หน้าที่ของ *NUCLEOLIN1* จากข้าวต่อความสามารถในการต้านทานภาวะเค็มของ Arabidopsis

นางสาวธนิกานต์ อุดมชโลทร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์ชีวภาพ
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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FUNCTION OF RICE *NUCLEOLIN1* IN SALT-RESISTANT ABILITY OF ARABIDOPSIS

Miss Thanikarn Udomchalothorn

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By	Miss Thanikarn Udomchalothorn
Field of Study	Biological Sciences
Thesis Advisor	Associate Professor Supachitra Chadchawan, Ph.D.
Thesis Co-Advisor	Associate Professor Teerapong Buaboocha, Ph.D. Professor Luca Comai, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Tosak Seelanan, Ph.D.)

.....Thesis Advisor
(Associate Professor Supachitra Chadchawan, Ph.D.)

.....Thesis Co-Advisor
(Associate Professor Teerapong Buaboocha, Ph.D.)

.....Thesis Co-Advisor
(Professor Luca Comai, Ph.D.)

.....Examiner
(Assistant Professor Pongchai Harnyuttanakorn, Ph.D.)

.....External Examiner
(Pataradawn Pinyopich, Ph.D.)

ธนิกานต์ อุดมชโลทร : หน้าที่ของ *NUCLEOLIN1* จากข้าวต่อความสามารถในการต้านทานภาวะเค็มของ Arabidopsis (FUNCTION OF RICE *NUCLEOLIN1* IN SALT-RESISTANT ABILITY OF ARABIDOPSIS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศุภจิตรา ชัชวาลย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ธีรพงษ์ บัวบูชา, ลูก้า โคไม, 181 หน้า.

วิทยานิพนธ์นี้มีจุดประสงค์หลักสองข้อ ประเด็นแรกคือการเปรียบเทียบข้าว LPT123 และสายพันธุ์ทนเค็มที่เกิดจากการกลายพันธุ์ LPT123-TC171 ในระดับจีโนมเพื่อระบุความแปรปรวนที่ส่งผลให้เกิดความสามารถในการทนต่อภาวะเค็ม ประเด็นที่สองคือการระบุหน้าที่ของยีน *Nucleolin1* จากข้าว (*OsNUC1)* ต่อกระบวนการปรับตัวต่อภาวะเค็ม โดยยีน *OsNUC1* ปรากฏสองรูปแบบ คือ *OsNUC1-S* และ*OsNUC1-L* ในการอธิบายหน้าที่ของยีน *OsNUC1* อาศัยการสร้างพืชดัดแปลงพันธุ์ที่เพิ่มการแสดงออกของยีน *OsNUC1*ภายใต้การควบคุมของโปรโมเตอร์ชนิดแสดงออกตลอดเวลา

ข้าว LPT123-TC171 เป็นข้าวทนเค็มและทนแล้งที่ได้มาจากการคัดเลือกจากการแปรของเซลล์ร่างกายของข้าวไทยพันธุ์ LPT123 โดยจีโนมของข้าว LPT123 และ LPT123-TC171 ถูกศึกษาเปรียบเทียบในสามระดับคือ ระดับโครโมโซม (polyploidy, aneuploidy) ระดับรูปร่างของโครโมโซม และระดับดีเอ็นเอ ด้วยวิธี whole genome sequencing ผลการศึกษาชี้ชัดว่า ไม่พบการเปลี่ยนแปลงในชุดของโครโมโซม (polyploidy) หรือจำนวนของโครโมโซม (aneuploidy) ของข้าว LPT123-TC171 ซึ่งเป็นที่น่าแปลกใจเมื่อศึกษารูปร่างของโครโมโซม พบความบกพร่องอย่างมีนัยสำคัญบริเวณปลายของแท่งโครโมโซม (telomere) ของข้าวพันธุ์กลายโดยการเปลี่ยนแปลงนี้เป็นผลมาจากภาวะ oxidative stress ที่จากกระบวนการคัดเลือก ยิ่งไปกว่านั้นการศึกษาด้วยวิธี functional genomics หรือ exome sequencing ได้ให้มุมมองใหม่ในการตอบสนองของจีโนมข้าวต่อการคัดเลือกในหลอดทดลอง exome sequencing ได้เปิดเผยรูปแบบของการเปลี่ยนแปลงที่เกิดจากการแปรของเซลล์ร่างกาย โดยการเปลี่ยนแปลงแทนที่ของนิวคลีโอไทด์แบบหลักที่เกิดจากการแปรของเซลล์ร่างกายคือ A↔G and T↔C มากไปกว่านั้นการกลายพันธุ์แบบ point mutation ถูกระบุไว้ทั้งสิ้น 493 ตำแหน่ง ใน 17 ยีนที่มีการรายงานว่าตอบสนองทั้งภาวะเค็มและภาวะแล้ง และมี 100 ตำแหน่ง ภายใน 6 ยีนที่ตอบสนองต่อความเค็ม

NUC เป็นโปรตีนที่มีหน้าที่หลากหลาย พบบริเวณนิวคลีโอลัสของเซลล์ยูแคริโอต มีความเกี่ยวข้องกับกระบวนการเมแทบอลิซึมของอาร์เอ็นเอ ความเสถียรของเอ็มอาร์เอ็นเอ และกระบวนการสร้างไรโบโซม ในการศึกษาเพิ่งค้นพบว่ายีน *NUC* มีหน้าที่ควบคุมการเจริญเติบโตใน Arabidopsis ส่วนในข้าวนั้นยีน *OsNUC1* ปรากฏในสองรูปแบบของเอ็มอาร์เอ็นเอคือ *OsNUC1-S* and *OsNUC1-L* ซึ่งทั้งคู่มีการเพิ่มระดับการแสดงออกของยีนภายหลังการได้รับภาวะเค็ม

จากการใช้ Arabidopsis ดัดแปลงพันธุ์ในการอธิบายหน้าที่ใหม่ของยีน *OsNUC1-S* โดยการเพิ่มการแสดงออกของยีน *OsNUC1-S* ใน Arabidopsis ดัดแปลงพันธุ์แสดงลักษณะ hypersensitive ต่อกรดแอบไซซิกขณะการทดสอบการงอก และจากการศึกษาการตอบสนองของยีนทั้งหมดพบว่ายีน *9-cis-epoxycarotenoid dioxygenase* (*NCED*) ซึ่งเข้ารหัสให้เอนไซม์สำคัญในกระบวนการสังเคราะห์กรดแอบไซซิก มีการแสดงออกเพิ่มขึ้น 22 เท่าซึ่งเป็นการเพิ่มอย่างมีนัยสำคัญ บ่งชี้ว่ายีน *OsNUC1-S* อาจจะมีส่วนเพิ่มความสามารถในการทนเค็มของพืชดัดแปลงพันธุ์ผ่านการเพิ่มปริมาณกรดแอบไซซิก นอกจากนี้ยังพบการเพิ่มขึ้นของยีนที่เพิ่มการแสดงออกโดยการชักนำของกรดแอบไซซิกเมื่อพืชแปลงพันธุ์ที่เติบโตในภาวะเค็ม

ยีน *OsNUC1-L* เพิ่มความสามารถในการทนเค็มของ Arabidopsis ดัดแปลงพันธุ์ นอกจากนั้นยังแสดงการเจริญที่เปลี่ยนแปลง เช่น ออกดอกเร็ว และมีวงชีวิตสั้น จากการศึกษาการตอบสนองของยีนทั้งหมดพบว่า Arabidopsis ดัดแปลงพันธุ์เพิ่มกลุ่มยีนที่เกี่ยวข้องกับกระบวนการสังเคราะห์ด้วยแสง ไรโบโซม และองค์ประกอบของพลาสมาเมมเบรน อย่างโดดเด่น ระบุถึงหน้าที่ของยีน *OsNUC1-L* ต่อการปรับตัวในภาวะความเครียดที่เกิดจากความเค็ม ผลที่ได้จากการทดลองได้ชี้ชัดว่าการเพิ่มขึ้นของยีน *OsNUC1-L* ในต้น Arabidopsis ดัดแปลงพันธุ์ปรับปรุงการทนต่อภาวะเค็มโดยการควบคุมระดับการแสดงออกของยีนในวิถีที่จำเพาะ

สาขาวิชา วิทยาศาสตร์ชีวภาพ
ปีการศึกษา 2557

ลายมือชื่อนิสิต .....
ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....
ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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THANIKARN UDOMCHALOTHORN: FUNCTION OF RICE *NUCLEOLIN1* IN SALT-RESISTANT ABILITY OF ARABIDOPSIS. ADVISOR: ASSOC. PROF. SUPACHITRA CHADCHAWAN, Ph.D., CO-ADVISOR: ASSOC. PROF. TEERAPONG BUABOOCHA, Ph.D., PROF. LUCA COMAI, Ph.D., pp.

The objectives of this dissertation have two main goals; the first is to compare the LPT123 and its salt-tolerant mutation line, LPT123-TC171, at the genomic level to indicate the variations that contribute to stress-tolerant character. Second objective is to characterize the function of *Nucleolin1* gene from rice (*OsNUC1*) on salt-adaptive mechanism. *OsNUC1* presents in two splicing forms which are *OsNUC1-S* and *OsNUC1-L*.To elucidate the function of *OsNUC1*, I generated the transgenic Arabidopsis, with *OsNUC1* over-expression under the control of constitutive promoter.

LPT123-TC171 is the salt and drought tolerant rice line which was selected from somaclonal variation of the original Thai rice, LPT123. Genome of LPT123 and LPT123-TC171 were comparatively studied in 3 levels which are chromosome level (polyploidy, aneuploidy), chromosome structure and DNA sequence changes by whole genome sequencing analysis. The results showed that LPT123-TC171 did not show the changes in the set of chromosome (polyploidy) or number of chromosome (aneuploidy). Interestingly, in the study of chromosome structure, the significant deficiency of chromosome ends (telomeres) in the mutant line were found. This change could be resulted from the oxidative stress in the screening process. In addition, the functional genomic approach or exome sequencing gave the new aspects of genome response to *in vitro* condition. Exome sequencing revealed the molecular spectrum and pattern of change of somaclonal variant. The major nucleotide substitutions in somaclonal variant were A↔G and T↔C transitions. Moreover, the total point mutation were found which are 493 positions within the known genes responding to both 17 drought and salt stress genes and 100 positions within 6 salt-responsive genes.

NUC is the multifunctional nucleolar protein found in all eukaryotes that involved in RNA metabolism, mRNA stability and ribosome biogenesis process. The recent study in Arabidopsis showed that *NUC* has functions in plant growth and development. In rice, *OsNUC1* presents in two mRNA species, *OsNUC1-S* and *OsNUC1-L*, which were increased the expression after salt treatment.

By using transgenic Arabidopsis, it revealed the new role of *OsNUC1-S*. The transgenic Arabidopsis with the overexpression of *OsNUC1-S* showed hypersensitive to ABA during seed germination. Based on the transcriptome analysis, 9-cis-epoxycarotenoid dioxygenase (*NCED*), the gene encoding the key enzyme in ABA biosynthesis, was dramatically up-regulated in transgenic Arabidopsis up to 22 fold, suggesting that *OsNUC1-S* may increase the salt-tolerant ability via the increase of ABA production. These were also supported with the increase of the ABA inducible gene expression in transgenic Arabidopsis grown in salt condition

The *OsNUC1-L* gene enhanced salt-tolerant ability in transgenic Arabidopsis. Besides, they also displayed some developmental changes such as early flowering and short life-cycle. Based on transcriptomic study, the transgenic Arabidopsis dominantly enriched group of genes in photosynthetic process, ribosome and plasma membrane component leading to the role of *OsNUC1-L* in salt stress adaptation. These results suggested that overexpression of the *OsNUC1* gene in transgenic Arabidopsis improved salt stress tolerance by regulating expression the genes in some specific pathways.

Field of Study: Biological Sciences
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Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....
Co-Advisor's Signature .....

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Chapter I

INTRODUCTION

Tissue culture is the technique broadly used in plant research for numerous applications including gene transformation and mutagenesis for plant improvement. In crop improvement programs, tissue culture was used to induce genetic variation among regenerated clones and also to reduce time of selection (Neelakandan and Wang 2012). The genetic changes that result from tissue culture propagation known as somaclonal variation had been reported, which include a wide range of mutations and chromosome aberrations (Jain 2001). Somaclonal variation has been applied to select plants with beneficial agronomic traits such as high yield (Gao *et al.* 1991), disease resistance and abiotic tolerance (Rai *et al.* 2011), early flowering (Xu and Chen 1995), and physical stress resistance (Adkins *et al.* 1995).

LPT123-TC171 is the salt and drought tolerant variant that was generated from somaclonal variegated callus of 'LPT123' rice, which is more susceptible to abiotic stress (Thikart *et al.* 2005, Udomchalothorn *et al.* 2009, Pongprayoon *et al.* 2013). A series of experiments on this somaclonal variant had been performed and some adaptive mechanisms to salt stress were reported (Udomchalothorn *et al.* 2009, Sripinyowanich *et al.* 2013). The expression level of at least two genes, *fructose-6-phosphate-2-kinase/fructose-2,6-bisphosphatase* (Udomchalothorn *et al.* 2009) and *OsNUC1* (Sripinyowanich *et al.* 2013), was different between LPT123 and LPT123-TC171 and it may contribute to salt tolerant ability in the mutant line.

