รายงานวิจัยฉบับสมบูรณ์

โครงการ การ screen หา mutant และศึกษาโปรตีนที่เกี่ยวข้องกับการแสดงออกของยีนที่ระดับ epigenetic ในสาหร่ายเซลล์เดียว *Chlamydomonas reinhardtii*

Screening and characterization of components required for epigenetic regulation in *Chlamydomonas reinhardtii*

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สนันสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

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Abstract

Title : Screening and characterization of components required for epigenetic regulation in *Chlamydomonas reinhardtii*

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Project Period : 2 years

Epigenetics is the changes of gene expression without any alteration of the underlying DNA sequences. These changes of gene expression come about by the modifications of DNA and histone proteins by addition of various functional groups such as methyl or acetyl groups. Epigenetics allows rapid alterations of gene expression under various environmental stresses. However, the knowledge of mechanisms involved in epigenetic regulation is far from being complete. New components implicated in epigenetic regulation are yet to be discovered. The aim of this research project is to understand epigenetic regulation in *Chlamydomonas reinhardtii*. Transgenic *C. reinhardtii* actively expressing paromomycin resistant gene were generated (Par^A). The Par^A lines were used as a background for random insertional mutagenesis and screened for silenced version of the paromomycin resistant gene (Par^S). Thirty seven Par^S lines were isolated from the screen. These candidate mutants are currently being characterized for their insertion as well as the underlying mutated genes. In addition, differential protein expression under salinity stress has been studied by 2-dimensional electrophoresis followed by LC/MS-MS mass spectrometry. The differential protein profiles were observed in the cells treated with short-term and long-term response to salinity stress.

Keywords : epigenetic, salinity stress, epigenetic mutant, microRNA, *Chlamydomonas reinhardtii*

บทคัดย่อ

รหัสโครงการ : MRG5280035

ชื่อโครงการ : การ screen หา mutant และศึกษาโปรตีนที่เกี่ยวข้องกับการแสดงออกของยีนที่ระดับ epigenetic ในสาหร่ายเซลล์เดียว *Chlamydomonas reinhardtii*

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ระยะเวลาโครงการ : 2 ปี

การควบคุมการแสดงของยีนที่ระดับ epigenetic หมายถึงการควบคุมที่ทำให้การแสดงออกของยีนเปลี่ยนแปลงไป โดยไม่เกิดความบกพร่องของยีน แต่มีการควบคุมบางประการที่ทำให้ยีนนั้นมีการแสดงออกที่ต่างไปจากเดิม ซึ่งมักเกิดจากการที่ดีเอ็นเอและโปรตีนฮีสโตนถูกเติมด้วยหมู่ฟังก์ชันต่างๆ เช่น หมู่เมทิลหรืออะเซทิล นอกจากนี้การเปลี่ยนแปลงการแสดงออกของยีนอย่างรวดเร็วนั้นสามารถส่งผลให้สิ่งมีชีวิตสามารถปรับตัวให้ทนต่อสภาวะเครียดจากสิ่งแวดล้อมภายนอกได้ อย่างไรก็ดีองค์ความรู้เกี่ยวกับกระบวนการ epigenetic นั้นยังไม่สมบูรณ์ ยังมีการค้นพบโปรตีนชนิดใหม่ๆ ซึ่งทำหน้าที่ในกระบวนการนี้อยู่เสมอ ดังนั้นวัตถุประสงค์ของโครงการวิจัยนี้ คือ การศึกษากระบวนการควบคุมการแสดงของยีนที่ระดับ epigenetic ในสาหร่ายเซลล์เดียว *Chlamydomonas reinhardtii* โดยทำการสร้างสาหร่ายที่มีการแสดงออกของยีนต้านทานต่อยา paromomycin (Par^A) จากนั้นใช้สายพันธุ์ Par^A ในการทำการกลายพันธุ์แบบสุ่มและทำการคัดเลือกสาหร่ายที่ไม่มีการแสดงออกของยีนต้านทานต่อยา paromomycin (Par^S) จากการคัดเลือกพบว่ามีสาหร่าย Par^S ทั้งสิ้น 37 สายพันธุ์ ซึ่งในขณะนี้กำลังทำการศึกษายีนที่ได้รับการกลายพันธุ์รวมถึงลักษณะอื่นๆ ในสาหร่ายเหล่านี้ นอกจากนี้ได้ทำการศึกษาโปรตีนที่มีการแสดงออกแตกต่างกันภายใต้สภาวะเครียดจากความเค็มโดยเทคนิค 2-DE และ LC/MS-MS mass spectrometry ทั้งนี้ผู้วิจัยพบโปรตีนที่มีการแสดงออกแตกต่างกันในสาหร่ายที่เลี้ยงในสภาวะที่มีเกลือเป็นระยะเวลาสั้นและระยะเวลานาน

คำหลัก : epigenetic, salinity stress, epigenetic mutant, microRNA, *Chlamydomonas reinhardtii*

เนื้อหางานวิจัย

บทนำ

Chromatin structure

Eukaryotic nuclear DNA is organized into a compact structure called chromatin. A basic building block of chromatin is nucleosome which contains histone octamer (two copies of each H2A, H2B, H3 and H4) wrapped around by 147 base pairs of DNA (Chakravarthy et al. 2005, Luger et al. 2006). Two types of chromatins could be observed by cytological analysis; euchromatin and heterochromatin (Grewal et al. 2007, Heitz et al. 1928). Euchromatin is a region of loosely-packed chromatin and weakly stained nuclear area (Heitz et al. 1928). It generally composes of actively transcribed genes (Grewal et al. 2007). In contrast, heterochromatin is a highly condensed chromatic region in the nucleus observed by cytological analysis (Heitz et al. 1928). Heterochromatin is condensed throughout the cell cycle. It is typically consisted of repetitive DNA such as pericentric, centromeric and telomeric sequences, transposable elements as well as transcriptionally silent loci (Grewal et al. 2007). Apart from their cytological structures, euchromatin and heterochromatin also differ in the types and/or density of chemical modifications that affect both DNA and histone proteins. Chromatin structure can be modulated by methylation of DNA, post-translational modifications of histone tails, and replacement of histone proteins by histone variants (Hsieh et al. 2005). Chromatin configuration can be altered by ATP-dependent chromatin remodeling complexes. These processes involve in modulating chromatin structure and they play key roles in regulation of gene expression.

Epigenetics and gene regulations

Historically the term “epigenetics” was introduced by Conrad Waddington as “the interactions of genes with their environment that bring the phenotype into being” (Waddington et al. 1942). Presently epigenetics is refered to as heritable, but potentially reversible changes in gene expression that occur without a corresponding change in the primary DNA sequence (Bird et al. 2007). Regulation of gene expression by epigenetic is associated with alterations in various epigenetic marks such as DNA methylation and histone modifications. Combination of epigenetic marks with the actions of chromatin remodeling complexes maintains the epigenetic balance. Various organisms control gene expression by exploiting epigenetic mechanisms (Grewal et al. 2007, Yang et al. 2007, Zaratiegui et al. 2007). For instance, the well-studied targets of epigenetic control in yeast are the mating type loci (HML and HMR genes), rDNA, telomeric and centromeric repeats. Position-effect variegation (PEV) described alteration of

gene expression when the gene is located nearby heterochromatin is another example of epigenetic control found in *Drosophila*. In mammals the X-chromosome inactivation is a classic example of epigenetic regulation. The epigenetic control in plant was first reported as paramutation in maize by Alexander Brink in the 1950s. Paramutation described the allelic interaction which the expression of one allele in heterozygous state is altered by the presence of the other allele. This expression status is heritable to successive generations (Chandler et al. 2007). Moreover, silencing of transgene in plant was discovered in the transgenic petunia over-expressing the sense chalcone synthase (*CHS*) gene under the control of 35S Cauliflower Mosaic Virus (*35SCaMV*) promoter. The purple flowers were expected due to over-expression of *CHS* enzyme however 42% of the transgenic plants showed white or variegated petals. The white flower resulted from loss-of-expression of both endogenous as well as transgenic *CHS* leading to the termed “cosuppression” (Napoli et al. 1990).

In plants, epigenetic gene silencing can be described into two categories, transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) even though now it is clear that both pathways are interconnected. Transcriptional gene silencing is the repression of transcription occurred due to suppressive chromatin structure. It is associated with high level of DNA methylation and specific histone modifications. Additionally TGS can be transmitted mitotically and meiotically and inherited over many generations. TGS involves in the control of both endogenous loci as well as transgenes. For example, silencing at phosphoribosylanthranilate isomerase (*PAI*) genes, an enzyme of tryptophan biosynthesis pathway is controlled by TGS. Four PAI genes (*PAI1-4*) are presented at 3 unlinked loci in *Arabidopsis* wild type accession Wassilewskija (Ws). However *PAI2* gene is silenced by the presence of high DNA methylation level at its promoter region (Bender et al. 1995). Flowering wageningen (*FWA*) is another example of endogenous gene regulated by DNA methylation. FWA is heavily methylated and silenced in all plant tissue. It is only reactivated in the endosperm where DNA methylation is removed (Kinoshita et al. 2004). For transgenes, the genes can undergo silencing when multiple copies are integrated into the plant genome. It is usually associated with hypermethylation which is directed by a process called RNA-directed DNA methylation (RdDM).

Post-transcriptional gene silencing is usually associated with sequence specific mRNA degradation. In this case, the transcription rate of a particular gene is not affected. In plants, PTGS requires the accumulation of short, 21-26 nucleotides, RNA species known as small interfering RNAs. These siRNAs are produced through RNA interference (RNAi) pathway

(Baulcombe et al. 2007, Brodersen et al. 2006). It is believed that PTGS is reset in each generation.

Epigenetic modifications

Epigenetic modifications can be divided into different categories. However, the proteins that are involved in generating these modifications to the chromatin are of focused here. These proteins can be classified into:

1. DNA methylation dependent silencing components
2. Histone modifying enzymes
3. Chromatin remodeling complexes

DNA methylation dependent silencing components

DNA methylation is the best characterized evolutionary conserved epigenetic mark which can be found in fungi, plants and mammals (Selker et al. 2003, Bender et al. 2004, Goll et al. 2005, Bernstein et al. 2007). In mammals, DNA methylation occurs mainly at cytosine residues in CpG sequence contexts and almost no methylation is observed at CpNpG and CpNpN (where N = A, C, or T). In plant, though methylation can be found predominantly at CpG sites but significant level of cytosine methylation are found at CpNpG and CpNpN sequence contexts (Bender et al. 2004). Six to thirty percent of cytosine residues are methylated depending on plant species; for example, as high as 19% of cysotine in *Arabidopsis* is methylated (Chen et al. 2004). DNA methylation is concentrated in the heterochromatic region. However, one third of the actively transcribed genes also contain methylated DNA (Zhang et al. 2006). Mechanism of DNA methylation can be divided into maintenance and *de novo* methylation catalyzed by maintenance and *de novo* methyltransferases (MTases) respectively. The maintenance methylation occurs on the hemimethylated templates after DNA replication or repair. Therefore, DNA methylation pattern can be stably inherited through both mitosis and meiosis.

