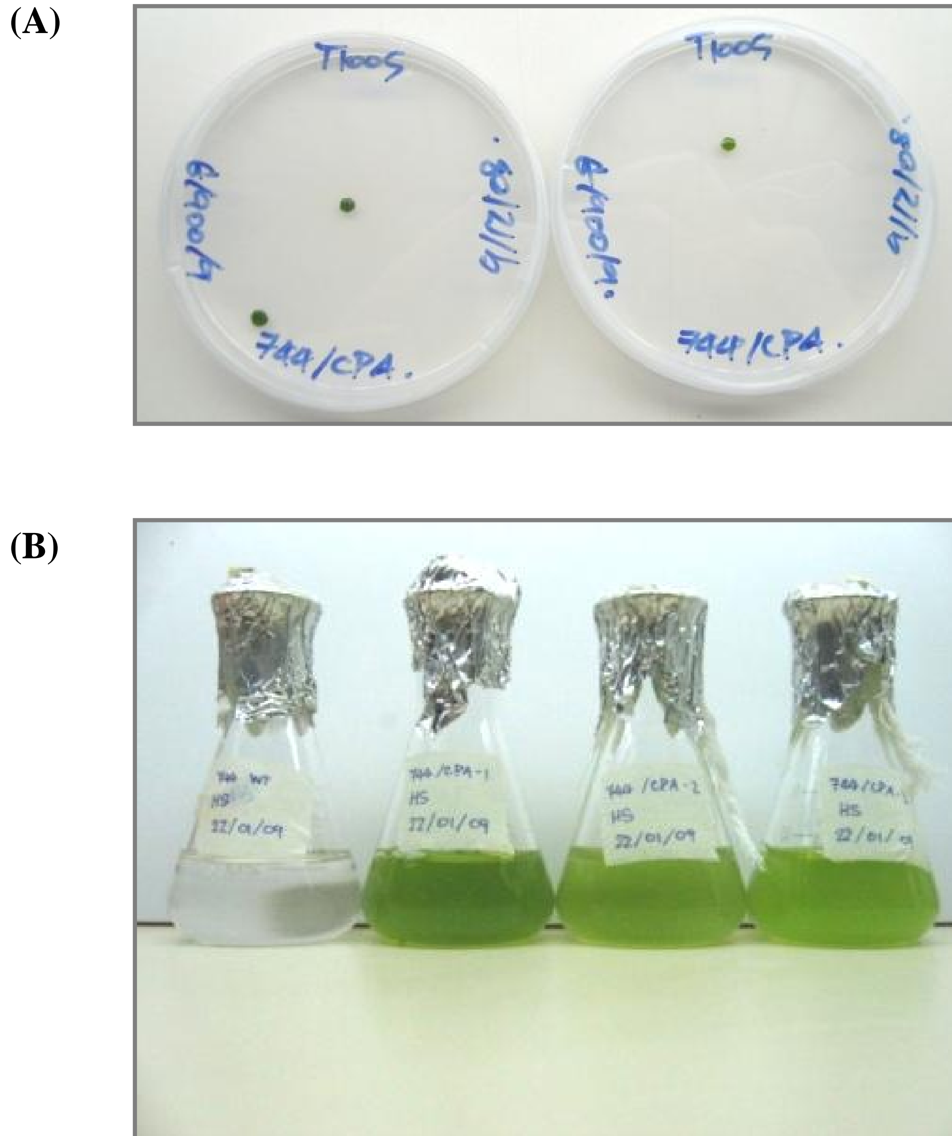


Figure 4.19 Dual-phenotypic screening *via* exhibition of resistance to spectinomycin and restoration of the photosynthetic activity

(A) Recovered transformant colonies on the selective TAP agar **(B)** Compatible with the first result, the further screening by photoautotrophic growth showed that all transformants could perform photosynthesis and and so could survive and scale up in the minimal medium. The CC744 strain acted as a negative control could not survive on minimal medium as the cell culture was clear.

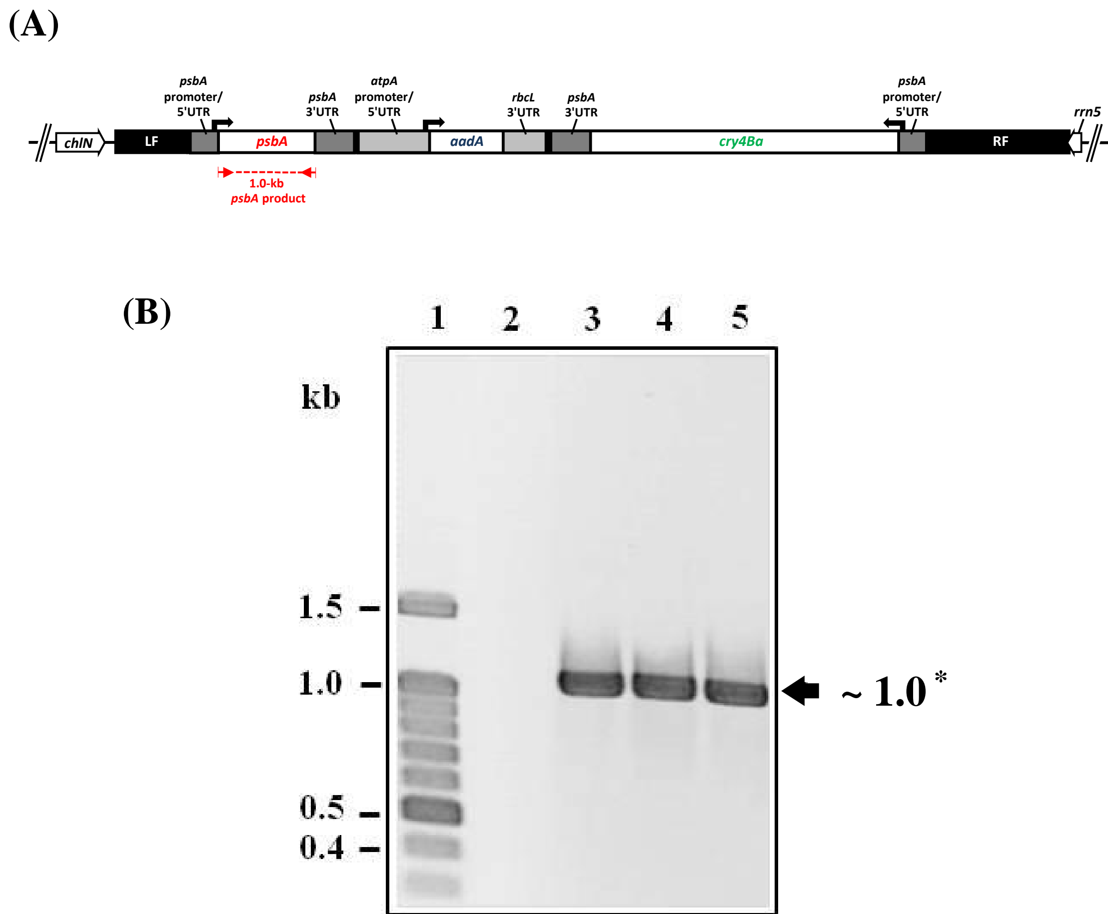


Figure 4.20 Amplification of the *psbA* transgene from total genomic DNA of transformant lines

(A) A diagram illustrates primer-binding sites and the expected product of the *psbA* transgene on the IR_b of *C. reinhardtii* transgenic chloroplast genomes.

(B) 50 ng of the total genomic DNA extracted from non-transformed and transgenic lines were subjected for PCR verification using *psbA*-f and *psbA*-r primers with expected products at 1,059 bp (asterisk). Amplification products were analyzed by 0.8% agarose gel electrophoresis.

Lane 1: 100-bp DNA ladder

Lane 2: PCR product of *psbA* from CC744 strain

Lanes 3-5: PCR product of *psbA* from transformant lines 1-3, respectively

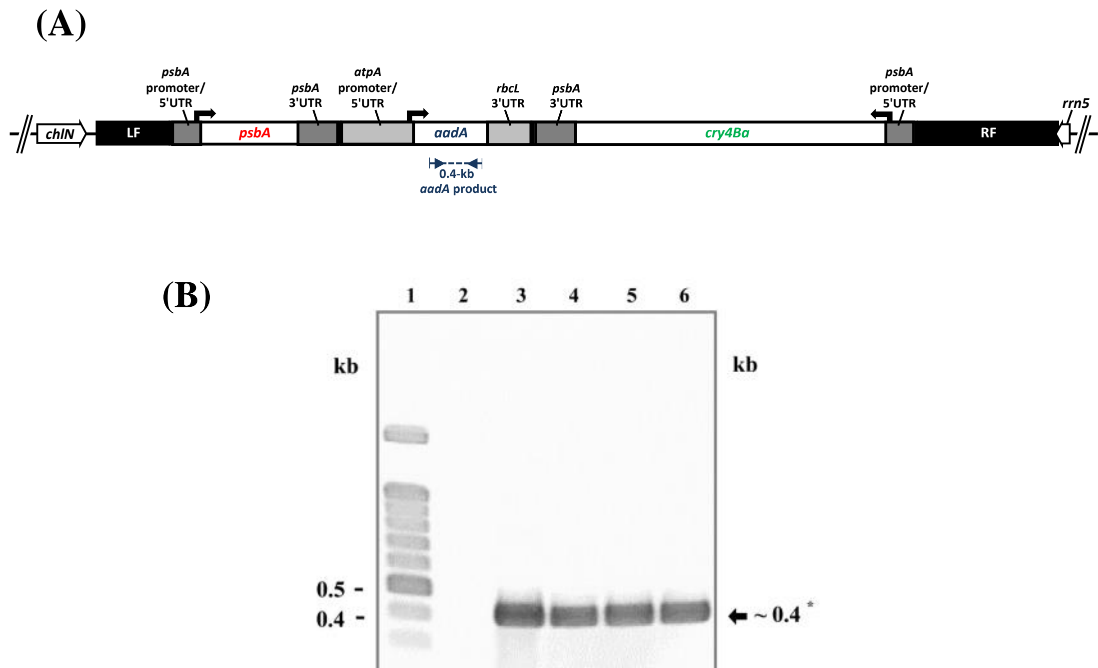


Figure 4.21 Amplification of the *aadA* transgene from the total genomic DNA of transgenic lines

(A) A diagram illustrates primer-binding sites and the expected product of the *aadA* transgene on the IR_b of *C. reinhardtii* transgenic chloroplast genomes.