The mechanisms of genome responses in somaclonal variants are associated with polyploidy, aneuploidy, structural abnormality of chromosome, single DNA sequence change, transposable element induction and the epigenetics (Neelakandan and Wang 2012). To understand the genetic variation and the adaptation of this somaclonal line at the molecular level, the next-generation sequencing technology was used to examine the genetic basis of difference between LPT123 and LPT123-TC171 rice. The results provided us with the information of the salt-tolerant rice genome that gave new insights of salt-tolerant mechanisms in rice.

OsD1B18-18 (GENBANK accession no. DQ012289) was found to be one of the gene showing the difference in gene expression, when it was detected by differential display method (Maneeprasopsuk 2004). The differential expression of the fragment *OsD1B18-18* (GENBANK accession no DQ012289) in rice leaves of two closely related rice cultivars that possess different salt tolerance showed that this fragment both had a higher apparent expression level in the salt-tolerant rice cultivar (LPT123-TC171) and was up-regulated in the two cultivars in response to salt stress (Maneeprasopsuk 2004). The cloned *OsD1B18-18* fragment was sequenced and BLAST algorithms searching on the ExPASy server (Gasteiger *et al.* 2003) with the sequence was performed. The predicted amino acid sequence showed 97 % sequence homology to *Nucleolin1* gene (*OsNUC1*) in Japonica rice genome.

In eukaryotes, nucleolin (NUC) plays the major function in ribosome biogenesis such as rDNA transcription, processing and maturation, so it is known to be involved in various metabolic processes in the cell. Moreover, some studies in Arabidopsis recently revealed that nucleolin affected growth and development (Kojima *et al.* 2007, Petricka and Nelson 2007, Pontvianne *et al.* 2007). In rice, two *OsNUC1* full-length cDNA species were submitted to GENBANK database. The longer form *OsNUC1-L*, GENBANK accession no. AK103446, has three domains that are: acidic N-terminal region, the central domain contains two bipartite RNA-recognition motifs (RRM) and the glycine-arginine rich (GAR) domain at the C-terminal end, while its alternative form, GENBANK accession AK063918 or shorter form *, OsNUC1-S*, has only RNA-binding motifs and the GAR domain.

Based on the experiments done in our laboratory, the transgenic Arabidopsis with the overexpression of *OsNUC1-S*, the shorter form, showed the higher salt-tolerant characters than the wild-type plant when they were challenged with salt stress (Sripinyowanich *et al.* 2013). In my preliminary study, these transgenic plants also showed the hypersensitive response to ABA in both seed germination and root development phenotypes.

As the overexpression of *OsNUC1-S* can improve the salt stress ability (Sripinyowanich *et al.* 2013), it leads to the hypothesis that the expression of *OsNUC1* gene is involved in the adaptation to the stress environment by action in ABA signaling process. However, the mechanism of *OsNUC1* function in abiotic stress response has not been assessed. We hypothesized that this protein may involve in the ABA biosynthesis or may play a part as an ABA signaling component. Therefore, I conducted the series of physiological, biochemical and molecular analyses by using the overexpressing *35SCaMV::OsNUC1-L* in Arabidopsis system to indicate some insight of the cellular function(s) of *OsNUC1* gene in salt-stress response.

Objectives:

1. To compare the difference at the genomic level of LPT123 and LPT123-TC171 rice
2. To compare of salt resistant role of two *OsNUC1* forms
3. To study the function of salt resistant domains of *OsNUC1* in ABA responses
4. To study the impact of *OsNUC1* overexpression on other gene expression in transgenic lines

Contents of the thesis:

The organization of this dissertation is started with an introduction of the research including rational, problems, and objectives. Then, chapter II presents the literature reviews about how plant response to salt stress, genome response to stress, *Nucleolin1* gene structure and functions, transcriptome study using next-generation sequencing, role of abscisic acid on abiotic stress response and lastly, the effect of salt stress on photosynthetic activity. Materials and methods obtained in this study are also mentioned in the following chapter. Chapter IV, results and discussion, is divided into 5 parts corresponding to:

(i) Comparison the salt-sensitive rice, LPT123, and its mutation line, LPT123-TC171, by using DNA sequencing-based method

(ii) Comparison of salt-resistant role of two *OsNUC1* splicing forms (*OsNUC1*-S and *OsNUC1-L*) during salt condition

(iii) Transcriptomics of overexpression *OsNUC1-S* transgenic Arabidopsis

(iv) Characterization of transgenic *35SCaMV::OsNUC1-L* Arabidopsis

After that, all results are summarized in chapter V and future perspectives are also described. Appendices indicated other information that did not included in the previous chapters.

Chapter II

LITERATURE REVIEWS

1. Plant response to salt stress

In agricultural field, abiotic stress such as drought and hypersaline condition dramatically reduced crop yield (Kawasaki *et al.* 2001). Understanding the response and adaptive mechanism of plant to stress is the principle knowledge that may apply to plant improvement program in the future. When investigated the response of plant to salinity stress by using microarray approach, Kawasaki and colleagues found that over thousand genes were response to salt stress including signal transduction, transcription factors, transporter proteins and proteins that involved in cell growth and division (Kawasaki *et al.* 2001). The salt stress affected plant through the combination of osmotic stress and ion toxicity which disused below.

1.1. ion stress

The regulation of ion balance in the cell is important character of plant under hypersaline condition, so they had several strategies to cope this unavoidable condition. Plant cell attempt to balance the ion homeostasis in the cell by reduce the Na^+ ion and maintain K^+ in the cell. The regulation of ion homeostasis resulted from the control of gene expression and activity of ion transporter protein such as Na^+ transporters and H^+ pumps (Zhu 2003). High concentration of salt affected to the activity of by directly ruined protein secondary structure (Neumann 1997). Moreover, the imbalance of Na^+ and K^+ ion also impacted to cellular metabolism (Greenway and Munns 1980)

1.2. osmotic stress

At the high concentration of salt, plant reduced capacity to up take the water from soil solution to plant cell which is the key factor that altered the physiology and metabolism and limited plant growth (Munns 2002). Because the lower osmotic potential outside the cell decreased the water absorption, plant cell accumulated the low molecular weight organic molecule such as sugar, polyol and amino acid which called osmolyte. The osmolyte regulated the water transport into the plant by reduced the osmotic potential in the cell. Moreover, some osmolyte directly stabilize the macromolecule such as protein and cell membrane (Hare *et al.* 1998). Osmolyte accumulation is the one strategy of adaptive character to hypersaline condition (Udomchalothorn *et al.* 2009)

2. Genome response to stress

McClintoch had noted that genome has a capacity to adapt itself when faces the unflavored environments including *in vitro* culture conditions (Philips *et al.* 1994). The mechanisms of genome responses in somaclonal variants are associated with polyploidy, aneuploidy, structural abnormality of chromosome, single DNA sequence change, transposable element induction and the epigenetics (Neelakandan and Wang 2012). At the chromosome level, tissue culture of the ex-plant of banana increased the degree of polyploidy (Giménez *et al.* 2001). The long term propagation of welsh onion (*Allium fisulosum* L.) showed chromosome abnormality such as branched chromosome, increase in chromosome number and also micronuclei formation (Joachimiak and Ilnicki 2003). Moreover, the *in vitro* propagation had been reported as one of the factors that affected the telomere structure. The long term barley callus culture (6-12 months) significantly increased the

telomere length when compared to the original germ-line callus (Kilian *et al.* 1995).

Some region of chromosome showed the sensitivity to stress such as telomere. Telomeres are special structures of the end of eukaryote linear chromosomes, which are composed of tandem repeats of DNA that form a complex structure with specific telomeric proteins (Kass-Eisler and Greider 2000). In most plants, telomeres have the consensus sequence of $[TTTAGG]_n$, while the length depends on species and varieties (Shakirov and Shippen 2004). Telomeres protect the chromosome from the cleavage of exonuclease and also regulated in chromosome stability (Greider 1996). Although telomeres compose of the simple repeats with almost no gene found, the change of telomere length can cause alteration of stress response. The *AtKU70* gene, which encodes a DNA binding protein, uniquely locates around the end of chromosome. $AtKU70^{-/-}$ plant lacking the control of telomere length regulation, which resulted in telomere elongation, showed hypersensitive to stresses (Bundock *et al.* 2002, Riha *et al.* 2002).

At the nucleotide level, *in vitro* culture triggered single nucleotide mutations (indel and nucleotide substitution) (Noro *et al.* 2007) and DNA methylation (Mirouze and Paszkowski 2011). Furthermore, *in vitro* conditions induced the expression of retrotransposons, *Tos10*, *Tos17* and *Tos19* and the copy number of *Tos17* was increased in the long-term rice culture (Hirochika *et al.* 1996)

3. Nucleolin structure and functions

In eukaryotes, nucleolin plays the major function in ribosome biogenesis such as rDNA transcription, processing and maturation, so it is known to be involved in various metabolic processes in the cell. Moreover, some studies in Arabidopsis recently revealed that nucleolin affected growth and development (Kojama et al, 2007; Petricka and Nelson, 2007).

The gene structure of *Nucleolin1* from rice, *OsNUC1*, is composed of 4 main parts which are nuclear localization signal, acidic-rich region, RNA-recognition motif and glycine-arginine rich region. In eukaryotes, nucleolin plays the major function in ribosome biogenesis such as rDNA transcription, processing and maturation, so it is known to be involved in various metabolic processes in the cell. Moreover, some studies in Arabidopsis recently revealed that nucleolin affected growth and development (Kojima *et al.* 2007, Petricka and Nelson 2007).

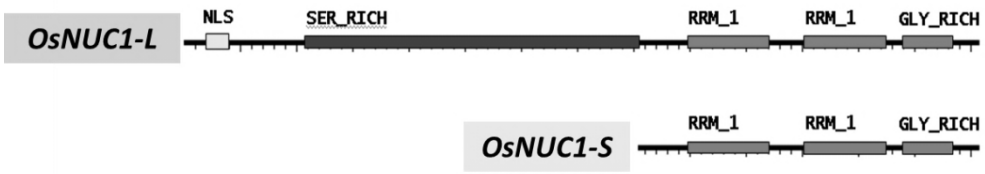


Figure 2. 1The illustration of two splicing mRNAs form of rice *OsNUC1* gene

The differential expression of the gene fragment *OsD1B18-18* (GENBANK accession no. DQ012289) in rice leaves of two closely related rice cultivars that possess different salt tolerance showed that this fragment had a higher apparent expression level in the salt-tolerant rice cultivar (LPT123-TC171) and was up-regulated in the both LPT1 23 and LPT123-TC171 rice under salt stress condition (Maneeprasopsuk 2004). The predicted amino acid sequence showed 97 % sequence homology to *OsNUC1* gene in Japonica rice genome (Sripinyowanich *et al.* 2013). According to the Rice Genome Annotation Project (http://rice .plantbiology.msu.edu/), *OsNUC1* gene was predictably reported in two splicing mRNA species that located on chromosome 4 and chromosome 8 respectively. The first splicing form (GENBANK accession number: AK103446) completely composed of 4 main

parts of gene while another splicing form has only RNA-recognition motif and glycine-arginine rich region. For this reason, they were called longer form *OsNUC1* (*OsNUC1-L*) and shorter form (*OsNUC1-S*) respectively. When investigated the ultracellular localization, the OsNUC1-L mainly deposited in nucleus while OsNUC1-S dispersed both nucleus and cytosol (Sripinyowanich *et al.* 2013) which reflected the different function of these two splicing forms.

To study the function of *NUC* in Arabidopsis, The knockout mutant Δ*ATNUC-L1* Arabidopsis revealed growth retardation, abnormal veination primary root growth inhibition and yield reduction (Kojima *et al.* 2007, Petricka and Nelson 2007). Furthermore, NUC affected to ribosome biosynthesis. The Δ*ATNUC-L1* defected in rRNA processing (Kojima *et al.* 2007). Besides, Yeast that defected the *NUC* gene function dramatically decreased in 40S/60S ribosomal subunit ratio (Kondo and Inouye 1992).

The expression analysis revealed that the expression of *NUC* gene was mostly found in meristematic zone and tightly correlated with mitotic activity of cells (Bogre *et al.* 1996). In addition, the study in soybean root revealed that the transcriptional activity of *NUC* gene significantly not only coordinated with cell division and ribosome production, but also correlated with cellular metabolism and root growth (Stepinski 2004).

4. Transcriptome study using next-generation sequencing

The transcriptomic approach become the new tool that use to study entire transcripts including mRNAs, non-coding RNAs and small RNA (Wang *et al.* 2009). The workflow of RNA-Seq method is divided into three steps; sample preparation, sequencing and data analysis as shown in Figure 2.2 (Wilhelm and Landry 2009). In brief, total RNA was extracted and removed rRNA using rRNA removal kits such as AMPure® XP beads (Beckman Coulter Inc., USA) then mRNAs were converted to cDNA by Revert transcriptase. The library preparation process begins with fragmentation of cDNA, purification.

The end repair of cDNA fragments process was performed using 3' -> 5' exonuclease activity of Klenow fragment then ligated blunt-end cDNA fragment with specific adaptor.