Plant DNA MTases can be classified into four main families; methyltransferase 1 (MET1), Domain-rearranged methyltransferase (DRM), Chromomethylase (CMT) and DNA methyltransferase 2 (Dnmt2) based on their structures and similarity of conserved amino acid motifs (Finnegan et al. 2000, Zhang et al. 2010). Members of MET1 families are mainly responsible for the maintenance of DNA methylation at CpG sites (Finnegan et al. 1996, Ronemus et al. 1996, Kankel et al. 2003, Saze et al. 2003). But maintenance activity at non-CpG could be observed (Lindroth et al. 2001). Depletion of MET1 in *met1* mutants and

antisense-*METI* plant resulted in the drastic loss of CpG methylation at the centromeric repeats, 5S rDNA repeats, transposon-related sequence, and the silent transgenic loci accompanied with transcription activation at these regions (Vong et al. 1993, Finnegan et al. 1996, Ronemus et al. 1996, Kankel et al. 2003, Saze et al. 2003, Vaillant et al. 2008). DRM is a homolog of Dnmt3-class in mammals (Cao et al. 2000, Cao et al. 2002a). *Arabidopsis* DRM2 is the major DNA methyltransferase in the establishment of RNA-directed DNA methylation (RdDM) pathway (Cao et al. 2002a, Cao et al. 2002b, Cao et al. 2003). Plant-specific DNA methyltransferases, CMT3, function in maintenance of CpNpG methylation (Henikoff et al. 1998, Bartee et al. 2001, Pavlopoulou et al. 2007).

Loss of DNA methylation can be occurred through passive and/or active demethylation mechanisms. The passive demethylation happens when there is a failure to maintain DNA methylation during DNA replication whereas the active loss of DNA methylation occurs through the function of the enzyme DNA glycosylases (Zhang et al. 2010). Four genes encoding for DNA glycosylases have been reported in Arabidopsis; Repressor of silencing (ROS1), Demeter (DME), Demeter-like 2 (DML2) and Demeter-like 3 (DML3) (Zhu et al. 2009). Based on biochemical analysis all *Arabidopsis* enzymes remove methylated cytosine through a base excision pathway for active DNA demethylation (Gong et al. 2002, Agius et al. 2006, Gehring et al. 2006, Morales-Ruiz et al. 2006, Penterman et al. 2007, Zhu et al. 2007). Hypermethylation together with silencing of transgenic locus as well as endogenous targets were observed in *ros1* mutant (Gong et al. 2002, Zhu et al. 2007). DME has been implicated in the allele-specific reactivation of Medea (*MEA*), Fertilization Independent Seed 2 (*FIS2*) and FWA (Choi et al. 2002, Kinoshita et al. 2004).

Histone modifying enzymes

Histone proteins are the basic components of chromatin. They are composed of globular domain carboxy-terminal and amino-terminal domain protrudes out from the nucleosomes. The N-terminal histone tails can be subjected to post-translational modifications including methylation (me), acetylation (Ac), phosphorylation (P), and ubiquitination (Ub) (Jenuwein et al. 2001). As a consequence, transcription can be controlled leading to the open-and-close nucleosomal array for activation/inactivation of transcription (Strahl et al. 2000, Shilatifard et al. 2006, Kouzarides et al. 2007). These modifications of histones are referred to as the histone code (Jenuwein et al. 2001, de la Cruz et al. 2005). As mentioned above there are several histone marks; however, only histone methylation and histone acetylation will be discussed here.

Histone methyltransferase and histone demethylase

Histone methylation occur on lysine (K) or arginine (R) residues such as histone H3K4, -9, -27, -36, -79 and K20 of H4 or at H3R2, -8, -17, -26 and H4R3. Furthermore, methylation on lysine residues can be mono-(me), di-(me2), or tri-methylated (me3) while methylation on arginine residues can be mono-(me) or di-(me2) (Kouzarides et al. 2007). Methylation of histone tails is correlated with the transcriptional activity of the underlying sequences. For instance, H3K9me2 is usually associated with the silent chromatin while H3K4me2 is found at the actively transcribed chromatic region (Jenuwein et al. 2001). The relationship between H3K9 methylation and gene silencing was observed in yeast (Nakayama et al. 2001), Neurospora crassa (Tamaru et al. 2001), and mammals (Peter et al. 2001). The H3K9 methylation is required for transposon silencing in *Arabidopsis* (Gendrel et al. 2002)

Methylation of histone tails is carried out by histone methyltransferases (HMTs) originally identified as SET-domain containing proteins. The name SET domain came from the fact that this domain is found in Drosophila suppressor of variegation (Su(var) 3-9) (Tschiersch et al. 1994, Rea et al. 2000), enhancer of Zeste (E(Z)), and trithorax (Qian et al. 2006). HMTs have been identified as Clr4p in *S. pombe* (Nakayama et al. 2001), Dim-5 in *N. crassa* (Tamaru et al. 2001), SUV39H1 and SUV39H2 in mammals (O'Carroll et al. 2000; Rea et al. 2000). In *Arabidopsis* at least 37 genes are predicted to encode for SET-domain containing proteins (Baumbusch et al. 2001, Naumann et al. 2005, Ebbs et al. 2006). Kryptonite (KYP/SUVH4) is a H3K9 mono- and di-methyltransferase (Jackson et al. 2002, Malagnac et al. 2002, Jackson et al. 2004).

Removal of methyl groups from histone N-tails is carried out by histone demethylase. Lysine-specific demethylase 1 (LSD1) is the first identified mammalian histone demethylase (Shi et al. 2004). This enzyme specifically demethylates mono- and di- H3K4 and H3K9 methylation (Shi et al. 2004). Afterward several histone demethylases have been identified from other organisms (Shi et al. 2007).

Histone acetyltransferase and deacetyltransferase

Histone acetylation occurs at lysine residues. Acetylation of histone N-terminal tails results in the decrease affinity of histone to DNA. It affects chromatin architecture and gene expression by altering the accessibility of nucleosomal DNA to transcriptional activators, chromatin modifying enzymes or chromatin remodeling complexes. Additionally acetylated histones can recruit regulatory protein as well as transcriptional machinery. In general histone

acetylation is correlated to gene expression whereas gene silencing is associated with histone deacetylation (Jenuwein et al. 2001). Immunolocalization reveals that acetylated histone H4 is present only in euchromatin but absent in the heterochromatic regions of *Arabidopsis* nuclei (Jasencakova et al. 2003, Probst et al. 2003).

Histone acetylation and deacetylation are very dynamic processes catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. The H3K9, -14, -18, -23, -56 H3 and H4K5, -8, -12 are the sites for acetylation (Kouzarides et al. 2007, Shahbazian et al. 2007). HAT enzymes can be classified into four different families based on their catalytic domains. These are GNATs (Gcn5 N-acetyltransferase), MYST, p300/CBP (CREB-binding proteins), and nuclear receptor co-activator families (Lee et al. 2007). Three HDAC families are categorized as RPD3 (Reduced potassium dependency 3), HDA1 (Histone deacetylase 1), and Sir2 (Silent information regulator 2) families (Pandey et al. 2002).

Arabidopsis genome contains at least 12 putative HATs and 18 putative HDACs (Pandey et al. 2002). Several studies have been carried out to examine function of these enzymes. For instance, AtHAC1 is involved in the regulation of flowering time (Deng et al. 2007). The *Arabidopsis* homolog of Gcn5 plays a role in light-regulated gene expression (Benhamed et al. 2006). Down-regulation of histone deacetylase 1 (HD1) by antisense HD1 resulted in accumulation of acetylated histones and ectopic expression of silent genes (Tian et al. 2001, Tian et al. 2005). The *athda6* mutant is defective for maintenance of transgene as well as rRNA silencing (Murfett et al. 2001, Probst et al. 2004, Earley et al. 2006).

Chromatin remodeling complexes

In addition to the modifications of DNA and histones, structure of chromatin can be altered. Modulation of chromatin structure which influences gene expression is carried out by ATP-dependent chromatin remodeling complexes (Narlikar et al. 2002, Smith et al. 2005, Reyes et al. 2006, van Vugt et al. 2007). These complexes are composed of multiple subunits and are very well conserved from yeast to humans. ATP-dependent chromatin remodeling complexes can be divided into 4 subfamilies based on conserved motifs of core ATPases, that is SWI2/SNF2 (mating type switching 2/ sucrose nonfermenting 2), ISWI (imitation switch), Mi-2/CHD (chromodomain helicase DNA binding), and INO80 (inositol auxotroph 80) families (Eberharter et al. 2004, Smith et al. 2005). Distinct domains implicated in recognition of histone modifications can be found in different families of ATPases. The SWI2/SNF2 ATPases contain bromodomain that recognizes acetylated lysine (Narlikar et al. 2002, Hassan et al. 2007, van Vugt et al. 2007). The ISWI ATPases enclose SANT (Swi3, Ada2, N-Cor, and TFIIIB) and

SLIDE (SANT-Like ISWI Domain) domains were predicted to interact with histone tails (Boyer et al. 2004, Dang et al. 2007, Horton et al. 2007). Chromodomains presented in the Mi-2/CHD ATPases possess methylated histone binding activity (Hall et al. 2007, Marfella et al. 2007, Kunert et al 2009). The INO80 ATPases contain DBINO domains which were predicted to bind DNA (Bakshi et al. 2004).

Epigenetic in Chlamydomonas reinhardtii

Epigenetic studies in *Chlamydomonas reinhardtii* started in 1997 when Cerutti and team tried to study nuclear transformation. The *C. reinhardtii* cells were transformed with *eubacterial aminoglycoside 3"-adenyltransferase* gene (*aadA*) under the control of *Chlamydomonas RbcS2* (small subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase) regulatory region (Cerutti et al. 1997a). The authors expected to recover *Chlamydomonas* cells conferring spectinomycin resistance however after culturing the cells under non-selective condition, they found the instability of the resistant phenotype in some transgenic lines. No *aadA* transcript could be detected in these lines (Cerutti et al. 1997a). Further characterization of the clones led to the conclusion that the *aadA* gene was silenced by epigenetic phenomenon. Moreover, the silencing of the *aadA* transgene could be inherited to next generations (Cerutti et al. 1997b). The transgenic lines with silencing phenotype were used as a background for random mutagenesis and screen for mutants regaining the spectinomycin resistant phenotype. The screen led to the discovery of genes involved in the regulation of gene expression. Some of these genes were already identified and studied in other organisms such as DEAH-box RNA helicase (Wu-Scharf et al. 2000), serine/threonine protein kinase (Jeong et al. 2002), histone H3K4 methyltransferase (van Dijk et al. 2005), and WD40-repeat containing protein (Jeong et al. 2002, Zhang et al. 2002). The WD40-repeat containing protein has sequence homology to the C-terminal domain of Tup1p, a global transcriptional repressor in *Saccharomyces cerevisiae*.

