

รายงานวิจัยฉบับสมบูรณ์

การศึกษาทรานสคริปโตมิกส์และไมโครอาร์เอ็นเอในสาหร่ายเซลล์เดียว *Chlamydomonas reinhardtii* ในสภาวะเครียดจากความเค็ม

Transcriptomic and genome-wide profiling of microRNAs in *Chalmydomonas reinhardtii* under salinity stress

โดย

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สนันสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

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Abstract

Title : Transcriptomic and genome-wide profiling of microRNAs in *Chalmydomonas reinhardtii* under salinity stress

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Salinity stress is one of the major problem for agriculture in Thailand and worldwide. Plants response to the saline condition by various mechanisms in order for them to survive under an unsuitable growth condition. The response of plant to salt stress was address here using *Chlamydomonas reinhardtii* as a plant model. An alga cells were induced to adapt to saline condition by culturing them in salt containing media. The salt-adapted cells were able to grow in the medium supplemented with relatively high salt concentration as 300 mM NaCl. The alteration of genes expression was assessed by transcriptomic analysis. About 1,261 genes were up-regulated and 664 genes were down-regulated genes more than 2-fold. These genes fall into various metabolic pathway as well as in the stress and defense mechanism. Deep analysis of the transcriptomic sequences displayed 2,098 SNPs in the salt-adapted compared to the wild type as well as the sequences in the database. In addition, the microRNAs of both wild type and the salt-adapted were examined. 50 potential microRNAs which matched to the MIRBASE were obtained.

Keywords : salinity stress, transcriptome, microRNA, *Chlamydomonas reinhardtii*

บทคัดย่อ

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ชื่อโครงการ : การศึกษาทรานสคริปโตมิกส์และไมโครอาร์เอ็นเอในสาหร่ายเซลล์เดียว *Chlamydomonas reinhardtii* ในสภาวะเครียดจากความเค็ม

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ปัญหาดินเค็มเป็นปัญหาสำคัญที่ส่งผลกระทบต่อการเกษตรกรรมทั้งในประเทศไทยและในต่างประเทศ พืชมีการตอบสนองต่อสภาวะดินเค็มผ่านกระบวนการต่างๆ หลากหลายกระบวนการด้วยกันเพื่อให้พืชสามารถดำรงชีวิตอยู่ได้ภายใต้สภาวะแวดล้อมที่ไม่เหมาะสมต่อการเจริญเติบโต ในการศึกษาวิจัยครั้งนี้ได้ศึกษากระบวนการตอบสนองของพืชต่อสภาวะเครียดจากความเค็มโดยอาศัยสาหร่ายสีเขียวเซลล์เดียว *Chlamydomonas reinhardtii* เป็นพืชโมเดล สาหร่าย *Chlamydomonas reinhardtii* ได้รับการเหนี่ยวนำให้สามารถทนเค็มได้ โดยการเลี้ยงสาหร่ายในอาหารเลี้ยงที่มีการเติมเกลือเป็นระยะเวลาหนึ่ง จนกระทั่งสาหร่ายสามารถทนต่ออาหารที่มีเกลือความเข้มข้นสูงถึง 300 มิลลิโมลาร์ NaCl ได้ การเปลี่ยนแปลงการแสดงออกของยีนในสาหร่ายทนเค็มนี้ได้รับการศึกษาโดยเทคนิคทรานสคริปโตมิกส์ พบว่า มียีนทั้งสิ้น 1,261 ยีน ที่มีการแสดงออกสูงขึ้นและมียีน 664 ยีนที่มีการแสดงออกลดลงกว่าในสาหร่ายสายพันธุ์ปกติถึง 2 เท่า เมื่อจัดจำแนกยีนเหล่านี้แล้ว พบว่ายีนเหล่านี้มีหน้าที่เกี่ยวข้องในกระบวนเมแทบอลิซึมต่างๆ รวมไปถึงการตอบสนองต่อความเครียดอีกด้วย เมื่อทำการศึกษาเพื่อหาลำดับเบสที่เปลี่ยนแปลงไป (SNP) ในสาหร่ายทนเค็ม พบว่ามีทั้งสิ้น 2,098 SNPs ในสาหร่ายทนเค็ม นอกจากนี้ยังมีการวิเคราะห์เพื่อหา microRNAs ในสาหร่ายทนเค็ม โดยจากการศึกษาพบไมโครอาร์เอ็นที่มีการรายงานในฐานข้อมูลไมโครอาร์เอ็นเอ MIRBASE อยู่ทั้งสิ้น 50 ชนิดด้วยกัน

Keywords : salinity stress, transcriptome, microRNA, *Chlamydomonas reinhardtii*

Literature review

Introduction

About 20% of agriculture area is salt effected (Munns, 2005). One of the major problem for agriculture is the saline soil which are classified when it contains approximately 40 mM NaCl (ECe is 4 dS/m). This concentration can generate osmotic pressure of about 0.2 mPa (Munns & Tester, 2008). While most of the crop plants are not salt tolerance. Salt effect plants by two distinct ways. The first and rapid effect of salt is the hyperosmotic stress followed by the hyperionic stress caused by toxicity of Na+ and Cl-. The change in osmotic pressure cause the reduction of water absorption by roots. The responses of plants to salinity stress including reduction of leaf expansion, stomata closure, impairment of photosynthetic activity, reduction of nitrogen assimilation, biomass loss (Zhang and Shi, 2013; Munns et al., 2006). During salinity stress, the production of reactive oxygen species (ROS) is increased causing oxidative damage to various cellular components. Sodium can also enter via KUP/HAK/KT potassium transporters, cyclic-nucleotide-gated channels, glutamate-activated channels, LCT transporters, and HKT transporters.

Salt tolerance

Some halotypes i.e. *S. salsa*, *Aeluropus littoralis* have been studied in order to understand the mechanisms that plants utilized to survive under high salt condition. Many pathways have been identified to be involved in the stress tolerance including (1) regulation of ion homeostasis, (2) ion uptake and transport, (3) synthesis of osmoprotectants/ compatible solutes, (4) synthesis of antioxidant enzymes. Alteration of membrane lipid composition is involved in the salt tolerance (Zhou et al., 2014). Proteomic analysis displays change in protein profiles in many plants in response to salt stress. Many up-regulated protein have been found such as chloroplastic ATP synthase, V-ATPase, nucleoside diphosphate kinase 1, chlorophyll a/b-binding protein, and ABC transporter I family (เพิ่ม ref Li et al., 2011)

Regulation of ion homeostatsis

Plants usually have to balance the ratio of Na+/K+ within the cell. In general, these ions are kept at a low concentration in cytoplasm. Concentration of K+ are roughly 100 mM in the cytosol and ranging between 10-200

mM in the vacuole (Binzel et al., 1988). While the Na+ ions are kept at 1 mM in the cytoplasm. High salt condition perturb the balance ratio of Na+/K+ ion in the cells. In addition, the salt stress causes the accumulation of Ca^{2+} ions within cytoplasm. Many transport proteins have been identified to function in the K+/Na+ homeostasis and salt tolerance for instance histidine kinase transporter (HKT), Na+/H+ antiporters (NHXs), Ca^{2+} ATPases (Schroeder et al., 2013; Chinnusamy et al., 2005; Hasegawa et al., 2000; Horie et al., 2009). The evidence that AtHKT is involved in salt stress came from genetic screen for mutants that cause the hyper-accumulation of Na+ leading to the conclusion that AtHKT1 functions in Na+ influx (Rus et al., 2001; Berthomieu et al., 2003; Rus et al., 2006; Horie et al., 2007). There are two class of HKT transporters, class I and class II HKT (Horie et al., 2001; Garciadebla et al., 2003; Platten et al., 2006; Horie et al., 2007). The class I HKT generally selectively transport Na+ while the class II HKT can transport both Na+ and K+ but preferentially K+ over other cations (Uozumi et al., 2000; Maser et al., 2002). In rice, there are 9 predicted HKT in Japonica rice (Garciadeblás et al., 2003). Similarly the Indica rice have also been studied. The salt-tolerance strain, Pokkali, displayed the down-regulation of OsHKT1;2 compared to the salt-sensitive strain, cv. BRRI Dhan29 (Kader et al., 2006; Horie et al., 2007).

Na+ sequestration into vacuole

Successful generation of salt-tolerance plants involved the sequestration of toxic ions into vacuole. Na+ is uptakes and transport into vacuole through Na+/H+ antiporter. And the H+ pump will further transport the Na+ ion into vacuole via an energy-dependent transport system. There are two types of H+ pumps. The first one is vacuolar type H+-ATPase (V-H+-ATPase) and the second type is vacuolar pyrophosphatase (V-H+-PPase) (Gupta and Huang, 2014; Apse and Blumwald, 2007; Gaxiola et al., 2007). The dominant H+ pump in plants is V-H+-ATPase. *S. salsa*, a salt-tolerance plant, contain 2 low-affinity Na+ transporters. The Na+/H+ antiporter is regulated by H+ gradient generated by V-H+-ATPase and V-H+-PPase (Wang et al., 2001). The first V-H+-PPase was cloned from Arabidopsis (Sarafian et al., 1992) followed by the cloning of its homolog from other plants such as Barley (Tanaka et al., 1993), rice (Sakakibara et al., 1996), and wheat (Brini et al., 2005). In addition to the function of H+ pump, another transport protein, NHX, has been found to function in the sequestration of Na+ into the vacuole (Hasegawa P, 2013). The first NHX1 was cloned from Arabidopsis (Gaxiola et al., 1999). By overexpressing *AtNHX1* in Arabidopsis, tomato and Brassica, the transgenic plants showed more tolerance to saline condition and accumulated higher level of Na+ in the vacuole (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001). The NHX1 homologs in other plants have shown to be function in Na+ sequestration

to vacuole similar to that of Arabidopsis homolog such as in wheat (Cuin et al., 2009), rice OsNHX1 (Fukuda et al., 1999), Barley (Fukuda et al., 2004), citrus (Porat et al., 2002) as well as in the halotype *S. salsa* (Song and Wang, 2015).

Plant signaling

The signaling of salt stress is involved in the over accumulation of Ca2+ in the cytoplasm. Isolation of the protein kinases involved in the salt stress signaling came from the genetic complementation of the yeast mutant that are salt-hypersensitive leading to the discovery of several Mitogen-Activated Protein Kinases (MAPKs), MAPKKs and MAPKKKs (Piao et al., 1999).

SOS signaling pathway

The Salt Overly Sensitive (SOS) pathway have been studied to play role in signaling during salt stress in plants. The SOS3 is a myristoylated EF hand type Ca+-binding protein (Ishitani et al., 2000). When the plants expose to salt stress, cytoplasmic Ca+ level is increased. The SOS3 protein binds to Ca+ ion and further activates the function of SOS2, a serine/threonine kinase (Guo et al., 2004). SOS2 contains kinase domain at its N-terminal and regulatory domain at the C-terminal (Lui et al., 2000). SOS3 as positive regulator binds to SOS2 through the NAF domain (also known as FISL) on SOS2 protein (Halfter et al., 2000). In addition, the SOS2 is negatively regulated by type 2C protein phosphatase ABI2 (Guo et al., 2001; Sanchez-Barrena et al., 2007). Activated SOS2-SOS3 complex will then phosphorylated SOS1, a plasma membrane Na+/H+ antiporter protein (Shi et al., 2000; Qui et al., 2002). The activity of phosphorylated SOS1 is increased (Quintero et al., 2002). The Na+ is transported out of the cell thus the ionic effect of Na+ is decreased.

SOS1 is a Na+/H+ antiporter protein containing a large C-terminal domain (Shi et al., 2000). A cyclic nucleotide-binding motif (residue 764-849) and an auto-inhibitory domain (residue 998-1146) are presented at its carboxyl-terminal. Without salt stress, the activity of SOS1 protein is inhibited through the interaction of cyclic nucleotide-binding motif and an auto-inhibitory domain. Activation of SOS1 occurs through phosphorylation at the cyclic nucleotide-binding motif by SOS2 (Quintero et al., 2011). Expression of SOS1 gene is observed in both shoot and root of the plants. Not only is the conformation of SOS1 protein regulated under salt stress, but also its transcription. The SOS1 genes is up-regulated under saline condition (Shi et al., 2000). The protein which has

highest similarity to SOS1 is AtNHX8. This protein lacks the long C-terminal domain. AtNHX8 plays roles in $LiCl_2$ tolerance but not NaCl suggesting the functional different in their C-terminal domain (An et al., 2007). In salt-tolerant plants, *Thellungiella halophila*, inhibition of ThSOS1 gene by RNAi techniques resulted in salt-sensitive phenotype (Oh et al., 2009).

Production of compatible solutes

The compatible solutes or osmoprotectants are produced in higher plants under salinity stress. These osmolytes are usually hydrophilic and non-toxic. The compatible solutes are accumulated in the cytoplasm to balance the osmotic potential in the cell (Hall et al., 1978; Gagneul et al., 2007). They are groups of organic compound such as amino acids (proline), quaternary ammonium compounds (glycine betaine, alanine betaine, proline betaine), sugars (sucrose and fructose) sugar alcohols (glycerol, mannitol, sorbitol, inositol, methylated inositol), and complex sugars (trehalose, raffinose, fructans) and polyols. Proline is hyper-accumulated in the plants under saline stress (Kavi Kishor et al., 2005). Biosynthesis of proline in higher plants exist in two pathways; the “ornithine” or “glutamate” pathway (Delauney et al., 1993). The main pathway in plants is the synthesis of proline from glutamate (Szabados and Savoure´, 2010). The pyrroline carboxylic acid synthetase (P5CS) and pyrroline carboxylic acid reductase (P5CR) are the key enzymes involving in production of proline in plants. For biosynthesis of glycine betaine, choline monooxygenase (CMO) uses choline as substrate and converts it to betaine aldehyde which further catalyzed by betaine aldehyde dehydrogenase (BADH) to be glycine betaine (Fitzgerald et al., 2009; Park et al., 2009; Turkan and Demiral, 2009). Accumulation of glycine betaine and the up-regulation of CMO as well as BADH genes are observed in various organisms under salinity stress including the halophytes (Ashraf et al., 2007; Khan et al., 2000). Other quaternary ammonium compounds such as alanine betaine, proline betaine are suggested to be a better osmoprotectant than glycine betaine as they required ubiquitous primary metabolites (Hanson et al., 1991).