To detect the gene expression, there two approaches of high throughput analysis which is sequencing-based approach and hybridization-based approach. The benefit of sequencing-based technology over the hybridization-based method is the limitation to detect the new transcripts. Moreover, the sequencing-based method clearly mapped the short reads to the reference genome and the short reads also reflected the amount of gene expression even the low expression gene while DNA microarray has less sensitivity to detect the small amount of low expressed gene (Wang *et al.* 2009)

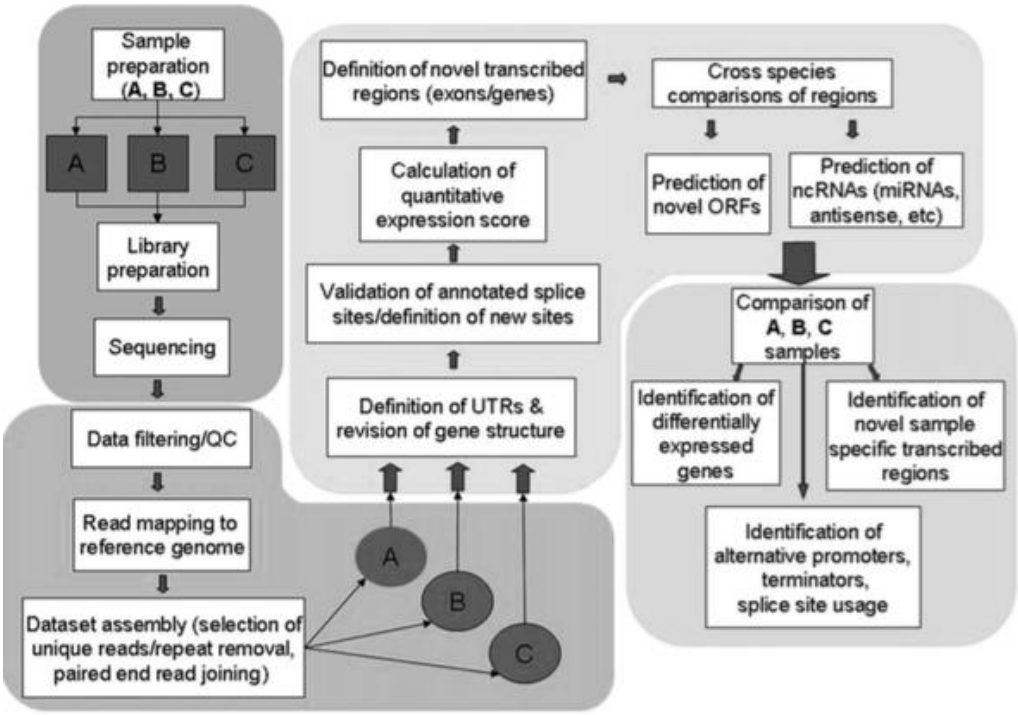


Figure 2. 2The diagram represented RNA-Seq workflow (Wilhelm and Landry 2009)

5. Role of abscisic acid on abiotic stress response

The plant hormone abscisic acid (ABA) regulate several response in plant such as stomatal closure, plant development such as seed maturation, germination, seedling growth, gene expression. It has been noted that ABA involved in adaptive responses to physical stress, especially the response to water status such as drought and salinity (Zhang *et al.* 2006).

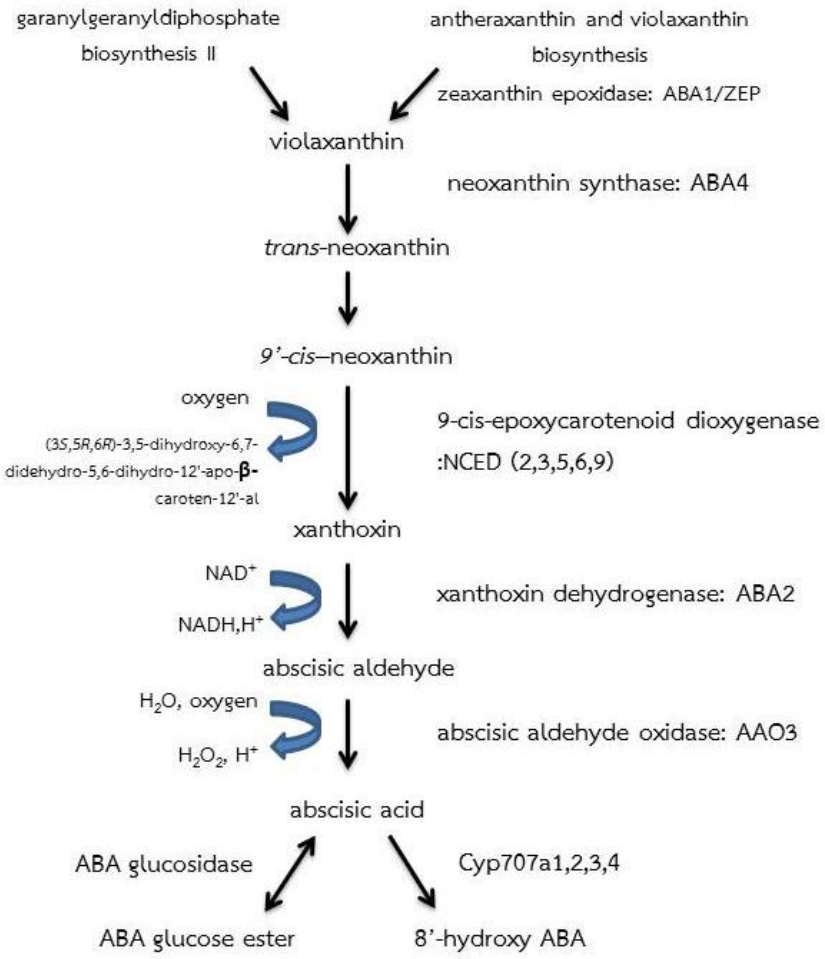


Figure 2. 3 The illustration showed the ABA biosynthesis and catabolism which showed all enzymes in this process

The ABA content in the cell is resulted from the rate of ABA synthesis and catabolism. In the ABA biosynthesis pathway, 9-cis-epoxycarotenoid dioxygenase (NCED) is the enzyme that controls the cleavage step of 9-cis-violaxanthin to xanthoxin which is the rate-limiting step in ABA biosynthesis (Qin and Zeevaart 2002).

Arabidopsis genome contains five *NCED* genes (*AtNCED2, AtNCED3, AtNCED5, AtNCED6, and AtNCED9*) that controlled ABA biosynthesis. When consider the relationship between ABA metabolism and salt stress, only *AtNCED3* was response to osmotic stress (Nambara and Marion-Poll 2005). Luci and colleagues discovered that the overexpressing *AtNCED3* significantly increased the ABA content in transgenic Arabidopsis and also improved the osmotic tolerance by reduction the leave transpiration rate (Luchi *et al.* 2001). Furthermore, Frey and colleagues (Frey *et al.* 2012) also found the *nced3* and *nced5* knock-out mutants, which decreased the ABA content, were susceptible to drought.

Recent studies of RNA-binding proteins mutants showed that they had the ABA-sensitive phenotypes such as delayed seed germination, inhibited root growth and rapid stomatal closure upon the ABA application that led to the correlation of ABA signal transduction and post-transcription RNA processing (Kuhn and Schroeder, 2003).

ABA-hypersensitive 1 (*abh1*) was identified as ABA-hypersensitive mutant. The *ABH1* gene encodes the large subunit of nuclear cap-binding protein complex that was showed to participate in several steps of RNA processing, nuclear export and mRNA decay (Makarov *et al.* 2002). The *abh1* mutant showed delay seed germination and rapid stomatal closure and less wilting phenotype during drought stress (Hugouvieux *et al.* 2001). The knock-out mutant Arabidopsis of another glycine-rich RNA-binding protein, *atgrp7-1*, showed the hypersensitive to ABA and salt stress in germination and root

development (Kim *et al.* 2008). Moreover, Cao and colleagues (Cao *et al.* 2006) revealed that the *atgrp7-1* mutant conferred the expression of some ABA-induced genes.

6. Effect of salt stress on photosynthetic activity

Salt stress effected wide range of metabolic process in the cell to adapt themselves to unavoidable condition (Kim *et al.* 2007). The early biochemical response after faces with salt condition is oxidative burst, the production of reactive oxygen species (ROS) in the cell (Allakhverdiev *et al.* 2005). In chloroplast, the oxidative burst was directly produced by photosystem II (PSII) (Asada 1999) that resulted to reduction of photosynthetic capacity and yield loss.

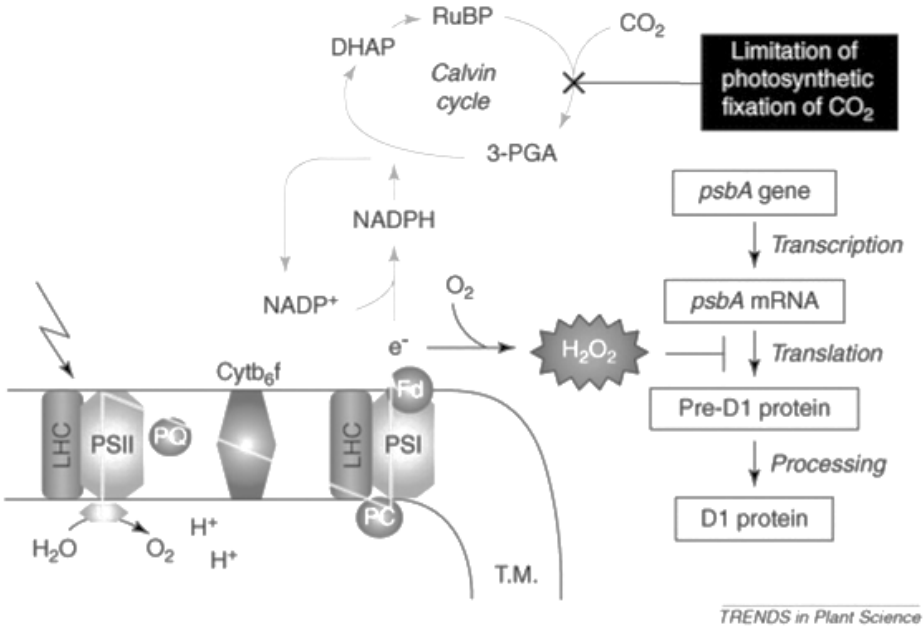


Figure 2. 4 The illustration showed the process of photoinhibition. The D1 protein synthesis was suppressed by the limitation of photosynthetic fixation of CO_2 generating hydrogen peroxide that repressed the translation of D1 protein. (Takahashi and Murata 2008)

The photoinhibition resulted from the balance between rate of photodamage and rate of repair to photosystem II (PSII). Likewise, the photodamage was strongly correlated with the light intensity (Allakhverdiev *et al.* 2005). Moreover, the photoinhibition is the process that suppressed the activity of PSII during high light condition and wide range abiotic stress including salt stress (Murata *et al.* 2007). However, the mechanism of salt stress affected to PSII still debated.

The mechanism of salt-induced photoinhibition was proposed in 3 possible processes. Firstly, the high concentration of salt suppressed the protein synthesis by reduced the activity of translation machinery. At the high concentration of salt, it significantly reduced ribosome stability and polysome (Brady *et al.* 1984). Secondly, when facing the hypersaline condition, it suppressed the activity of Rubisco that directly affected to CO_2 fixation process. (Solomon *et al.* 1994). Lastly, the salt condition induced the passively transport of sodium ion into the cell which repressed the function of ATP synthase involving in the repair mechanism of PSII (Allakhverdiev *et al.* 2005).

CHAPTER III

METERIALS AND METHODS

MATERIALS

1. Plant materials

1.1. Rice (*Oryza sativa* L.)

1.1.1.Leung Pra Tew 123 (LPT123) rice

LPT123 rice is the original Thai rice seed was obtained as seed stock from the Rice Department, Ministry of Agriculture and Cooperatives, Thailand.

1.1.2.Leung Pra Tew 123- TC171 (LPT123-TC171) rice

LPT123-TC171 rice was selected from LPT123 calli grown on selective medium supplemented with 85mM NaCl. Only survival calli were chosen (Thikart *et al.* 2005). LPT123-TC171 was grown at Department of Botany, Faculty of Science, Chulalongkorn University.

Both seeds were germinated in moist sand for a week then transferred to the modified WP nutrient solution (Vajrabhaya and Vajrabhaya 1991) for another week in greenhouse under natural lighting before DNA extraction process.

1.2. Arabidopsis (*Arabidopsis thaliana* L.)

1.2.1. *Arabidopsis thaliana* L., ecotype Columbia

1.2.2. Two independent lines of transgenic Arabidopsis that overexpressed *OsNUC1* gene in L-form (*OsNUC1-L*) and S-form (*OsNUC1*-S) (Sripinyowanich *et al.* 2013) under the controlled of constitutive promoter, *35SCaMV*.