(B) 50 ng of the total genomic DNA extracted from non-transformed and transgenic lines were subjected for PCR verification by using *aadA*-f and *aadA*-r primers with expected products at 413 bp (asterisk). Amplification products were analyzed by 0.8% agarose gel electrophoresis.

Lane 1: 100-bp DNA ladder

Lane 2: PCR product of *aadA* from CC744 strain

Lane 3: PCR product of *aadA* from pCh2T-3

Lanes 4-6: PCR product of *aadA* from transformants 1-3, respectively

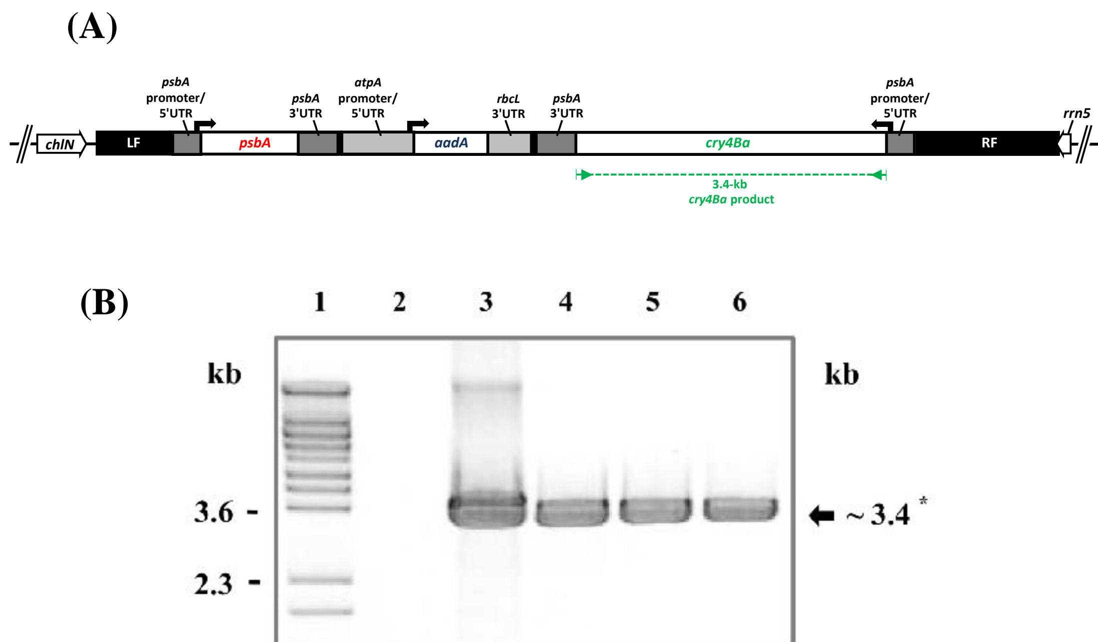


Figure 4.22 Amplification of the *cry4Ba* transgene from the total genomic DNA of transgenic lines

(A) A diagram illustrates primer-binding sites and the expected product of the *cry4Ba* transgene on the IR_b of *C. reinhardtii* transgenic chloroplast genomes.

(B) 50 ng of the total DNA extracted from non-transformed and transgenic lines were subjected for PCR verification by using 4B-ORF and R1 primers, which were respectively specific to start and stop codon of the *cry4Ba* gene, with expected products at 3,405 bp (asterisk). Amplification products were analyzed by 0.8% agarose gel electrophoresis.

Lane 1: /*Bst*EII DNA marker

Lane 2: PCR product of *cry4Ba* from CC744 strain

Lane 3: PCR product of *cry4Ba* from pCh2T-3

Lanes 4-6: PCR product of *cry4Ba* from transformants 1-3, respectively

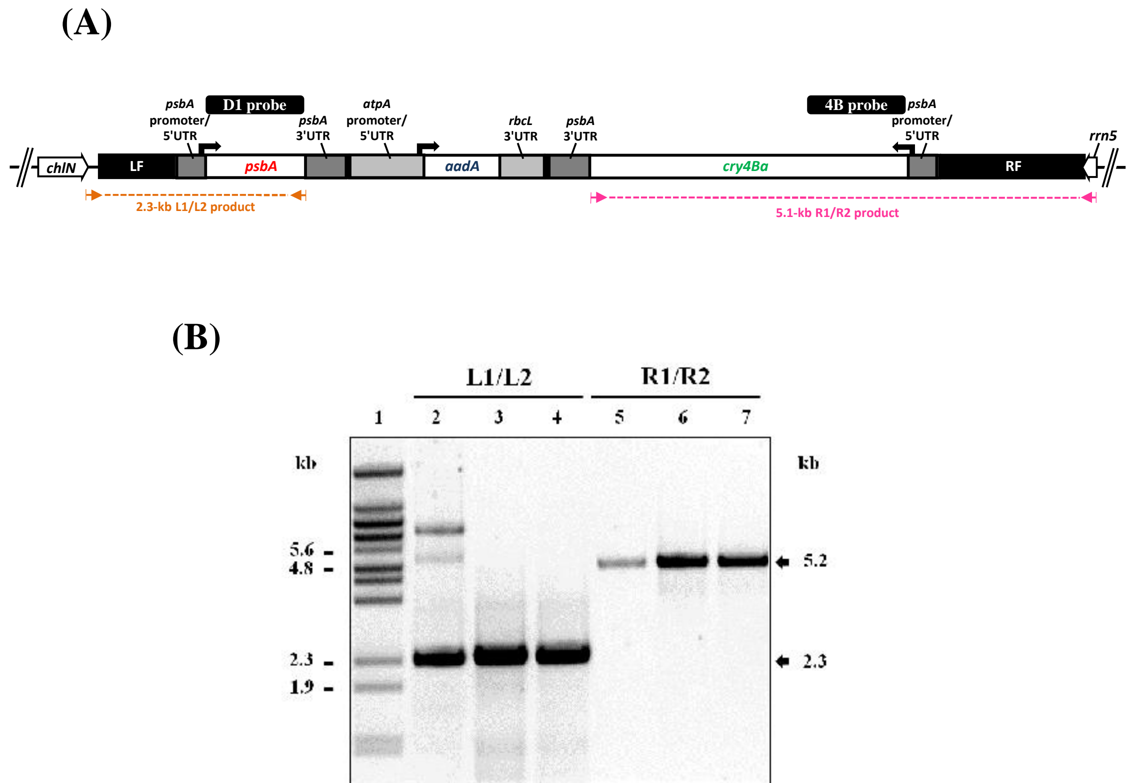


Figure 4.23 Identification of specific transgene integration into chloroplast genomes by PCR analysis

(A) A diagram illustrates primer-binding sites and the expected products of L1/L2 and R1/R2 amplifications on the IR_b of *C. reinhardtii* transgenic chloroplast genomes. Two black bars, D1 and 4B, indicated the binding site of each probe.

(B) 0.8% agarose gel showed the result of PCR analysis of transgene integration in transformants 1-3 indicating ~ 2.3-kb L1/L2 and ~ 5.2-kb R1/R2 products.