Synthesis of antioxidant enzymes

Some plants develop anti-oxidant enzymes to scavenge Reactive Oxygen Species (ROS) in order to protect the importants biomolecules from being destroyed such as glutathione peroxidase, glutathione S-transferase, Cu/Zn-SOD, and a cytosolic APX. These enzymes are found to be up-regulated in the salt stress plants (Zhang et. al., 2005; Mallik et al., 2011).

Table 1. List of transformed genes in salt-tolerance transgenic plants

Over-expressed genes	Reference
Arginine decarboxylase, ADC	Review in Flower, TJ 2004
Betaine aldehyde dehydrogenase, BADH; betB, choline dehydrogenase (CDH); 15 choline oxidase, codA (glycinebetaine)	Review in Flower, TJ 2004
Ca2+-dependent protein kinase, CDPK,	Review in Flower, TJ 2004
Ca/H antiporter, CAX1,	Review in Flower, TJ 2004
Ca protein kinase, OsCDPK7	Review in Flower, TJ 2004
Glutathione S-transferase, GST and glutathione peroxidase, GP	Review in Flower, TJ 2004
Glyceraldehyde-3-phosphate dehydrogenase	Review in Flower, TJ 2004
Glutamine synthetase	Review in Flower, TJ 2004
Heat shock protein, DnaK/HSP70 1	Review in Flower, TJ 2004
High-affinity potassium transporter, HKT1	Apse et al., 1999; Møller et al., 2009
Late embryo abundant protein	Review in Flower, TJ 2004
Myo-inositol O-methyltransferase	Review in Flower, TJ 2004
Delta (1)-pyrroline-5-carboxylate synthetase	Review in Flower, TJ 2004
Proton sodium exchanger, HNX1	Fukuda et al., 2004, Chen et al., 2007
S-adenosylmethionine decarboxylase, SAMDC (spermine, spermidine)	Review in Flower, TJ 2004
Serine/threonine kinase	Review in Flower, TJ 2004
Sorbitol-6-phosphate dehydrogenase, SPD (sorbitol) 1	Review in Flower, TJ 2004
SOS1 overexpression	Shi et al., 2003
SR-like, putative splicing protein 1	Review in Flower, TJ 2004
Trehalose-6-phosphate synthase/phosphatase, TPSP (trehalose) 1	Review in Flower, TJ 2004
superoxide dismutase, Mn-SOD 1	Review in Flower, TJ 2004
Vacuolar H+-pyrophosphatase, AVP1	Gaxiola et al., 2001; Li et al., 2005

Photosynthesis of plants under salt stress

In general, the proteins involve in the photosynthesis are largely effected by salt stress (Chaves et al., 2009). The genes in photosynthesis and energy productions are slightly down-regulated at the transcription level (Chaves et al., 2009; Kilian et al., 2007). Genes in Calvin cycle and photorespiration are affected. Fructose-bisphosphatase, fructose-bisphosphate aldolase, phosphoribulokinase, transketolase, ribose-5-phosphate

isomerase, phosphoglycolate phosphatase and glycine hydroxymethyltransferase are down-regulated except some genes in the family are found to be up-regulated such as one gene in glycine hydroxymethyltransferase family, AT1G36370 (Chaves et al., 2009). As the photosynthesis rate is dropped, the production of ROS is increased. Therefore, the plants need to develop the detoxifying enzymes to prevent the oxidative damage.

Transcription regulation of genes under salt stress

Genes are altered their expression under saline condition. Several works have been carried out on the transcriptomic analysis in order to identify the salt-responsive genes. One group of the genes that are important for gene expression is transcription factor. Many of these transcription factor are stress-regulated such as AP2, bZIP, DREB, NAC, WRKY and zinc finger families (Ito et al., 2006; Nakashima et al., 2007; Song et al., 2011; Mizoi et al., 2012; Fujita et al., 2013). The AtbZIP17 is degraded when the plants were cultured under high salt concentration and the up-regulation of salt-responsive genes were observed (Lui et al., 2007).Some transcription factors are induced under salt stress in a tissue specific manner. The salt-responsive ERF1 in rice has been reported to be induced in the roots of salt-treated plants. The down-regulation of this transcription factor resulted in the reduction in expression of its down-stream genes such as MAPK cascade (Schmidt et al., 2013).

Some transcription factors are also play roles in the salt-adaptation. Arabidopsis overexpressed WRKY25 and WRKY33 were more tolerant to saline condition (Jiang and Deyholos, 2009). These proteins function in both oxidative as well as osmotic stress response (Miller et al., 2008).

MicroRNAs

MicroRNAs are evolutionary conserved small non-coding RNAs. The microRNAs are present in various organisms from green algae to land plants (Floyd et al., 2004: Arazi et al., 2005: Griffiths-Jones et al., 2008: Kozomara et al., 2011). Several classes of 21-24 nucleotide small non-coding RNAs are present in plants based on their biogenesis and functions including short interfering RNAs (siRNAs), microRNAs (miRNAs), trans-acting siRNAs (ta-siRNAs), natural antisense siRNAs (nat-siRNAs) and repeat-associated siRNAs (rasiRNAs) (Vaucheret et al., 2006). The siRNAs and miRNAs are the major classes of small RNAs. Both types of RNAs function to control gene expression at the transcriptional and post-transcriptional level (Vaucheret et al., 2006; Voinnet et al., 2009). MicroRNAs are a conserved class of small regulatory RNAs. In plant, endogenous MIR

genes are encoding for microRNAs that are 20-24 nucleotide in length. The MIRNA loci can be several kilobases in length. In plant, MIRNA loci are located in the intergenic region. These microRNA precursors are transcribed by RNA Pol II producing primary microRNAs (pri-miRNAs). The pri-miRNAs are further processed in a nucleus to stem-loop intermediates called pre-microRNAs by Dicer-like 1 (DCL1) together with Hyponastic leave1 (HYL1) and Serrate (SE) proteins (Kurihara et al., 2004; Kurihara et al., 2006; Lobbe et al., 2006). Before exporting to cytoplasm by a plant exportin 5 and HASTY (HST1), pre-microRNAs are methylated at the 3' terminus by Hua Enhancer1 (HEN1) (Park et al., 2002). The microRNAs bind to Agonaute (AGO) guiding RNA-induced silencing complex (RISC) to the target transcripts (Baumberger et al., 2005). The perfectly or near-pefectly binding of microRNAs to their target sequences bring about specific cleavage of the target mRNAs. MicroRNAs can also cause translational inhibition when the binding of microRNAs is imperfect to the cognate sequence. Moreover, microRNAs modulate gene expression by inducing an alteration of epigenetic modifications such as DNA methylation and histone modifications (Jones-Rhoades et al., 2006, Voinnet et al., 2009; Sun et al., 2011). The specific degradation of the target mRNA is the main mechanism in plants.

In an attempt to study plant small RNAs, more than a hundred microRNAs were firstly identified in Arabidopsis by three independent group in a year 2002 (Llave et al., 2002; Park et al., 2002; Reinhart et al., 2002). Afterward more microRNAs have been identified from various plant species through computational prediction, forward genetic, direct cloning and genome-wide analysis in *Arabidopsis thailiana* (Sunkar et al., 2004; Fahlgren et al., 2007; Zhou et al. 2011), *Brassica napus* (Xie et al., 2007), *Oryza sativa* (Sunkar et al., 2005; Sunkar et al., 2008; Sanan-Mishra et al., 2009; Zhou et al., 2011), cotton (Zhang et al., 2007; Pang et al., 2009), soybean (Subramanian et al., 2008), tomato (Yin et al., 2008; Zhang et al., 2008b), wheat (Wei et al., 2009), Populus (Lu et al., 2008; Li et al., 2009, Li et al., 2011, Zhou et al., 2011), *Nicotiana tabacum* (Frazier et al., 2010), switchgrass (Matts et al., 2010), apple (Yu et al., 2011), barley (Schreiber et al., 2011), maize (Wang et al., 2011), and Sorghum (Zhang et al., 2011; Zhou et al., 2011). According to the microRNA database Release 17 April 2011, 3,362 plant microRNA sequences were deposited (Griffiths-Jones et al., 2008; Kozomara et al., 2011). MicroRNAs are highly conserved among plant species indicating their functional significance. It has been reported that microRNAs play vital roles in plant growth and development (Lui et al., 2009; Nonogaki et al., 2010), flowering time (Chen et al., 2004; Schwab et al., 2005; Wu et al., 2006; Wang et al., 2008), hormonal response (Guo et al., 2005; Mallory et al., 2005; Li et al., 2010), nutrient limitation and adaptation to various environmental stimuli (Sunkar et al., 2004; Fujii et al., 2005; Arenas-Huertero et al., 2009; Li et al., 2010). Many gene targeted by microRNA are transcription factors which are the key components that modulate an adaptation of plant to environmental stresses (Jones-Rhoades et al., 2006; Lui et al., 2007, Reyes et al., 2007; Chen et al.,

2009; Golldack et al., 2011). By regulating the function of the transcription factors, microRNAs therefore regulate diverse downstream genes. For example, accumulation Arabidopsis AtMYB33 and AtMYB101 transcription factors are controlled by microRNA miR159 (Reyes et al., 2007).

MicroRNAs and stress response

It has been shown that microRNAs function in stress response by regulating different biological and metabolic pathways (Sun et al., 2011). A number of stress-regulated microRNAs have been cloned and identified in plants grown under various stress conditions including salinity (Sunkar et al., 2004; Liu et al., 2008; Sunkar et al., 2008; Ding et al., 2009), drought (Sunkar et al., 2004; Reyes et al., 2007; Zhao et al., 2007; Lui et al., 2008; Zhao et al., 2010), cold (Sunkar et al., 2004; Lui et al. ,2008; Zhou et al., 2008; Lv et al., 2010), and nutrient deficiency (Fujii et al., 2005). For instance, 17 out of 117 microRNAs were induced under salinity, drought and cold stresses (Lui et al., 2008). The miR159 is increased under dehydration indicating the link between microRNA and drought stress (Reyes et al., 2007). Moreover Arabidopsis mutants of genes involved in microRNA biogenesis and metabolism shown altered phenotypes under diverse stresses. The dicer-like, hen1 and hasty mutants exhibit hypersensitivity to salt and osmotic stresses (Zhang et al., 2008a).

MicroRNAs and salinity stress

Numbers of genes are differentially expressed under salt stress. The alterations of gene expression are specifically controlled by microRNAs. Zhou and colleagues were the first to identify microRNA during salinity and drought stress (Sunkar et al., 2004). A number of microRNA families responsive to salinity stress have been characterized afterward (Covarrubias et al., 2010). Recently microRNAs responsive to salt stress are intensively reviewed (Khraiwesh et al., 2011). Several works have been reported on changes of microRNA profiles under salinity stress (Jung et al., 2007; Liu et al., 2008; Lu et al., 2008; Jagadeeswaran et al., 2009; Jia et al., 2009; Jian et al., 2010; Shen et al., 2010; Qin et al., 2011). In response to salt stress, some microRNAs are up-regulated for example Arabidopsis miR156, miR158, miR159, miR165, miR167, miR168, miR169, miR171, miR319, miR393, miR394, miR396, and miR397 were up-regulated. In contrast the miR169a miR169c and miR398 were down-regulated (Li et al., 2008; Lui et al., 2008). Similar result was observed in *Nicotiana tabacum* treated with various concentration of NaCl. The miR159, miR167, and miR169 were down regulated while miR172 and miR396 were up-regulated in salt-treated tobacco (Frazier et al., 2011). The changes of microRNA

expression were dose-dependent. The change of microRNA expression results in an alteration of target genes expression. Reduction of miR169a and miR196c resulted in an over-accumulation of its targets, NFYA5 (Li et al., 2008). NFYA5 is a subunit of the nuclear factor Y transcription factor. Alteration of NFYA5 abundance indicates that expression of many downstream genes might be changed as well. However the diverse effect of microRNAs is observed. Under salinity and drought stress, the transcripts of NFYA are reduced as a result of over-expression of rice microRNA miR169g and miR169n/o (Zhao et al., 2009). Apart from NFYA5 protein other transcription factors belonging to growth-regulating factor (GRL), scarecrow-like (SCL), squamosa promoter binding protein (SBP), APETALA2 (AP2) and TCP are targeted by salt-inducible microRNA (Lui et al., 2008).

One interesting work has been carried out on studying microRNA profile in maize. MicroRNAs were isolated from salt-sensitive and salt-tolerance maize lines. From this work many microRNA families have been identified (Ding et al., 2009). Interestingly induction of zma-miR168 was observed in the salt-tolerant line. On the contrary zma-miR168 was repressed in the salt-sensitive line (Ding et al., 2009). The osa-MIR393 and osa-MIR396c was reduced when growing under salt containing medium (Gao et al., 2010; Gao et al., 2011). Over-expression of osa-MIR393 resulted in the reduction of salinity tolerance in rice (Gao et al., 2011). In an independent experiment, transgenic rice over-expressing osa-MIR396c was also more sensitive to salinity stress (Gao et al., 2010). Both microRNAs target abiotic stress related genes suggesting their roles in abiotic stress response (Gao et al., 2010; Gao et al., 2011). 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).