2. Bacterial strains

2.1. *Eschericia coli* strains DH5-alpha

2.2. *Agrobacterium tumerfaciens* strains GV3101 harboring the activation-tagging plasmid pSKI015 (Weigel *et al.* 2000)

3. Instruments

3.1. Equipment for genome analysis of salt-tolerant rice

3.1.1.Equipment for molecular karyotyping and exome sequencing

- Bioanalyzer (Agilent Technology, USA)
- Centrifuge5424/5424R (Eppendorf, USA)
- DynaBeads (Life Technology)
- Genome Analyzer II (Illumina Inc., USA)
- Micropipette (Pipetman, USA)
- Nanodrop 1000 spectrophotometer (Thermo Scientific, USA)
- Thermal Cycler (Bio-Rad, USA)
- The Qubit® 2.0 Fluorometer (Invitrogen, USA)
- Thermomixer®R (Eppendorf, USA)
- UVP imaging (Labworks software, Germany)

3.2 Gene expression study of *OsNUC1*

3.2.1 Equipment for rice and Arabidopsis growing

- 4" pot
- 8"x12"x4" plastic tray
- Glass bottle 100ml
- Peat moss
- Perlite

- Sand
- Vermiculite

3.2.2 Equipment for RNA extraction

- Deep freezer -80°C (Thermo Scientific, USA)
- Microcentrifuge tube (Labcon North American, USA)
- Micropipette (Pipetman, USA)
- Mortars and pestles
- Refrigerated centrifuge (Univeral 16, Hettich, Germany)
- Spatula
- Spectrophotometer (Agilent Technology, USA)
- Vortex mixture (Labnet, USA)
- Water bath (Labtech, India)

3.2.3 Equipment for formaldehyde gel for fractionating RNA

- GelDoc^{TM} 2000 and UV transsilluminator (Bio-Rad, USA)
- Horizontal gel electrophoresis (Amersham Pharmacia Biotech UK limited, UK)
- Microcentrifuge tube (Labcon North American, USA)
- Micropipette (Pipetman, USA)
- Ruler
- Water bath (Labtech, India)

3.2.4 Equipment for northern blotting and detection

- Bio-Rad GS Gene Linker^{TM} UV Chamber

- Film cassette with intensifying screen: 35x43 cm (Amersham Pharmacia Biotech UK limited, UK)
- Filter paper: Whatman No.1 (Whatman International Ltd.)
- Forceps
- Hot air oven (Binder, USA)
- Hot plate (Fisher Scientific, USA)
- Hybond N^+ (Amersham Pharmacia Biotech UK limited, UK)
- Hybridization oven (Techne Hybridizer HB-1D, Lab Extreme, Inc., USA)
- Microcentrifuge (Biofuge pico, UK)
- Microcentrifuge tube (Labcon North American, USA)
- Micropipette (Pipetman, USA)
- Water bath (Labtech, India)
- X-ray film (Kodak(Australia) PTY, LYD, Australia)

3.3 Comparison of salt-resistant role of two OsNUC1 splicing forms during salt stress

3.3.1 Equipment for plasmid construction and bacterial transformation

3.3.1.1 Equipment for molecular cloning

- Cuvette (Agilent technology, USA)
- Incubator (Memmert, Germany)
- Lamina flow (Astec Microflow, UK)
- Microcentrifuge (Microfuge pico, UK)
- Microcentrifuge tube (Labcon North American, USA)
- PCR thin wall microcentrifuge tube 0.2ml (Axygen Scientific, Inc., USA)

- PTC-100™ programmable thermal controller (MJ research, USA)
- Spectrophotometer
- Water bath (Labtech, India)

3.3.1.2 Equipment for bacterial culture

- 0.22μm Millipore membrane filter (Millipore, USA)
- Glass plate
- Incubator (Memmert, Germany)
- Lamina flow (Astec Microflow, Bioquell Medical Limited, UK)

3.3.1.3 Equipment for DNA plasmid extraction

- Deep freezer -80°C (Thermo Scientific, USA)
- Microcentrifuge (Biofuge pico, UK)
- Microcentrifuge tube 1.5ml, (Labcon North American, USA)
- Vortex mixture (Labnet, USA)

3.1.2.Equipment for Arabidopsis transformation

- Microcentrifuge (Biofuge pico, UK)
- Vortex mixture (Labnet, USA)

3.1.3.Equipment for *OsNUC1* expression quantification

- 96-well plate (Bio-Rad, USA)
- CFX96™ Real-time PCR detection system (Bio-Rad, USA)
- Microcentrifuge (Biofuge pico, UK)
- PCR thin wall microcentrifuge tube 0.2ml (MJ research, USA)
- Vortex mixture (Labnet, USA)

3.1.4.Equipment for study of salt stress response in transgenic Arabidopsis

3.1.4.1. Equipment for growth measurement

- Aluminum foil
- Balance, Sartorius CP423s (Scientific Promotion C., USA)
- Forceps
- Hot-air oven (Binder, USA)
- Scissor

3.1.4.2. Equipment for primary root length

- Digital camera (Olympus, Japan)
- Ruler

3.1.5.Equipment for photosynthetic measurement and stomatal response detection

- 6400-15 extended reach 1cm chamber (Li-Cor Inc., USA)
- LI-6400XT Portable Photosynthesis System (Li-Cor Inc., USA)
- Aquarium lighting 55W (Hopar™, China)

3.1.6.Equipment for study of the impact of *OsNUC1* expression on the other gene expression in transgenic Arabidopsis by RNA-Seq

3.1.6.1. Equipment for library preparation

- Bioanalyzer (Agilent Technology, USA)
- Centrifuge5424/5424R (Eppendorf, USA)
- DynaBeads (Life Technology, USA)
- Genome Analyzer II (Illumina Inc., USA)
- Micropipette
- Nanodrop 1000 spectrophotometer (Thermo Scientific, USA)

- Thermal Cycler (Bio-Rad, USA)
- The Qubit® 2.0 Fluorometer (Invitrogen, USA)
- Thermomixer®R (Eppendorf, USA)
- UVP imaging (Labworks software, Germany)

3.2. Equipment for study the relationship between *OsNUC1* and ABA

3.2.1.Equipment for study the effect of ABA on seed germination

- 1000ml Tip
- Glass plate
- Surgical tape (3M, USA)

4. Chemicals and reagents

4.1. Chemicals and reagents for genome analysis of salt-tolerant rice

4.1.1.Chemical and reagent for molecular karyotyping and exome sequencing

4.1.1.1. Genomic DNA library preparation

- 5X TBE (Appendix A)
- 100mM dATP (New England Biolabs, USA)
- Absolute ethanol (Merck, Germany)
- AMPure® XP beads (Beckman Coulter Inc., USA)
- Plant RNA extraction (Invitrogen, USA)
- DNA polymerase I (New England Biolabs, USA)
- Dynabeads® mRNA Purification Kit (Invitrogen, USA)
- Elution buffer (Qiagen, Germany)
- End repair module (New England Biolabs, USA)
- Ethidium bromide (Promega, USA)
- Fragmentase enzyme (New England Biolabs, USA)

- GeneRulerTM 1kb DNA ladder plus (Fermentas, USA)
- Genomic DNA extraction buffer (Appendix A)
- Hypure™ molecular biology grade water (HyClone, USA)
- Isopropanol (Merck, Germany)
- Klenow fragment (New England Biolabs, USA)
- Phenol: Chloroform: Isoamyl alcohol (25:24:1) (V/V)
- Phusion DNA polymerase (New England Biolabs, USA)
- Quick ligation kit (New England Biolabs, USA)

4.1.1.2. Exome library preparation

- 5X TBE (Appendix A)
- 100mM dATP (New England Biolabs, USA)
- Absolute ethanol (Merck, Germany)
- AMPure® XP beads (Beckman Coulter Inc., USA)
- Capture probes (Bioo Scientific, USA)
- Plant RNA extraction (Invitrogen, USA)
- DNA polymerase I (New England Biolabs, USA)
- Dynabeads® mRNA Purification Kit (Invitrogen, USA)
- Elution buffer (Qiagen, Germany)
- End repair module (New England Biolabs, USA)
- Ethidium bromide (Promega, USA)
- Fragmentase enzyme (New England Biolabs, USA)
- GeneRuler™ 1kb DNA ladder plus (Fermentas, USA)
- Genomic DNA extraction buffer (Appendix A)
- Hypure™ molecular biology grade water (HyClone, USA)
- Isopropanol (Merck, Germany)

- Klenow fragment (New England Biolabs, USA)
- Phenol: chloroform: isoamyl alcohol (25:24:1) (V/V)
- Phusion DNA polymerase (New England Biolabs, USA)
- Quick ligation kit (New England Biolabs, USA)

4.2. Chemicals and reagents for gene expression study of *OsNUC1*

4.2.1.Chemicals and reagent for RNA extraction

- 3M sodium acetate, CH_3COONa (Sigma-Aldrich Co., USA)
- 5M sodium chloride, NaCl (Merck, Germany)
- 5X TBE (Appendix A)
- 80% ethanol
- Absolute ethanol (Merck, Germany)
- Chloroform (Merck, Germany)
- Cloned DNase I (RNase-free) (Takara Bio Inc., Japan)
- Diethly pyrocarbonate, DEPC (Sigma-Aldrich Co., USA)
- RQ1 RNase-free *DNase*I (Takara Bio Inc., USA)
- Elution buffer (Qiagen, Germany)
- Ethidium bromine (Promega, USA)
- Hydrogen peroxide (Fisher Scientific, USA)
- iScript reverse transcription supermix (Bio-Rad, USA)
- Isopropanol (Merck, Germany)
- Liquid nitrogen
- Lithium chloride (Sigma, USA)
- Phenol: choloform: isoamyl alcohol (25:24:1) (V/V)
- Plant RNA Reagent (Invitrogen, USA)
- RNA loading dye for formaldehyde gel (Appendix A)

- TE buffer (Appendix A)

4.2.2.Chemicals and reagents for formaldehyde gel for fractionating RNA

- 0.5M EDTA
- 10X MOPS (Appendix A)
- 40% formaldehyde (Carlo Erba Reagents, France)
- Agarose (Research Organics, USA)
- DEPC-treated water
- Ethidium bromide (Promega, USA)
- RNA loading dye for formaldehyde gel (Appendix A)
- RNA marker (RNA ladder, New England Biolabs, USA)

4.2.3.Chemicals and reagents for northern blotting

- 20X SSC (Appendix A)
- Hybridization solution (Appendix A)
- Prehybridization solution (Appendix A)
- Random hexamer
- Washing buffer (Appendix A)
- alpha-[^{32}P]-dCTP
- Developer and fixer solution (Kodak, Australia PTY. LTD., Australia)

4.3. Chemicals and reagents for comparison of salt-resistant role of two *OsNUC1* splicing forms during salt stress

4.3.1.Chemicals for rice and Arabidopsis growing

- Distilled water
- Modified WP no.2 nutrient solution (Appendix A)

- MS media (Sigma, USA)
- NaCl (Merck, Germany)

4.3.2.Chemicals and reagents for plasmid construction and bacterial transformation

- LB media (Appendix A)
- Kanamycin
- Glycerol (Ajex Finechem Pty Ltd., Australia)
- Calcium chloride
- Zero Blunt® TOPO PCR cloning vector (Invitrogen, USA)
- Restriction endonuclease: *Xba*I, *Sac*I and *Hind*III (New England Biolabs, USA)
- *pfu* polymerase reaction mixture (Invitrogen, USA)
- 80% ethanol
- Sodium acetate, CH_3COONa (Merck, Germany)

4.3.3.Chemicals and reagents for plasmid DNA extraction

- Absolute ethanol (Merck, Germany)
- Phenol: chloroform (1:1)(v/v) (Merck, Germany)
- RNase A (Sigma-Aldrich Co., USA)
- Sodium acetate (CH_3COONa) (Sigma-Aldrich Co., USA)
- Solution I (Appendix A)
- Solution II (Appendix A)
- Solution III (Appendix A)
- TE buffer (see in Appendix A)

4.3.4.Chemicals and reagents for Arabidopsis transformation

- DNA extraction buffer (Appendix A)
- LB media (Appendix A)
- Rifampicin
- Silwet L-77 (Lehle Seeds, USA)
- Sucrose (Sigma, USA)

4.3.5.Chemicals and reagents for *OsNUC1* expression quantification

- Hypure™ molecular biology grade water (HyClone, USA)
- SsoFast™ EvaGreen® Supermix (Bio-Rad, USA)

4.3.6.Chemicals and reagents for study the salt response on transgenic Arabidopsis

4.3.6.1. Chemicals and reagents for growth determination

- Agar
- MS media (Sigma, USA)
- Sodium chloride, NaCl (Merck, USA)
- Sodium hypochlorite (Avantor™ Performance Materials, Inc., USA)
- Sucrose (Sigma, USA)
- Tween20 (Promega, USA)

4.3.7.Chemicals and reagents for photosynthetic measurement and stomatal response detection

- Sodium chloride, NaCl (Merck, Germany)

4.3.8.Chemicals and reagents for study the impact of *OsNUC1* expression on other gene expression in transgenic Arabidopsis by RNA-Seq

4.3.8.1. Chemical and reagent for RNA-Seq library preparation

- 5X TBE (Appendix A)
- 100mM dATP (New England Biolabs, USA)
- 3M Sodium acetate, CH3COONa
- 5M Sodium chloride, NaCl
- 5x Second strand synthesis buffer (Appendix A)
- Absolute ethanol (Merck, Germany)
- AMPure® XP beads (Beckman Coulter Inc., USA)
- Chloroform (Merck, Germany)
- Plant RNA extraction (Invitrogen, USA)
- DNA polymerase I (New England Biolabs, USA)
- Dynabeads® mRNA Purification Kit (Invitrogen, USA)
- Elution buffer (Qiagen, Germany)
- End repair module (New England Biolabs, USA)
- Ethidium bromide (Promega, USA)
- Fragmentase enzyme (New England Biolabs, USA)
- GeneRuler^{TM} 1kb DNA ladder plus (Fermentas, USA)
- Hypure^{TM} molecular biology grade water (HyClone, USA)
- Isopropanol (Merck, Germany)
- Klenow fragment (New England Biolabs, USA)
- Phenol: choloform: isoamyl alcohol (25:24:1) (V/V)
- Phusion DNA polymerase (New England Biolabs, USA)
- Quick ligation kit (New England Biolabs, USA)
- RNA loading dye (Appendix A)

- RNase A (Sigma-Aldrich, USA)
- SuperScript™ III First-Stand Synthesis System (Invitrogen, USA)

4.4. Chemicals and reagents for the study of the relationship between *OsNUC1* gene and ABA

4.4.1.Chemical and reagent for germination test

- Abscisic acid (Sigma, USA)
- Agar
- MS media (Sigma, USA)
- Sodium hypochlorite (Avantor™ Performance Materials, Inc., USA)
- Sucrose (Sigma, USA)
- Tween20 (Promega, USA

METHODS

1. Genome analysis of salt-tolerant rice

1.1. Molecular karyotyping

1.1.1 DNA extraction and genome library preparation

For genomic library, DNA was extracted from 3-4 week-old rice seedlings by modified CTAB method (Thikart *et al.* 2005). One microgram of DNA was cleaned up with AMPure (Beckman Coulter) and the DNA was fragmented by dsDNA Fragmentase (New England Biolabs, USA). Sheared DNA was modified using End repaired enzyme (New England Biolabs, USA) and deoxyadenosine was added at the 3´ end using Klenow fragment enzyme (New England Biolabs, USA). Next, the unique DNA barcodes (Bioo Scientific) were jointed to each of the DNA libraries by using DNA ligase (New England Biolabs, USA). Fragments with approximate size of 300 bp were selected and cleaned using AMPure method (Beckman Coulter). The DNA fragments were amplified by PCR for 14 cycles. Four libraries were loaded to Illumina Genome Analyzer and ran together.