Lane 1: /*Bst*EII DNA marker

Lanes 2-4: L1/L2 PCR products from transformants 1-3

Lanes 5-7: R1/R2 PCR products from transformants 1-3

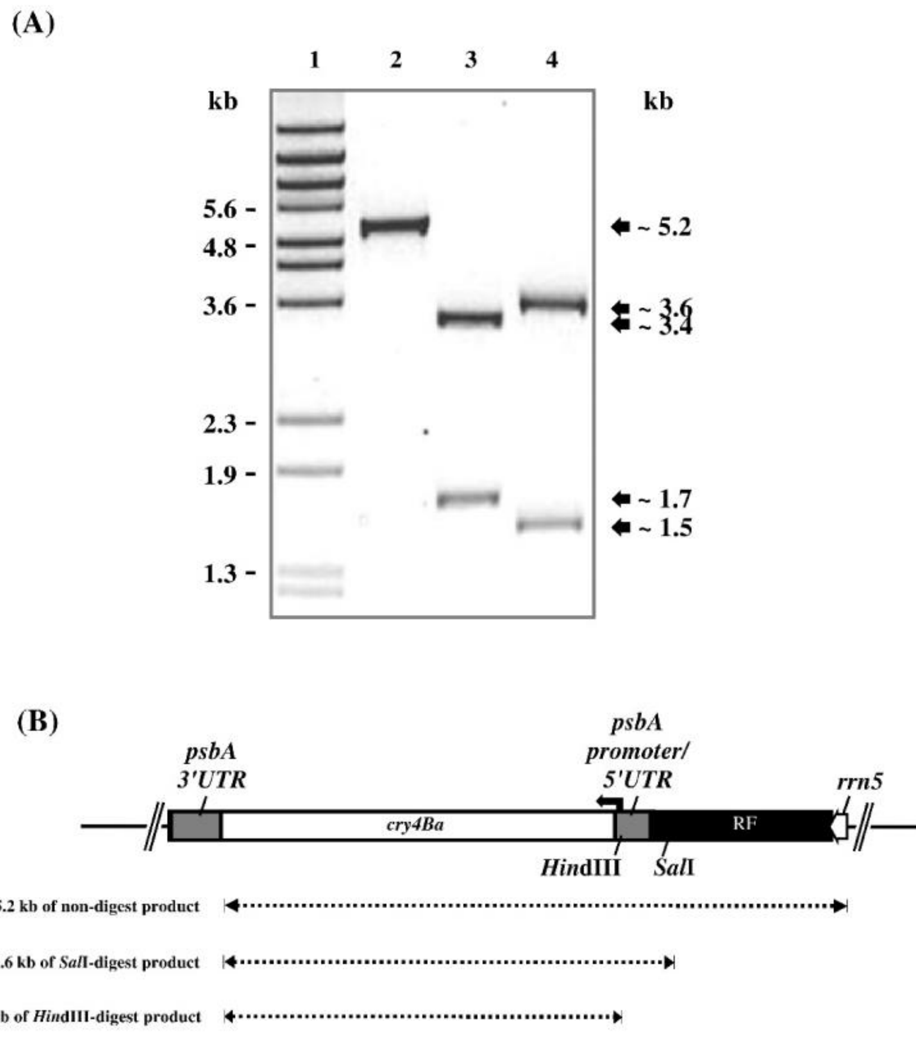


Figure 4.24 Verification of the R1/R2 PCR product by *Sal*I and *Hin*dIII restriction digestion analyses

(A) The 5.2-kb R1/R2 product was separately digested with *Sal*I and *Hin*dIII prior to analyzing by 0.8% agarose gel electrophoresis.

Lane 1: /*Bst*EII DNA marker

Lane 2: Non-digest R1R2 PCR product

Lane 3: Digestion analysis of the R1/R2 PCR product by *Hin*dIII

Lane 4: Digestion analysis of the R1/R2 PCR product by *Sal*I

(B) A diagram illustrates the DNA fragment covering from the expression cassette of recombinant *cry4Ba* to the nearest wild-type chloroplast gene, *rrn5*, with the position of relevant restriction enzymes.

4.11 Verification of the recombinant *cry4Ba* transcript by the RT-PCR analysis

The accumulation of recombinant *cry4Ba* transcript was analyzed *via* the RT-PCR technique with *cry4Ba* specific 4B-ORF and R1 primers generating the product covering the ORF of *cry4Ba* gene. Results showed the 3.4-kb amplified product of the *cry4Ba*-cDNA equal to that of pCh2T-3 which was using the same primer set and served as a positive control. The negative RT contained no RNA-dependent DNA polymerase enzyme, confirming the absence of DNA from the RNA template, whereas the negative PCR containing no template verified the existence of cDNA template for the subsequent PCR step (**Figure 4.25**).

4.12 Verification of recombinant *cry4Ba* and *psbA* transcripts by Northern blot analysis

The *cry4Ba*-specific DIG-probe (4B-probe) (**Figure 4.26A**), specific to the approximately 0.9 kb immediately upstream of the start codon, was generated in order to confirm the accumulation of *cry4Ba* transcript by Northern blot analysis. After total RNA samples were transferred from the denaturing agarose gel onto the nylon membrane, the detection step was performed. The results showed that there was a majority band at approximately 3.4-kb fragment as expected (**Figure 4.27B**).

The *psbA*-specific DIG-probe (D1-probe) (**Figure 4.26B**), complementary to the approximately 1.0 kb full-length of *psbA*-cDNA, was generated in order to confirm the accumulation of *psbA* transcript by Northern blot analysis. The results showed a majority band at approximately 1.0-kb fragment from total RNA samples of all transformants and from that of the CC400 strain which served as a positive control (**Figure 4.27A**). The ethidium-bromide staining of the corresponding agarose gel served as the loading control of each sample (**Figure 4.27C**).

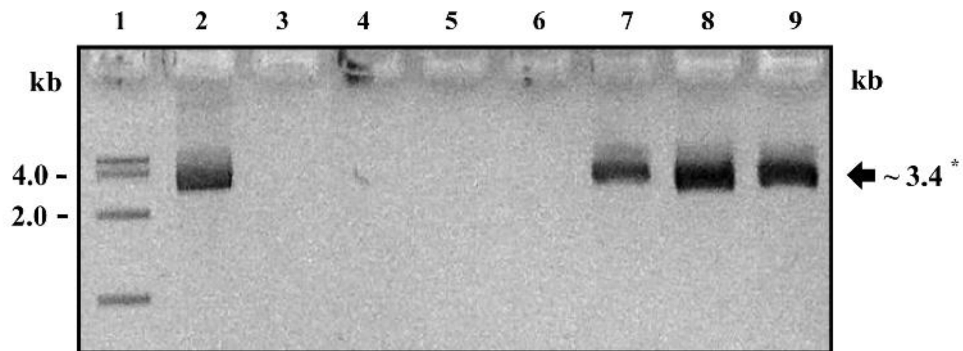


Figure 4.25 Identification of the recombinant *cry4Ba* transcript by RT-PCR analysis

The 250 ng of DNA-free RNA of transgenic lines was subjected for RT by *cry4Ba*-specific reverse primer R1. The *cry4Ba*-cDNA from RT-product was then amplified by *cry4Ba*-specific primers, 4B-ORF and R1, which generated the expected products of 3405 bp (asterisk). The amplification product was analyzed by 0.8% agarose gel electrophoresis with ethidium bromide staining.

Lane 1: Gene ladder fast2, DNA marker

Lane 2: PCR product of *cry4Ba* from pCh2T-3

Lane 3: PCR product from the reaction without template

Lanes 4-6: The RT negative products of transformants 1-3, respectively

Lanes 7-9: RT-PCR products of transformants 1-3, respectively

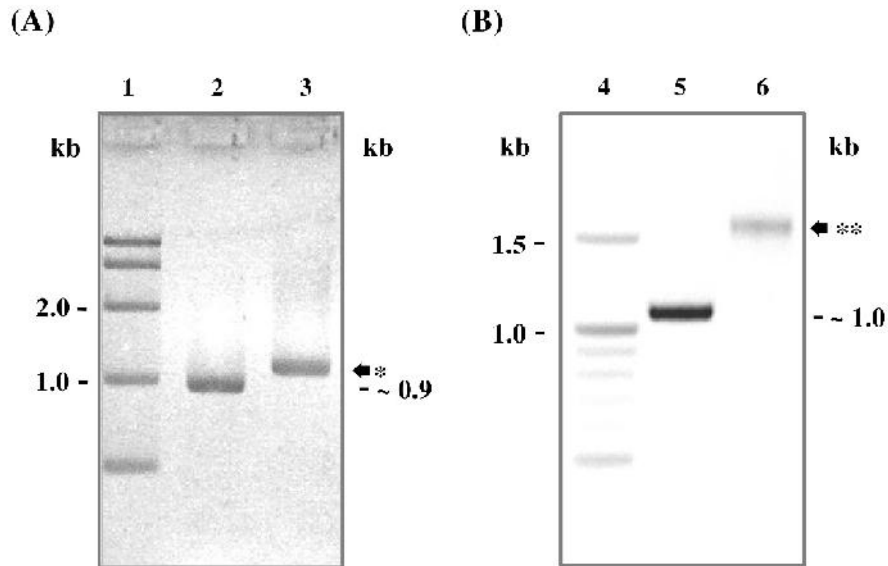


Figure 4.26 Amplification of *cry4Ba* and *psbA* specific DIG probes

The 50 ng of pCh2T-3 harboring *cry4Ba* and *psbA* gene was subjected as a template to generate DIG probes. Amplification products were analyzed by 0.8% agarose gel electrophoresis **(A)** The DIG-incorporate *cry4Ba*-specific probe (4B probe) was amplified by using 4B-946 and 4B-ORF primers. **(B)** The DIG-incorporate *psbA*-specific probe (D1-probe) was amplified by using *psbA*-f and *psbA*-r primers. The DIG incorporation into 4B (*) and D1 (**) probes were migrated through the agarose gel at a slower rate than those of unlabelled PCR product as expected.