Changes of microRNA expression might be due to the effect to modulation in genes or proteins involved in microRNA biogenesis and metabolism. The AGO1 expression was decreased in salt-tolerance but increased in salt-sensitive lines (Ding et al., 2009). This indicates that the metabolism of microRNA might be different in dissimilar plant backgrounds. It is also showed that plants might utilize microRNAs in order to response to salinity stress ultimately leading to its resistance. Stress-responsive microRNAs have been identified by sequencing libraries of small RNAs isolated from sample subjected to stress conditions (Sunkar et al., 2004; Khraiwesh et al., 2011).

MicroRNAs in Chlamydomonas

The existence of RNA silencing system in *C. reinhardtii* was first reported by an experiment carried out by Cerutti and team. The *aadA* gene conferring spectinomycin resistance was transform into *C. reinhardtii*. After culturing transformed cells under nonselective condition, the transformed cells became sensitive to the drug. Analysis of *aadA* transcripts showed that the transcripts were degraded suggesting that the silencing was occurred post-transcriptionally. Components in the RNA silencing system were then suggested to be present in *C. reinhardtii* genome. Three Dicer-like proteins and two AGO proteins were found by bioinformatics analysis suggesting that *C. reinhardtii* could employ microRNA system to regulate gene expression (Schroda et al., 2006).

The presence of microRNAs in *C. reinhardtii* was first published only recently. In 2007 Zhoa and colleague were the first to report the existence of microRNAs in this single cell green alga. Using parallel pyrosequencing technology the authors identified 4,182 unique small RNA sequences which were mapped to nuclear genome (Zhao et al., 2007). These small RNA sequences mostly matched to the intergenic regions similar to those found in higher plants (Zhao et al., 2007; Zhou et al., 2011). Interestingly some small RNA cognate sequences were found to locate within intronic region. Further bioinformatics analysis predicted that 200 small RNAs could form hairpin structure, a signature for microRNA. The expression of some predicted microRNAs were examined and the corresponding microRNAs were cloned from vegetative cells. The cloned microRNAs can direct cleavage of target mRNA in vitro indicating that the microRNAs might be indeed functional in *C. reinhardtii* (Zhao et al., 2007). In the same year Baulcombe and coworkers constructed small RNAs library and identified 68 microRNA candidate loci (Molnar et al., 2007).

As mentioned earlier, microRNA functions in a large number of biological processes in plants including development, flowering time, hormonal response, and response to various environmental stresses. MicroRNA expressions are altered under different developmental and environmental conditions, even to a very specific developmental program such as nodulation (Yu et al., 2008). These changes of microRNA production modulate the target gene expression. In *C. reinhardtii*, microRNA expressions were found to be under developmental program similar to those found in higher plants. Differentially expressed microRNAs were observed in vegetative compared to gametes indicating that microRNAs are involved in gametogenesis (Zhao et al., 2007). Light-induced microRNAs were also discovered. No detectable expression of these microRNAs was observed in culture growing under dark condition (Molnar et al., 2007). Therefore it is certain that there must be microRNAs responsive to other cellular processes including microRNAs responsive to different environmental conditions.

Methods

Strain and culture conditions

C. reinhardtii strain CC503 (mt+) was used throughout this study. The culture was maintained on Tris-acetate-phosphate (TAP) medium (Harris et al., 1978). For the CC503 salt-adapted culture, the cells were maintained in Tris-acetate-phosphate (TAP) medium supplement with 300 mM NaCl. All samples were cultured under 16-h light and 8-h dark with cool-white fluorescent bulbs at 22-25°C.

Result

Generation of salt-acclimated *Chlamydomonas reinhardtii* cells

The project has been designed to investigate differentially expressed transcripts and microRNAs in *Chlamydomonas reinhardtii* during the responses to salinity stress under short-term as well as long-term saline treatments. Generally *C. reinhardtii* can grow under very low 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 have to be constructed. During the first year after receiving MRG grant, the salt-acclimated cells were generated by culturing *C. reinhardtii* and challenged the cells under various concentrations of NaCl beyond the previously maximum reported concentration of NaCl. Figure 1 illustrates an experimental set up to generate the salt-acclimated cells of *C. reinhardtii*. The cells were cultured in Tris-Acetate-Phosphate (TAP) medium (Harris et al. 1989) containing 0 mM NaCl. The log-phase cells were transferred into new culture medium containing 0 mM, 50 mM, 100 mM and 200 mM NaCl, respectively. As expected, when the *C. reinhardtii* cells were firstly exposed to salt, the growth of the algae was inhibited compared to the cells cultured in a medium without any supplement of NaCl. The cells were cultured for 14 days prior to next sub-culturing. Initially the cells in the medium containing 200 mM NaCl could not grow which was consistent to previously report by Herma et al. (2007). However, to our surprise, after 10

days of culturing, the culture medium began to turn green; indicating that populations of cells were successfully adapted to the high salt concentration (Figure 2).

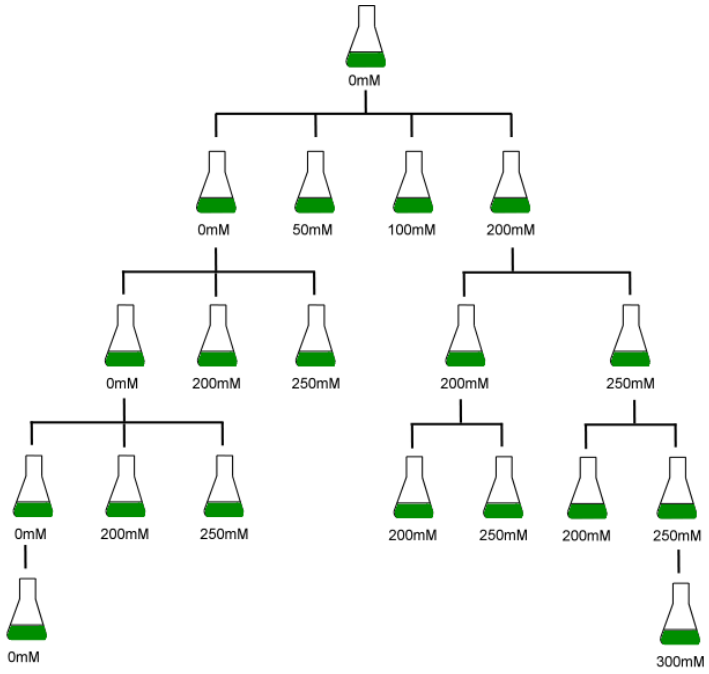

Figure 1. Experimental outline for generation of salt-acclimated cells. Initially *C. reinhardtii* cells were cultured in TAP medium containing 0 mM NaCl. The cells were transferred into new culture medium containing 0 mM, 50 mM, 100 mM, and 200 mM NaCl. After 14 days the cells were further transferred 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).

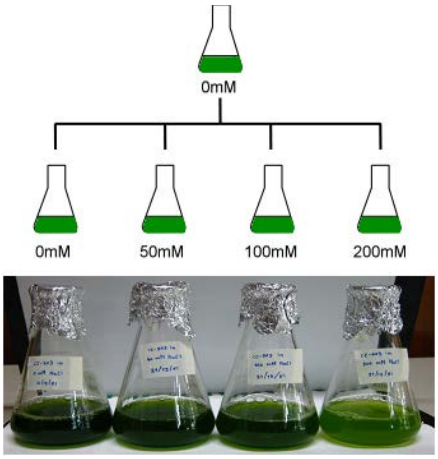

Figure 2. Growth inhibition of *Chlamydomonas reinhardtii* by the presence of NaCl in the culture medium. *C. reinhardtii* cell were grown in TAP liquid medium in the presence of 0 mM, 50 mM, 100 mM and 200 mM NaCl. Photo was taken 14 days after inoculation.

The cells cultured in 0 mM and 200 mM NaCl were chosen for further experiment. These cells were sub-cultured into new culture medium containing 0 mM, 200 mM and 250 mM NaCl as shown in figure 1. 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 3). The cells were sub-cultured under these conditions for 2 more generations. Subsequently the cells which were pre-exposed to 250 mM NaCl were then transferred to TAP medium containing 300 mM NaCl to further challenge the cells. These pre-exposed 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 4). Therefore these cells were considered as “salt-acclimated” cells and represented the long-term salinity stress responses.

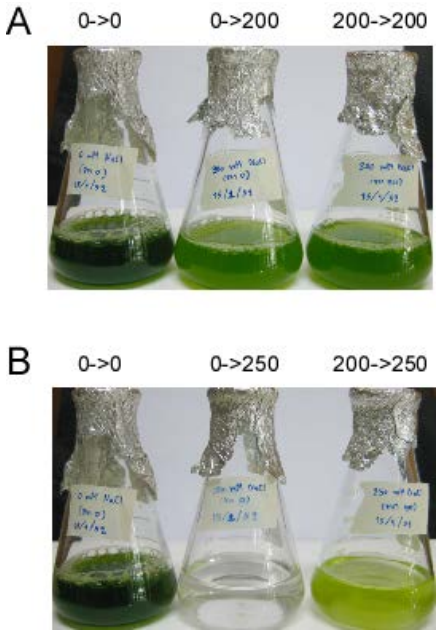

Figure 3. Adaptation of *Chlamydomonas reinhardtii* to salinity stress. A. *C. reinhardtii* cells were transferred from TAP medium containing 0 mM to 0 mM NaCl (0->0), from TAP medium containing 0 mM to 200 mM NaCl (0->200) and from TAP medium containing 200 mM to 200 mM NaCl (200->200). B. The cells 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).

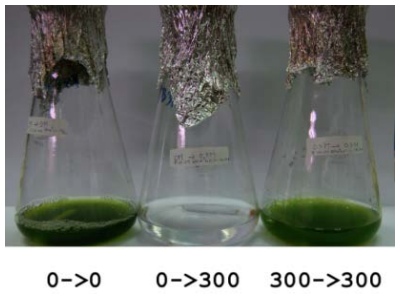

Figure 4. Salt-acclimated *Chlamydomonas reinhardtii* cells. *C. reinhardtii* cells were transferred from TAP medium containing 0 mM to 0 mM NaCl (0->0), from TAP medium containing 0 mM to 300 mM NaCl (0->300) and from TAP medium containing 300 mM to 300 mM NaCl (300->300).

The salt-acclimated cells appear to have no significant difference in term of its phenotype compared to that of wild type (Figure 5). However, they tend to show more sensitivity to higher light intensity than the wild type control.

Figure 5. Salt-acclimated cells. The salt-acclimated cells were grown in liquid TAP medium containing 200 mM NaCl compared to wild type (WT).

Purification and transcriptomic analysis

Total RNA was extracted from vegetative cells growing under normal growth (wild type) and salt-acclimated (SA) culture using TriReagent in accordance with the manufacturer's instructions. The mRNA was purified using Megnosphere™ UltraPure mRNA Purification Kit (Takara) following manufacturer's instructions. In collaboration with Dr. Sithichoke Tangphatsornruang, Head of Sequencing Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC), the purified mRNA was submitted to the Genome Institute and the transcriptomes of both cells grew under normal condition as well as salt-acclimated cells were analyzed using Ion Torrent (Life technologies) high-throughput sequencing.

The RNA sequence data was analyzed by mapping it either with Chlamydomonas genome (CDS and the corresponding UTR) and CDS sequences based on phytozome (http://www.phytozome.net/chlamy.php) as a Chlamydomonas sequence reference. Approximate 96% of the RNA-Seq data were mapped with the genome, which contains 54 scaffolds (110 Mbp). The data obtained from the run was 86,468,286 reads which the number of reads ranged from 16,762,915 to 26,198,408. The mean read length was ranging from 73-81 bp for each sample (Table 2). Of these 85% of total bases can be aligned to the reference sequences (Figure 6).

Table 2 Summary of high-throughput sequencing of salt-treated *C. reinhardtii* cells mapped with Chlamydomonas genome reference sequence. WT: cells grew under normal growth condition; SA: salt-acclimated cells. The sequencing was carried out on 2 biological replicates.

Condition	>=Q20 Bases	Number of read	Mean read Length (bp)
WT1	1,682,672,367	26,198,408	73
WT2	1,352,807,487	20,686,243	74
SA1	1,182,764,052	16,762,915	81
SA2	1,428,683,862	21,940,868	75

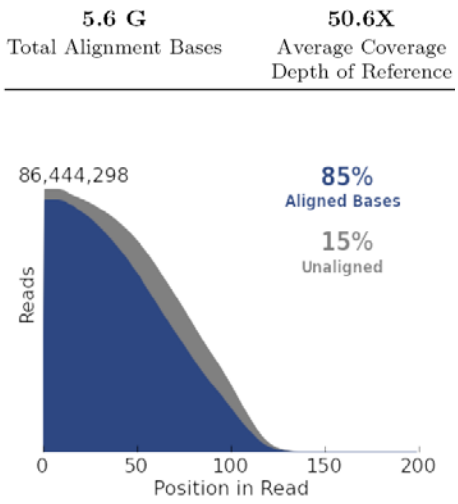

Figure 6. The RNA-Seq data mapped with Chlamydomonas genome sequence reference.

When analyzed the sequenced data by mapping the sequences with CDS of Chlamydomonas, the mapped sequences contain 19,526 contigs (42.5 Mbp). The data obtained from the run was 86,465,885 reads which the number of reads ranged from 16,764,917 to 26,200,387. The mean read length was also ranging from 73-81 bp for each sample (Table 3). Of these 49% of total bases can be aligned to the reference sequences (Figure 7).

Table 3 Summary of high-throughput sequencing of salt-treated *C. reinhardtii* cells mapped with Chlamydomonas CDS reference sequence. WT: cells grew under normal growth condition; SA: salt-acclimated cells. The sequencing was carried out on 2 biological replicates.