The DNA barcodes of all short-sequence reads from Illumina Genome Analyzer were removed and pooled into the right categories then the sequence reads were uniquely aligned and mapped to the Rice genome database (RGAP6.0) using BWA algorithm (Li and Durbin 2009).

1.1.2 Molecular karyotyping

The molecular karyotyping was performed according to Henry and colleague's method (Henry *et al.* 2010). The small sequencing read of each library was pooled into the 1000 base pairs bins according to the position on reference genome.

For the normalization method, since the ranges of raw data in DNA libraries were varied in term of depth, each dataset was rescaled to the ranges of1000 counts. This is to adjust all DNA libraries into the same scale for the comparable measurement.

The normalization formula is given as:

$$x_i' = \left\lceil \frac{x_i}{x_{max}} \times 1000 \right\rceil$$

Where x_i' = the rescaled data, x_i = the original data, and x_{max} = the maximum value of all values, x_i.

To identify large indels, three independent replications of the normalized reads for both rice lines were compared in term of the amount of reads found in a considered region with those in the rest of the whole genome by using Fisher's exact test. The obtained *p* values were adjusted for multiple testing errors with the Benjamini and Hochberg FDR correction. Significance level for an adjusted *p* value was set at 0.01.

1.2 Exome approach

1.2.1 Exome library preparation

For exome library preparation, libraries were hybridized with the capture probes designed based on the *Oryza sativa* var. Nipponbare database (MSU Rice Genome Annotation Project, version 6) for 72 hours. The capture libraries were cleaned and amplified by PCR using post-capture primers for 8 cycles. The final yields were quantified by the Bioanalyzer (Agilent Technologies). Exome capture libraries were pooled and sequenced followed the Illumina HiSeq2000's protocols (Illumina, USA).

The DNA barcodes of all short-sequence reads from Illumina Genome Analyzer were removed and pooled into the right categories then the sequence reads were uniquely aligned and mapped to the Rice genome database (RGAP6.0) using BWA algorithm (Li and Durbin 2009).

1.2.2 Mutation discovery and evaluation of the degree of mutation

Mutation detection was performed using Mutations And Polymorphisms Surveyor (MAPS, (Henry *et al.* 2014)). MAPS pipeline was used to estimate the mutation rate and identify mutation. In brief, MAPS screened all libraries and detected the distinct nucleotide by which each library played as a control for others (Henry *et al.* 2014). Mutation rate was calculated from the number of position divided by the total sequenced space (Mbp). To evaluate the degree of mutation, we used SnpEffect version 2.0.5 (Reumers *et al.* 2006) to identify the location of the mutation on the reference genome. Next, SIFT score (Sorting Intolerant From Tolerant) (Ng and Henikoff 2003) was used to estimate the impact of mutation on protein function in the LPT123-TC171 genome.

2. Northern blot analysis of *OsNUC1*

Seeds of wild-type LPT123 rice and its derived salt-tolerant mutant line, LPT123-TC171 (Thikart *et al.* 2005) were germinated on sand and then transferred to WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya 1991) and they were grown in the greenhouse under natural condition. Total RNA was extracted from leaf tissues of 2 week-old plants after 0 and 48 hour after subjected salt stress condition (nutrient solution supplemented with 85mM NaCl) according to Thikart and colleagues (Thikart *et al.* 2005). Northern blot analysis was done by using Os*NUC1-S* cDNA as a probe.

3. Investigation of the role of the two *OsNUC1* forms during salt stress

To investigate the role of the two *OsNUC1* forms, transgenic Arabidopsis lines with the overexpression construct of *OsNUC1-L* and *OsNUC1-S* cDNA were used to characterize the physiological responses and transcriptomes under normal and salt stress conditions. The transgenic lines with the overexpression construct of *OsNUC1-S* cDNA was provided by Dr. Siriporn Sripinyowanich (Sripinyowanich *et al.* 2013) and for the transgenic lines with the overexpression construct of *OsNUC1-L* were created in this dissertation.

3.1 Plasmid construction

To create the overexpression construction, the *OsNUC1-L* (GENBANK accession number: AK103446) was amplified with forward and reverse gene-specific primers, as followed; 5′GGCTCTAGACGCTTCCTCGTTTGGAATTA 3′and 5′CCTGAGCTCTCGGGCAAATTTACCAACAC 3′ respectively. The PCR condition was as follows: denaturation at 94°C for 5 minutes then followed with 40 cycles of 94°C for 30 seconds, 57°C for 30 seconds and 72°C for 45 seconds. Then the final extension was performed at 72°C for 7 minutes. The blunt-end PCR product was cloned into pCR®- Blunt II-TOPO® vector (Invitrogen, USA) before cloned into plant transformation vector, pJim19 plasmid to generate the plant gene expression vector with the regulation of *35SCaMV* promoter and kanamycin resistance marker gene (*nptII*). The map of the construct was shown in Figure 3.1

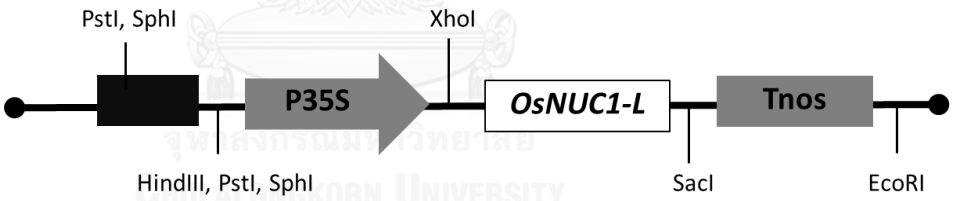


Figure3. 1The schematic diagram of the overexpression construct of *OsNUC1-L* under the control of *35SCaMV* promoter.

3.2 Arabidopsis transformation

The recombinant construct was transferred into *Agrobacterium tumefaciens* strain GV3101 for plant transformation and introduced into wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) by floral dipping method (Clough and Bent 2008).

The T_0 seeds were screened on the MS medium (Murashige and Skoog 1962) supplemented with 50μg/ml kanamycin and grew in tissue culture room and the resistant seedlings were collected as T_1 generation. The T_1 plants were subjected for PCR detection to confirm the existence of the *OsNUC1* overexpression construct by using gene specific primer for *OsNUC1-L*. DNA was used as the template for PCR detection. The genomic DNA extraction was done by using modified CTAB method (Thikart *et al.* 2005). The thermal cycle was performed at 98°C for 30 seconds, then 40 cycles of 98°C for 30 seconds, 58.2°C for 30 seconds and 72°C for 45 seconds followed by a final extension at 72°C for 5 seconds.

3.3 OsNUC1-L expression quantification

Total RNA was isolated from whole plants of two-week-old wild-type and transgenic Arabidopsis with *OsNUC1* expression using Plant RNA Reagent (Invitrogen, USA). One μg of DNase-treated RNA was used as the template to synthesize the cDNA with iScript™ cDNA Synthesis Kit (Bio-Rad). The expression of *OsNUC1* and *AtEF-1alpha*, as the internal control, was performed by quantitative PCR using Ssofast Evagreen supermix (Bio-Rad). The quantitative RT-PCR was performed using gene specific primer for *OsNUC1-L* and *AtEF-1alpha* as followed:

AtEF-1alpha forward primer: 5′TTCTCCGAGTACCCACCTTT 3′
AtEF-1alpha reverses primer: 5′A TTTGGCACCCTTCTTCACT 3′

OsNUC1-L forward primer: 5′ ATGGATCTGACCTCGGTGGA 3′
OsNUC1-L reverses primer: 5′ GTCTTCCTCCTCTCTCAGTG 3′

The thermal cycle was performed at 98°C for 30 seconds, then 49 cycles of 98°C for 5 seconds, 58.2°C for 10 seconds followed by a final extension at 58.2°C for 5 seconds. After the PCR amplification step, the PCR

products were subjected to melting-curve profile to certify the product uniformity. The relative expression of *OsNUC1* was calculated using Pfaffl's approach (Pfaffl 2001)

$$expression\ ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

3.4 Study of the salt-stress responses in transgenic Arabidopsis

3.4.1 Relative growth rate

Seeds of wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) and transgenic lines were surface sterilized with 10% bleach. The seeds were placed on half-strength MS media (Murashige and Skoog 1962) and stratified at 4°C for 2 days and then transferred and vertically placed to culture room that set at 25°C, with a light intensity of 100 μmole $m^{-1}s^{-1}$ and 16-hour a day. The relative growth rate (RGR) was calculated using the formula is given;

$$RGR = \frac{(ln_{W2} - ln_{W1})}{(t_2 - t_1)}$$

Where;

ln = natural logarithm

t_1= time at the original stage

t_2 = time at the later stage

$W1$ = dry weight at the t_1

$W2$= dry weight at the t_2

3.4.2 Primary root growth measurement

Seeds of wild-type Arabidopsis and transgenic lines were germinated on MS plates as indicated in 3.4.1. For the primary root length measurement, the five-day-old seedlings were placed on MS media with and without 100mM NaCl supplemented and took a picture every day for 10 days. The primary root length was measured using Image J program.

3.5 Determination of gas exchange parameters

Wild type and transgenic Arabidopsis were grown under the controlled condition; 25^{o}C, 180 µmole photon $m^{-1}s^{-1}$, and 16-hour a day. The four-week-old plants were used to measure photosynthetic activity, stomatal conductance, transpiration rate by using Gas Analysis System (Li-Cor, LI-6400) with 6400-15 extended reach 1cm diameter chamber. The gas exchange measurement was performed at 25^{o}C under external light source 650 µmole photon $m^{-1}s^{-1}$, with the reference carbon dioxide concentration 350 µmole $m^{-1}s^{-1}$ and relative humidity of 40-44 %. For the salt treatment, wild type and transgenic Arabidopsis were treated with 25ml of 100mM NaCl/pot by irrigation.

3.6 The impact of OsNUC1-L overexpression on other gene expression in the transgenic Arabidopsis

The impact of *OsNUC1* overexpression on other gene expression in the transgenic Arabidopsis was determined by transcriptome analysis. Three biological replicates of the wild-type and transgenic Arabidopsis were used for RNA-seq library generation. Two-week old plants grown on the MS medium (Murashige and Skoog 1962) were transferred on the MS medium containing 100mM NaCl for salt stress condition and transferred onto the freshly prepared MS medium for the control condition. After 5 hours of the treatment, total RNA was isolated with Plant RNA Reagent (Invitrogen, USA).

The mRNA was isolated with Dynabeads mRNA purification (Invitrogen, USA) and converted to cDNA with SuperScript III (Invitrogen, USA). The cDNA was fragmented by dsDNA fragmentase (NEB). Fragment sizes of 300 bp were selected and purified using AMPure protocol. The DNA fragments were enriched by PCR for 10 cycles. The unique DNA barcodes were ligated to each cDNA libraries. All six libraries were loaded to Genome Analyzer (Illumina) and ran together.

The transcriptome analysis was done to compare the transcripts from the wild type and transgenic plants under salt stress and control condition. Briefly, all short-sequence reads from Genome Analyzer were removed the DNA barcode and grouped them in the right category using the pipeline created by Missirian and colleagues (Missirian *et al.* 2012). The sequence reads were aligned and mapped to Arabidopsis genome database (TAIR10) using Bowtie aligner (Langmead *et al.* 2009) and TopHat (Trapnell *et al.* 2009). The DESeq (Anders and Huber 2010) was used to verify the differentially gene expression.

4. Study the relationship between *OsNUC1-L* and ABA

4.1 The responses of transgenic Arabidopsis to ABA on seed germination

The germination assay was carried with 4 replications of 45 seeds/replication. Seeds of wild-type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) and 3 independent transgenic lines (*35SCaMV::OsNUC1-L*) were surface sterilized with 10% Clorox. The seeds were placed on half-strength Murashige and Skoog media (Murashige and Skoog 1962) and stratified at 4°C for 2 days and then transferred to culture room that set at 25 $^{\circ}$C, with a light intensity of 100 μmole $m^{-1}s^{-1}$ and 16-hour day. For the germination test, seeds of *OsNUC1* transgenic and wild-type Arabidopsis were germinated on half-strength MS media supplemented with 0, 0.2, 0.4 and 0.6μM ABA (Sigma).

CHAPTER IV

RESULTS AND DISCUSSION

1. Genome analysis of salt-tolerant rice

To understand the genetic variation and the adaptation of this somaclonal line at the molecular level, we used the next-generation sequencing technology to examine the genetic basis of difference between LPT123 and LPT123-TC171 rice. The results provided us with the information of the salt-tolerant rice genome that gave new insights of salt-tolerant mechanisms in rice.