Other known proteins involved in epigenetic regulation have been studied in *Chlamydomonas* as well. For instance, the functions of DNA methyltransferase, CrMETI which methylates maternal chloroplast DNA (Nishiyama et al. 2002, Nishiyama et al. 2004), histone H3K9 methyltransferase (Casas-Mollano et al. 2007), histone H3T3 kinase (Casas-Mollano et al. 2008a), and polycomb repressive complex 2 (PRC2) which is a H3K27 methyltransferase (Shaver et al. 2010). Moreover components involved in post-transcription gene silencing as well as small interfering RNAs (siRNAs) and microRNAs (miRNAs) have also been identified and

studied in *Chlamydomonas* (Ibrahim et al. 2006, Zhao et al. 2007, Casas-Melano et al. 2008b, Molnar et al. 2008, Ibrahim et al. 2010).

Epigenetic studies in *Chlamydomonas* have only begun. Little is known about epigenetic components in *Chlamydomonas* compared to other organisms that have been studied hitherto.Thus, by studying epigenetics using *Chlamydomonas* as model would provide us a better understanding on epigenetic regulation in plants.

วิธีการทดลอง

Strain and growth condition

C. reinhardtii cells strain cc-503 were grown mixotrophically in Tris-Acetate-Phosphate (TAP) medium (Harris, 1989). The medium was supplemented with 10 mg/l paromomycin or 10 mg/l hygromycin when required. For salinity stress response, *C. reinhardtii* cells were cultured in TAP liquid medium containing various concentration of NaCl as required.

Generation of Chlamydomonas expressing paromomycin resistant gene

C. reinhardtii cells were grown in TAP liquid culture until log phase. The culture was transformed with pSL18 plasmid carrying paromomycin resistant cassette (S. Lemaire, J.D. Rochaix, personal communication) using glass bead transformation method (Kindle et al. 1990). The transformed cells were selected on TAP medium supplemented with 10 mg/l paromomycin for 2 weeks. Single colonies which were grown on selective medium were then transferred to new selective plate and were grown for another 2 weeks. The positive transformants were called Par^{Active} or Par^{A} hereafter.

DNA and RNA Analyses of Chlamydomonas Transformants

DNA was isolated according to “QUICK AND EASY” genomic prep for PCR analysis (Pollock et al. 2003). PCR was carried out using gene specific primers. The primer sequences are upon request.

Random mutagenesis and screen for epigenetic mutants

The *C. reinhardtii* Par^{A} cells were randomly mutagenized using plasmid pHyg3 conferring hygromycin B resistant gene (Berthold et al. 2002). For mutagenesis, glassbead transformation method was employed (Kindle et al. 1990). The transformed cells were selected on TAP medium supplemented with 10 mg/l hygromycin B for 2 weeks. Single colonies which were

grown on selective medium were then transferred to new selective plate and were grown for another 2 weeks. The positive transformants were called Hyg^{Active} or Hyg^{A} hereafter.

Screening of epigenetic mutants was carried out by culturing the Hyg^{A} on medium containing 10 mg/l hygromycin and medium containing both 10 mg/l hygromycin and 10 mg/l paromomycin. The cells which could be grown only on medium supplemented with 10 mg/l hygromycin were selected. These cells were called Hyg^{Active} $Par^{Silenced}$ or Hyg^{A}/Par^{S} henceforth. Figure 1 illustrates the overall experimental design.

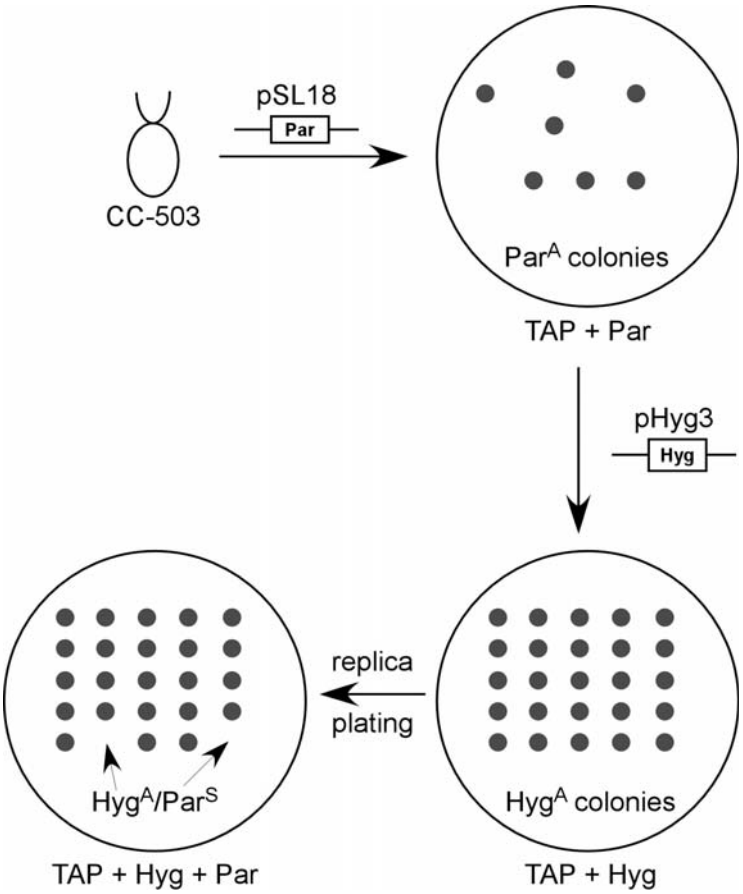

Figure 1. Diagram illustrates the experimental design. Wild type *C. reinhardtii* cc-503 cells were transformed with pSL18 to generate Par^{A} cells. The Par^{A} cells were selected on TAP medium containing 10 mg/l paromomycin as were used as a background for random mutagenesis by transforming pHyg3. The Hyg^{A} cells were selected on TAP medium supplemented with 10 mg/l hygromycin B. The Hyg^{A}/Par^{S} were selected on TAP medium supplemented with both 10 mg/l hygromycin and 10 mg/l paromomycin. TAP; Tris-Acetate-Phosphate medium, Hyg; hygromycin B, Par; paromomycin.

Characterization of mutants altering paromomycin expression

The copy number of pHyg in Hyg^{A}/Par^{S} cells will be analyzed by Southern blot analysis using hygromycin resistant gene as probe. The colonies with single copy insert will be selected. Linkage analysis will be carried out by crossing cc-503 (mating type plus, mt+) with wild type cc-3491 (mating type -, mt-). Haploid progenies will be grown on TAP medium supplemented with 10 mg/l paromomycin and TAP medium containing 10 mg/l paromomycin and 10 mg/l hygromycin. If insertion of pHygs3 resulting in the HygA/ParS phenotype, thus, all the haploid progenies will not be able to grown on TAP medium containing both 10 mg/l paromomycin and 10 mg/l hygromycin (Figure 2). These cells will be further analyzed for the pHyg3 insertion site using plasmid rescue technique (Tam et al. 1995).

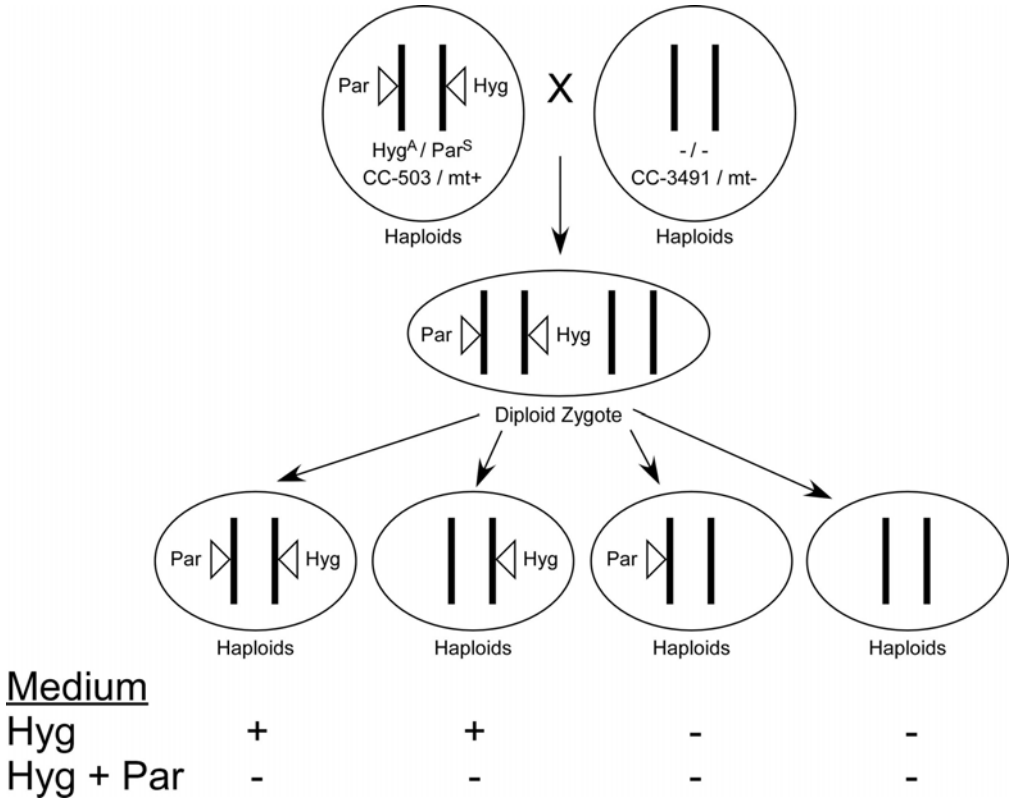

Figure 2. Diagram illustrates genetic cross to test that the insertion of pHygs3 in ParA resulting in Hyg^{A}/Par^{S}. The haploid progenies cannot be grown on TAP medium supplemented with 10 mg/l paromomycin and 10 mg/l hygromycin. TAP; Tris-Acetate-Phosphate medium, Hyg; hygromycin B, Par; paromomycin

Protein isolation and separation by two-dimensional electrophoresis

Cell aliquots were subjected to centrifugation at 2,000 g for 2 min at room temperature and the supernatant was discarded. The pellet was washed twice with distilled water before lyzing with buffer containing 8 M urea, 4% CHAP, 2% thiourea. Cell debris and unsolubilized materials were separated by centrifugation at 10,000 g for 5 min, the resulting green supernatant was transferred to a new microcentrifuge tube while the pellet was discarded. To eliminate photosynthetic pigments and other hydrophobic compounds that may interfere with two-dimensional electrophoresis (2–DE), the supernatant was added with 3–4 volumes of ice–cold acetone and kept at –20 °C overnight. The precipitated material was harvested by centrifugation at 10,000 g for 5 min and was resuspended in rehydration buffer containing 8 M urea, 4% CHAPS and 2% thiourea, 2% IPG buffer, 40 mM Dithiothreitol, 0.002% bromophenol blue. Protein concentration of the extraction was determined using Bradford protein assay kit (Bio–Rad Laboratory). Approximately 500 µg of protein samples were subjected to 2–DE using Immobiline dry strip pH 4–7 (GE Healthcare) with running condition set according to the protocol recommended by the manufacturer (GE Healthcare). Protein spots were visualized upon staining the resolved 2–DE gel with colloidal Coomassie blue G. Proteins from at least 3 independent biological replicates were isolated. For each of the biological replicate, the isolated proteins were resolved on at least three or four 2–DE gels, called sample replicates.