Condition	>=Q20 Bases	Number of read	Mean read Length (bp)
WT1	1,680,005,379	26,200,387	73
WT2	1,350,776,336	20,685,421	74
SA1	1,181,221,583	16,764,917	81
SA2	1,426,927,992	21,937,775	75

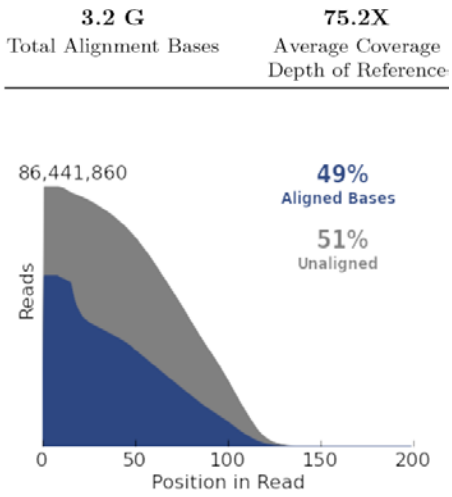

Figure 7. The RNA-Seq data mapped with Chlamydomonas CDS sequence reference.

Gene expression between Chlamydomonas grew under normal condition and under long-term salinity stress was compared by using Cuffdiff software. With the cut-off at more than 2-fold difference, we found that there were 1,261 up-regulated gene and 664 down-regulated genes in salt-acclimated cells compared to those in wild type cells. Out of these 1,925 genes, 9 of them were exclusively expressed in the WT while the other 8 genes were expressed only in the salt-adapted cells.

Figure 8 shows the genes that were more than 4-fold up-regulated in the SA compared to WT consisting of 303 genes. The genes with 4-fold changes in expression level were selected, annotated and classified into

various biological functional groups such as carbohydrate and energy metabolism, cell division, membrane and transport as well as stress and defense etc. While about 102 genes showed 4-fold down-regulated compared to the expression in WY background. These genes are annotated and classified as shown in figure 9. Interestingly most of the annotated genes were fall into signaling group. This might mean that some proteins involved in signaling were to be repressed while some proteins in signaling can be activated under saline stress. The gene list that show differentially expression level are shown in the appendix 1.

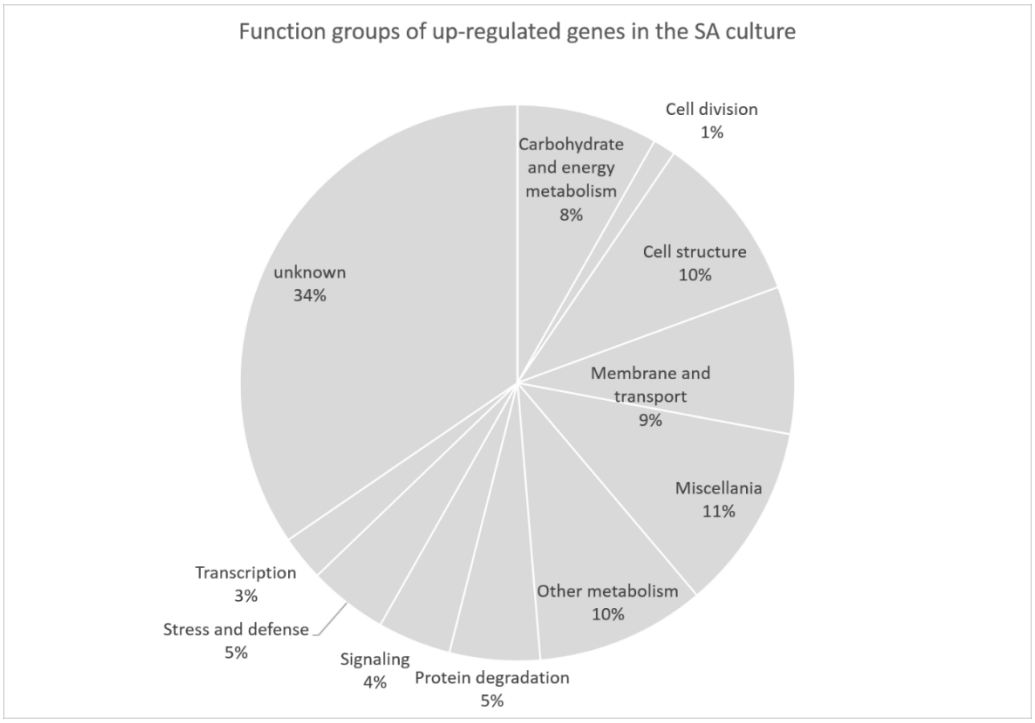

Figure 8. Functional group of genes that showed 4-fold upregulation in the salt-acclimated *Chlamydomonas reinhardtii*.

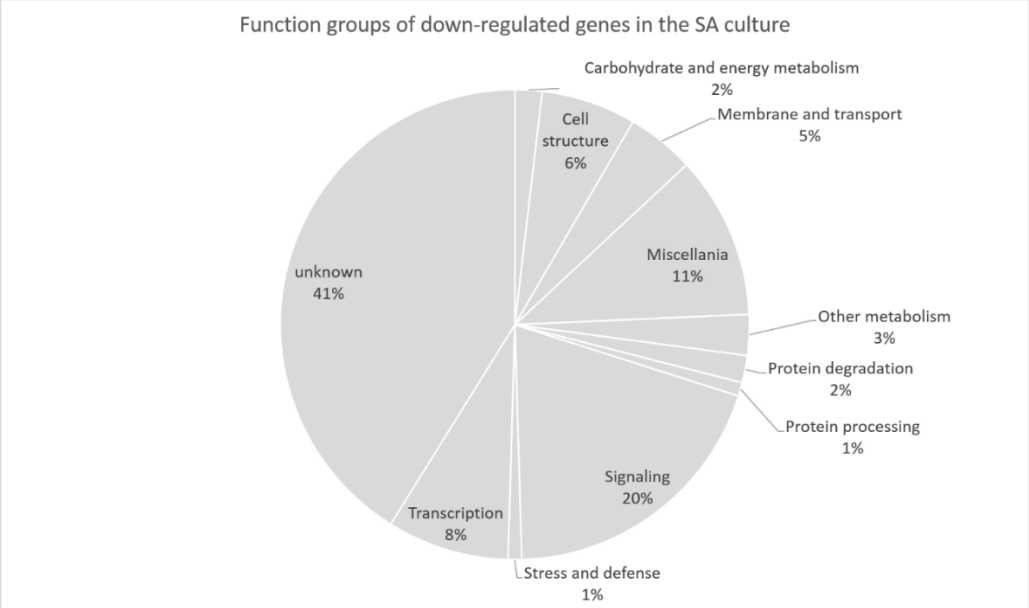

Figure 9. Functional group of 4-fold down-regulated genes in the salt-acclimated *Chlamydomonas reinhardtii*.

Conservation between Chlamydomonas genes with the higher plant genes

Even though the genome of Chlamydomonas has been fully sequenced but the genes have not yet been fully annotated. Therefore, the gene sequences were search against Arabidopsis genome and other gene ontology program such as Pfam, Panther, KOG, KEGG ec, KEGG Orthology in order to further annotate the genes. Here we report the genes that have homology to the Arabidopsis genome. As shown in table 4, the genes from Chalmydomonas matched to the same function of proteins in Arabidopsis.

Table 4. List of orthologue genes in Arabidopsis

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Glycerol-3-phosphate dehydrogenase/ dihydroxyacetone-3-phosphate reductase	NAD-dependent glycerol-3-phosphate dehydrogenase family protein	AT5G40610.1	7.7
Glycerol-3-phosphate dehydrogenase/ dihydroxyacetone-3-phosphate reductase 2	NAD-dependent glycerol-3-phosphate dehydrogenase family protein	AT5G40610.1	6.9
Fe-assimilating protein 2	-	-	6.1
Peroxisomal membrane protein MPV17	Peroxisomal membrane (Mpv17/PMP22)	AT4G03410.2	5.9

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
	family protein		
Sodium/phosphate symporter PTB6b/a	Phosphate transporter 2;1	AT3G26570.2	5.9
Glycosyltransferase family 92	Zinc finger (C3HC4-type RING finger) family protein	AT3G27330.1	5.9
Flagellar Associated Protein FAP211	-	-	5.5
Expansin	Expansin A1	AT1G69530.1	5.4
Carbonic anhydrase	Alpha carbonic anhydrase 4	AT4G20990.1	5.4
Unknown protein	-	-	5.2
Zygote-specific protein ZYS3 ZYS3-1	XBAT31/XB3 ortholog 1 in *A. thaliana*	AT2G28840.2	5.2
Gametolysin peptidase M11	-	-	4.9
Predicted protein -	-	-	4.6
Gametolysin peptidase M11	-	-	4.6
CHRD domain containing protein	-	-	4.5
C-type lectin CTL3	-	-	4.5
Hypothetical protein	-	-	4.5
predicted protein	-	-	4.5
Low-CO_2 response regulator, Myb-like transcription factor LCR1 SNT domain	Myb domain protein 115	AT5G40360.1	4.5
Glyoxal oxidase GOX1	Glyoxal oxidase-related protein	AT1G19900.1	4.4
Predicted membrane protein/ peptidase superfamily	Protein of unknown function (DUF2012)	AT2G25310.1	4.4
Mitochondrial carnitine-acylcarnitine carrier protein	Mitochondrial substrate carrier family protein 2	AT4G11440.1	4.4
Glutamate 5-kinase	Delta 1-pyrroline-5-carboxylate synthase P5CS2	AT3G55610.1	4.4
Proprotein convertase subtilsin/kexin - (peptidase)/ VLE gene	-	-	4.2
Fasciclin-like protein FAS2	-		4.1
Gametolysin peptidase M11	-	-	4.1
Expansin A- related	Expansin A24	AT5G39310.1	4.1
CGI-141-related/ lipase containing protein - Lipase (class 3)TGL21	alpha/beta-Hydrolases superfamily protein (DAD1)	AT2G44810.1	4.1
Glucan endo-1,3-beta-D-glucosidase / Laminarinase	-	-	4.1
Pherophorin	-	-	4.1
Lipoxygenase	Lipoxygenase 1 ATLOX1,LOX1	AT1G55020.1	4.0
Predicted protein with ankyrin repeats ANK20	Ankyrin repeat family protein	AT2G03430.1	3.9
Predicted protein	-	-	3.9
Hypothetical protein (similar to 23 kDa-	-	-	3.9

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Jasmonate induced protein-like)			
SARS coronavirus main proteinase / Severe acute respiratory syndrome coronavirus main protease	-	-	3.9
Pherophorin	-	-	3.8
Glyoxal oxidase GOX2	Glyoxal oxidase-related protein	AT3G57620.1	3.8
Pherophori	-	-	3.8
Xaa-Pro dipeptidase	Metallopeptidase M24	AT4G29490.1	3.8
Urate oxidase II UO UOX UOX1	uricase / urate oxidase / nodulin 35, putative	AT2G26230.1	3.8
GTP-BINDING PROTEIN 6-RELATED	-	-	3.7
Cell wall protein pherophorin-C21 PHC21	-	-	3.7
Dynamin family	-	-	3.7
hypothetical protein CHLREDRAFT_194760	-		3.7
Predicted protein CHLREDRAFT_189957	-		3.7
Predicted protein CHLREDRAFT_169044	-	-	3.6
predicted protein, partial CHLREDRAFT_95982	-	-	3.6
Metallophosphoesterase	Purple acid phosphatase 29	AT5G63140.1	3.5
Expansin A1 related	Expansin A15	AT2G03090.1	3.5
Hydroxyproline-rich cell wall protein	-	-	3.5
Equilibrative nucleotide transporter 1	Equilibrative nucleotide transporter 1	AT1G70330.1	3.4
Predicted protein CHLREDRAFT_175752 contain ARC105 or Med15 subunit of Mediator complex	-	-	3.4
Hypothetical protein CHLREDRAFT_188455	-	-	3.4
Glyoxal oxidase GOX17	Glyoxal oxidase-related protein	AT1G67290.1	3.4
Predicted protein CHLREDRAFT_184100	-	-	3.4
3-oxo-5-alpha-steroid 4-dehydrogenase	3-oxo-5-alpha-steroid 4-dehydrogenase family protein ATDET2,DET2,DWF6	AT2G38050.1	3.3
Hypothetical protein CHLREDRAFT_179453	-	-	3.3
Hypothetical protein CHLREDRAFT_167236	-	-	3.3
Non-specific serine/threonine protein kinase / Threonine-specific protein kinase	-	-	3.3
Gametolysin peptidase M11			3.3
Predicted protein CHLREDRAFT_182549	-	-	3.3
Lactonase	-	-	3.3
Gamma-glutamyl hydrolase GGH1	Gamma-glutamyl hydrolase 3	AT1G78670.1	3.2
Low-CO_2-inducible membrane protein	-	-	3.2
Predicted protein CHLREDRAFT_143757	-	-	3.2