Lane 1: Gene ladder fast2, DNA marker

Lane 2: The unlabelled PCR product of 4B-probe

Lane 3: The DIG-incorporate PCR product of the 4B-probe

Lane 4: 100-bp DNA ladder

Lane 5: The unlabelled PCR product of D1-probe

Lane 6: The DIG-incorporate PCR product of the D1-probe

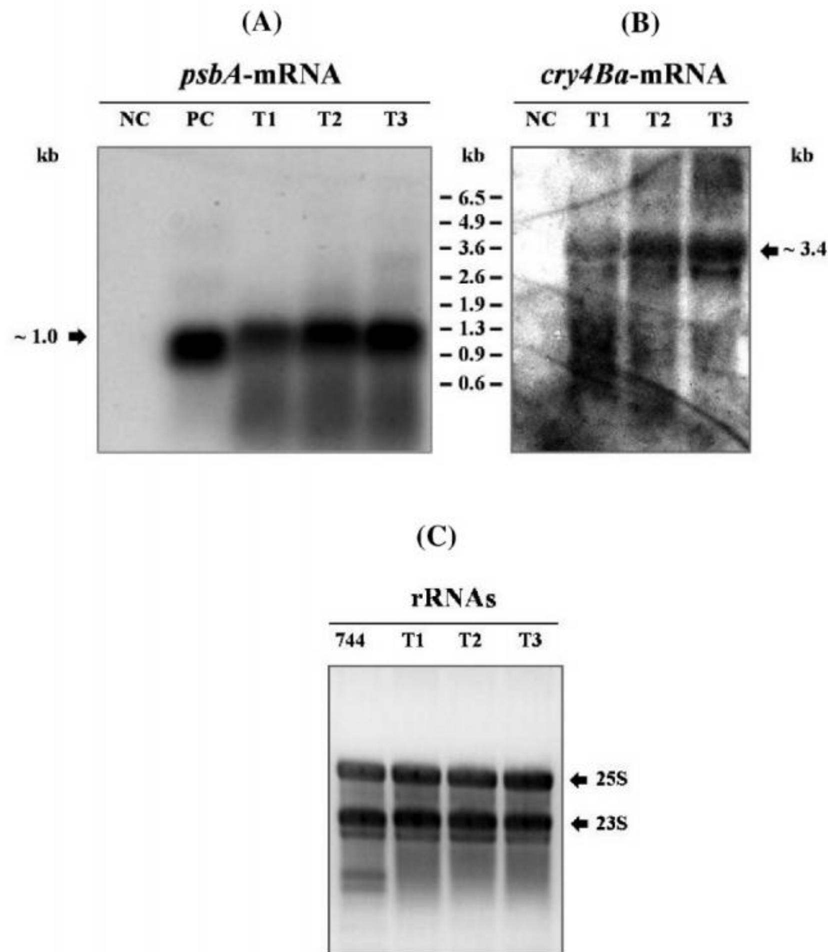


Figure 4.27 Identification of the recombinant *psbA* and *cry4Ba* transcripts by Northern blot analysis

The 5 µg of total RNA transferred onto the nylon membrane was incubated with the specific probes. **(A)** By using the D1 probe, a majority band at approximately 1.0 kb of the *psbA* transcript was observed from all transformant lines (T1-T3) equal to that from the wild-type CC400 strain served as a positive control. **(B)** By using the 4B probe, a majority band at approximately 3.5 kb of the *cry4Ba* transcript was observed from all transformant samples. Sample from the CC744 strain was used as a negative control. **(C)** The corresponding agarose gel showed the distribution of the cytoplasmic 25S and the plastid 23S rRNAs from all samples indicating an equal amount of loading samples.

4.13 Verification of the recombinant Cry4Ba protein by Western blot analysis

The expression of recombinant *cry4Ba* was analyzed by Western blot employing the specific Cry4Ba-domain III monoclonal antibody. The SDS-PAGE results showed no difference of protein pattern between all transformant lines and the *psbA*-deleted CC744 strain (**Figure 4.28A**). The corresponding SDS gel was continually blotted to the nitrocellulose membrane prior to performing the detection step. Nevertheless, the result showed no expression signal of 130-kDa Cry4Ba whereas the others just showed the lower signals of non-specific as also observed from that of non-transformant line (**Figure 4.28B**). In order to identify the components of the non-specific protein bands, the corresponding bands from SDS-gel were excised and submitted for mass spectrometry analysis. The results from mascot search software indicated that most of the predict proteins were belong to *C. reinhardtii* and no one was matched that of the *Bti*-Cry4Ba protein (**Appendix 5**).

4.14 Analysis of feeding mosquito larvae with the *C. reinhardtii* cells

The experiment was conducted to examine whether mosquito larvae can feed on *C. reinhardtii* cells by comparing the phenotype between the starved and the fed larvae groups. Results demonstrated that the fed larva was comparatively larger in size with a green color of engulf algae cells along its digestive tract. This result suggested that the mosquito larvae can consume *C. reinhardtii* cells (**Figure 4.29**).

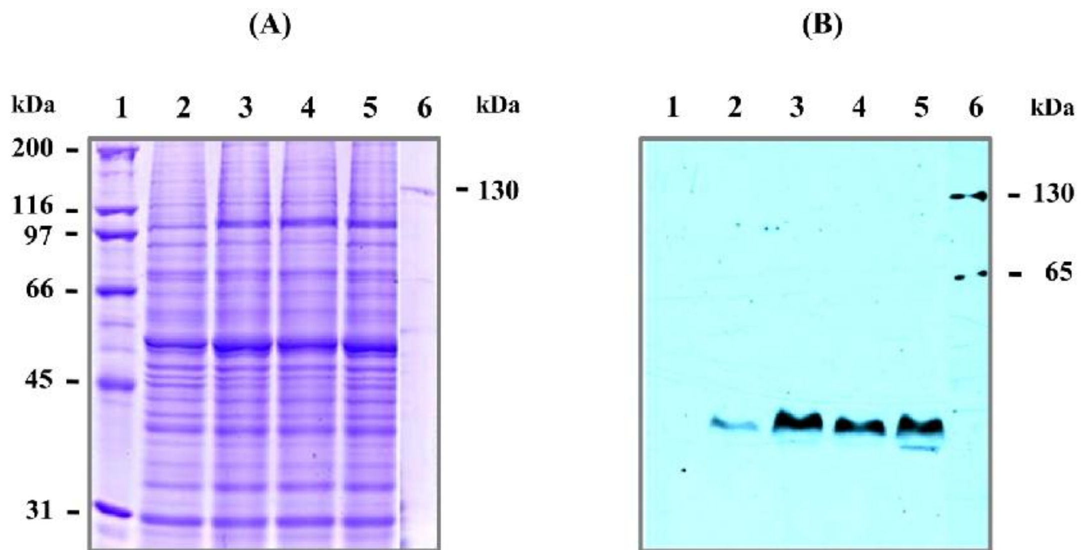


Figure 4.28 Detection of the recombinant Cry4Ba protein by Western blot analysis

(A) The SDS gel showed the protein pattern from the 3-nmol total chlorophylls of each sample comparing with the purified Cry4Ba expressed from *E. coli* served as a positive control.

(B) The signal from the exposed X-ray film showed the corresponding signal detected by *cry4Ba*-specific antibody. Strong signals of non-specific were observed from both non-transformant and transfromant lines. The positive control showed the signal of 130 kDa and 65 kDa of full-length and activated form of the *E. coli* expressed Cry4Ba, respectively.

Lane 1: The standard protein marker

Lane 2: Total protein extracted from CC744 strain

Lanes 3-5: Total protein extracted from transformant lines 1-3

Lane 6: The purified Cry4Ba protein expressed from *E. coli*

Figure 4.29 Algae-consuming analysis of mosquito larvae

Effect on *A. aegypti* larval growth after feeding mosquito larvae with algal *C. reinhardtii* (upper larva), as compared with a control group of starving mosquito larva (lower larva) for 1 week (scale bar: 1 mm).

CHAPTER V
DISCUSSION

5.1 Advantages of harboring the recombinant *cry4Ba* gene in the cell wall-less and the *mt⁻* *C. reinhardtii* strains

It is interesting to note that cell-wall deficient *C. reinhardtii* cells, either inherently or as a result of enzymatic degradation during gametogenesis, are available. Therefore, the possible problem that the algal cell wall might not be digested by the susceptible mosquito larval gut enzymes could perhaps be avoided. It is also worth mentioning that chloroplast genes are only inherited from the maternal parent mt^{+} as paternal mt^{-} chloroplast genes are selectively eliminated in young zygotes [113], further suggesting that the problem of transgene escape to the native strain could possibly be prevented by harboring recombinant genes in the chloroplast genome of the mt^{-} strain.

5.2 Establishment of chloroplast transformation vector

There were two series of chloroplast transformation vector, pCh1T-2 contained single fragment of recombination target and pCh2T-3 contained double fragments of recombination targets, respectively. Fragments of recombination targets of both constructs were identical to the non-coding regions between the *rrn5* and *chlN* genes of chloroplast genomes. Therefore, after specific insertion of recombinant genes, coding sequences of all native genes inside the chloroplast genomes were still intact. It is also noteworthy that the integration regions of both constructs located within the IRs of the *C. reinhardtii* chloroplast genome. Double copies of transgene integration into the cognate sequence in the chloroplast genome were thus expected (**Figure 5.1**).

Recombinant *aadA* gene, but not the *cry4Ba* gene could be detected by PCR in the transformant lines recovered from pCh1T-2 transformation. Optimization by doubling fragments of recombination targets as in pCh2T-3 however could support the insertion of larger recombinant genes. This result indicated that plasmid with single recombination target has the hindrance in limitation size of transgenes which can be solved *via* doubling the fragments of recombination targets. In addition, by employing the vector with double fragments of recombination targets, only genes of interest were delivered into the chloroplast genome (**Figure 5.1**) but not the whole vector as usually occurred in the single target usage.