Image analysis and spot comparison

Gel images were scanned and analyzed electronically with computer software PDQuest (Bio–Rad Laboratory). The best 2–DE image from each of the sample replicates was selected as a representative of the corresponding biological sample. Protein spot patterns from independent biological replicates of the same time point were compared. The spots that consistently appear on every biological replicates of each time were marked by the software for construction of a master image and the spot intensities were averaged. Any protein spot inconsistently appears in different biological samples was not included in the master image and was also ignored from the subsequent cross–comparison with the spot patterns from other time points. Master images from different time intervals were compared and statistically analyzed by two–way student t–test for significant difference (P value < 0.05). The spots that showed significant up– or down–regulation were subjected for subsequent tryptic digestion and protein identification by mass spectrometry.

In-gel digestion

After protein spots were excised, the gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Jaresitthikunchai et al. 2009). The gel plugs were dehydrated with 100% acetonitrile, reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 h and alkylated at room temperature for 1 h in the dark in the presence of 100 mM iodoacetamide in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100% ACN for 5 min. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50% ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 min, and then 20 µl of 30% ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37°C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50% ACN in 0.1% formic acid was added into the gels, and then the gels were incubated at room temperature for 10 min in a shaker. Peptides extracted were collected and pooled together in the new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

HCT Ultra LC-MS analysis

The digested peptides were injected into Ultimate 3000 LC System (Dionex, USA) coupled to ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray at flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 80% 0.1% formic acid in 80% acetonitrile was run in 40 min.

Protein identification

All retrieved MS/MS mass spectra were reached via online mascot tool by several following parameters; Data base NCBI, MSDB, and Swiss Prot (mostly achieved protein searching by NCBI), allowing up to 1 missed cleavages, fixed carbamidomethyl variable, variable methionene oxidation modification, peptide tolerance ±1.2 Da, MS/MS tolerance ± 0.6 Da, peptide charge 1+,2+, and 3+. Result searching were agreed that mass spectrum from a spot protein correlate hit protein from data bases if probability based Mowse score is more over indicate identity value or easily interpret by red bar which represent candidate protein appearance out site green area.

ผลการทดลอง

Effective concentration for selection of paromomycin resistant and hygromycin resistant transformants

In order to study epigenetic regulation of gene expression, marker genes are required for easy and rapid screening. A paromomycin resistant gene as well as hygromycin resistant gene was chosen as the marker genes. Since the resistance to paromomycin and hygromycin B will be used for generation of transgenic *C. reinhardtii* as well as for screening of epigenetic mutants, effective concentration of paromomycin and hygromycin for selection of the positively transformed cells were analyzed. The wild type *C. reinhardtii* strain CC-503 were grown on TAP medium supplemented with different concentrations of required antibiotic: 0, 1, 2.5, 5 and 10 mg/l. After culturing the algae for 3 weeks, the wild type cells could grow on all media except on the TAP medium containing 10 mg/l paromomycin (Figure 3A) and TAP medium supplemented with 10 mg/l hygromycin (Figure 3B). Therefore, the concentration of 10 mg/l paromomycin and 10 mg/l hygromycin will be used for further screening of the transformed cells.

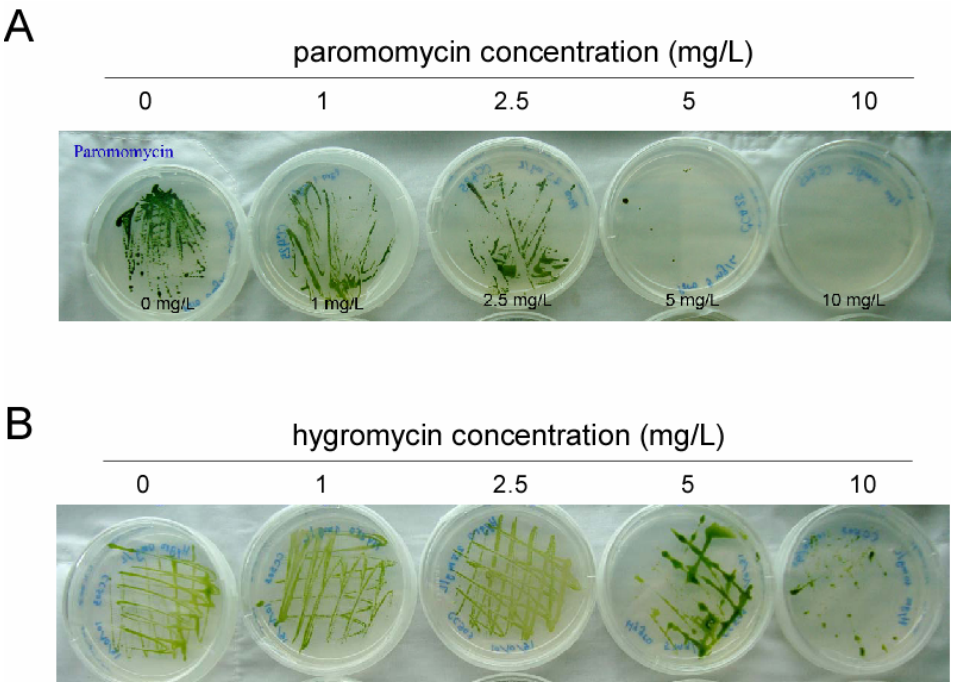

Figure 3. Effective concentration of paromomycin and hygromycin B for screening the transformed *C. reinhardtii* cells. A; wild type *C. reinhardtii* cc-503 were grown on TAP medium supplemented with 0, 1, 2.5, 5 and 10 mg/l paromomycin for 3 weeks, B; wild type *C. reinhardtii* cc-503 were grown on TAP medium supplemented with 0, 1, 2.5, 5 and 10 mg/l hygromycin B for 3 weeks.

Generation of transgenic Chlamydomonas carrying paromomycin resistant gene

With courtesy from Professor Jean-David Rochaix at the Department of Plant Biology and Department of Molecular Biology, University of Geneva, Switzerland, we obtained the plasmid pSL18 containing the paromomycin resistant gene (Figure 4).

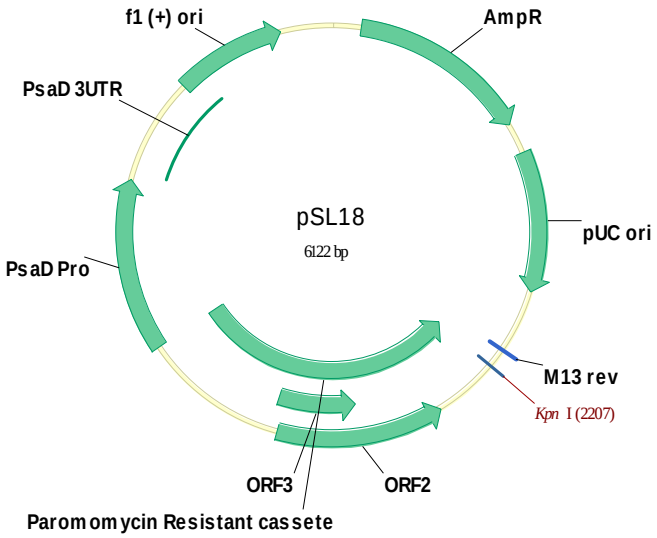

Figure 4. Diagram for plasmid pSL18 used for generation of transgenic *Chlamydomonas reinhardtii*. The plasmid pSL18 contains paromomycin resistant gene for selection in *C. reinhardtii* and ampicillin resistant gene for selection in *E. coli*. *Kpn*I restriction site is indicated.

As shown in the experimental outline in Figure 1, *C. reinhardtii* strain CC-503 cells were cultured in liquid medium with acetate as a carbon source (Tris-Acetate-Phosphate, or commonly known as TAP medium) (Harris et al. 1989). As the pSL18 plasmid contain single *Kpn*I restriction site (Figure 4), the plasmid was digested with *Kpn*I to generate a linearized plasmid in order to increase the transformation efficiency. Linearized pSL18 plasmid was used for nuclear transformation by glass bead transformation method, which is a well-established protocol commonly used worldwide (Kindle et al. 1990). After transformation, the cells were subsequently plated on the TAP medium agar containing 10 mg/l paromomycin and culture for 3 weeks. Only the transformed cells expressing paromomycin resistant gene would be able to grow as a single colonies on the selective medium. Each colony grown on TAP+Paro plate came from independent transformation events therefore these positive transformants are considered as independent transgenic lines. These cells are called Par^{Active} or Par^{A} (Figure 5).

Figure 5. The primary transformants. *C. reinhardtii* cells were transformed with pSL18 plasmid. The transformed cells with active paromomycin (Par^A) were grown as single colonies on the TAP medium supplemented with 10 mg/l paromomycin.

The Par^A colonies were further subcultured by re-streaking the cells and maintained on selective media plates. In order to verify the presence of the paromomycin resistant gene, the primary transformed cells were grown in TAP liquid media and their DNA was extracted by "QUICK AND EASY" genomic prep for PCR analysis (Pollock et al. 2003). PCR was carried out using paromomycin specific primer pair (Paro-F and Paro-R). As shown in figure 6, the selected colonies contained paromomycin resistant gene while no amplicon was amplified in the wild type control.

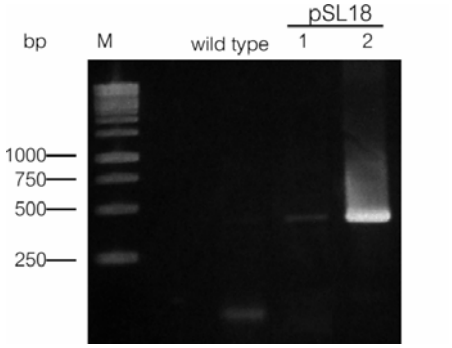

Figure 6. The presence of paromomycin resistant gene in the primary transformants. M; DNA marker, wild type; untransformed *C. reinhardtii* cells, pSL18; transformed colonies 1 and 2

Spontaneous induced paromomycin silencing in the transformants with Par^A phenotype

One obstacle for generation of transgenic organisms is the spontaneous suppression of the transgene such as the classic example in expression of chalcone synthase (*CHS*) gene in petunia (Napoli et al. 1990). This suppression might be due to epigenetic modifications such as

methylation of DNA and/or histone N-terminal tail modifications. As previously reported, in the transgenic *C. reinhardtii* lines expressing spectinomycin, the spectinomycin resistant phenotype was unstable after culturing transgenic lines for a few weeks under nonselective condition (Cerutti et al. 1997b). The transgenic lines with silenced spectinomycin gene served as a good resource for random mutagenesis. Cerutti and colleague used one of the silenced lines as a background in the screen for mutants which can reactivate the silenced spectinomycin resistant gene. All of the mutants reported to be recovered from the screen were defective in epigenetic regulators such as SET3p H3K9 methylase (Casas-Mollan et. al. 2007) and H3Thr3 kinase, MUT9p (Casas-Mollan et. al. 2008a).