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
"Chlamydomonas specific protein, induced by iron deficiency			
Hypothetical protein CHLREDRAFT_167236	-	-	3.1
Lactonase	-	-	3.1
Predicted protein CHLREDRAFT_171678	-	-	3.1
Calcium dependent MEKK	MAP kinase 10 ATMPK10,MPK10	AT3G59790.1	3.0
Cytochrome P450 gene CYP744C1, CYP3 superfamily	Cytochrome P450 superfamily protein CYP97C1,LUT1	AT3G53130.1	3.0
C-type lectin CTL2	-	-	3.0
Hypothetical protein CHLREDRAFT_167880	-	-	
Hypothetical protein CHLREDRAFT_115272	-	-	3.0
Hypothetical protein CHLREDRAFT_149776	-	-	3.0
Iron permease, membrane protein FTR1	-	-	3.0
Nucleotide-diphospho-sugar transferase	-	-	3.0
Predicted protein CHLREDRAFT_185442	-	-	3.0
Rieske iron-sulfur cluster 55 kDa protein of chloroplast inner membrane translocon	Pheophorbide a oxygenase family protein with Rieske [2Fe-2S] domain ACD1, LLS1, PAO	AT3G44880.1	3.0
RNA exonuclease EXN11	Small RNA degrading nuclease 2 SDN2	AT5G05540.2	3.0
Gamma-interferon inducible lysosomal thiol reductase (GILT)	-	-	2.9
Hypothetical protein CHLREDRAFT_187842	-	-	2.9
Hypothetical protein CHLREDRAFT_170956	-	-	2.9
Hypothetical protein CHLREDRAFT_194245 (similar to acyltranferase)	-	-	2.9
Predicted protein CHLREDRAFT_193913	-	-	2.9
Putative UDP-glucuronate 5'-epimerase NAD-dependent epimerase/dehydratase	UDP-D-glucuronate 4-epimerase 2 GAE2	AT1G02000.1	2.9
Pherophorin	-	-	2.9
Transmembrane protein 2-related	-	-	2.9
WSC domain containing protein	-	-	2.9
Ankyrin repeat	-	-	2.8
Cysteine-rich secretory protein-related, PR-related	Basic pathogenesis-related protein 1 ATPRB1, PRB1	AT2G14580.1	2.8
Endothelin-converting enzyme 1 / ECE-1	-	-	2.8
Glutathione S-transferase GST12	Glutathione S-transferase F3 ATGSTF3, GST16, GSTF3	AT2G02930.1	2.8
Heparan sulfate 3-O sulfotransferase	-	-	2.8
Hypothetical protein CHLREDRAFT_192823	-	-	2.8

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
contain CTP synthase (UTP-ammonia lyase) MLD1 MLDP1			
Hypothetical protein CHLREDRAFT_205652	-	-	2.8
Hypothetical protein CHLREDRAFT_186807	-	-	2.8
Hypothetical protein CHLREDRAFT_183905	-	-	2.8
Hypothetical protein CHLREDRAFT_175013	-	-	2.8
Hypothetical protein CHLREDRAFT_193137 similar to Low CO_2 indicible protein	-	-	2.8
Low-CO_2-induced aldose reductase LCI28	NAD(P)-linked oxidoreductase superfamily protein	AT2G37770.2	2.8
Low CO_2 inducible protein LCI6 CHLREDRAFT_184094	-	-	2.8
Pherophorin	-	-	2.8
Sugar transporter sweet1 RAG1-ACTIVATING PROTEIN 1	Nodulin MtN3 family protein	AT4G10850.1	2.8
Transmembrane protein 2-related, PHD-finger	Methyl-CPG-binding domain 9 ATMBD9, MBD9	AT3G01460.1	2.8
Ammonium transporter AMT4	Ammonium transporter 1;2 AMT1;2, ATAMT1;2	AT1G64780.1	2.7
DNA polymerase epsilon subunit 4	-	-	2.7
Glyoxal oxidase GOX3	Glyoxal oxidase-related protein	AT3G57620.1	2.7
Guanine deaminase	-	-	2.7
Hypothetical protein CHLREDRAFT_150931	-	-	2.7
Hypothetical protein CHLREDRAFT_189378	-	-	2.7
Long-chain-alcohol O-fatty-acyltransferase / Wax synthase domain	Acyl-CoA sterol acyl transferase 1 ASAT1, ATASAT1, ATSAT1	AT3G51970.1	2.7
NAD(P)-binding Rossmann-fold superfamily protein Oxidoreductase	NAD(P)-binding Rossmann-fold superfamily protein	AT3G20790.1	2.7
Predicted protein CHLREDRAFT_190468 protein kinase catalytic domain	-	-	2.7
Stress-related chlorophyll a/b binding protein 2 LHCSR2 LHCSR3.1	Chlorophyll A/B binding protein 1 AB140, CAB1, CAB140, LHCB1.3	AT1G29930.1	2.7
Stress-related chlorophyll a/b binding protein 3 LHCSR3 LHCSR3.2	Chlorophyll A/B binding protein 1 AB140, CAB1, CAB140, LHCB1.3	AT1G29930.1	2.7
Unknown protein	RING/U-box superfamily protein	AT3G26730.1	2.7
Alpha-amylase AMA3	Alpha-amylase-like 3 AMY3,ATAMY3	AT1G69830.1	2.6
GCC2 and GCC3	-	-	2.6
Glucosyl/ Glucuronosyltransferases	UDP-Glycosyltransferase superfamily protein	AT3G02100.1	2.6
Hydroxyproline-rich glycoprotein component of	-	-	2.6

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
the outer cell wall-			
Hypothetical protein CHLREDRAFT_189638	-	-	2.6
Hypothetical protein CHLREDRAFT_145397	-	-	2.6
Lectin C-type domain (Lectin_C) // Pherophorin	-	-	2.6
Pherophorin	-	-	2.6
Predicted protein CHLREDRAFT_160684			2.6
Sodium-dependent phosphate transporter PHT6	Phosphate transporter 4;5 PHT4;5	AT5G20380.1	2.6
Tyrosine kinase specific for activated (GTP-bound) p21cdc42Hs	Protein kinase superfamily protein with octicosapeptide/Phox/Bem1p domain	AT3G46920.1	2.6
Ubiquitin UBQ1	Polyubiquitin 10 UBQ10	AT4G05320.2	2.6
Vegetative cell wall protein gp1	-	-	2.6
12-oxophytodienoate reductase	12-oxophytodienoate reductase 2	AT1G76690.1	2.5
Arylsulfatase ARS18	-	-	2.5
Carbonic anhydrase, alpha type, periplasmic	Alpha carbonic anhydrase 4 ACA4, ATACA4	AT4G20990.1	2.5
Cathepsin B / Cathepsin B1	Cysteine proteinases superfamily protein	AT1G02300.1	2.5
Copper transport accessory protein CTR3	-	-	2.5
Cytochrome P450, CYP55 superfamily	Cytochrome P450, family 88, subfamily A, polypeptide 3	AT1G05160.1	2.5
Ferric-chelate reductase/ oxidoreductase FRE1	Ferric reduction oxidase 2 ATFRO2, FRD1, FRO2	AT1G01580.1	2.5
Flagellar Associated Protein FAP102	-	-	2.5
His(2)-Cys(2) zinc finger (zf-H2C2)	Methyl-CPG-binding domain 9 ATMBD9, MBD9	AT3G01460.1	2.5
Hypothetical protein CHLREDRAFT_176878	-	-	2.5
Hypothetical protein CHLREDRAFT_189257 RING FINGER PROTEIN 10	-	-	2.5
Lipid-phosphate phosphatase / Soluble epoxide hydrolase	soluble epoxide hydrolase ATSEH, SEH	AT2G26740.1	2.5
Multicopper ferroxidase FOX1	-	-	2.5
Pirin Transcription co-activator	RmlC-like cupins superfamily protein	AT2G43120.1	2.5
Predicted extracellular protein similar to pathogenesis-related protein 1 gene PRL10	Pathogenesis-related gene 1 ATPR1, PR 1, PR1	AT2G14610.1	2.5
Serine/threonine protein kinase STK23 STPK23	MAP kinase 9 MPK9	AT3G18040.1	2.5
9-O-Acetylneuraminic acid deacetylase-related	Protein containing Domain of unknown function (DUF303)	AT3G53010.1	2.4

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Amino acid transporter AOT4	Amino acid transporter family protein	AT2G41190.1	2.4
Beta-galactosidase	-	-	2.4
Calpain	Calcium-dependent protein kinase 34 CPK34	AT5G19360.1	2.4
Flagellar membrane protein, paralog of AGG2 detergent resistant membrane protein-2 PLAC8 domain	PLAC8 family protein	AT3G18470.1	2.4
His(2)-Cys(2) zinc finger (zf-H2C2)	Methyl-CPG-binding domain 9 ATMBD9, MBD9	AT3G01460.1	2.4
HVA22-like protein (Stress, ABA-induced protein)	HVA22 homologue E ATHVA22E, HVA22E	AT5G50720.1	2.4
Hypothetical protein CHLREDRAFT_191760	-	-	2.4
Hypothetical protein CHLREDRAFT_192689	-	-	2.4
Low-CO_2-inducible protein LCI12 CHLREDRAFT_160482	-	-	2.4
Non-specific serine/threonine protein kinase / Threonine-specific protein kinase	-	-	2.4
NUDIX domain containing protein	-	-	2.4
Pherophorin-C2 PHC2	-	-	2.4
Predicted protein CHLREDRAFT_194524	-	-	2.4
Protein similar to proteosome assembly chaperon 2	Clast3-related protein	AT3G18940.1	2.4
Sterol esterase / Triterpenol esterase	-	-	2.4
Stress-related chlorophyll a/b binding protein 1 LHCSR1	Chlorophyll A/B binding protein 1 AB140, CAB1, CAB140, LHCB1.3	AT1G29930.1	2.4
Arylsulfatase ARS5	-	-	2.3
Cysteine-rich secretory protein-related	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	AT4G33730.1	2.3
Cytochrome c oxidase assembly factor, SCO1/SenC	Electron transport SCO1/SenC family protein	AT3G08950.1	2.3
Gametolysin peptidase M11	-	-	2.3
Glyoxal oxidase GOX4	Glyoxal oxidase-related protein	AT1G19900.1	2.3
Glyoxal oxidase GOX5	Glyoxal oxidase-related protein	AT1G67290.1	2.3
Hypothetical protein CHLREDRAFT_141138	-	-	2.3
Hypothetical protein CHLREDRAFT_185746	-	-	2.3
Hypothetical protein CHLREDRAFT_149209	-	-	2.3
Hypothetical protein CHLREDRAFT_19457	-	-	2.3
Hypothetical protein CHLREDRAFT_187963	-	-	2.3
Hypothetical protein CHLREDRAFT_194668	Phloem protein 2-B8 AtPP2-B8, PP2-B8	AT2G02340.1	2.3

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Hypothetical protein CHLREDRAFT_159875 similar to Low-CO2 inducible protein	-	-	2.3
Hypothetical protein CHLREDRAFT_205597	-	-	2.3
Hypothetical protein CHLREDRAFT_195183	-	-	2.3
IAA-amino acid hydrolase ILR1-LIKE 3 PTHR	Peptidase M20/M25/M40 family protein	AT1G51760.1	2.3
Leucine carboxyl methyltransferase SOM4	-	-	2.3
Lysine carboxypeptidase / Lysine (arginine) carboxypeptidase	ฉarboxypeptidase D, putative	AT1G71696.2	2.3
Monooxygenase	-	-	2.3
MAPK	MHK protein kinase superfamily protein	AT4G13020.1	2.3
Pherophorin-C8		-	2.3
Predicted protein CHLREDRAFT_175081	-	-	2.3
Predicted protein CHLREDRAFT_172805	-	-	2.3
Programmed cell death protein 2 PCDC2	Ubiquitin-specific protease 16 UBP16	AT4G24560.1	2.3
Proton gradient regulation 5	Proton gradient regulation 5 PGR5	AT2G05620.1	2.3
Serine/threonine phosphatases, family 2C	Protein phosphatase 2C family protein	AT5G53140.1	2.3
Sodium/potassium-exchanging ATPase / Sodium/potassium-transporting ATPase	Endoplasmic reticulum-type calcium-transporting ATPase 3	AT1G10130.1	2.3
Transmembrane protein 2-related	-	-	2.3
Unknown protein	Methyltransferases	AT5G01710.1	2.3
Zeaxanthin epoxidase	zeaxanthin epoxidase (ZEP) (ABA1)	AT5G67030.2	2.3
Cytochrome P450 CYP197 superfamily CYP20 CYP743B1	Cytochrome P450, family 97, subfamily A	AT1G31800.1	2.2
Dehydrogenases	NAD(P)-binding Rossmann-fold superfamily protein	AT4G23430.2	2.2
Exostosin-like glycosyltransferase ELG8	Exostosin family protein	AT3G57630.1	2.2
Histidine phosphatase	-	-	2.2
Ionotropic glutamate receptor	Glutamate receptor 3.4	AT1G05200.1	2.2
FAD NAD oxidoreductase	-	-	2.2
Fasciclin-like protein FAS3	-	-	2.2
dTDP-glucose 4,6-dehydratase SNE5 NAD-dependent epimerase/dehydratase	Rhamnose biosynthesis 1 ATRHM1, RHM1, ROL1	AT1G78570.1	2.2
Glycosyltransferase family 92	Zinc finger (C3HC4-type RING finger) family protein	AT3G27330.	2.2
Hypothetical protein CHLREDRAFT_169394	-	-	2.2
Hypothetical protein CHLREDRAFT_148896	-	-	2.2
MAPK	Protein kinase superfamily	AT1G71530.1	2.2
Nitrogen-starved gametogenesis 13	-	-	2.2
P-type ATPase/cation transporter	H(+)-ATPase 1	AT2G18960.1	2.2