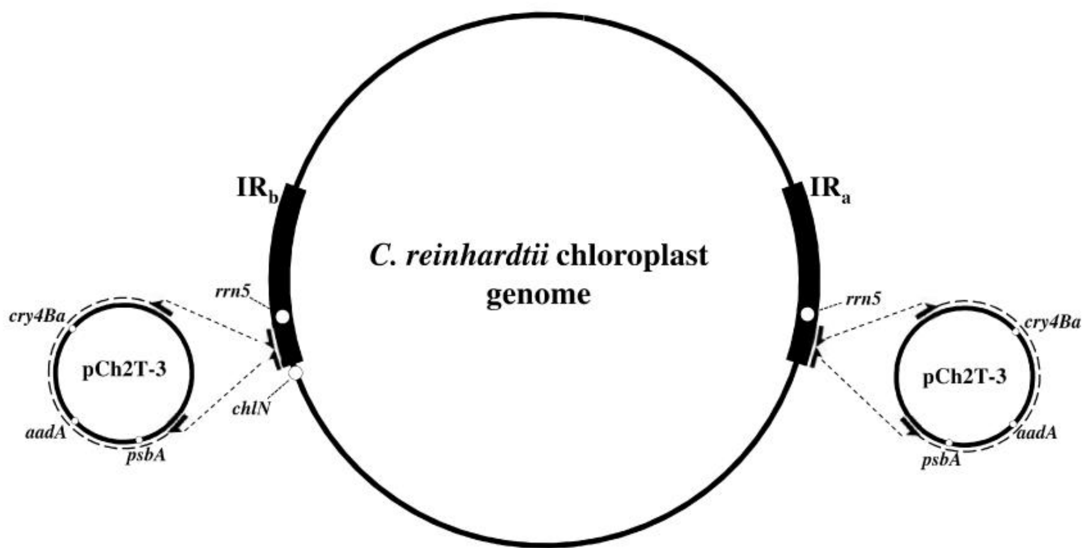


Figure 5.1 Integration sites of expression cassette into CC744 *C. reinhardtii* chloroplast genomes

Physical map of the CC744 *C. reinhardtii* chloroplast genome indicated the location of specific target regions within both inverted repeats. Two cassettes of transgenes integrated into *C. reinhardtii* chloroplast genome. The orientation of recombination sites were indicated by half arrows. The dash line on pCh2T-3 indicated regions that would be inserted into the chloroplast genome including recombinant *psbA*, *aadA* and *cry4Ba* genes. The positions of *psbA*, *aadA*, *cry4B*a, *rrn5* and *chlN* were indicated by circles.

5.3 Dual-phenotypic screening and transformation efficiency of transgenic chloroplasts

Apart from the initial screening for the *C. reinhardtii* transgenic lines *via* resistance to spectinomycin treatment, additional screening *via* the restoration of photosynthetic *psbA* gene would be very helpful in ruling out the faulted positive lines that might have resulted from spontaneous mutation induced by frequent drug screening [47]. It is interesting to note that both spectinomycin resistance and restored-photosynthetic phenotypes for those transformant lines remained stable since all lines have been monthly subcultured for more than a year on fresh TAP agar supplemented with antibiotic spectinomycin.

Only three transformant lines could be recovered from double screenings. The possible reason of low transformation efficiency could be explained by the fact that the host strain CC744 is a photosynthetic mutant which has slower growth rate as well as less favourable physical status even the enrich heterotrophic medium such as TAP was used. Another reason might be due to the fact that recombination event within the inverted repeat of chloroplast genomes was preferentially occurred inside the 0.7-kb sequences spanning immediately the 3'-end of *psbA* gene known as hotspots [114]. Given that the mutant host strain used in our work was originally knocked out the whole set of *psbA* gene including the 0.7-kb hotspots (**Figure 4.16**), the remaining regions might therefore deteriorate the frequency of recombination events and hence decrease in the number of transgenic lines.

5.4 Specific integration of intact transgenes in chloroplast genomes and homoplasmy

The result from PCR analysis using L1/L2 and R1/R2 primer pairs specific to *psbA* and *cry4Ba* transgenes and their corresponding adjacent-endogenous chloroplast genes indicated that transgenes were specifically integrated into the target IRs of the chloroplast genome (**Figure 4.23**). These results further suggested that the two identical sequences of *psbA* promoter and terminator used in both *cry4Ba* and *psbA* expression cassettes did not cause a deletion-looping out phenomenon [115]. The

difference in amount of amplification products among each line as shown by the DNA-band intensity (**Figure 4.23**) could be reasoned that each transformant line contains the difference in the copy number of chloroplast genomes.

As mentioned earlier that the two transgenic lines steadily exhibited both spectinomycin resistance and restore photosynthetic phenotypes that were offered respectively by *aadA* and *psbA* transgenes. Therefore, this phenotypic stability along with the gene integrity of the chloroplast transgenes would most likely lead to the homoplasmic plastome as the incompatible co-existing plasmid molecules would be segregated to yield homoplasmic isolates after a long period-repeatedly subculture under selective conditions [28].

5.5 High yield and integrity of *cry4Ba* transcripts in transgenic chloroplasts

Results from RT-PCR revealed the expected 3.4-kb high yield product suggesting an indicative of the transcription integrity and the absence of any deletion or truncated product of the unexpected splicing (**Figure 4.25**). Consistent with the RT-PCR results, Northern blotting (NB) also revealed a major ECL-positive band of approximately 3.4 kb (**Figure 4.27B**), further strengthen the efficient transcription of the recombinant *cry4Ba* gene in transgenic chloroplasts. It was worth mentioning that an intense NB band of approximately 1.0-kb *psbA* transcript was specifically revealed in all transgenic lines (**Figure 4.27A**), establishing the adequacy of basal transcription factors utilized to efficiently co-transcribe the *cry4Ba* and *psbA* transgenes driven by each individual *psbA*-controlling cassette.

5.6 Expression of the recombinant *cry4Ba* gene under control of the algal chloroplast promoter in *E. coli*

It is interesting to note that expression machineries in *E. coli* and algal chloroplast share some similarities which allow a variety of chloroplast promoters, *i.e. psbA*, *rbcL* and *atpB* promoters, to operate in *E. coli* [33]. In this work, the result

clearly showed that the recombinant *cry4Ba* gene under control by the promoter, 5 UTR and 3 UTR of the *psbA* gene (*psbA-cry4Ba*) was able to be expressed in *E. coli* **(Figure 5.2A)**. In addition, by using the same expression cassette, the corresponding results was obtained with recombinant 29-kDa *psbA-cyt2Aa2* gene (construction of the chloroplast expression vector pCh2T-4 harborring *psbA-cyt2Aa2* was illustrated in **Appendix 6**) **(Figure 5.2B)**. It is therefore further suggesting of the availability of a dual expression vector which allows to briefly check the traslation of recombinant gene in *E.coli* and expectedly provide a greater yield of protein expression in the *C. reinhardtii* chloroplast.

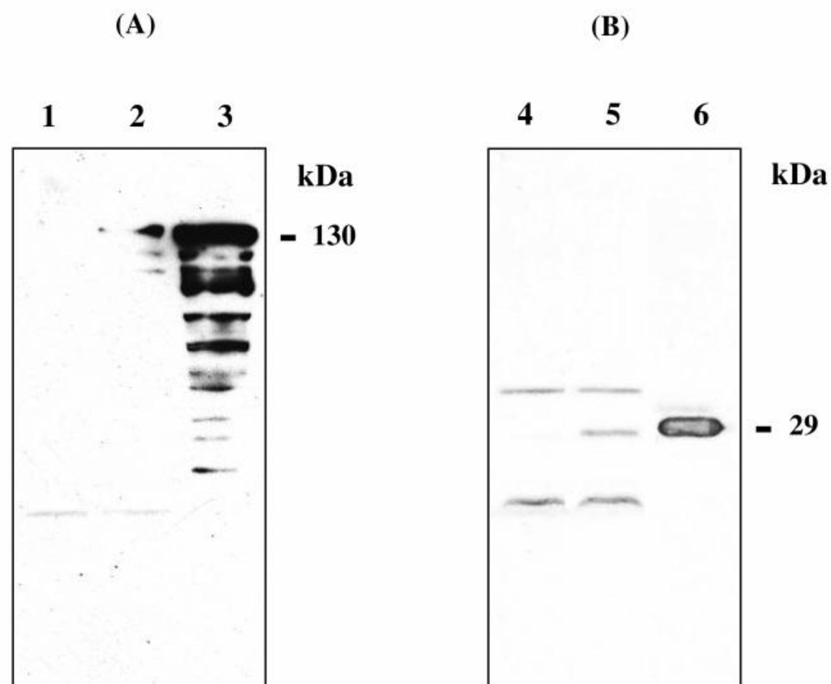


Figure 5.2 Western blot analyses of recombinant *psbA-cry4Ba* and *psbA-cyt2Aa2* genes expressed in *E.coli* cells

Whole cell lysates of *E. coli* cells harborring the recombinant *psbA-cry4Ba* gene and *psbA-cyt2Aa* gene were loaded in SDS–PAGE and detected by immunoblotting using anti-Cry4Ba **(A)** and anti-Cyt2Aa2 **(B)**.

Lane 1: Whole cell lysate of *E. coli* competent cells, JM109 strain

Lane 2: Whole cell lysate of *E. coli* harboring the recombinant *psbA-cry4Ba*

Lane 3: Whole cell lysate of *E. coli* harboring the pMU388

Lane 4: Whole cell lysate of *E. coli* competent cells, JM109 strain

Lane 5: Whole cell lysate of *E. coli* harboring the recombinant *psbA-cyt2Aa2*

Lane 6: The purified Cyt2Aa2 protein expressed from *E. coli*

5.7 Deficient production of the recombinant Cry4Ba protein in transgenic chloroplasts

Despite the efficient transcription of the *cry4Ba* gene was achieved, it was still discerned about the translation of this high-yield transcript. The result from Western blotting revealed no detectable accumulation of this recombinant protein (**Figure 4.28**). These results therefore make a note of a poor correlation between protein accumulation and high-yield transcription product in *C. reinhardtii* chloroplast as also previously observed [53, 116]. The reasons why this particular heterologous transcript is defectively translated in the transgenic chloroplast are still unclear. Nevertheless, base on the achievement of *psbA-cry4Ba* transgene expression in *E. coli*, the cause of deficient expression product might be narrowed up to the Cry4Ba-coding sequence *per se.*

Therefore, we were assumed that the highest possibility of the deficient production of the recombinant Cry4Ba protein in transgenic chloroplasts might be affected by the bias seen in Cry4Ba codon usage. Interestingly, it was previously shown that un-optimization of the codon usage showed a profound effect on recombination protein accumulation in the algal chloroplasts, generally resulted in very low [31] or no accumulation [117, 118] of translation product. While, optimization of the transgene codon usage to match with the pattern of synonymous codons used by expression hosts could dramatically increase the translation product as demonstrated up to 80 folds in the codon-optimized *gfp* transgene [34]. Therefore, the codon usage of the *Bti*-Cry4Ba protein-coding gene was evaluated to check its preference for the utilization of synonymous persistent codons in comparison with that of the *C. reinhardtii* chloroplast genome (www.kazusa.org.jp/codon). Although the patterns of synonymous codon usage in both the bacterial *cry4Ba* transgene and the *C. reinhardtii* chloroplast genome are quite similar as almost all codons ending in A or U are preferred, there is a strong bias in the Cry4Ba-coding gene towards the use of glycine GGG and histidine CAC codons (**Table 5.1**). It is therefore possible that the deficient translation of the high-yield *cry4Ba* transcript in transgenic chloroplast could perhaps due to biases seen in glycine and histidine codons used in this recombinant protein-coding gene. Hence, further studies *via* codon optimization of this non-native gene are of great interest since a codon-optimized *cry4Ba* gene might be indeed a

requirement for improving the heterologous production of the Cry4Ba insecticidal protein in *C. reinhardtii* chloroplasts.