We therefore attempted to induce spontaneous silencing of paromomycin resistant genes in the Par^{A} cells by culturing the transgenic lines on the TAP medium plate without the paromomycin antibiotic. As shown in figure 5, the primary transformed cells were selected on the TAP+Paro plate. After 3 weeks, the Par^{A} cells were then transferred to a new TAP+Paro plate and culture for further 2 weeks. All of the Par^{A} lines grown on second round of selection were subsequently transferred onto grid plates. The cells were cultured on both selective (TAP+Paro) and nonselective (TAP) conditions (Figure 7, second re-streak). Subsequently the cells were transferred onto new medium plate under both selective and nonselective conditions for second round of induction (Figure 7, third re-streak). After growing the cells under nonselective condition, if the paromomycin resistant gene in Par^{A} lines was silenced the cells should not be able to grow when transferred back to selective condition. Indeed, a number of the Par^{A} lines were silenced as illustrated by the absence of growth on the TAP+Paro plate (Figure 8, circle). These transgenic lines were called Par^{S} hereafter. We also observed the Par^{A} lines which expression of the paromomycin resistant gene was stable even though the cells were cultures on nonselective condition for several passages (Figure 8, square). About 45% of the transgenic lines were found to be spontaneously silenced after two rounds of culturing under nonselective condition (Figure 9, third re-streak). While growing only on the selective condition, all of the transgenic lines did not have any silencing (Figure 9). The Par^{S} lines served as a good genetic background for random mutagenesis as well as the Par^{A} lines. We, therefore, utilized both Par^{A} and Par^{S} lines for random insertional mutagenesis afterward. For the Par^{S} lines, the mutants will be screened for reactivation of the silenced paromomycin resistant phenotype after random mutagenesis.

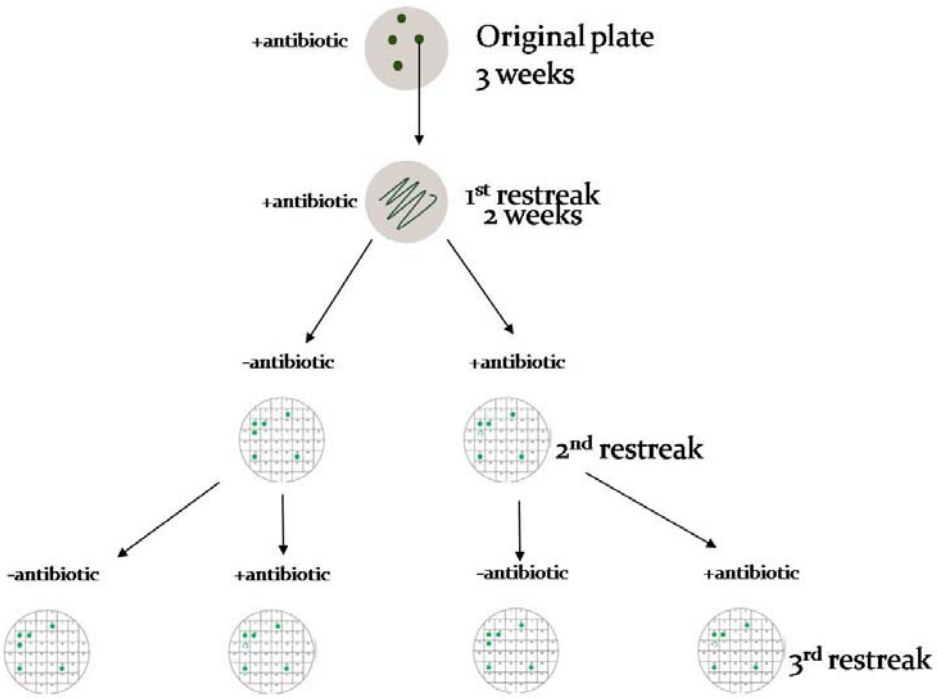

Figure 7. Outline diagram for the clonal analysis of paromomycin silencing in transgenic Par^{A} *Chlamydomonas rienhardtii*. The primary transformed cells were cultured on selective TAP+Paro plate then transferred onto selective (TAP+Paro) and nonselective (TAP) conditions.

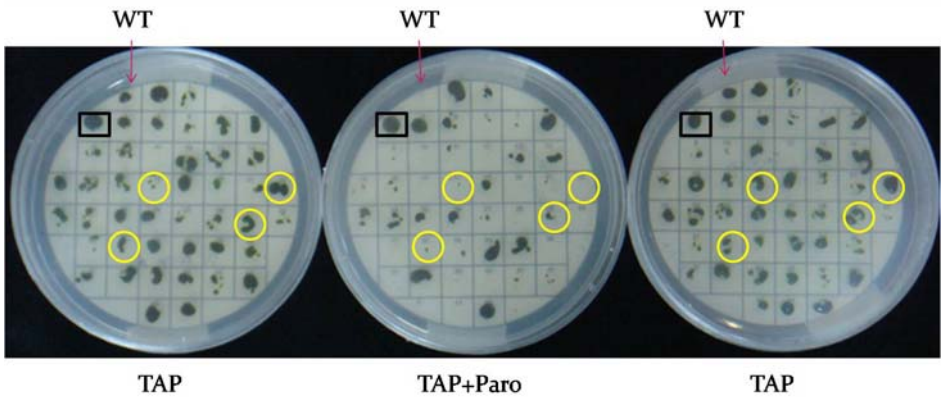

Figure 8. Clonal analysis of induction of silencing in Par^{A} *Chlamydomonas reinhardtii* cells. The Par^{A} cells were cultured on selective medium (TAP+Paro) prior the induction of Par silencing by growing the cells on nonselective conditions (TAP). The same colonies were replicated on selective (TAP+Paro) plate. WT; wild type cells, square box; the stable Par^{A} line, circle; silenced Par (Par^{S})

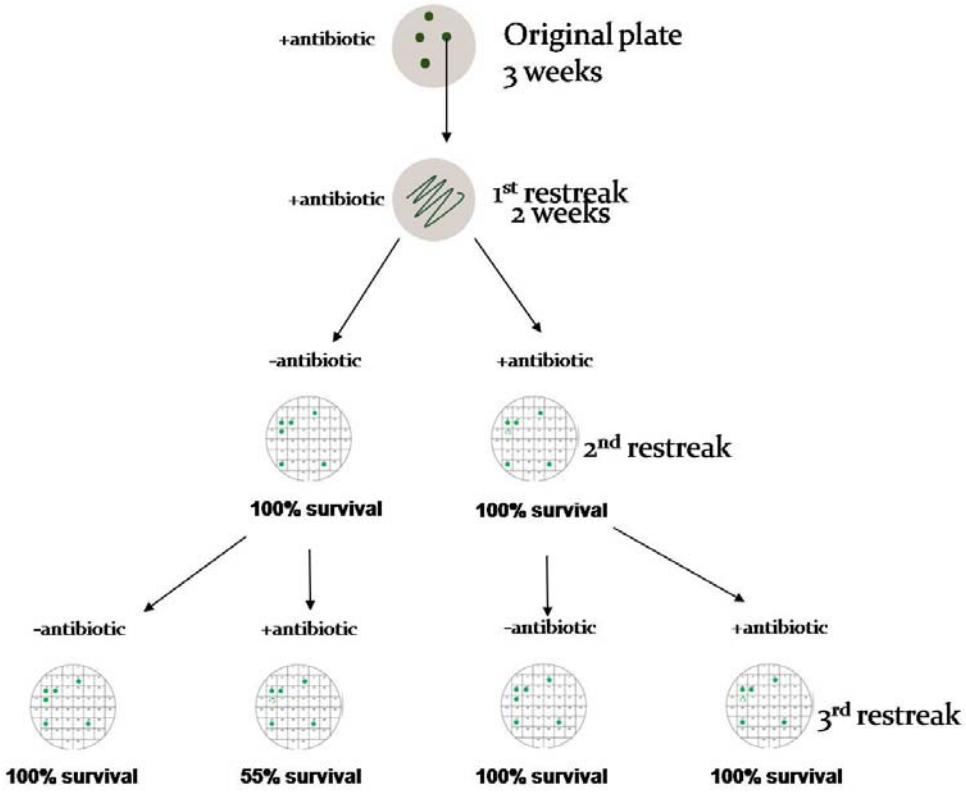

Figure 9. The clonal analysis of paromomycin silencing in transgenic Par^{A} *Chlamydomonas rienhardtii*. After culturing transgenic lines under nonselective pressure, only 55% of transgenic lines survived when transferred back to selective condition. +antibiotic; TAP medium supplemented with 10 mg/l paromomycin, -antibiotic; TAP medium without paromomycin supplemented

Random mutagenesis and screen for mutants defective in epigenetic regulation of paromomycin expression

For the random mutagenesis, the pHyg3 plasmid containing *Streptomyces hygroscopicus* aminoglycoside phosphotransferase gene (*aph7"*) conferring resistant to hygromycin was used (Figure 10) (Berthold et al. 2002). The plasmid pHyg3 was kindly provided by Professor Wolfgang Mages, Lehrstuhl für Genetik, Universität Regensburg, Germany.

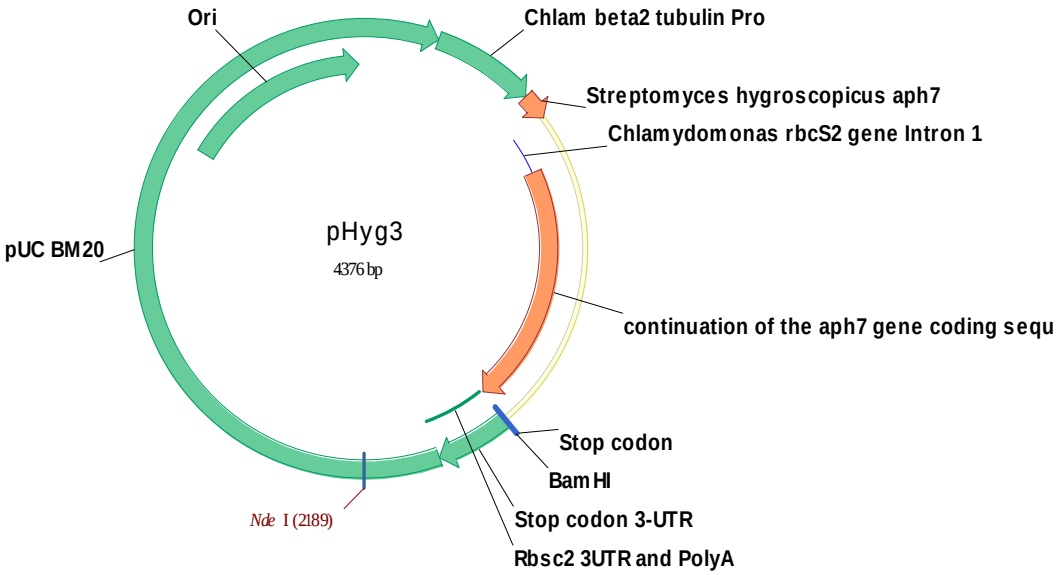

Figure 10. Physical map of plasmid pHyg3 used for random mutagenesis of Par^{A} and Par^{S} *Chlamydomonas* cells. The plasmid pHyg3 contains hygromycin resistant gene for selection in *Chlamydomonas* and ampicillin resistant gene for selection in *E. coli*. Single NdeI restriction site is indicated.