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Pherophorin	-	-	2.2
Predicted protein with mechanosensitive ion channel domain MSC4 MSCL1	Mechanosensitive channel of small conductance-like 10 ATMSL10, MSL10	AT5G12080.1	2.2
Pyrazinamidase/ nicotinamidase	-	-	2.2
RAG1	AtVEX1 Nodulin MtN3 family protein	AT5G62850.1	2.2
Sulfate anion transporter SUL2 SULTR2	Sulfate transporter 4;2 SULTR4;2	AT3G12520.1	2.2
Uncharacterized membrane protein	Saccharopine dehydrogenase	AT5G39410.1	2.2
Allantoate deiminase	Allantoate amidohydrolase	AT4G20070.1	2.1
Ankrin repeat and protein kinase domain containing protein	XBAT31 XB3 ortholog 1 in *Arabidopsis thaliana*	AT2G28840.1	2.1
Dicarboxylate/amino acid cation sodium transporter DAT1	-	-	2.1
EGF-like domain containging protein	-	-	2.1
Endothelin-converting enzyme 1	-	-	2.1
Exostosin –like glycosyltransferase	Exostosin family protein	AT3G57630.1	2.1
Flagellar Associated Protein FAP233	-	-	2.1
Gametolysin peptidase M11	-	-	2.1
Glycosyltransferase 14 family	-	-	2.1
Glyoxalase	-	-	2.1
Granule-bound starch synthase I GBBSI GBS1 STA2	UDP-Glycosyltransferase superfamily protein	AT1G32900.1	2.1
Heparan sulfate 3-O sulfotransferase	-	-	2.1
Histone-lysine N-methyltransferase	-	-	2.1
Hydroxyproline-rich glycoprotein, cell wall protein VSP4	-	-	2.1
Hypothetical protein CHLREDRAFT_177730	-	-	2.1
Hypothetical protein CHLREDRAFT_152521	-	-	2.1
Hypothetical protein CHLREDRAFT_175108	-	-	2.1
Hypothetical protein CHLREDRAFT_150030	-	-	2.1
Matrix metalloproteinase	-	-	2.1
PDDEXK-like family of unknown function (PDDEXK_6)	Protein of unknown function (DUF506)	AT1G77145.1	2.1
Phosphate metabolism protein RSN1	ERD (early-responsive to dehydration stress) family protein	AT4G02900.1	2.1
Predicted protein CHLREDRAFT_146582,	Protein with domain of unknown function (DUF220)	AT1G70480.1	2.1
Scavenger receptor cysteine rich (SRCR) protein	-	-	2.1

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
SGNH hydrolase	-	-	2.1
Starch phosphorylase PHO2 PHOA	Alpha-glucan phosphorylase 2 ATPHS2, PHS2	AT3G46970.1	2.1
Thioesterase superfamily TEH3	-	-	2.1
Transient receptor potential ion channel protein	-	-	2.1
Zygote-specific protein EZY9	chloroplast signal recognition particle component (CAO) CAO, CPSRP43	AT2G47450.1	2.1
Arylsulfatase ARS11	-	-	2.0
Ascorbate peroxidase APX1	SAPX stromal ascorbate peroxidase	AT4G08390.1	2.0
Beta-galactosidase	-	-	2.0
BLZ20	-	-	2.0
Defense protein L(2)34F	Auxin-responsive family protein	AT3G25290.1	2.0
ERD4-related membrane protein ERM1	ERD (early-responsive to dehydration stress) family protein	AT4G02900.1	2.0
Ferredoxin FDX2 Apoferredoxin	A 2Fe-2S ferredoxin-like superfamily protein ATFD2, FED	AT1G60950.1	2.0
Similar to Flagellar Associated Protein FAP16 (FAL9)	-	-	2.0
Gamma-interferon inducible lysosomal thiol reducatse (GILT)	-	-	2.0
Gametolysin peptidase M11	-	-	2.0
Hydroxyproline-rich cell wall protein ISG-C4 ISG4 FAP137	-	-	2.0
Hypothetical protein CHLREDRAFT_153310	-	-	2.0
Hypothetical protein CHLREDRAFT_171687 contain replication factor A domain	-	-	2.0
Hypothetical protein CHLREDRAFT_171614	-	-	2.0
Hypothetical protein CHLREDRAFT_167623	-	-	2.0
Hypothetical protein CHLREDRAFT_192903	-	-	2.0
Hypothetical protein CHLREDRAFT_171678	-	-	2.0
Hypothetical protein CHLREDRAFT_156911	Conserved expressed chloroplast protein	AT2G36145.1	2.0
Hypothetical protein CHLREDRAFT_192467	-	-	2.0
Hypothetical protein CHLREDRAFT_171176	-	-	2.0
Hypothetical protein CHLREDRAFT_190404	-	-	2.0
Interferon-inducible GTPase 5	-	-	2.0
L-threonine 3-dehydrogenase	Elicitor-activated gene 3-2 ATCAD8, CAD-B2, ELI3, ELI3-2	AT4G37990.1	2.0
Leucine-rich repeat containing protein	-	-	2.0

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Metalloproteinase of VMP family	-	-	2.0
Pheophorbide a oxygenase, Rieske iron-sulfur cluster protein TIC55-2 PAO2	ACD1-LIKE PTC52, TIC55-IV	AT4G25650.2	2.0
Pherophorin	-	-	2.0
Phosphate-repressible alkaline phosphatase PHO5 PHOX	-	-	2.0
Protein contain domain of DNA polymerase III subunits gamma and tau	-	-	2.0
Xyloglucan fucosyltransferase	-	-	2.0
Hypothetical protein CHLREDRAFT_193324	-	-	-2.0
Hypothetical protein CHLREDRAFT_18339	-	-	-2.0
Leucine-rich repeat containing protein	-	-	
Non-specific serine/threonine protein kinase / Threonine-specific protein kinase	-	-	2.0
Predicted protein CHLREDRAFT_194317	-	-	-2.0
Predicted protein CHLREDRAFT_186300	-	-	-2.0
Predicted protein CHLREDRAFT_173298	-	-	-2.0
Ribonuclease III	-	-	-2.0
Squamosa promoter binding protein-like 10-related	Squamosa promoter binding protein-like 10 SPL10	AT1G27370.1	-2.0
Tam3-transposase (Ac family) Transcription	-	-	-2.0
3',5'-cyclic-nucleotide phosphodiesterase PDE25	-	-	-2.1
Ankrin repeat-containing protein	Ankrin repeat-containing protein	AT5G14230.1	-2.1
Cation antiporter NAH1	-	-	-2.1
Exostosin-like glycosyltransferase ELG3	Exostosin family protein	AT3G57630.1	-2.1
Flagellar Associated Protein with ankyrin repeats FAP33 ANK17	Ankyrin repeat family protein	AT2G03430.1	-2.1
Gametolysin / Lysin MMP19	-	-	-2.1
Hypothetical protein CHLREDRAFT_181385	-	-	-2.1
Hypothetical protein CHLREDRAFT_188532	-	-	-2.1
Hypothetical protein CHLREDRAFT_145844	-	-	-2.1
Lactosylceramide 4-alpha-galactosyltransferase	alpha 1,4-glycosyltransferase family protein	AT2G38152.1	-2.1
Leucine-rich repeat containing protein	Leucine-rich receptor-like protein kinase family protein	AT4G36180.1	-2.1
Non-specific protein-tyrosine kinase	Protein kinase superfamily protein AtCTR1, CTR1, SIS1	AT5G03730.1	-2.1
Predicted protein CHLREDRAFT_149697	-	-	-2.1

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Predicted protein CHLREDRAFT_19519	-	-	-2.1
WD40 repeat protein	Transducin family protein TOZ / WD-40 repeat family protein	AT5G16750.1	-2.1
Hypothetical protein CHLREDRAFT_173908	-	-	-2.2
Hypothetical protein CHLREDRAFT_195218	-	-	-2.2
Las17-binding protein actin regulator (Ysc84)		-	-2.2
Nitrogen network kinase	AGC kinase 1.5	AT3G12690.1	-2.2
Phosphate transporter	Phosphate transporter 2;1 ORF02, PHT2;1	AT3G26570.1	-2.2
Predicted protein CHLREDRAFT_167259	-	-	-2.2
Protein E03H4.4-related	Protein of unknown function (DUF288)	AT3G57420.1	-2.2
Protein kinase superfamily protein with octicosapeptide/Phox/Bem1p domain centriole proteome protein	-	-	-2.2
Pyroglutamyl-peptidase I / Pyrrolidone-carboxylate peptidase	Pyroglutamyl peptidase I-like peptidase C15	AT1G56700.1	-2.2
Ribonuclease III	-	-	-2.2
Serine threonine protein kinase	Protein kinase	AT3G24715.1	-2.2
Tumor Necrosis factor receptor super family member	-	-	-2.2
Calcium/calmodulin dependent protein kinase II association domain (CaMKII_AD)	-	-	-2.3
Cytochrome C1 heme lyase	-	-	-2.3
Hypothetical protein CHLREDRAFT_181385	-	-	-2.3
Non-specific protein-tyrosine kinase / Cytoplasmic protein tyrosine kinase	Protein kinase superfamily protein with octicosapeptide/ Phox/ Bem1p domain	AT3G46920.1	-2.3
Predicted protein CHLREDRAFT_18659			-2.3
Adenylate/guanylate cyclas CYG12	-	-	-2.4
Chitin binding domain containing protein	-	-	-2.4
Guanylate cyclase	-	-	-2.4
Hypothetical protein CHLREDRAFT_152091	-	-	-2.4
Hypothetical protein CHLREDRAFT_158973		-	-2.4
Predicted protein CHLREDRAFT_186626	-	-	-2.4
Predicted protein CHLREDRAFT_166201	-	-	-2.4
Predicted protein CHLREDRAFT_190755	-	-	-2.4
Putative transposase DNA-binding domain	-	-	-2.4
Aryl sulfotransferase / Sulfokinase	P-loop containing nucleoside triphosphate hydrolases superfamily protein	AT2G03770.1	-2.5
Ion transport protein KCN7	Gated outwardly-rectifying K+ channel GORK	AT5G37500.1	-2.5

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Serine-threonine protein kinase	ACT-like protein tyrosine kinase family protein	AT4G35780.1	-2.5
Serine-threonine protein kinase	PAS domain-containing protein tyrosine kinase family protein	AT5G49470.3	-2.5
Serine-threonine protein kinase	Protein kinase superfamily protein	AT5G50000.1	-2.5
12-oxophytodienoate reductase 1-related NADH oxidoreductase related	12-oxophytodienoate reductase 1 ATOPR1, OPR1	AT1G76680.1	-2.6
Cellulase / Endoglucanase	Glycosyl hydrolase 9C3 AtGH9C3, GH9C3	AT4G11050.1	-2.6
Flagellar Associated Protein similar to calmodulin FAP272	Calmodulin 4 ACAM-4, CAM4	AT1G66410.1	-2.6
Hydroxyproline-rich cell wall protein ISG-C3 ISG6	-	-	-2.6
Hypothetical protein CHLREDRAFT_195055	-	-	-2.6
Polyketide cyclase / dehydrase and lipid transport	-	-	-2.6
Protein kinase domain (Pkinase) // Protein tyrosine kinase (Pkinase_Tyr)	Protein kinase superfamily protein ATMRK1	AT3G63260.1	-2.6
Cathepsin H / N-benzoylarginine-beta-naphthylamide hydrolase cysteine endopeptidase	Cysteine proteinases superfamily protein	AT3G45310.1	-2.7
Hypothetical protein CHLREDRAFT_146760	-	-	-2.7
Hypothetical protein CHLREDRAFT_187136	-	-	-2.7
Hypothetical protein CHLREDRAFT_205951	-	-	-2.7
Hypothetical protein CHLREDRAFT_174438	-	-	-2.7
Tiny microcysts protein B-related	-	-	-2.7
Adenylate and Guanylate cyclase catalytic domain	Histidine kinase 1 AHK1, ATHK1, HK1	AT2G17820.1	-2.8
Lactosylceramide 4-alpha-galactosyltransferase / Histo-blood group P(k) UDP-galactose	alpha 1,4-glycosyltransferase family protein	AT2G38152.1	-2.8
Hypothetical protein CHLREDRAFT_188752	-	-	-2.8
3',5'-cyclic-nucleotide phosphodiesterase PDE23	-	-	-2.9
Protein of unknown function (DUF1524)	-	-	-2.9
Tyrosine kinase specific for activated (GTP-bound) p21cdc42Hs MEKK	-	-	-2.9
5'-nucleotidase and Flagellar Associated Protein FAP215 FPN1	-	-	-3.0
Peptide-methionine (S)-S-oxide reductase /	-	-	-3.0

Chlamydomonas gene name	Arabidopsis ortholog	Arabidopsis TAIR number	Expression level (log2 FC)
Peptide methionine sulfoxide reductase			
RWP-RK transcription factor RWP6	RWP-RK domain-containing protein	AT4G35590.1	-3.0
Serine-threonine protein kinase	PAS domain-containing protein tyrosine kinase family protein	AT1G67890.1	-3.0
Sodium/phosphate symporter PTB5	Phosphate transporter 2;1 ORF02, PHT2;1	AT3G26570.1	-3.1
Cytochrome C1 heme lyase	-	-	-3.2
Hypothetical protein CHLREDRAFT_175676	-	-	-3.2
Predicted PWR protein PWR4	Protein of unknown function (DUF506)	AT3G54550.1	-3.2
Adenylate cyclase / ATP pyrophosphate-lyase // Guanylate cyclase / Guanylyl cyclase CYG65	-	-	-3.3
Transposase family tnp2	-	-	-3.3
Hypothetical protein CHLREDRAFT_188531	-	-	-3.4
FAL11 Similar to Flagellar Associated Protein FAP154	-	-	-3.5
Hypothetical protein CHLREDRAFT_143129	-	-	-3.5
Reverse transcriptase	-	-	-3.5
Hydroxyproline-rich glycoprotein HRP3	-	-	-3.9
Protein of unknown function	-	-	-4.0
RWP-RK transcription factor/minus dominance protein MID RWP5Transcription	RWP-RK domain-containing protein	AT4G35590.1	-4.1
Cell wall protein pherophorin-C21 PHC21	-	-	-4.2
Sodium/phosphate symporter PTB4	Phosphate transporter 2;1 ORF02, PHT2;1	AT3G26570.1	-4.4
Calcium-binding EGF domain	WAK3 wall associated kinase 3	AT1G21240.1	-5.7

Genetic variation of the salt-adapted Chlamydomonas

The polymorphism between salt-adapted and wild type were analyzed. The sequences of differentially expressed genes were analyzed by matching the sequences with 2 types of sequences on the databases. The first one was the sequences about 1,500 bp upstream of the coding region. These sequences were assigned as “genomic sequence”. The second SNP identification was carried out by matching the differentially expressed genes with the CDS sequences from the database. From the search with the genomic sequences, 23 SNPs were observed between wild type and the salt-adapted cells. The wild type sequences were identical to the sequence from the database only the sequences obtained from the salt-adapted cells displayed the changes. Table 5 shows the SNPs found on the genomic sequences. While the comparison of sequences between salt-

adapted cells to the WT and the CDS sequences on the database. Altogether 2,098 SNPs which caused the amino acid changes were observed (data not shown). Similar to the comparison to genomic sequences, only the sequences obtained from the salt-adapted cells displayed SNPs when compared to the database sequences. The 2,098 SNPs represented in 1,636 genes.