1.1 Karyotyping analysis

After approximately 22,728,672 and 35,097,873 sequence reads of LPT123 and LPT123-TC171 with three replicates were mapped to the rice reference genomes (RGAP6.0). To study large indels, all the short sequence reads were counted, rescaled, and compared between LPT123 and LPT123-TC171 genomes.

Based on the above hypothesis, 3 replicates of read counts for our two rice cultivars were tested using Fisher's Exact Test. After the *p* value correction, we found deletion at telomeres of the rice chromosomes including chromosomes 1, 2, 4, 5, 6, 7, 8 and 10. The telomere regions were found significantly deleted in LPT123-TC171 ($p < 0.01$) and reported with asterisks in Figures 4.1 and 4.2.

Beside the natural senescence process, the oxidative stress had been noted that the external factor that affects the telomere shortening. Von Zglnicki and colleagues (von Zglinicki *et al.* 1995) studied the telomere shortening rate in human fibroblast cell culture under hyperoxia condition which known as one of the oxidative stress conditions. They found that, under lower 40% of ambient oxygen, the telomere shortening rate was 5 times higher than the control group. Moreover, when the cell cultures were treated with alpha-phenyl-*t*-butyl-nitrone, free-radical scavenging molecule, the rate of telomere shortening significantly decreased in the treatment group

(von Zglinicki 2000, von Zglinickia *et al.* 2000). In conclusion, telomere length is sensitive to oxidative stress and the intensity of oxidative stress correlates with the telomere shortening rate.

Telomeres are special structures of the end of eukaryote linear chromosomes, which are composed of tandem repeats of DNA that form a complex structure with specific telomeric proteins (Kass-Eisler and Greider 2000). For the functions, telomeres protect the chromosome from exonuclease and involve in chromosome stability (Greider 1996). In most plants, telomeres have the consensus sequence of $[TTTAGG]_n$, while the length depends on species and varieties (Shakirov and Shippen 2004). Although telomeres compose of the simple repeats with almost no gene found, the change of telomere length can cause alteration of stress response. The *AtKU70* gene, which encodes a DNA binding protein, uniquely locates around the end of chromosome. AtKU70$^{-/-}$ plant lacking the control of telomere length regulation, which resulted in telomere elongation, showed hypersensitive to stresses (Bundock *et al.* 2002, Riha *et al.* 2002). LPT123-TC171 was selected on NaCl-supplemented media, which induced the condition of oxidative stress (Fadzilla *et al.* 1997) suggesting that the telomere shortening in LPT123-TC171 genome resulted from the oxidative stress during screening process.

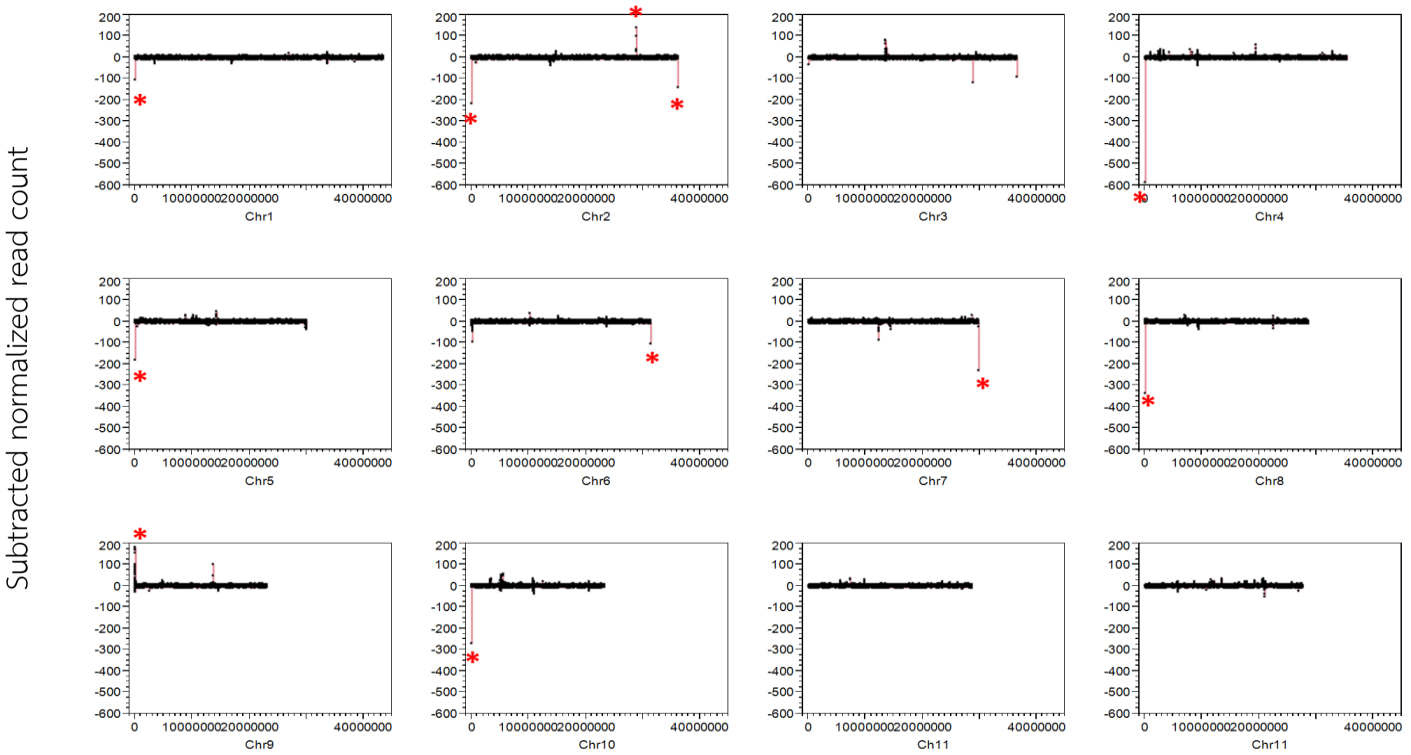


Figure 4. 1 The molecular karyotype displayed the subtraction of average sequencing reads of LPT123-TC171 and LPT123. The x-axis represented the position on chromosome and y-axis represented the subtracted normalize read count. The deletion and duplication of DNA were detected by Fisher's exact test. An adjusted *p*-value of 0.01 was considered significant, $p<0.01$ (asterisk).

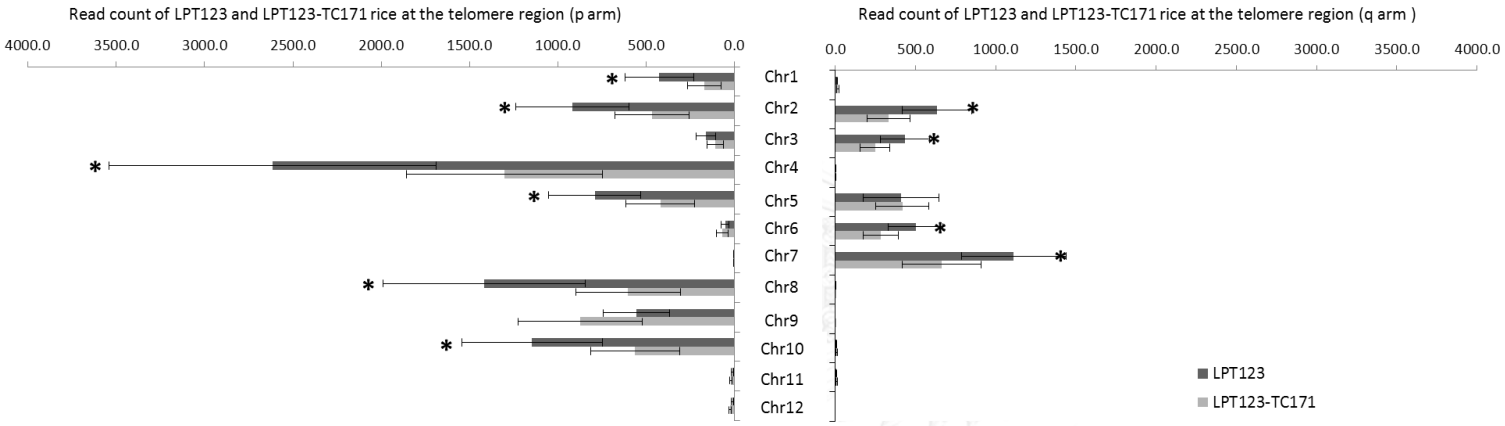


Figure 4. 2 The comparison of read count between LPT123 and its somaclonal variegated line, LPT123-TC171 (mean±SE) on the telomere region (p<0.01)

1.2 Exome approach

LPT123 and LPT123-TC171 exome data were analyzed and the effects of base changes on protein sequence and localization of those changes were classified using SnpEffect version 2.0.5 (Reumers *et al.* 2006). From our survey most mutations found were non-synonymous mutation (243 positions, 41.32%), synonymous mutation (201 positions, 33.11%) and in intron (103 positions, 16.89%) (Figure4.5). From previous study of DNA mutagenesis in *Panax ginseng* cell culture, non-synonymous mutation was reported as the dominance mutation in all genes studied which were, *Actin, PAL, DDS* and *SERK*. Furthermore, based on the comparison of mutation types occurred in short- and long-term cultures of *Panax ginseng*, it showed a higher level of non-synonymous substitution in the long-term culture (Kiselev *et al.* 2011).

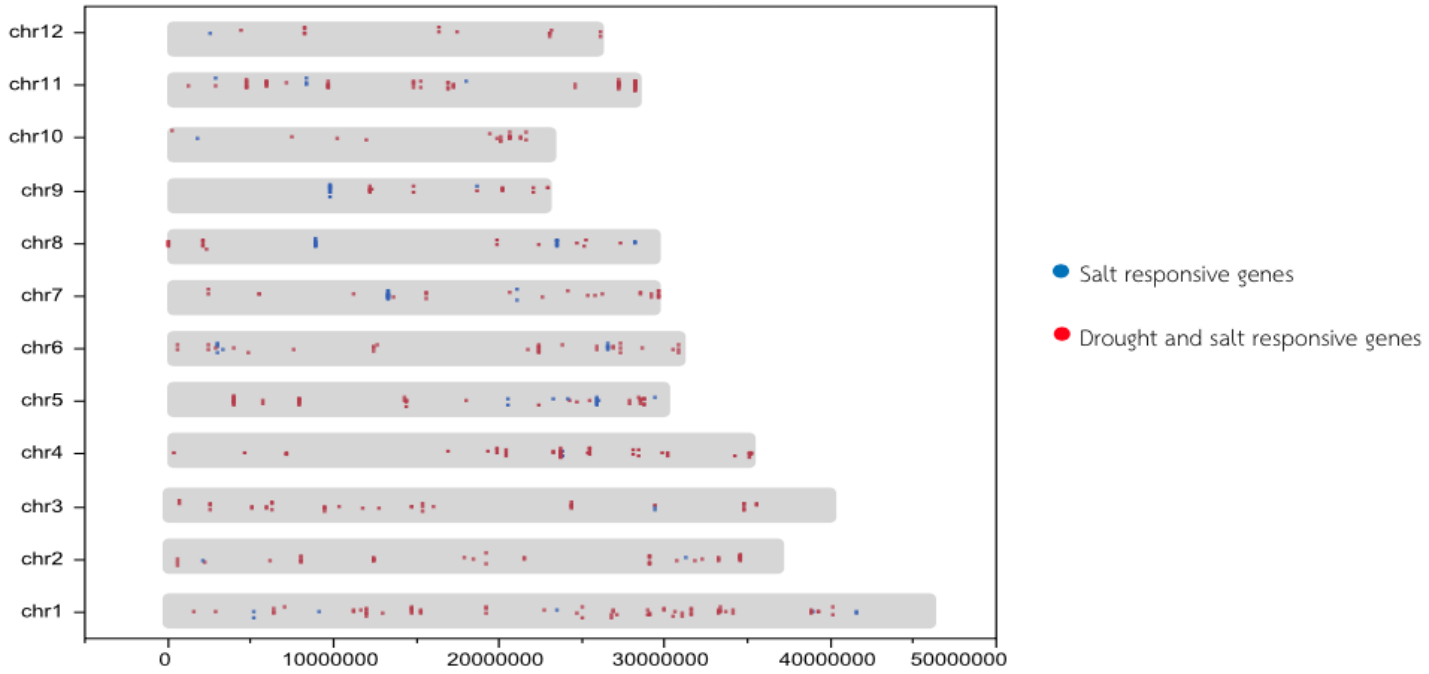


Figure4. 3 The distribution of single nucleotide mutation in LPT123-TC171's genome. Mutations on salt-responsive genes and drought and salt stress-responsive genes were indicated by blue dot and red dot, respectively (Childs *et al.*