The screen for epigenetic mutants was divided the screen into two types. The first one was screening the Par^{S} phenotype using Par^{A} as a genetic background for random mutagenesis. The second one was carried out by transformation of Par^{S} lines and screened for the reactivated Par^{A} phenotype. For both screens, *C. reinhardtii* Par^{A} or Par^{S} cells were cultured in an appropriated liquid medium (TAP+Paro for Par^{A} and TAP for Par^{S}). The log phase cells were transformed with pHyg3 by glass bead transformation method. The positively transformed cells were selected on the TAP medium supplemented with 10 mg/l hygromycin.

From the transformation of pHyg3, we obtained in total 161 Hyg^{A} primary transformants from 2 independent Par^{A} lines (115 transformants from line 18-9 and 46 transformants from 18-10). For the screening of Hgy^{A}Par^{S} cells, all of the Hyg^{A} transformants (with previously active paromomycin resistant gene) were then transferred onto new TAP medium supplemented with 10 mg/l hygromycin (TAP+Hyg). The same colonies were replicated onto TAP medium containing 10 mg/l hygromycin and 10 mg/l paromomycin (TAP +Hyg+Paro) as shown in the experimental design (Figure 1). The cells with Hgy^{A}Par^{S} phenotype would be able to grown only on TAP+Hyg plate while on the TAP+Hyg+Paro, the cells will turn pale in color and finally die. From the screen, 37 Hgy^{A}Par^{S} lines were obtained from line 18-9 background (Figure 11). Currently these lines are being analyzed for the transcript of paromomycin resistant gene as

well as the copy number of pHyg3 insert. Afterward further characterization of the mutated gene will be carried out in order to identify the affected gene in these putative mutants.

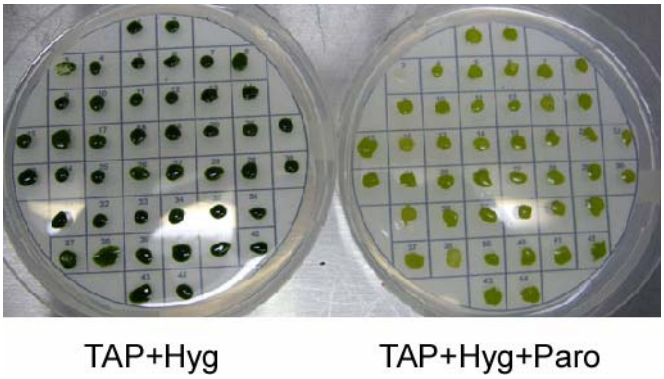

Figure 11. Screening of Par^{A} silencing in *Chlamydomonas reinhardtii* cells. The Hyg^{A} cells were cultured on TAP medium supplemented with 10 mg/l hygromycin (TAP+Hyg). The same colonies were replicated on TAP medium supplemented with 10 mg/l hygromycin and 10 mg/l paromomycin plate (TAP+Hyg+Paro).

According to the research plan, we aimed to screen mutants of which expression of paromomycin resistant marker gene is altered. However, only recently we were able to isolate the putative mutants with the desired phenotype. In parallel, using parts of the fund from TRF, we have conducted another research project related to the original proposal. It has been reported that epigenetic regulation plays pivotal roles in plant response and adaptation under different environmental changes especially under various stress conditions. Salinity stress is a major problem for agriculture in Thailand and worldwide. At the cellular level, adaptation to salinity stress requires significant changes in protein expression pattern. The rapid alteration of protein expression both increase in proteins essential for the adaptation and decrease in proteins that are unnecessary at the time were observed. In a lot of cases, the changes of protein expression are due to the alterations of microRNA accumulation. Several works have been reported on changes of microRNA profiles under salinity stress for example osa-MIR393 was reduced under salinity stress. Over-expression of osa-MIR393 resulted in the reduction of salinity tolerance in rice (Gao et al. 2010). The miR396 from maize root is reduced under salt stress. This microRNA targets cytochrome oxidase and is thought to protect photosynthesis in the cells leading to metabolic adaptation (Ding et al. 2009). As the ultimate goal of the proposed project is to identify genes that are involved in epigenetic regulation in

Chlamydomonas, ultimately we hope that the candidate genes isolated from the mutant screen might play important roles during environmental adaptation. Therefore, another approach was chosen to study the global changes of proteins under salinity stress by two-dimensional electrophoresis followed by mass spectrometry.

Generation of salt-acclimated Chlamydomonas reinhardtii cells

The project has been designed to study responses of *C. reinhardtii* to salinity stress under both short-term and long-term treatments. Normally *C. reinhardtii* cannot grow under very high concentration of NaCl. Only as low as 50 mM NaCl could already inhibit the growth of *C. reinhardtii*. In addition, the salt concentration at 150 mM NaCl was the maximum reported level tested with *C. rerinhardtii* (Hema et al. 2007). Therefore, in order to study long-term responses to salinity stress in *C. reinhardtii*, salt-acclimated *C. reinhardtii* cells were generated by culturing *C. reinhardtii* and challenged the cells under various concentrations of NaCl beyond the reported level of 150 mM NaCl. Figure 12 illustrates an experimental set up to generate the salt-acclimated cells of *C. reinhardtii*. The cells were cultured in TAP medium containing 0 mM NaCl and were transferred into new culture medium containing 0 mM, 50 mM, 100 mM and 200 mM NaCl, respectively. These cells were cultured for another 14 days prior to next sub-culturing. The cells were challenged to adapt to higher concentration of NaCl by transferring the cells into new TAP medium containing 0 mM, 200 mM and 250 mM NaCl. The cells were sub-cultured under these conditions for 2 more generations. Subsequently the cells which were pre-exposed to 250 mM NaCl were transferred and maintained in TAP medium containing 300 mM NaCl. These cells were considered as salt-acclimated cells.

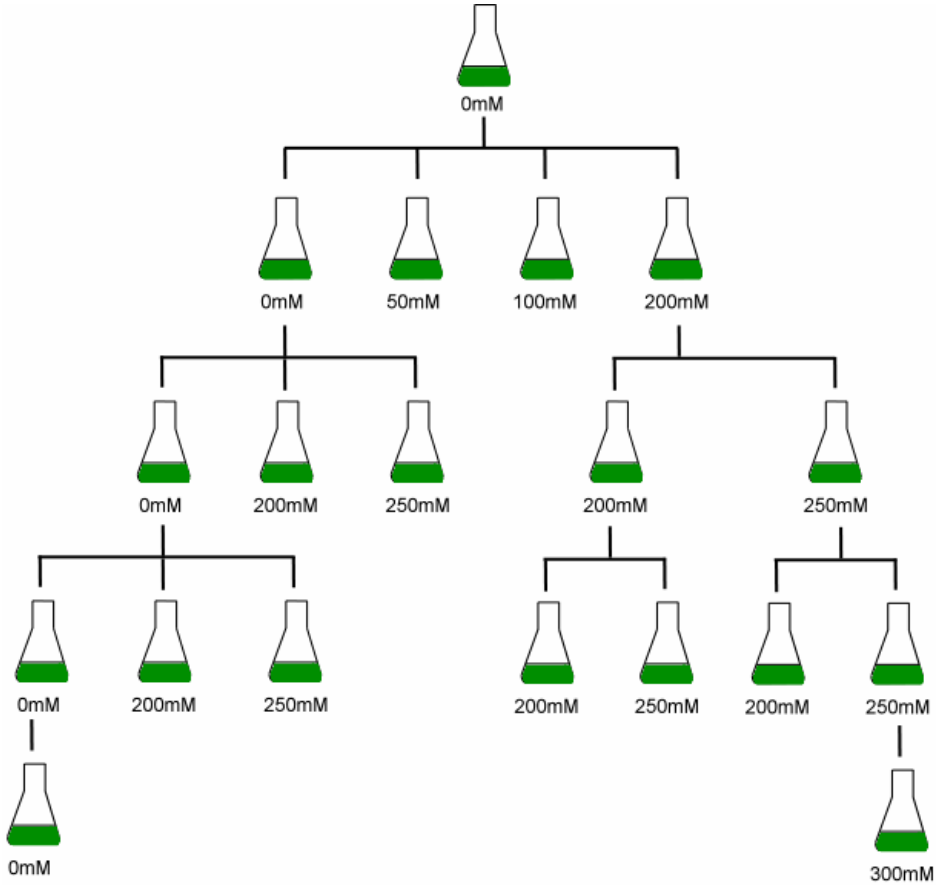

Figure 12. Experimental outline for generation of salt-acclimated cells. Initially *C. reinhardtii* cells were cultured in TAP medium containing 0 mM NaCl. The cells were transferred to new culture medium containing 0 mM, 50 mM, 100 mM, and 200 mM NaCl. After 14 days the cells were further transferred into new TAP medium containing 0 mM, 200 mM and 250 mM. The sub-culturing was repeated for another time. Lastly the cells were transferred and maintained in TAP medium containing 300 mM NaCl (salt-acclimated cells).

When the *C. reinhardtii* cells were firstly exposed to NaCl, the presence of NaCl in the medium inhibited the growth of the algae as expected. To our surprise, *Chlamydomonas* can tolerate and grow under 200 mM NaCl even though the growth rate was much slower as represented by the lower cell density with the lighter green color of the culture (Figure 13).

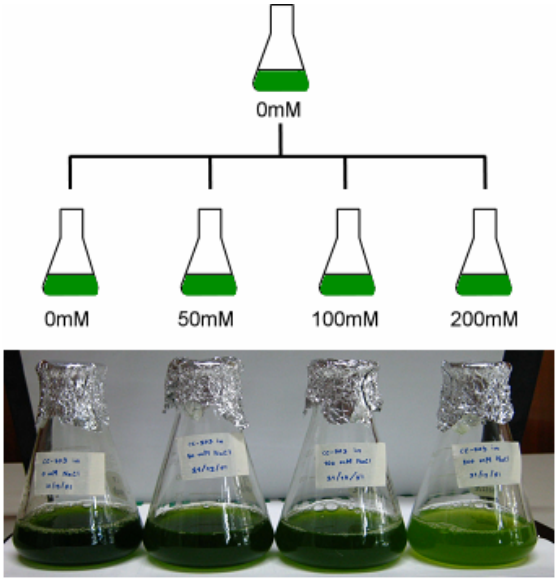

Figure 13. Growth inhibition of *Chlamydomonas reinhardtii* by the presence of NaCl. *C. reinhardtii* cell were grown in TAP liquid medium in the presence of 0 mM, 50 mM, 100 mM and 200 mM NaCl. Photo was taken 14 days after inoculation.