Another possibility that the Cry4Ba protein might contain the cleavage site of chloroplast transit peptide (cTP) is also of interest as it might be the cause to truncate the target proteins. The prediction of chloroplast transit peptides and their cleavages on the Cry4Ba protein sequences was performed by the application of the predictive tools Predotar and ChloroP 1.1. However, results from both prediction servers indicated that the Cry4Ba protein sequences do not contain any recognition site of the chloroplast transit peptide (**Appendix 7**).

Besides the Cry4Ba-coding sequence *per se,* the minor possibility that the accumulation of translation products is regulated by the auto-regulatory feedback repression remains to be investigated. Previously, it has been demonstrated that the expression product of native *psbA* genes might deteriorate the translation of recombinant genes in which the corresponding *psbA* promoter/5'UTR was employed [58, 39]. On the other hand, transgene expressions driven by those corresponding sequences were increased in case that the native *psbA* gene was knocked out [39]. The facts that dramatically reduced amounts of those expression products might be explained by the feedback regulation of D1 protein, or the competition for limited transcription and translation machineries [39, 119]. From our data presented here in which the particular heterologous transcript was defectively translated, it is therefore provided the further evidence suggesting that there was no shortage of transcriptional machineries although the recombinant *cry4Ba* and *psbA* genes were controlled by separated expression cassettes of the *psbA* promoter/5 UTR.

On the other hand, the unlikely reason of the defective translation in transgenic chloroplasts was the possibility that the Cry4Ba protein might be poisonous to the *C. reinhardtii* cells. Although this aspect has been previously demonstrated by unavailing to recover the transformant line expressing the recombinant DILP-2 protein [38], it appears contradictory to our observations in which the transgenic lines harborring the recombinant *cry4Ba* gene still could be recovered. In addition, the argument against the toxicity issue has been supported by the previous achievements in expression of some other *Bt-cry* genes, *i.e. cry1Ab, cry1Ac and cry2Aa2*, into plant chloroplasts [101].

Table 5.1 Comparison of the bias usage between the *Bti-cry4Ba* transgene and the *C. reinhardtii* chloroplast genome (Kazusa database, www.kazusa.org.jp/codon)

Amino acid/Codon usage		Usage percentage [a]	
		The native *cry4Ba* gene	Kazusa database
Alanine	GCG	13	5
	GCC	14	9
	GCA	41	32
	GCU	32	53
Valine	GUC	8	4
	GUG	14	9
	GUA	49	41
	GUU	29	46
Leucine	CUC	4	1
	CUG	7	3
	UUG	11	4
	CUA	10	6
	CUU	22	13
	UUA	46	72
Isoleucine	AUU	56	77
	AUC	13	12
	AUA	31	10
Phenylalanine	UUU	75	66
	UUC	25	34
Tryptophan	UGG	100	100
Methionine	AUG	100	100
Proline	CCU	40	35
	CCC	3	8
	CCA	40	53
	CCG	17	5
Aspartic acid	GAU	88	72
	GAC	12	28
Glutamic acid	GAA	69	88
	GAG	31	12
Glycine	GGU	27	70
	GGC	10	10
	GGA	29	14
	GGG	34	6
Serine	UCU	37	25
	UCC	6	4
	UCA	23	33
	UCG	5	6
	AGU	21	24
	AGC	8	8
Threonine	ACU	27	37
	ACC	10	8
	ACA	46	49
	ACG	17	6
Cysteine	UGU	80	84
	UGC	20	16
Tyrosine	UAU	79	71
	UAC	21	29
Asparagine	AAU	75	70
	AAC	25	30
Glutamine	CAA	88	90
	CAG	12	10
Lysine	AAA	89	92
	AAG	11	8
Arginine	CGU	21	70
	CGC	7	9
	CGA	21	7
	CGG	0	1
	AGA	43	11
	AGG	7	2
Histidine	CAU	7	53
	CAC	93	47

[a] The most usage frequency of individual codons in each coding sequence is underlined

CHAPTER VI
CONCLUSIONS

1. The chloroplast transformation vector harboring the 3.4-kb coding sequence of *Bti* mosquito-larvicidal *cry4Ba* gene under the control of promoter and terminator of chloroplast *psbA* gene was successfully constructed and was delivered into the *C. reinhardtii* chloroplast by the bombardment technique.

2. Upon screening by antibiotic resistance and photosynthetic complementation, three putative lines were obtained. Verification by PCR analysis indicated that the recombinant *cry4Ba* expression cassette was precisely integrated into the non-coding region on inverted repeats of recipient chloroplast genome.

3. The recombinant *cry4Ba* gene was successfully transcribed as indicated by RT-PCR analysis. The transcription product covered the ORF of recombinant *cry4Ba* gene. This evidence denotes that the *cry4Ba* expression cassette could properly operate.

4. Transcriptions of recombinant *cry4Ba* and *psbA* genes could be indicated by Northern blot analysis. It was therefore suggested that the recovery of *psbA* gene did not affect the stability of the recombinant *cry4Ba* transcript although the same *psbA* promoters were employed to control in both cassettes

5. Expression of the heterologous *cry4Ba* transcript could not be demonstrated by Western blot analysis which was further suggesting of a poor correlation between protein and transcript accumulation in the *C. reinhardtii* chloroplast.

6. Base on the fact that recombinant *cry4Ba* gene (harbored in pCh2T-3 vector) and *cyt2Aa2* gene (harbored in pCh2T-4 vector) under controlled by the promoter and

terminator sequences of *psbA* gene were able to be expressed in *E. coli* cells, these results provide the further suggesting evidence that the Cry4Ba-protein sequences *per se* were likely to be the cause of the deficient translation production in transgenic chloroplasts.

7. The highest possibility of the deficient production of the recombinant Cry4Ba protein in transgenic chloroplasts might be affected by the unfavorable codon usages seen in Cry4Ba coding sequences. After the *cry4Ba* codon usage was evaluated to compare the utility of synonymous codons with that of the *C. reinhardtii* chloroplast genome, a strong bias could be detected at glycine GGG and histidine CAC codons.

8. Data in this study provided the further supporting evidence that algal chloroplasts have adequacy of basal transcriptional factors utilized to efficiently co-transcribe the *cry4Ba* and *psbA* transgenes.