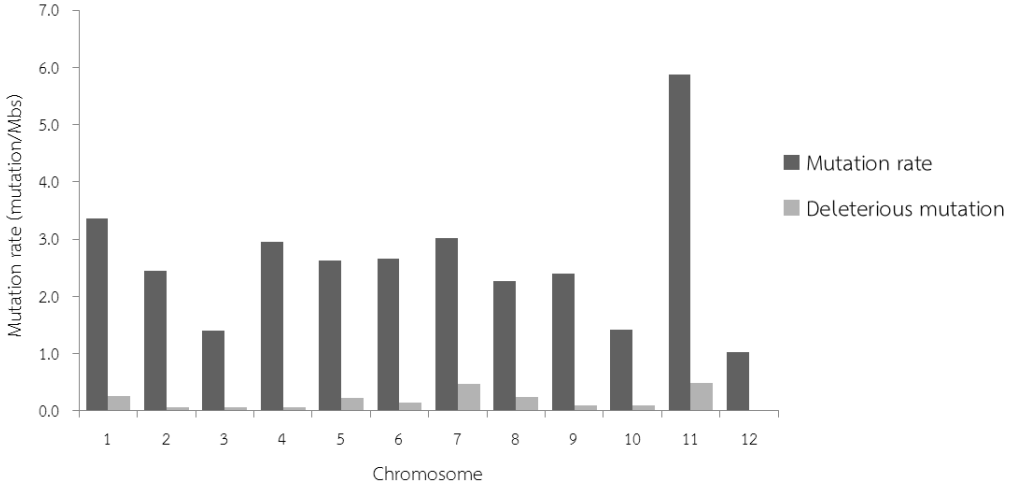


Figure4. 4 The overview of nucleotide mutations in LPT123-TC171 (a) Mutation rate and deleterious mutations on LPT123-TC171 chromosomes

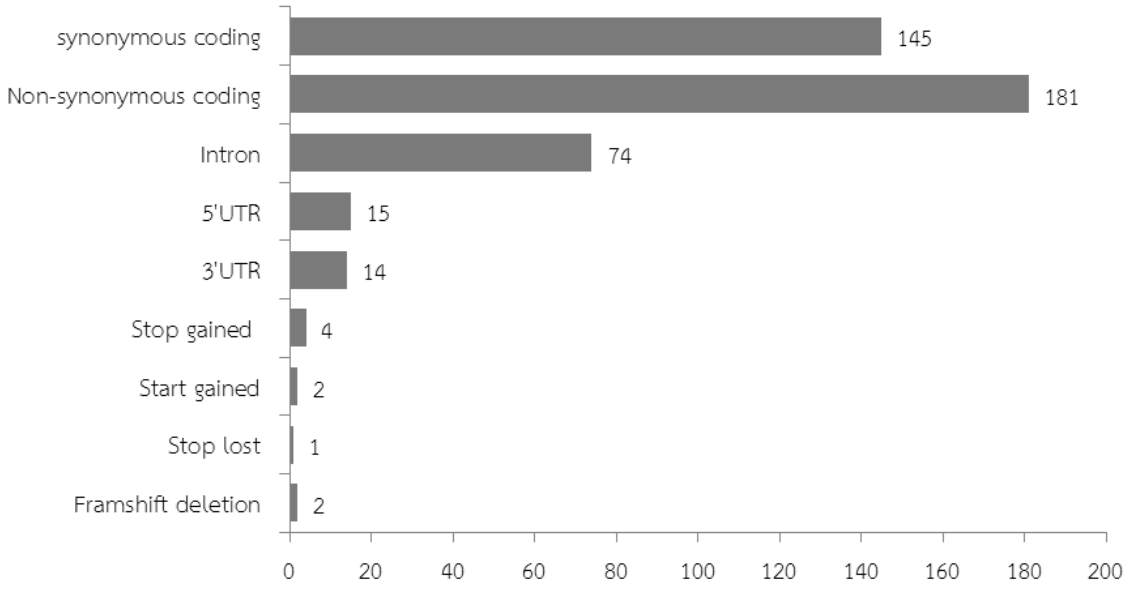


Figure4. 5 Type of mutation in LPT123-TC171 determined by SnpEffect version 2.0.5 (Reumers *et al.* 2006).

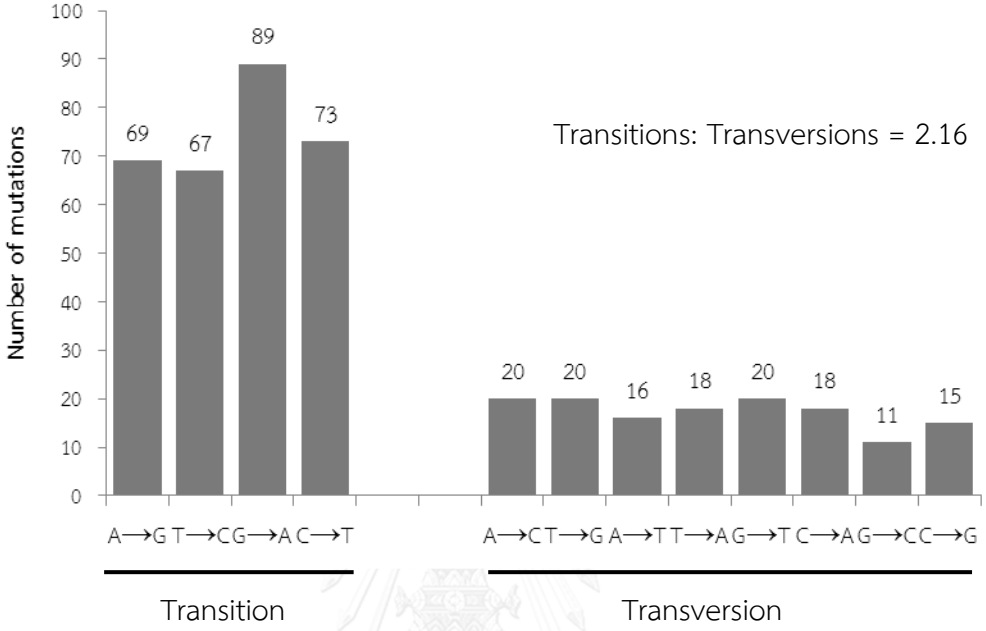


Figure4. 6 Nucleotide substitution frequency of LPT123-TC171 genome determined by MAPS pipeline (Henry *et al.* 2014)

MAPS pipeline (Henry *et al.* 2014) was used to study mutation in LPT123 and LPT123-TC171 genomes. Most of nucleotide substitutions discovered in LPT123-TC171 rice were A↔G and T↔C transitions (Figure4.6). Moreover, the nucleotide substitutions N⟶A, N⟶T, N⟶C and N⟶G were equally found in 154, 147, 150 and 142 positions, respectively. The transition/transversion ratio of LPT123-TC171 was 2.16 (389/204, Figure4.6). This ratio was close to transition/transversion ratio found in natural mutation of AA-genome *Oryza* species which was 1.92 (Kobayashi *et al.* 2005, Noro *et al.* 2007). In contrast, transition and transversion mutations in LPT123-TC171 were found in 389 and 204 positions, while the numbers of spontaneous mutation in rice were 25 and 13, respectively (Noro *et al.* 2007). Based on nucleotide substitution, the genetic pattern of somaclonal variation in rice was similar to spontaneous mutation but different in term of mutation rate.

Unlike whole genome sequencing, the exome capture sequencing specifically sequences only the functional region of a genome (Majewski *et al.* 2011). The hybridization capture was designed from the *Oryza sativa* var. Nipponbare database. From the 100 base pair paired-end Illumina next-generation sequencing, 4 and 14 million reads of LPT123 and LPT123-TC171 were mapped to the Nipponbare reference genome (MSU Rice Genome Annotation Project, version 6). Figure4.3 showed the diagrammatic distribution of nucleotide mutation in the somaclonal line, LPT123-TC171 chromosomes. SNPs thoroughly distributed over the genome of LPT123-TC171 rice. Nucleotide mutation was categorized into two groups by the mode of expression (Childs *et al.* 2011). Exome-based investigation, 35,431 mutations were found within LPT123 and LPT123-TC171 genome in which 212 genes were the salt- and drought-responsive genes. The list of all the salt/drought responsive loci with mutation was showed in appendix C.

The salt-tolerant rice, LPT123-TC171, was selectively cultured on NaCl-supplemented media from LPT123 rice callus (Vajrabhaya and Vajrabhaya 1991). LPT123-TC171 retained normal growth and development and showed better adaptation under salt condition as described in previously report (Udomchalothorn *et al.* 2009). SIFT score (Ng and Henikoff 2003) was applied to calculate the impact of mutation on protein performance. The idea of this method, it searched the conserve region by sequence alignment and score the mutation by the degree of change.

Total of 35,431 pointed mutations in tissue-culture selected rice LPT123-TC171 were discovered the mutation was totally accumulated in 10,000 genes in which 2.12% (212 genes) was the abiotic-associated genes. When compared the mutation in abiotic-associated genes to total mutation by using the enrichment analysis, it revealed no significant enrichment in salt- and drought-associated genes.

Type of mutation	Module	Locus number and gene description	
Loss of Function	Salt responsive genes	LOC_Os05g14220	tyrosine protein kinase domain containing protein
		LOC_Os06g37750	S-locus-like receptor protein kinase
		LOC_Os01g47050	OsFBK1 - F-box domain and kelch repeat containing protein
		LOC_Os11g17380	protein kinase domain containing protein
	Drought and Salt responsive genes	LOC_Os01g21420	pre-mRNA-splicing factor SF2
		LOC_Os04g32480	zinc-finger protein
		LOC_Os05g44570	histidine-containing phosphotransfer protein
		LOC_Os06g44010	WRKY28
		LOC_Os07g23570	cytochrome P450 72A1
		LOC_Os07g26900	alpha-galactosidase precursor

Table4. 1 The deleterious mutated genes within LPT123-TC171 genome divided into two categories; The loss of function genes and gain of function genes. The loss of function genes had lack function in somaclonal rice, LPT123-TC171 while the gain of function genes reverted to their original function in LPT123-TC171.

Type of mutation	Module	Locus number and gene description	
Loss of Function	Salt responsive genes	LOC_Os11g10760	NBS-LRR disease resistance protein
		LOC_Os11g45740	MYB family transcription factor
		LOC_Os08g38990	WRKY30
		LOC_Os08g39694	Cytochrome P450
		LOC_Os07g05370	The CrRLK1L-1 subfamily has homology to the CrRLK1L homolog
		LOC_Os01g27390	Glutathione S-transferase
		LOC_Os09g20284	Amine oxidase, flavin-containing, domain containing protein
		LOC_Os10g04020	OsFBX356 - F-box domain containing protein
		LOC_Os02g47560	DNA-binding protein, putative
		LOC_Os05g34730	Ethylene-responsive transcription factor ERF020
		LOC_Os08g32060	Spotted leaf 11

Table 4.1 (cont.) The deleterious mutated genes within LPT123-TC171 genome divided into two categories; The loss of function genes and gain of function genes. The loss of function genes had lack function in somaclonal rice, LPT123-TC171 while the gain of function genes reverted to their original function in LPT123-TC171.

Type of mutation	Module	Locus number and gene description	
Gain of Function	Drought and Salt responsive genes	LOC_Os03g05200	DENN domain containing protein
		LOC_Os01g25920	Expressed protein

Table 4.1 (cont.) The deleterious mutated genes within LPT123-TC171 genome divided into two categories; The loss of function genes and gain of function genes. The loss of function genes had lack function in somaclonal rice, LPT123-TC171 while the gain of function genes reverted to their original function in LPT123-TC171.

Based on SIFT score calculation, 23 genes were considered as deleterious mutation which seriously affected to their function (Table4.1). The deleterious mutant genes were categorized into 14 groups according their biochemical function.

1. Transcriptional factor

Transcription factors, glutathione S-transferase, polyamine oxidase, cytochrome P450, F-box protein, U-box protein, Jasmonate-Zim-domain protein, NBS-LRR disease resistance protein, alpha-galactosidase precursor, kinase-containing proteins, DENN containing protein, mRNA splicing factor and uncharacterized proteins

Plant practically adapted themselves to the unavoidable environment by evolved a complex of regulatory network controlling gene expression. Transcription factor belongs to the DNA-binding protein that directly controls the gene expression by activation or repression *cis*-regulatory sequence (Bartels and Sunkar 2005). In this study, three types of transcription factor *JAmyb* (LOC_Os11g45740), *ERF* (LOC_Os05g34730) and *WRKY* (LOC_Os06g44010 and LOC_Os08g38990) were mutated which affected the gene functions.

a. JAmyb

MYB is the group of trans-acting factor that implicated in diverse stress response (Abe *et al.* 2003). *OsJAmyb* gene (LOC_Os11g45740) belonged to *MYB* gene family in rice which strongly induced by jasmonic acid, wounding and fungal infection(Lee *et al.* 2001). *OsJAmyb* played the key component of pathogen response signaling by drastically induced the pathogen-related genes (Nahar *et al.* 2013). *OsJAmyb* recently reported the new function in abiotic stress by overexpressing *OsJAmyb* gene in Arabidopsis. The authors revealed that increased of the *OsJAmyb* enhanced the salt stress tolerant. Moreover, the microarray analysis revealed that the transgenic plant induced the large numbers of defense-associated and stress-associated genes.