Afterward the cells in 0 mM and 200 mM NaCl were chosen for further experiment. The cells were sub-cultured into new culture medium containing 0 mM, 200 mM and 250 mM NaCl as shown in figure 12. Fourteen days later, similar results have been observed. After sub-culturing, if there is an adaptation of cells to the presence of salt in the culturing medium, the adaptation might occur with the cells which were pre-exposed to salt. The cells should show a better growing rate than the cells that are treated with NaCl for the first time. As expected, the growths of cells transferred from 0 mM to 200 mM NaCl and 0 mM to 250 mM NaCl were retarded when compared with the cells transferred from 200 mM to 200 mM NaCl and 200 mM to 250 mM NaCl, respectively (Figure 14). The cells which were grown in 250 mM NaCl were then transferred into new culture medium containing 300 mM NaCl to further challenge the cells. These cells could be able to grow in 300 mM NaCl. The cells were maintained in TAP liquid medium with 300 mM NaCl. By comparing the cells which have not been exposed to salt before (0 mM->300 mM NaCl) with the cells that were maintained in 300 mM (300->300 mM NaCl), the drastic growth retardation was observed in the cells freshly transferred to NaCl. In contrast, the salt pre-treated cells could be able to grow perfectly in the presence of salt (Figure 15). Therefore these cells were considered as “salt-acclimated” cells and were used in subsequent analysis.

The salt-acclimated cells represented the long-term salinity stress responses. The short-term response was also studied in the short-term treated cells. These cells were generated by

culturing the *C. reinhardtii* cells in TAP medium without NaCl. The cells were subjected to salinity stress by treating these cells with 300 mM NaCl for 3 hours. The short-term and long-term treaments are called 0->300mM and 300->300mM henceforth.

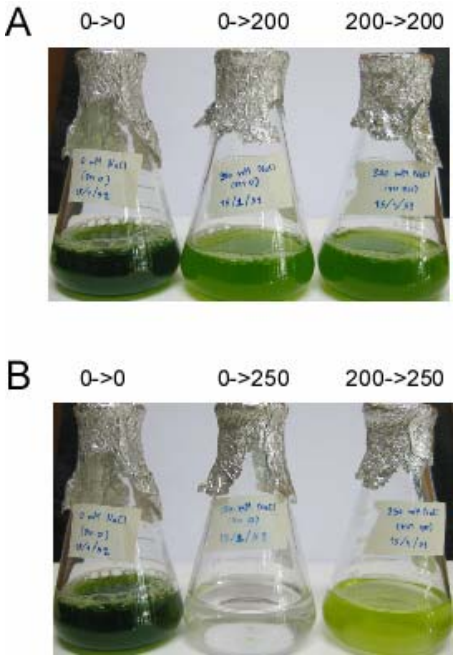

Figure 14. Adaptation of *Chlamydomonas reinhardtii* to salinity stress. A. The cells were transferred from TAP medium containing 0 mM to 0 mM NaCl (0->0), from TAP medium containing 0 mM to 200 mM NaCl (0->200) and from TAP medium containing 200 mM to 200 mM NaCl (200->200). B. The cells were transferred from TAP medium containing 0 mM to 0 mM NaCl (0->0), from TAP medium containing 0 mM to 250 mM NaCl (0->250) and from TAP medium containing 200 mM to 200 mM NaCl (200->250).

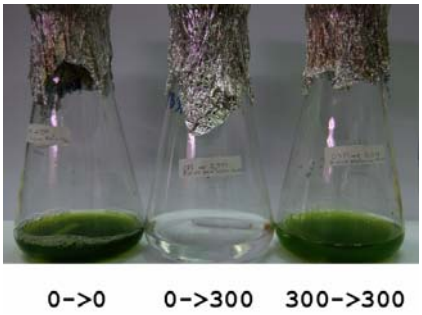

Figure 15. Salt-acclimated *Chlamydomonas reinhardtii* cells. The cells were transferred from TAP medium containing 0 mM to 0 mM NaCl (0->0), from TAP medium containing 0 mM to 300 mM NaCl (0->300) and from TAP medium containing 300 mM to 300 mM NaCl (300->300).

Differentially protein profiles of Chlamydomonas reinhardtii under salinity stress

Total proteins extracted from short-term salinity treatment and salt-acclimated *C. reinhardtii* cells were resolved on 2–DE and stained with Commassie Brilliant Blue G. Figure 16 shows representatives of the 2 dimensional electrophoresis resolved gels of the samples 0->300 mM, 300->300 mM and 0->0 mM as a control. Interestingly the differential protein profiles were clearly observed in all three samples. Some proteins were increased in a short-term response to salinity stress while some are specific only to the long-term response to salt (Table 1). The proteins present only in the cells growing in 0 mM NaCl are indicated in table 2. The short-term NaCl treatment induced a lot of proteins to be expressed. These proteins represent the short-term response to salinity stress and are shown in table 3. Interestingly many proteins were present only in salt-acclimated cells (Table 4). This might indicate the significance of these proteins for cells to be able to adapt in response to the salinity stress.

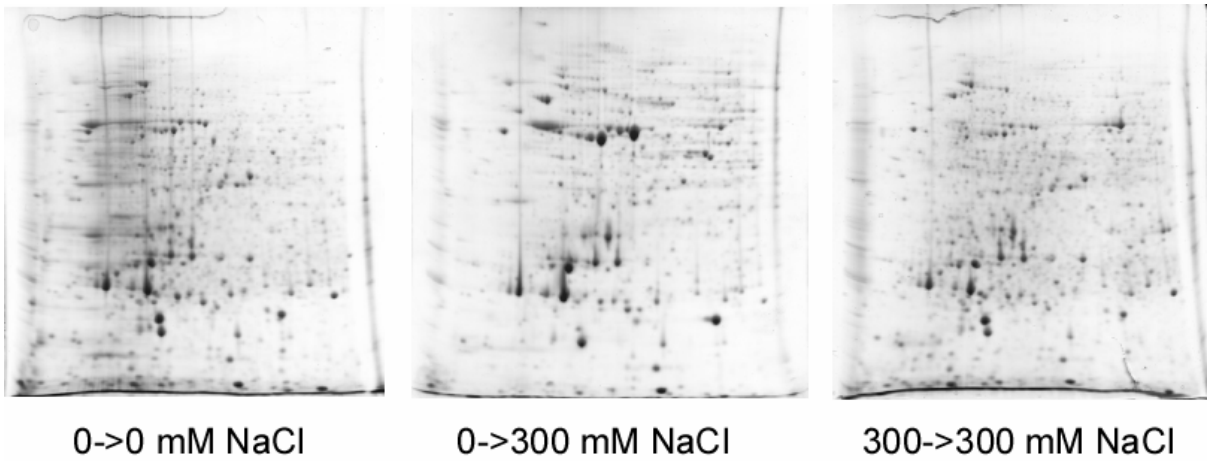

Figure 16. Differential protein profile of *Chlamydomonas reinhardtii* in response to salinity stress. Representatives of two-dimensional gel electrophoresis protein profiles of *C. reinhardtii* samples collected from the cells transferring from 0 mM to 0 mM NaCl (0->0), from 0 mM to 300 mM NaCl (0->300) and from 300 mM to 300 mM NaCl (300->300) for three hours.

Table 1. Proteins differentially express under both short- and long-term salinity treatments. These proteins were expressed only under the conditions indicated.

Number of proteins exclusively found in	Number of protein spots detected
0->0 mM NaCl	19
0->300 mM NaCl	61
300->300 mM NaCl	27

Table 2. list of identified *C. reinhardtii* proteins found only in 0->0 mM NaCl

Spot	Matched protein	NCBI accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score
139	predicted protein	gi|159463270	20.2/4	20.7/5.10	129
140	ribosomal protein L12, component of cytosolic 80S ribosome and 60S large subunit	gi|159477751	19.7/4	17.8/9.19	222
141	peptidyl-prolyl cis-trans isomerase	gi|159484660	19.1/4	18.6/7.66	48
416	CF1 ATP synthase: SUBUNIT gamma	gi|228698	44.1/4.6	39.1/9.08	150
1022	5-methyltetrahydropteroyltri glutamate--homocysteine methyltransferase	gi|3334258	18./4.9	87.2/6.02	80
1615	Rubisco chain A	gi|16975080	70.7/4.8	53.1/6.04	174
2131	light harvesting complex protein I-20	gi|18125	21.3/5	23.5/7.98	63
2613	beta subunit of mitochondrial ATP synthase	gi|159466892	65.8/5.1	62/5	241
3915	predicted protein	gi|159471910	103.7/5.6	67.6/6.41	156
5311	isocitrate lyase	gi|619932	39.5/5.7	45.8/5.78	73
5428	photosystem II stability/ assembly factor HCF136	gi|159476190	44.5/5.7	32.7/6.95	414
8226	G strand binding-protein 1/ telomere binding-protein	gi|74272657	29.6/6.5	24.5/6.78	132
9207	G strand binding-protein 1/ telomere binding-protein	gi|74272657	29.6/7	24.5/6.78	393
9209	phosphoenolpyruvate carboxykinase	gi|7272695	27.1/7	24.5/8.65	70
9213	G strand binding-protein 1/ telomere binding-protein	gi|74272657	26.6/7	24.5/6.78	212
9214	mitochondrial F1F0 ATP	gi|159470863	30.7/6.9	34.1/6.86	77

	synthase associated 31.2 kDa protein				
9217	predicted protein	gi|159487124	43.7/7	31/8.50	65
9612	dihydrolipoamide dehydrogenase	gi|159463380	68.4/7	60.1/8.73	103
9614	biotin carboxylase, acetyl-CoA carboxylase component	gi|159488652	67.7/7	52.3/8.96	222

Table 3. list of identified *C. reinhardtii* proteins found only in 0->300 mM NaCl

Spot	Matched protein	NCBI accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score
1417	chloroplast ATP synthase gamma chain	gi|159476472	44/4.9	39.1/9.08	336
1710	beta subunit of mitochondrial ATP synthase	gi|159466892	84.2/5	62/4.99	783
1815	binding protein 1	gi|159487349	91.7/4.8	72.7/4.99	597
2221	major light-harvesting complex II protein m1	gi|20269804	26.9/4.9	27.6/5.96	69
2415	peptidyl-prolyl cis-trans isomerase	gi|159467709	49.9/5.1	44.8/5.37	283
2416	chloroplast ATP synthase gamma chain	gi|159476472	44.1/5	39.1/9.08	551
2519	eukaryotic initiation factor	gi|159482426	53/5.1	37.7/4.96	212
2611	translocon component Tic40-related protein	gi|159465627	66.9/5	50/5.61	434
2729	beta subunit of mitochondrial ATP synthase	gi|159466892	84.6/5.1	62/4.99	205
3621	Chain A, Rubisco	gi|16975080	70.4/5.2	53/6.04	473
3622	Chain A, Rubisco	gi|16975080	70.6/5.2	53/6.04	450
3623	Chain A, Rubisco	gi|16975080	70.9/5.3	53/6.04	531
3264	ATP synthase CF1 beta subunit	gi|41179057	67.4/5.3	53.2/5.21	516
3715	chaperonin 60A	gi|159491478	78/5.2	61.9/5.49	944