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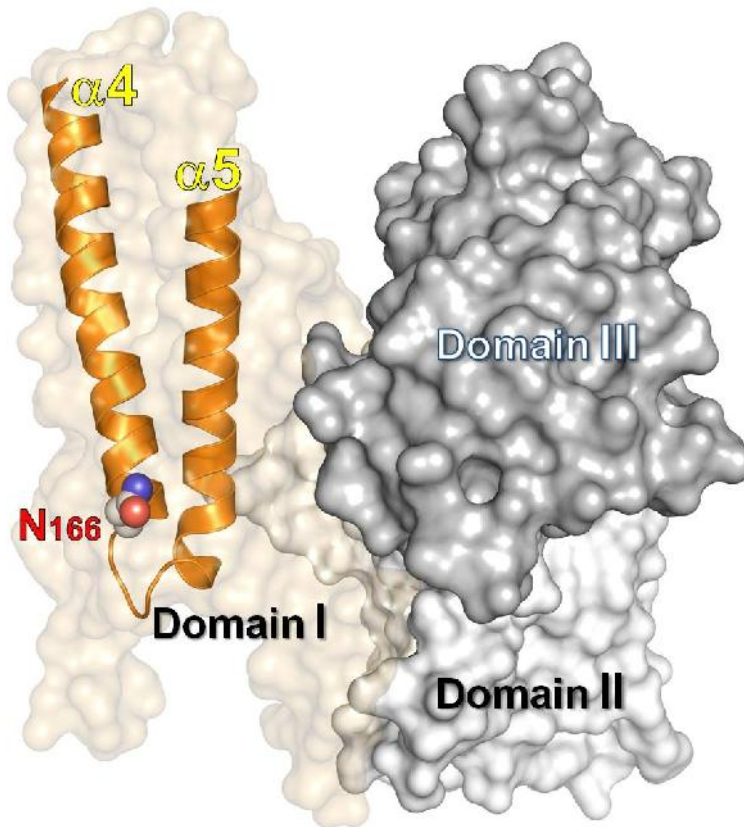
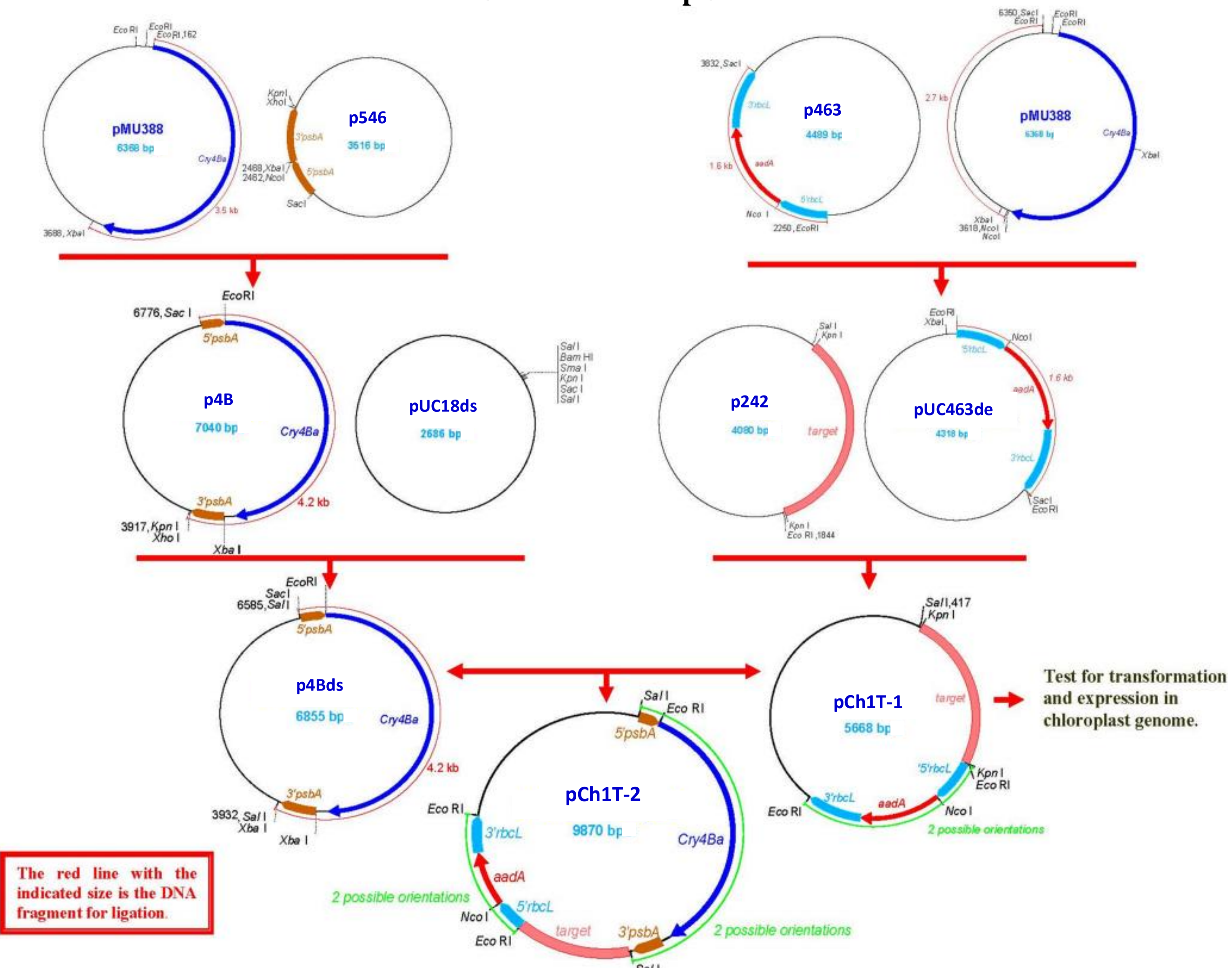
APPENDIX

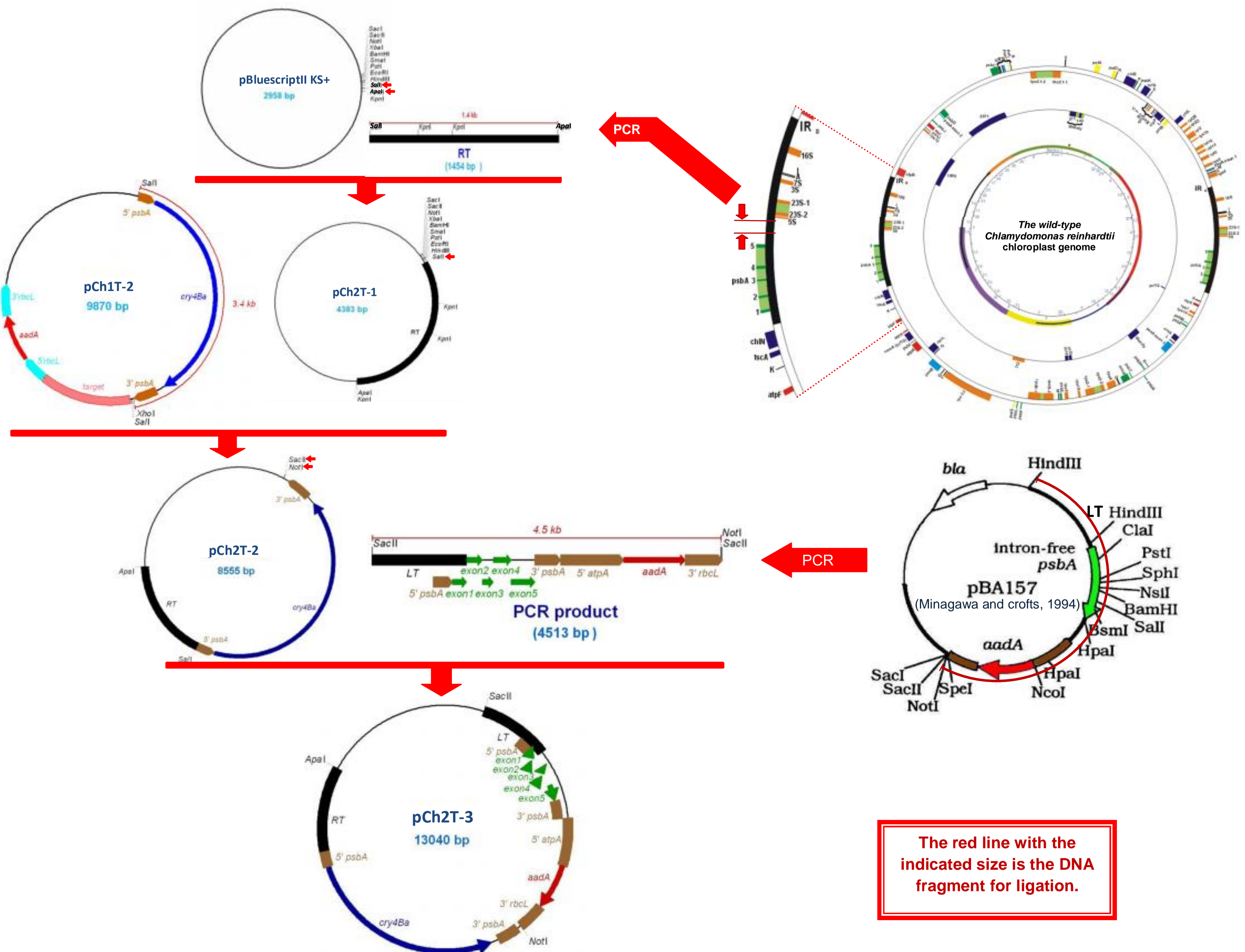
1. The Cry4Ba crystal structure and its Asn^{166} located in 4-loop- 5 hairpin within the pore-forming domain (Domain I).