Table 5. The single nucleotide polymorphisms observed in the salt-adapted cells when blast against the UTR.

Gene function	Chromosome number	Log 2 fold chnage
Cell wall protein pherophorin-C21 PHC21	4	-4.17
Hydroxyproline-rich glycoprotein HRP3	10	-3.88
Cation antiporter NAH1	6	-2.13
Zinc finger (C3HC4-type RING finger) family protein	12	1.10
Glutamate receptor 3.4	13	1.10
SET domain group 37	9	1.10
RNA-binding (RRM/RBD/RNP motifs) family protein	12	1.10
Photosystem II light harvesting complex gene 2.2	12	1.11
Arabidopsis thaliana protein of unknown function (DUF821) KDEL LYS-ASP-GLU-LEU CONTAINING - RELATED	12	1.14
Starch branching enzyme 2.2	10	1.18
Gamma-tubulin complex protein 2	3	1.18
Xyloglucanase 113	7	1.27
Cryptococcal mannosyltransferase 1	2	1.28
Unknown protein	8	1.53
Nucleotide-sugar transporter family protein	11	1.55
Cinnamyl-alcohol dehydrogenase / CAD ADH8	3	1.64
Serinc-domain containing serine and sphingolipid biosynthesis protein	7	1.8
Glyoxal oxidase-related protein GOX17	16	1.9
Glyoxal oxidase-related protein GOX17	16	1.9
L-threonine 3-dehydrogenase	3	2.0
Glyoxal oxidase-related protein GOX4	17	2.3
Predicted protein CHLREDRAFT_184100	12	3.4
GTP binding protein 6-related contain ADP-ribosylglycohydrolase contain	7	3.7

Alteration of sequences in glyoxal-oxidase genes

From the transcriptome analysis, one group of proteins, glyoxal oxidases, were observed to be up-regulated. Six genes encoding for the glyoxal oxidase proteins were up-regulated from 5 fold to 21 fold. These

genes displayed the SNPs in both UTR and CDS. Glyoxal oxidase is enzyme catalyzing the formation of H2O2 using glyoxal as substrate which might serve as stress siganlling molecule for the salt-adapted cells.

The expression of some glyoxal oxidase genes were analyzed by semi-quantitative RT-PCR. The expression of *GOX17* gene were observed only in the salt-adapted cells but not in the wild type sample. Figure 10 shows gradient RT-PCR for the *GOX17* transcript detection in the salt-adapted cells. This is consistent to the result obtained from the transcriptome analysis which we found very low amount of expression in the wild type and up-regulation about 4 fold in the salt-adapted cells.

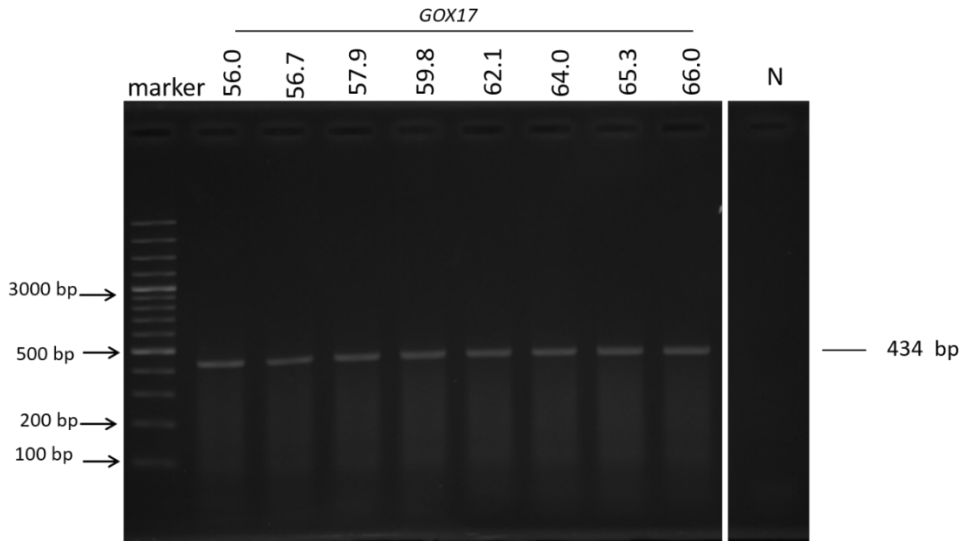

Figure 10. Expression of GOX17 genes in the salt-adapted cells detected by semi-quantitative RT-PCR.

Cloning and profiling of MicroRNA

According to the proposal, we were to prepare mRNA as well as microRNA sample for the whole-genome analysis within the first year after receiving MRG grant. The microRNA was prepared from the *C. reinhardtii* wild type as well as salt-acclimated cultures. Total RNA was extracted using TRI reagent (MRC, USA) in accordance with the manufacturer's instructions. The quality of total RNA was estimated by running the RNA in a Urea PAGE gel containing 15% polyacrylamide with 7 M urea (Figure 11).

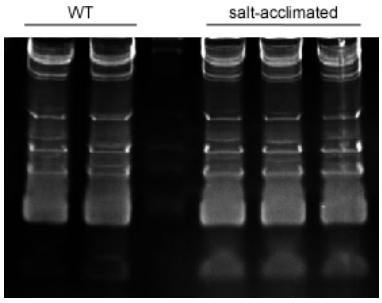

Figure 11. Total RNA profile from wild type (WT) and salt-acclimated cells.

The cloning of microRNA will be carried out using miRCat, microRNA cloning kit (Integrated DNA Technologies, Inc.) following the manufacturer's instructions (Figure 12). In brief, 100 μg of total RNA from wild type as well as salt-adapted cells were used for fractionation and purification of microRNA. Total RNA was run in a Urea PAGE gel containing 15% polyacrylamide with 7 M urea and the RNA with the size of 18-26 nucleotide were excised from the gel and purified. The purified small RNAs were then ligated to 3'-linker (Figure 7). Prior the next steps, the ligated small RNA have to be purified from the non-ligated RNA. The 3'-linked small RNAs were size fractionated by running in 15% denaturing polyacrylamide gel. The 3'-linked small RNAs were about 40 nucleotides long while the 3'-linker was 19 nucleotide in length. The 3'-linked small RNAs were cut from the gel and purified. Afterward, the small RNA was ligated to 5'-linker (Figure 7) and the 5'-linked small RNA was further purified by PAGE gel purification.

The small RNA with both 3' and 5' linker was further used as template to convert into cDNA by reverse transcription and amplification by PCR (Figure 13). The quality of PCR amplification was determined by gel electrophoresis (Figure 13). Currently the cloned microRNA is under the process of sequencing in collaboration with Dr. Sithichoke Tangphatsornruang, Head of Sequencing Laboratory, Genome Institute, National Center for Genetic Engineering and Biotechnology (BIOTEC).

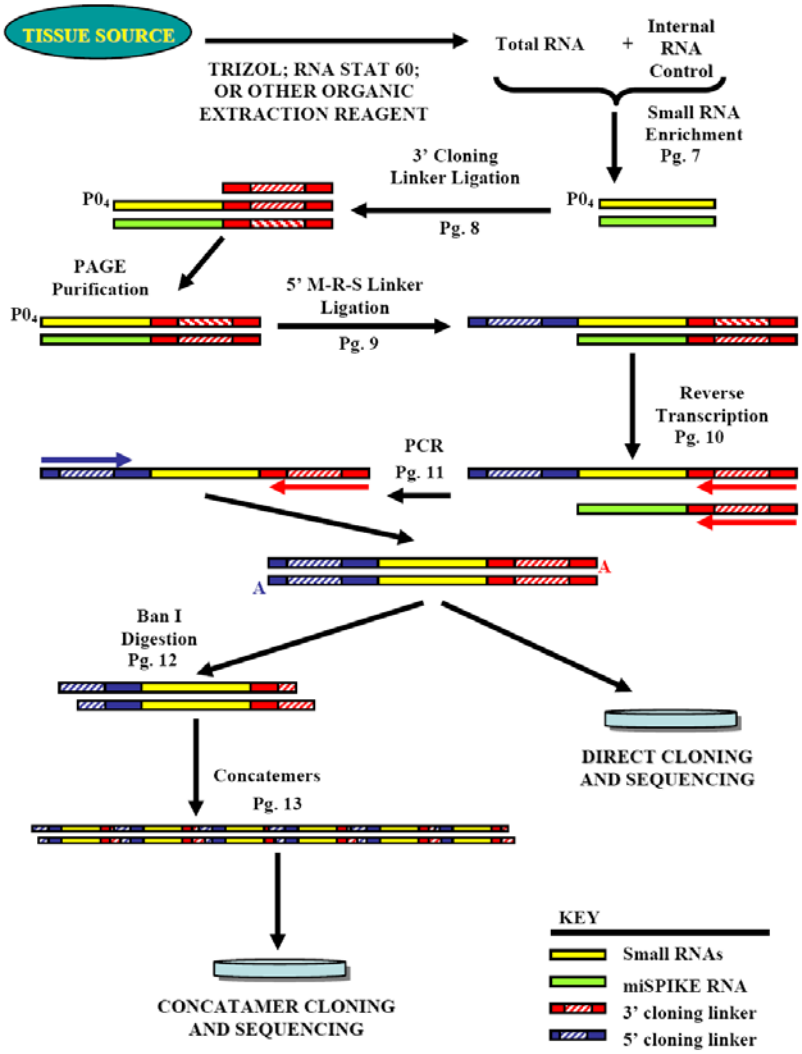

Figure 12. MicroRNA cloning procedure using the miRCat kit microRNA cloning kit (Integrated DNA Technologies, Inc.).

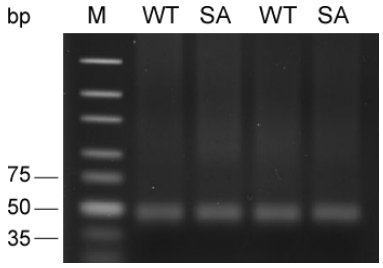

Figure 13. PCR product of cloned microRNA. M = GeneRuler™ Ultra Low Range DNA Ladder (ThermoScientific), WT = PCR product of cloned microRNA from wild type cells, SA = PCR product of cloned microRNA from salt-adapted cells.

Mapping of the microRNA to the genome of Chlamydomonas

The microRNA sequences were analyzed. We found 43 potential microRNA location which mapped on the Chlamydomonas genome (Chlre v3.0) (Table 6). However, no positive correlation between the expression level of microRNA and the transcript level of the target genes were observed. In addition, the microRNA sequences obtained from the analysis were BLAST against the microRNA database (MIRBASE). We found 50 microRNA hits to our sequences (Table 7). Further verification of the presence of microRNA and the expression of the target genes will be further analyzed.

Table 6. Genomic location of the microRNA found in the Chlamydomonas cells.

locus	Expression level WT: SA (log 2 fold change)
scaffold_10:1077584-1077788	-3.20486
scaffold_45:95143-95376	-2.54499
scaffold_1:496160-496308	1.1352
scaffold_36:313052-313072	1.19368
scaffold_6:555575-555628	1.29518
scaffold_9:244106-244356	1.31203
scaffold_22:1026657-1026683	1.5446
scaffold_2:1049124-1049365	1.55195
scaffold_7:2772397-2772682	1.57086
scaffold_20:231834-231931	1.59006
scaffold_78:198478-198578	1.63407
scaffold_4:2979413-2979492	1.69942
scaffold_5:1288236-1288444	1.77161
scaffold_54:536790-536859	1.87389
scaffold_83:240641-240840	1.90518
scaffold_93:192313-192517	1.94086
scaffold_1661:1505-1744	2.20414
scaffold_76:316388-316459	2.23975
scaffold_1:2712757-2712910	2.43722
scaffold_6:2466408-2466540	2.47354

scaffold_33:631036-631102	2.54216
scaffold_78:228031-228251	2.65903
scaffold_1:3891225-3891242	2.69338
scaffold_46:102144-102218	2.77804
scaffold_10:1980847-1980993	3.85719
scaffold_1:6411286-6411395	(-inf)
Scaffold_10:540965-540979	(-inf)
scaffold_10:1776310-1776324	(-inf)
scaffold_106:94873-94956	(-inf)
scaffold_15:1759844-1760085	(-inf)
scaffold_16:1808214-1808384	(-inf)
scaffold_21:1696928-1697064	(-inf)
scaffold_23:42282-42348	(-inf)
scaffold_25:1292815-1292829	(-inf)
scaffold_4:948471-948481	(-inf)
scaffold_43:525524-525533	(-inf)
scaffold_5:1111861-1111874	(-inf)
scaffold_670:99-708	(-inf)
scaffold_81:61130-61270	(-inf)
scaffold_83:163746-163916	(-inf)
scaffold_94:161193-161359	(-inf)
scaffold_45:792362-792464	(+inf)

Table 7. Identification of the known microRNA in the Chlamydomonas cell under salt stress