3817	5 kDa chloroplast membrane translocon 7	gi|159490640	90/5.5	87.7/5.55	256
3718	predicted protein	gi|159484662	84.6/5.3	14.5/7.79	69
3816	5 kDa chloroplast membrane translocon 7	gi|159490640	97.9/5.3	87.7/5.55	290
3817	5 kDa chloroplast membrane translocon 7	gi|159490640	97.7/5.3	87.7/5.55	256
3818	heat shock protein 90C	gi|159490014	102.6/52	89.5/5.24	574
3819	heat shock protein 90C	gi|159490014	103/52	89.5/5.24	743
3820	heat shock protein 90C	gi|159490014	102.7/5.2	89.5/5.24	765
3828	predicted protein	gi|159472442	94.6/5.3	58.6/8.62	224
4426	chloroplast glyceraldehyde-3-phosphate dehydrogenase	gi|74272659	48/5.4	40/9.08	207
4719	iron-sulfur cluster assembly protein	gi|159485362	79.8/5.4	57.2/9.16	136
4819	heat shock protein 70A	gi|159486599	90.9/5.4	71.5/5.25	980
4821	predicted protein	gi|159485022	97.3/5.5	75.3/5.82	211
4824	chloroplast elongation factor G	gi|159487669	97.8/5.4	79.9/5.23	255
4825	heat shock protein 90C	gi|159490014	95.2/5.4	89.5/5.24	134
5015	Chain C, Structure of the cytochrome B6f	gi|40889430	18.6/5.6	13.9/5.74	97
5222	soluble inorganic pyrophosphatase	gi|159473581	25.1/5.6	22.4/5.49	108
5320	heterogeneous nuclear ribonucleoprotein	gi|159486121	34.2/5.7	31.3/5.79	165
5321	L-ascorbate peroxidase	gi|159488379	32.8/5.7	36.5/9.23	149
5422	full-length thiazole biosynthetic enzyme	gi|159481205	44.8/5.7	37/6.72	112
5424	peptidyl-prolyl cis-trans isomerase	gi|159466422	47/5.7	42.3/5.69	346
5613	ATP synthase CF1 beta subunit	gi|41179057	66.8/5.7	53.2/5.21	390
5721	chaperonin 60C	gi|159466312	79.3/5.6	57.2/5.40	530
6218	phosphoribulokinase	gi|159471788	25.3/5.9	42.1/8.11	108
6319	L-ascorbate peroxidase	gi|159488379	32.7/6	36.5/9.23	208

6413	3,8-divinyl protochlorophyllide a 8-vinyl reductase	gi|159463876	46.1/5.9	44.8/9.01	627
6619	ATP synthase CF1 alpha subunit	gi|41179050	70.5/5.9	54.8/5.44	531
7323	mitochondrial F1F0 ATP synthase associated 31.2 kDa protein	gi|159470863	38.2/6.4	34.1/6.86	369
7526	NADP-malate dehydrogenase	gi|159477375	54.7/6.2	45.3/8.04	458
7626	elongation factor Tu	gi|226818	60/6.2	45.7/5.84	242
8222	hypothetical protein	gi|159470187	26/6.7	28.9/8.92	307
8318	prohibitin	gi|159477687	34.8/6.6	31.2/6.37	459
8319	hypothetical protein	gi|159479888	35.8/6.6	30.7/6.95	168
8419	eukaryotic initiation factor	gi|159470237	44/6.6	36.8/6.01	73
8420	adenosinetriphosphatase	gi|1334356	42.6/6.5	48.8/6.20	119
8518	phosphoglycerate kinase	gi|1172455	55/6.5	49.3/8.84	358
8625	argininosuccinate synthase	gi|159477301	62.9/6.4	49.2/8.41	382
8714	phosphoenolpyruvate carboxykinase, splice variant	gi|159473683	78.8/6.6	62.4/6.23	471
8822	cobalamin-independent methionine synthase	gi|159489910	100.2/6.7	87.3/5.94	689
8823	cobalamin-independent methionine synthase	gi|159489910	100.4/6.5	87.3/5.94	540
8824	cobalamin-independent methionine synthase	gi|159489910	100.7/6.4	87.3/5.94	628
8826	pyruvate formate-lyase	gi|92084842	93.3/6.7	93.7/6.40	512
8827	pyruvate-formate lyase	gi|159462978	95.8/6.6	91.4/6.49	306
8829	pyruvate-formate lyase	gi|159462978	96.1/6.6	91.4/6.49	278
8830	pyruvate-formate lyase	gi|159462978	93.7/6.6	91.4/6.49	434
9416	acidic ribosomal protein P0	gi|159477927	42.8/6.8	34.6/6.07	466
9616	cystathionine gamma-synthase	gi|159475262	59.3/6.9	51.1/7.28	90
9709	phosphoenolpyruvate carboxykinase, splice variant	gi|159473683	77.9/6.9	62.3/6.23	506

Table 4. list of identified *C. reinhardtii* proteins found only in 300->300 mM NaCl

Spot	Matched protein	NCBI accession	Observed MW (kDa) / pI	Theoretical MW (kDa) / pI	MOWSE Search Score
1327	predicted protein	gi|159491211	38.6/4.7	51.7/6.02	94
1330	pepsin-type aspartyl protease	gi|159468321	38.9/4.8	32.6/5.44	56
2132	predicted protein	gi|159478935	21.3/5	15/5.13	85
2720	heat shock protein 90B	gi|159487749	82.9/5.1	87.5/4.80	191
3415	ADP-glucose pyrophosphorylase small subunit	gi|159467349	51.9/5.3	55.9/8.38	275
3427	ADP-glucose pyrophosphorylase small subunit A	gi|159467349	49/5.2	55.9/8.38	131
3719	heat shock protein 70A	gi|159486599	75.9/5.4	71.5/5.25	381
3830	predicted protein	gi|159463132	90.9/5.3	76.2/5.31	51
4124	2-cys peroxiredoxin	gi|159483223	23/5.5	21.8/5.46	142
4720	phosphoglucomutase	gi|159479834	79.5/5.5	64.8/7.12	140
5429	elongation factor Tu	gi|41179007	45.1/5.8	45.8/5.90	74
7113	predicted protein	gi|159472955	24/6.4	15.5/5.57	130
7324	chlorophyll a-b binding protein of photosystem II	gi|159478202	36/6.3	30/6.22	173
7326	ascorbate peroxidase	gi|159487873	37.4/6.2	35.9/8.67	204
7425	phosphoserine aminotransferase	gi|159464395	44.2/6.2	44.4/8.88	128
7528	ATP synthase CF1 alpha subunit	gi|41179050	51.2/6.2	54.8/5.44	397
8115	hypothetical protein	gi|159486443	19.7/6.6	25.8/6.00	61
8322	hypothetical protein	gi|159464369	35.3/6.5	21.6/6.23	92
8416	catalase	gi|115705	51.8/6.5	57.6/6.72	133
8423	coproporphyrinogen III oxidase	gi|159487437	42.7/6.7	41.7/8.12	177
8514	phosphoenolpyruvate carboxykinase, splice variant	gi|159473683	56.9/6.5	62.4/6.23	497

8523	pyruvate formate-lyase	gi|92084842	53.5/6.5	93.7/6.40	53
9212	cytochrome c peroxidase	gi|159479120	32.8/6.8	40/8.63	287
9221	mitochondrial F1F0 ATP synthase associated 31.2 kDa protein	gi|159470863	33.4/6.7	34.1/6.86	166
9224	isocitrate dehydrogenase, NADP-dependent	gi|159481269	25.4/6.9	53.9/8.98	126
9309	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	gi|41179049	35.4/6.7	53.2/6.14	323
9516	ADP-glucose pyrophosphorylase large subunit	gi|159470605	58.4/6.8	55.7/7.41	124

Conclusion

rom this research project, we have separated the work into two parts. The first part was aimed to screen for mutants that are involved in the epigenetic regulation in *C. reinhardtii*. From this part of the project, we have isolated in total 37 candidate mutants which the expression of transgenic marker gene, paromomycin resistant gene, is altered. These mutants will serve as a good resource to identify the underlying mutated genes. Hopefully some of these genes are involved in the regulation of gene expression. However, the work cannot be finished in time for the 2 years granting period of TRF. Thus, more works on characterization of these candidate mutants need to be carried out in the future, which maybe a continuing new proprosal submitting for TRF consideration again.

Another part of the project has been focused on identification of differential protein profiles under salinity stress condition. We have observed indeed differentially expressed genes under both short-term and long-term treatment with NaCl. In dept analysis of the proteins including the involvement of these proteins in salt stress response will be studied. The latter proteomic work already gained a lot of information worth a publication in an international journal. Manuscript will soon be drafted and submitted for a peer-review process. TRF grant will be acknowledged in this prospective paper.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกอ. และ สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

เนื่องจากงานวิจัยหลักที่ดำเนินการโดยใช้ทุนสนับสนุนจาก สกอ. และ สกว. นั้นประสบปัญหาในการคัดเลือก mutant โดยผู้วิจัยเพิ่งสามารถจำแนกสาหร่ายที่อาจจะเป็น candidate mutant ในช่วง 6 เดือนสุดท้ายนี้เอง ผู้วิจัยจะต้องทำการวิจัยต่อเนื่องเพื่อวิเคราะห์หายีนที่ได้รับการ mutate ไปในสาหร่ายเหล่านี้ รวมทั้งยังต้องทำการศึกษาลักษณะ phenotype อื่นๆด้วย จึงทำให้ผลการวิจัยในขณะนี้ยังไม่เพียงพอสำหรับการตีพิมพ์ในวารสารวิชาการระดับนานาชาติได้ แต่เนื่องจากทุนวิจัยที่ได้รับการสนับสนุนจาก สกอ.และ สกว. บางส่วนนั้นได้ถูกนำไปใช้เพื่อดำเนินการในโครงการวิจัยที่เกี่ยวเนื่องกัน คือ การศึกษาโปรตีนที่มีการแสดงออกเปลี่ยนแปลงไปภายใต้สภาวะเครียดจากความเค็ม ซึ่งผู้วิจัยได้ขอเพิ่มแผนการทดลองในรายงานการวิจัยรอบ 18 เดือนไปแล้วนั้น งานวิจัยส่วนนี้ผู้วิจัยได้รับผลการวิจัยก้าวหน้าเป็นอย่างมาก ซึ่งผู้วิจัยคาดว่าจะสามารถเขียนร่าง manuscript และสามารถส่ง manuscript นี้ไปยังวารสารวิชาการเพื่อพิจารณาได้ภายในระยะเวลา 6 เดือน โดยผู้ดำเนินการวิจัยมีแผนที่จะส่งผลงานนี้ไปตีพิมพ์ในวารสาร Journal of Proteomics (Impact Factor 3.851) หรือวารสาร Planta (Impact Factor 3.372) เมื่อผู้วิจัยได้ทำการส่ง manuscript ไปยังวารสารและได้รับการยืนยันตอบรับเพื่อให้ตีพิมพ์ในวารสารดังกล่าว จะดำเนินการแจ้งให้ทาง สกอ. และ สกว. ทราบต่อไป

2. การนำผลงานวิจัยไปใช้ประโยชน์

N/A

3. อื่นๆ

N/A