2. Construction of pCh1T-2



3. Construction of pCh2T-3



4. Nucleotide sequences of the *psbA* promoter and 5 UTR in p546

p546

3520 bp

*amp*ʳ

*Kpn*I

*Xho*I

psbA 3'UTR

*Xba*I

*Nco*I

psbA promoter/ 5'UTR

*Sac*I

SacI

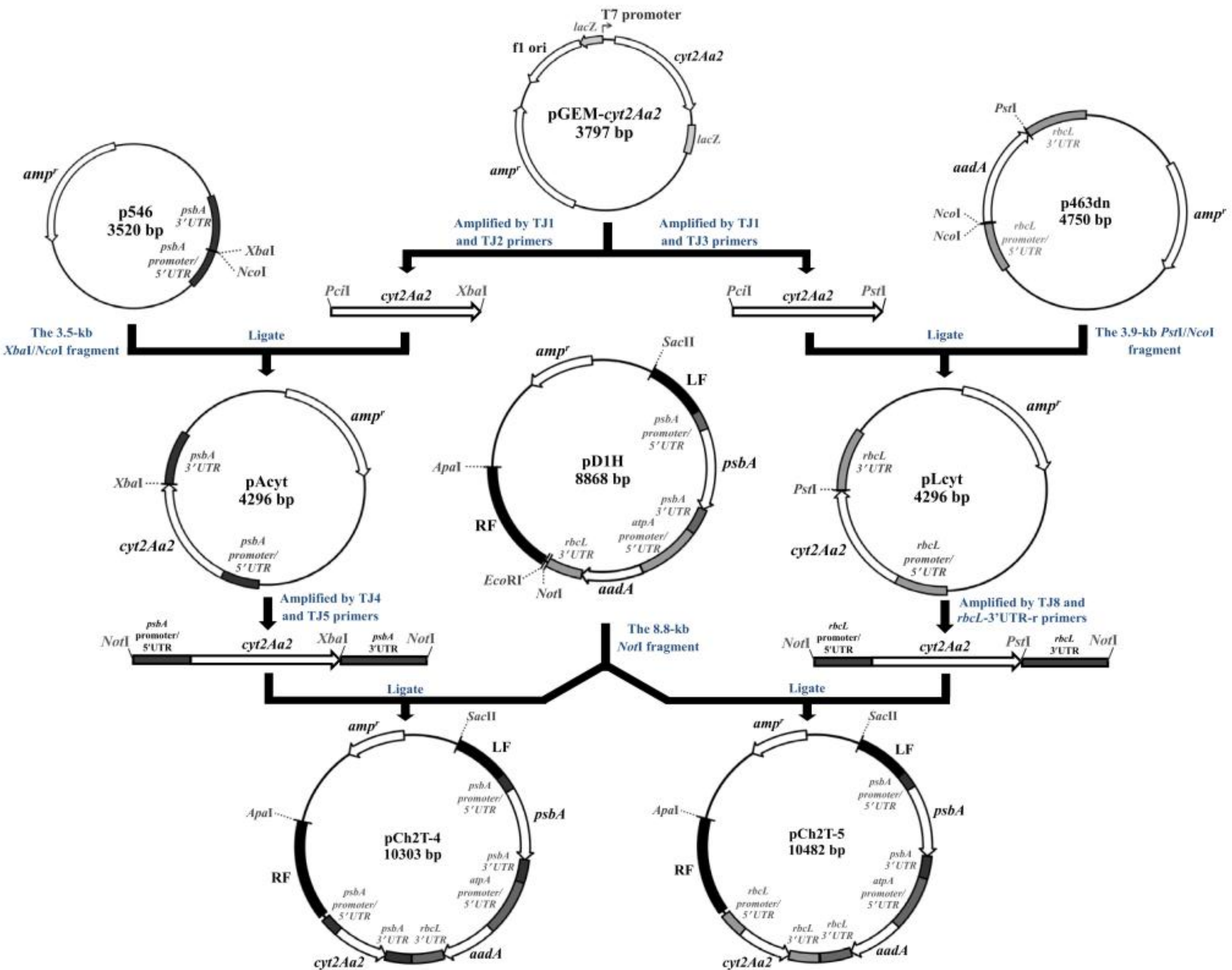
NcoI

XbaI

5. Mascot search results of the non-specific Western blotting bands

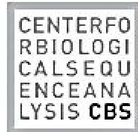
```
Protein hits    : gi|41179065   photosystem II 44 kDa protein [Chlamydomonas reinhardtii]
                  gi|159476472 chloroplast ATP synthase gamma chain [Chlamydomonas reinhardtii]
                  gi|159485250 fructose-1,6-bisphosphate aldolase [Chlamydomonas reinhardtii]
                  gi|159467635 sedoheptulose-1,7-bisphosphatase [Chlamydomonas reinhardtii]
                  gi|2459573   envelope protein [Chlamydomonas reinhardtii]
                  gi|46093489  calcium sensing receptor [Chlamydomonas reinhardtii]
                  gi|228267    carbonic anhydrase
                  gi|136429    RecName: Full=Trypsin; Flags: Precursor
                  gi|304322988 CP43 chlorophyll apoprotein of photosystem II [Floydiella terrestris]
                  gi|4929987   Chain A, Cytochrome F From Chlamydomonas Reinhardtii
                  gi|17224776  ATP synthase beta subunit [Thismia rodwayi]
                  gi|159477927 acidic ribosomal protein P0 [Chlamydomonas reinhardtii]
                  gi|302849386 hypothetical protein VOLCADRAFT_83516 [Volvox carteri f. nagariensis]
                  gi|18076104  glyceraldehyde-3-phosphate dehydrogenase [Spirogyra sp.]
                  gi|168012869 predicted protein [Physcomitrella patens subsp. patens]
                  gi|224116080 predicted protein [Populus trichocarpa]
                  gi|56750881  psbC gene product [Synechococcus elongatus PCC 6301]
                  gi|225457407 PREDICTED: malate dehydrogenase, chloroplastic [Vitis vinifera]
                  gi|113476898 F0F1 ATP synthase subunit beta [Trichodesmium erythraeum IMS101]
                  gi|303245589 ATP synthase F1, beta subunit [Desulfovibrio fructosovorans JJ]
                  gi|159462468 light-dependent protochlorophyllide reductase [Chlamydomonas reinhardtii]
                  gi|139522589 ATP synthase beta subunit [Siphocampylus affinis]
                  gi|302843850 malate dehydrogenase [Volvox carteri f. nagariensis]
                  gi|303272938 malate dehydrogenase [Micromonas pusilla CCMP1545]
                  gi|41179058  hypothetical protein ChreCp059 [Chlamydomonas reinhardtii]
                  gi|159469941 malate dehydrogenase [Chlamydomonas reinhardtii]
                  gi|231610    RecName: Full=ATP synthase gamma chain, chloroplastic; AltName: Full=F-ATPase gamma subunit; Flags: Precursor
                  gi|365823897 photosystem II CP43 [Najas flexilis]
                  gi|116793693 unknown [Picea sitchensis]
```

6. Construction of the chloroplast expression vector pCh2T-4 harborring the recombinant *psbA-cyt2Aa2* gene



7. The prediction results of chloroplast transit peptides on Cry4Ba protein sequences by ChloroP 1.1 (A) and Predotar (B) programs.

(A)



ChloroP 1.1 Server - prediction results

Technical University of Denmark

```
### chlorop v1.1 prediction results ##########################
Number of query sequences: 1

Name                    Length       Score   cTP

-----------------------------------------------
Translation_of_cry4B      1136       0.479    -
-----------------------------------------------
```

Name is the name of the submitted sequence
Length is the length of the submitted sequence.
Score is the output score from the second step network. The prediction cTP/no cTP is based solely on this score.
cTP tells whether or not this is predicted as a cTP-containing sequence; "Y" means that the sequence is predicted to contain a cTP; "-" means that is predicted not to contain a cTP.

(B)

A prediction service for identifying putative mitochondrial, plastid and ER targeting sequences

Predotar v. 1.03 using plastid prediction networks

Sequence	Mitochondrial	Plastid	ER	Elsewhere	Prediction
Cry4Ba	0,01	0,01	0,00	0,99	none

For each protein sequence, Predotar provides a probability estimate as to whether the sequence contains a mitochondrial, plastid or ER targeting sequence. The fourth number is simply the estimated probability that no targeting sequence is present.

BIOGRAPHY

NAME Mr Thanate Juntadech

DATE OF BIRTH 13 July 1982

PLACE OF BIRTH Pattani, Thailand

HOME ADDRESS 146/24 Moo 6, Lung Mor-Or Road, Rusamilae District, Amphur Muang, Pattani, Thailand, 73170
E-mail: tjuntadech@gmail.com

INSTITUTION ATTENDED

- 2001-2004 : Bachelor of Science (Plant Science), Mahidol University, Thailand
- 2005-2012 : Doctor of Philosophy (Molecular Genetics and Genetic Engineering), Mahidol University, Thailand

AWARD & SCHOLARSHIP

- 2005 : The Professor Dr. Tab Nilanidhi Foundation's Award for The Outstanding Graduate Student in Plant Science
- 2007-2012 : Royal Golden Jubilee Ph.D. Scholarship from Thailand Research Fund
- 2007-2012 : The Shell Centenary Educational Foundation

PUBLICATION AND INTERNATIONAL MEETING ABSTRACTS

1. **Juntadech T.**, Kanintronkul Y., Karnchanawarin C., Katzenmeier G., Angsuthanasombat C. (2014) Importance of polarity of the 4- 5 loop residue-Asn^{166} in the pore forming domain of the *Bacillus thuringiensis*

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2. **Juntadech T.**, Yokthongwattana K., Tangphatsornruang S., Yap Y.K., Katzenmeier G. and Angsuthanasombat C. (2012) Efficient transcription of the larvicidal *cry4Ba* gene from *Bacillus thuringiensis* in transgenic chloroplasts of the green algal *Chlamydomonas reinhardtii*. ***Advances in Bioscience and Biotechnology***. *3*:362-369.
3. Wongratana J., **Juntadech T.**, Sereeruk C., Angsuthanasombat C. and Yokthongwattana K. (2012) Generation and characterization of His-tagged-PsbA-expressing transformants of *Chlamydomonas reinhardtii* that are capable of photo-autotrophic growth. ***Journal of Applied Phycology***. *25*:445–452.
4. **Juntadech T.**, Yokthongwattana K., Tangphatsornruang S., Yap Y.K. and Angsuthanasombat C. Expression of The Cry4Ba Mosquito-Larvicidal Protein from *Bacillus thuringiensis* in The Chloroplast of *Chlamydomonas reinhardtii*. ***Oral Presentation in Abstract The 10th RGJ Congress***. Pattaya, Thailand. April 3-5, 2009.
5. **Juntadech T.**, Yokthongwattana K., Tangphatsornruang S., Yap Y.K. and Angsuthanasombat C. Expression of The Cry4Ba Toxin in algal chloroplast system. ***Poster Presentation in Abstract Bacterial Protein Toxins from Basic Research to Application***. Nakhonpathom, Thailand. March 23, 2009.
6. **Juntadech T.**, Yokthongwattana K., Tangphatsornruang S., Yap Y.K. and Angsuthanasombat C. Expression of The Cry4Ba Mosquito-Larvicidal Protein from *Bacillus thuringiensis* in The Chloroplast of *Chlamydomonas reinhardtii*. ***Oral Presentation in Abstract the 11th Biological Science Graduate Congress***. Bangkok, Thailand. December 15-17, 2006.