Micro_ID
cre_MIR905_MI0005698
cre_MIR906_MI0005699
cre_MIR907_MI0005700

cre_MIR908_MI0005701
cre_MIR909_MI0005702
cre_MIR910_MI0005703
cre_MIR911_MI0005704
cre_MIR912_MI0005705
cre_MIR913_MI0005706
cre_MIR914_MI0005707
cre_MIR915_MI0005708
cre_MIR916_MI0005711
cre_MIR917_MI0005710
cre_MIR918_MI0005697
cre_MIR919_MI0005709
cre_MIR1142_MI0006203
cre_MIR1143_MI0006204
cre_MIR1144a_MI0006205
cre_MIR1144b_MI0006235
cre_MIR1145_MI0006206
cre_MIR1146_MI0006207
cre_MIR1147_MI0006208
cre_MIR1148_MI0006209
cre_MIR1149_MI0006210
cre_MIR1150_MI0006211
cre_MIR1151a_MI0006212
cre_MIR1151b_MI0006213
cre_MIR1152_MI0006214
cre_MIR1153_MI0006215
cre_MIR1154_MI0006216
cre_MIR1155_MI0006217
cre_MIR1156_MI0006218
cre_MIR1157_MI0006219
cre_MIR1158_MI0006220
cre_MIR1159_MI0006236
cre_MIR1160_MI0006221
cre_MIR1161a_MI0006222
cre_MIR1161b_MI0015964

cre_MIR1162_MI0006223
cre_MIR1163_MI0006224
cre_MIR1164_MI0006225
cre_MIR1165_MI0006226
cre_MIR1166_MI0006227
cre_MIR1167_MI0006234
cre_MIR1168_MI0006228
cre_MIR1169_MI0006229
cre_MIR1170_MI0006230
cre_MIR1171_MI0006231
cre_MIR1172_MI0006233
cre_MIR1173_MI0006232

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Appendix 1 List of the genes that were 4x change in expression level

Up-regulated genes	Down-regulated genes

Carbohydrate and energy metabolism	***Carbohydrate and energy metabolism***
9-O-ACETYL-N-ACETYLNEURAMINIC ACID DEACETYLASE-RELATED Alpha-amylase AMA3 Beta-galactosidase Cytochrome c oxidase assembly factor, SCO1/SenC Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases) dTDP-glucose 4,6-dehydratase SNE5 NAD-dependent epimerase/dehydratase fucosyltransferase Glycerol-3-phosphate dehydrogenase/dihydroxyacetone-3-phosphate reductase Glycerol-3-phosphate dehydrogenase/dihydroxyacetone-3-phosphate reductase 2 GLYCOSYLTRANSFERASE 14 FAMILY MEMBER Glycosyltransferase family 92 GOX1 GOX3 GOX4 GOX5 Granule-bound starch synthase I GBBSI GBS1 STA2 HEPARAN SULFATE 3-O SULFOTRANSFERASE Lactonase NAD(P)-BINDING ROSSMANN-FOLD SUPERFAMILY PROTEIN PROTON GRADIENT REGULATION 5 L-THREONINE 3-DEHYDROGENASE UDP-glucuronate 5'-epimerase NAD-dependent Xyloglucan	CYTOCHROME C1 HEME LYASE
Cell division	***Cell division***
Metallophosphoesterase, phosphate-repressible gene MPA1 Leucine carboxyl methyltransferase SOM4 regulated by the PSR1 transcription factor Zygote-specific protein EZY9 ZYS3-2 ZYS4 Zygote-specific protein ZYS3 ZYS3-1	-
Cell structure	*Cell structure*
Cell wall protein pherophorin-C8 Cell wall protein pherophorin-C2 PHC2 Cell wall protein pherophorin-C21 PHC21 EXPANSIN-A1-RELATED EXPANSIN-A1-RELATED PF03330 - Rare lipoprotein A (RlpA)-like double-psi beta-barrel Flagellar Associated Protein FAP102	Cell wall protein pherophorin-C21 PHC21 Flagellar Associated Protein FAL11 Flagellar Associated Protein with ankyrin repeats FAP33 ANK17 Flagellar Associated Protein FAP215 FPN1 Hydroxyproline-rich cell wall protein ISG-C3 ISG6 Hydroxyproline-rich glycoprotein HRP3

Flagellar Associated Protein FAP137 Flagellar Associated Protein FAP211 Flagellar Associated Protein FAP233 Flagellar membrane protein, paralog of AGG2 detergent resistant membrane protein-2 [Chlamydomonas reinhardtii] Hydroxyproline-rich cell wall protein ISG-C4 ISG4 Hydroxyproline-rich glycoprotein, cell wall protein VSP4 Hydroxyproline-rich glycoprotein component of the outer cell wall Lectin C-type domain Sterol esterase / Triterpenol esterase Vegetative cell wall protein gp1	Las17-binding protein actin regulator (Ysc84)
Membrane and transport Amino acid transporter AOT4 Ammonium transporter AMT4 C-type lectin CTL2 C-type lectin CTL3 Copper transport accessory protein CTR3 Dicarboxylate/amino acid cation sodium transporter DAT1 ERD4-related membrane protein ERM1 HVA22-LIKE PROTEINS (Stress, ABA-induced protein) IONOTROPIC GLUTAMATE RECEPTOR Iron permease, membrane protein FTR1 Mechanosensitive ion channel domain MSC4 MSCL1 Mitochondrial carnitine-acylcarnitine carrier protein Multicopper ferroxidase FOX1 Nucleoside transporter Nucleotide-diphospho-sugar transferase P-type ATPase/cation transporter, plasma membrane, Low Co2 inducible Peroxisomal membrane protein MPV17 and related proteins RAG1 (recombination-activating gene 1)-ACTIVATING PROTEIN Sodium/potassium-exchanging ATPase / Sodium/potassium-transporting ATPase alpha subunit Sodium/phosphate symporter PTB6b/a SODIUM-DEPENDENT PHOSPHATE TRANSPORTER SUGAR TRANSPORTER SWEET1 RAG1-ACTIVATING PROTEIN 1 Sulfate anion transporter SUL2 SULTR2 Transient receptor potential ion channel protein TRP7	*Membrane and transport* Cation antiporter NAH1 KCN7 Ion transport protein PHOSPHATE TRANSPORTER Sodium/phosphate symporter PTB4 Sodium/phosphate symporter PTB5
Miscellania ANKYRIN REPEAT AND PROTEIN KINASE DOMAIN-CONTAINING PROTEIN	*Miscellania* 12-OXOPHYTODIENOATE REDUCTASE 1-RELATED NADH OXIDOREDUCTASE-RELATED

Arylsulfatase ARS5 ARYLSULFATASE ARS11 ARYLSULFATASE ARS18 CGI-141-RELATED/LIPASE CONTAINING PROTEIN - Lipase (class 3)TGL21 FAD NAD BINDING MONOOXYGENASE Fe-assimilating protein 2 Ferric-chelate reductase/ oxidoreductase FRE1 Gametolysin peptidase M11 (Peptidase_M11) His(2)-Cys(2) zinc finger LEUCINE-RICH REPEAT-CONTAINING PROTEIN Low-CO2-inducible protein Low-CO2-induced aldose reductase LCI28 Low-CO2-inducible membrane protein Pheophorbide a oxygenase, Rieske iron-sulfur cluster protein RING FINGER PROTEIN 10 SARS coronavirus main proteinase / Severe acute respiratory syndrome coronavirus main protease hypothetical protein	ANKYRIN REPEAT-CONTAINING PROTEIN Aryl sulfotransferase / Sulfokinase Cellulase / Endoglucanase Chitin binding domain Gametolysin / Lysin MMP19 LEUCINE-RICH REPEAT-CONTAINING PROTEIN Polyketide cyclase / dehydrase and lipid transport PWR protein PWR4 TINY MACROCYSTS PROTEIN B-RELATED WD40 REPEAT PROTEIN
Other metabolism 3-OXO-5-ALPHA-STEROID 4-DEHYDROGENASE 12-oxophytodienoate reductase ALLANTOATE DEIMINASE Carbonic anhydrase, alpha type, periplasmic Cytochrome P450 gene CYP744C1, CYP3 superfamily Cytochrome P450, CYP197 superfamily CYP20 CYP743B1 Cytochrome P450, CYP55 superfamily, CYP55A family CYP2 CYP55B1 EXOSTOSIN-LIKE GLYCOSYLTRANSFERASE Exostosin-like glycosyltransferase ELG8 Fasciclin-like protein FAS2 Fasciclin-like protein FAS3 Ferredoxin FDX2 Apoferredoxin Gamma-glutamyl hydrolase GGH1 Glucan endo-1,3-beta-D-glucosidase / Laminarinase GLUCOSYL/GLUCURONOSYL TRANSFERASES Glutamate 5-kinase Guanine deaminase HEPARAN SULFATE 3-O SULFOTRANSFERASE LIPOXYGENASE Long-chain-alcohol O-fatty-acyltransferase / Wax synthasedomain PHOSPHATE METABOLISM PROTEIN RSN1 (YEAST)-RELATED	*Other metabolism* Exostosin-like glycosyltransferase ELG3 Lactosylceramide 4-alpha-galactosyltransferase

PYRAZINAMIDASE/NICOTINAMIDASE rieske iron-sulfur cluster 55 kDa protein of chloroplast inner membrane translocon SGNH hydrolase Starch phosphorylase PHO2 PHOA Thioesterase superfamily TEH3 Urate oxidase II UO UOX UOX1	
Protein degradation CALPAIN Endothelin-converting enzyme 1 / ECE-1 GAMMA-INTERFERON INDUCIBLE LYSOSOMAL THIOL REDUCTASE (GILT) IAA-AMINO ACID HYDROLASE ILR1-LIKE 3 Lysine carboxypeptidase / Lysine(arginine) carboxypeptidase matrix metalloproteinase PF11617 Metalloproteinase of VMP family PEPTIDASE M20 FAMILY MEMBER Phosphate-repressible alkaline phosphatase PHO5 PHOX PROPROTEIN CONVERTASE SUBTILISIN/KEXIN (peptidase)/ VLE gene Scavenger receptor cysteine rich (SRCR) protein TUMOR NECROSIS FACTOR SUPERFAMILY, MEMBER 5-INDUCED PROTEIN 1 (CLAST3) Ubiquitin UBQ1 Xaa-Pro dipeptidase	*Protein degradation* Cathepsin H / N-benzoylarginine-beta-naphthylamide hydrolase cysteine endopeptidase Pyroglutamyl-peptidase I / Pyrrolidone-carboxylate peptidase
Protein processing -	*Protein processing* centriole proteome protein, partial
Signalling CALCIUM-DEPENDENT PROTEIN KINASE Dynamin family CHLREDRAFT_187905 contain ATPase GTP-BINDING PROTEIN 6-RELATED Histidine phosphatase INTERFERON-INDUCIBLE GTPASE 5 Lysosomal & prostatic acid phosphatases MITOGEN-ACTIVATED PROTEIN KINASE Serine/threonine phosphatases, family 2C, catalytic domain Non-specific serine/threonine protein kinase / Threonine-specific protein kinase Serine/threonine protein kinase STK23 STPK23 Tyrosine kinase specific for activated (GTP-bound) p21cdc42Hs MEKK and related serine/threonine protein kinases // Mitogen-	*Signalling* Calcium-binding EGF domain Adenylate cyclase / ATP pyrophosphate-lyase // Guanylate cyclase / Guanylyl cyclase CYG65 SERINE-THREONINE PROTEIN KINASE Tyrosine kinase specific for activated (GTP-bound) p21cdc42Hs // MEKK and related serine/threonine protein kinases 3',5'-cyclic-nucleotide phosphodiesterase PDE23 3',5'-cyclic-nucleotide phosphodiesterase PDE25 Adenylate and Guanylate cyclase catalytic domain Protein kinase domain (Pkinase) // Protein tyrosine kinase (Pkinase_Tyr) Flagellar Associated Protein similar to calmodulin FAP272 guanylate cyclase

activated protein kinase kinase (MAP2K)	Adenylate/guanylate cyclas CYG12 Non-specific protein-tyrosine kinase / Cytoplasmic protein tyrosine kinase // Non-specific serine/threonine protein kinase / Threonine-specific protein kinase Calcium/calmodulin dependent protein kinase II association domain TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER NITROGEN NETWORK KINASE 1
Stress and defense Ascorbate peroxidase APX1 CYSTEINE-RICH SECRETORY PROTEIN-RELATED DEFENSE PROTEIN L(2)34F Glutathione S-transferase GST12 Glyoxalase GOX2 GOX17 Lipid-phosphate phosphatase / Soluble epoxide hydrolase similar to pathogenesis-related protein 1 gene PRL10 nitrogen-starved gametogenesis 13 NSG13 Stress-related chlorophyll a/b binding protein 1 LHCSR1 Stress-related chlorophyll a/b binding protein 2 LHCSR2 LHCSR3.1 Stress-related chlorophyll a/b binding protein 3 LHCSR3 LHCSR3.2 zeaxanthin epoxidase	*Stress and defense* Peptide-methionine (S)-S-oxide reductase / Peptide methionine sulfoxide reductase
Transcription DNA POLYMERASE EPSILON SUBUNIT 4 Histone-lysine N-methyltransferase / Protein-lysine N-methyltransferase Low-CO2 response regulator, Myb-like transcription factor LCR1 SNT domain PCDC2 (PROGRAMMED CELL DEATH PROTEIN 2)-RELATED PIRIN Transcription co-activator TRANSMEMBRANE PROTEIN 2-RELATED PF00628 - PHD-finger RNA EXONUCLEASE NEF-SP-RELATED EXONUCLEASE EXN11	*Transcription* RWP-RK transcription factor/minus dominance protein MID RWP5 RWP-RK transcription factor RWP6 Reverse transcriptase (RNA-dependent DNA polymerase) // Endonuclease/Exonuclease/phosphatase Transposase family tnp2 (Transposase_21) Putative transposase DNA-binding domain RIBONUCLEASE III SQUAMOSA PROMOTER-BINDING-LIKE PROTEIN 10-RELATED Tam3-transposase (Ac family)