However, the overproduction of *OsJAmyb* displayed a negatively effect on normal growth. The transgenic Arabidopsis which contained *35S*:*OsJAmyb* exhibited retarded growth (Yokotani *et al.* 2013) suggested that the function of *OsJAmyb* need to be closely investigated in further study.

b. ERF

Ethylene-responsive transcription factor, *ERF20* (LOC_Os05g34730) is the member of the APETAL2/ ethylene element binding factor (AP2/ERF) family. Generally, the ERF transcription factor regulated the gene expression by binding to the specific *cis*-acting element, ethylene-responsive element (Mizoi *et al.* 2012). The survey of LPT123-TC171 genome, *ERF20* gene accumulated severe mutation that caused of loss-of-function of this gene. To date, there are was no report about OsERF20 function under hypersaline or hyperosmotic condition. However, several members of the ERF family were characterized about their role in the abiotic stress. In Arabidopsis, the member of ERF family, *AtERF3* and *AtERF7* were stated as the transcriptional repressors (Kazan 2006). Arabidopsis that overexpressed *AtERF3* showed the insensitive to ABA treatment and increased the salt sensitivity indicated that the *AtERF3* served as the negative regulator to the ABA signaling (Yang *et al.* 2005). Likewise, *AtERF7* also clearly revealed that it suppressed the ABA signaling. Moreover, the *AtERF7* also participated in stomatal closure that directly affected to transpirational water loss(Song *et al.* 2005). Summarily, the function of *ERF20* transcription factor in abiotic stress needs to elucidate in the detail.

c. WRKY

WRKY transcription factors involve in stress response by controlling the expression of genes via the interaction with the specific *cis*-acting element known as W-box sequence (Chujo *et al.* 2013). The WRKY involved in plant response to various environmental conditions including pathogen infection(Dong *et al.* 2003), wounding, drought and salt stress (Shen *et al.* 2012). Previously, WRKY transcription factors revealed their functions as a

crucial factor in ABA signaling (Chen *et al.* 2010, Shang *et al.* 2010). In our study, two *WRKY* genes, *OsWRKY28* (LOC_Os06g44010) and *OsWRKY30* (LOC_Os08g38990), accumulated the serious mutation that leaded to loss of their functional proteins.

OsWRKY28 was stated as a transcriptional repressor of pathogen response by regulated the pathogen-related genes (Chujo *et al.* 2013). According to MSU annotation (http://rice.plantbiology.msu.edu /index.shtml) the homologous gene of Rice *OsWRKY28* are *AtWRKY18* and *AtWAKY60* gene in Arabidopsis. In abiotic stress response, *AtWRKY60* and *AtWRKY18* played a part of downstream in ABA signaling (Chen *et al.* 2010, Shang *et al.* 2010). ABA is a plant hormone that regulates the development, stomatal movement and the control gene expression in order to response to abiotic stress (Wasilewska *et al.* 2008). The lack of function of *AtWRKY60* altered the ABA response in which the knockout mutant showed the ABA- hypersensitive phenotype in germination (Shang *et al.* 2010) and increased the drought and salt tolerant ability which associated with the expression of some ABA-responsive genes (Chen *et al.* 2010). Because *WRKY18* and *WRKY60* involved the stress tolerant mechanism by acting as the suppressor of ABA, the function of *OsWRKY28* in ABA signaling is the interesting gene for the further study.

The *WRKY30* closely associated functions as other members of the group of transcription factors such as the in pathogen defense mechanisms (Peng *et al.* 2012, Han *et al.* 2013). By overproduction of *OsWRKY30* gene expression, the transgenic rice enhanced the drought tolerant. The author suggested that WRKY30 acted as the downstream MAPK signaling (Shen *et al.* 2012).

WRKY gene family showed the diverse response to abiotic stress and ABA signaling.

2. Glutathione S-transferase

Hypersaline and hyperosmotic condition directly induced the production of ROS leading to cell damaging growth retardation by the inhibition of repair system (Bartels and Sunkar 2005). Glutathione S-transferase (GST) involved in the equilibrium of reactive oxygen species in the cell. In rice, the *GST* family contains 59 gene members (Soranzo *et al.* 2004). *GLUTATHIONE S-TRANSFERASE U17* (*AtGSTU17*) gene was proposed as the negative regulator of salt and drought response. Chen and colleagues demonstrated that the *atgstu17* mutant increased the level of glutathione. Glutathione is the tripeptide molecule that controlled the oxidative balance in the cell (Edwards *et al.* 2000). The increasing of glutathione content in *gstu17* mutant could be the key factor in salt and drought-adaptive mechanism. Moreover, the *gstu17* mutant also increased in ABA level leading to the ABA-hypersensitive characters including reduced stomatal apertures, inhibited seed germination and improved root elongation. Besides, the ABA accumulation affected the transcriptomic modification inducing the expression of *AREB1*, *XERICO*, *RAP2*.4, ENH1, and *AnnAt1* leading to the increased in salt tolerant ability in the mutant (Chen *et al.* 2012). In LPT123-TC171 genome, loss-of-function in LOC_Os01g27390 which encodes *GST* could be the key adaptive role of LPT123-TC171 rice that modulated the reactive oxygen scavenging in the cell.

3. Polyamine oxidase

Polyamines are small organic cations such as spermine, spermidine and putrescine. In plants, polyamines played the crucial role in adaptive mechanism to abiotic stress (Alca´zar *et al.* 2006). The polyamine metabolism responds to several abiotic stresses. (Rodrı´guez *et al.* 2009). The level of polyamine in cells was controlled by polyamine oxidase which catalyze the degradation of spermine (Alcázar *et al.* 2010). LOC_Os09g20284 encodes polyamine oxidase enzyme. In stress response mechanism, spermine level in the cell positively correlates with the stress susceptible. The double mutant *acl5/spms* that defectes in spermine production is susceptible to salt and

drought stresses (Kusano *et al.* 2007). Likewise, Kamada-Nobusada and his co-workers characterized the function of *AtPAO4*. The *AtPAO4* constitutively expressed both vegetative and reproductive organ but it was mostly found in roots. The null mutant of *AtPOA4* drastically accumulated high level of spermine while the spermidine content was significantly decreased especially in roots. This data confirmed that the *AtPOA4* functioned on spermine degradation. Interestingly, the *Atpoa4* null mutant were up-regulated the stress-related genes such as late embryogenesis abundant protein (LEA protein), Desiccation-responsive protein 29A (RD29A) and DRE-binding protein (Kamada-Nobusada *et al.* 2008).

4. Cytochrome P450

LOC_Os07g23570 and LOC_Os08g39694 belong to cytochrome P450 gene family which was predicted to potentially lack of function in the somaclonal rice, LPT123-TC171. Cytochrome P450 (P450) is the heme-containing protein involved in various biological processes such as lipid biosynthesis and oxidative reactions (Chapple 1998). Cytochrome P450 gene family compose 286 genes in Arabidopsis(Narusaka *et al.* 2004) and 727 genes in rice (Nelson *et al.* 2004) indicated the diverse evolution and the significant role of P450 gene family. Moreover, the expression of genes in cytochrome P450 family differently responded to the various external stimuli (Narusaka *et al.* 2004). Cytochrome P450 responded to pathogen infection by regulated the pathogen-related genes (Kim *et al.* 2006). However, there was no report the function of cytochrome P450 under abiotic stress. Therefore, LOC_Os07g23570 and LOC_Os08g39694 were up-regulated under abiotic stress (GSE6901,http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6901), they could participate in some stress-response.

5. F-box protein

F-box protein family shares the signature F-box motif which composed of 40-50 amino acid residues. In rice genome, F-box protein family contained 687 members (Jain *et al.* 2007) and approximately 700 members in Arabidopsis (Lechner *et al.* 2006). Generally, F-box protein interacted with the complex of Cullin1 (CUL1), SUPPRESSOR OF KINETOCHORE PROTEIN (SKP1) and RING-BOX1(RBX1)/REGULATOR OF CULLINS1 (ROC1) to form structure that controlled the protein degradation in the cells (Lechner *et al.* 2006, Jain *et al.* 2007). F-Box protein regulates diverse cellular processes including abiotic response. The F-box protein, COI1 was shown to act as the jasmonic acid signaling component which regulated the expression of defend-related genes (Devoto *et al.* 2005). Likewise, the DOR F-box protein was reported as the repressor to ABA signaling in guard cells. The *dor* mutant, the T-DNA insertion of DOR, elevated the ABA level in cell that associated with significantly decreased water loss resulted in drought tolerant in mutant (Kim and Delaney 2002).

The LOC_Os01g47050 and LOC_Os10g04020 were highly up-regulated when challenged with drought and salt stress respectively (Jain *et al.* 2007) suggesting that the F-box protein could play some adaptive mechanisms which improved the salt tolerant ability in LPT123-TC171 rice.

6. U-box protein

Spotted leaf11 (*SPL11*, LOC_Os08g32060) encodes the protein that contains U-box and six armadillo repeat motifs. From Table 4.1, the *SPL11*, was deleteriously mutated in LPT123-TC171, which resulted in the loss-of-function. *OsSPL11* enhanced the pathogen resistance by induced the expression of pathogen-related (PR) genes. Yin and colleagues (2000) selected *spl11* mutant from the EMS-mutagenized population of IR68 rice. The *spl11* mutant globally induced the expression of PR genes which positively correlated with level of bacterial and fungal resistance (Yin *et al.* 2000). Moreover, the study of *PUB22* and *PUB23* Arabidopsis homolog of Rice *SPL11,* showed that they acted as the negative regulator in drought response. The

null mutant of *PUB22* and *PUB23* genes enhanced drought tolerance in Arabidopsis (Cho *et al.* 2008). In conclusion, the *SLP11* played as the repressor both in biotic and abiotic stress responses, the absent of this gene may be the key factor that promoted the LPT123-TC171 rice survived when faced abiotic stress condition.

7. Jasmonate-Zim-domain protein

According the annotation from MSU database, this zinc-finger protein (LOC_Os04g32480) homologues to Arabidopsis Jasmonate-Zim-domain protein 10(JAZ10). In Arabidopsis, Jasmonate-Zim-domain (JAZ) family is composed of 12 genes which diversely interacted with the transcription factors regulating other genes (Kazan and Manners 2011). Based on yeast two-hybrid system, some members of OsJAZ protein family *in vivo* interacted with OsbHLH148 and suppressed the *OsbHLH148* performance.

The *OsbHLH148*, the Rice's basic helix-loop-helix transcription factor148, was up-regulated by drought stress and ABA. The transgenic plants that constitutively expressed *OsbHLH148* improved drought tolerant ability. The transcription factor *OsbHLH148* specifically activated *OsDREB1A,B,C,E* and *G* which associated to the salt response mechanisms (Seo *et al.* 2011). In conclusion, *OsJAZ* was hypothesized to be an inhibitor of drought stress signaling through the inhibition of OsbHLH148.

8. NBS-LRR disease resistance protein

In LPT123-TC171 genome, LOC_Os11g10760, encodig NBS-LRR disease resistance protein, was null mutated. Rice genome contained more than 400 members of Nucleotide-Binding Site Leucine-Rich Repeat (NBS-LRR) gene family (Meyers *et al.* 2003). NBS-LRR proteins are composed of three domains; the leucine-rich repeat domain, the nucleotide binding domain and c-terminus. NBS-LRR genes involve in plant response to pathogen (McHale *et al.*

2006). In biotic stress, the Arabidopsis's NBS-LRR protein *ADR1* was reported to be involve in pathogen defense mechanism by constitutively inducing the key defense maker genes and increasing the level of salicylic acid (Grant *et al.* 2003). In abiotic stress, constitutive expression of *ADR1* gene by the activation-tagged altered the drought stress tolerance, which was correlated with the induction of drought-associated genes (Chini *et al.* 2004). In conclusion, NBS-LRR gene family has diverse functions in the cells. The function of LOC_Os11g10760 could participate in some salt-adaptive mechanisms which needs to study more in further work.

9. Alpha-galactosidase precursor

Alpha-galactosidase catalyzed the hydrolysis of various macromolecules such as lipid, protein and cell wall component (Chrost *et al.* 2007). Moreover, the alpha-galactosidase also played the dominant role in abiotic stress response by controlled the degradation of raffinose. The antisense genetic mutation of alpha-galactosidase altered carbohydrate metabolism especially elevation of raffinose content and improved the freezing tolerance(Pennycooke *et al.* 2003). Likewise, raffinose broadly altered abiotic stress perception. The transgenic that accumulated raffinose content tolerated to freezing, drought and salt stress (Liu *et al.* 2007). Since the LPT123-TC171 genome lacked the function of alpha-galactosidase precursor gene (LOC_Os07g26900), the alpha-galactosidase activity and the raffinose content in LPT123-TC171 to clarify the function of this gene in salt-stress response.

10. Kinase-containing protein

Kinase protein is the key role of plant adaptive to unavoidable condition including stress sensing (Kim *et al.* 2009), ion homeostasis (Liu *et al.* 2000) and controling the expression of stress-associated genes (Teige *et al.* 2004). The histidine kinase *AHK1* is the positive regulator of drought signaling

which induces osmotic-related genes both ABA-dependent and ABA-independent pathways, while *AHK2*, *AHK3* and *CRE1* which function in cytokinin signaling act as the negative regulator of abiotic stress signaling. The *ahk2*, *akh3* and *cre1* null mutants increased osmotic and salt stress tolerant by inducing ABA-responsive genes such as *ANAC055* and *AT-MYC2*. The diverse functions of hitidine kinase family were reviewed by (Tran *et al.* 2007).

The LOC_Os05g44570 (histidine-containing phosphotransfer protein), LOC_Os05g14220 (tyrosine protein kinase domain containing protein) and LOC_Os11g17380 (histidine-containing phosphotransfer protein) were up-regulated under abiotic stress (Jain *et al.* 2007) indicated the important function in stress response mechanisms.

11. Receptor kinase

LOC_Os07g05370 encodes TKL_IRAK_CrRLK1L-1.15 - The CrRLK1L-1 subfamily. Receptor-like kinase (RLK) is the groups of integral membrane proteins, containing kinase domain on the c-terminus. The RLK regulates the diverse cellular processes including biotic and abiotic responses (Ouyang *et al.* 2010). In Arabidopsis, *Catharanthus roseus* RLK1-like kinase (CrRLK1Ls) subfamily was globally down-regulated under abiotic stress. Interestingly, salt and drought stress deactivates different member in *CrRLK1L* suggesting that different *CrRLK1L* played the distinct role in abiotic stress response (Lindner *et al.* 2012). FER encodes the FERONIA receptor-like kinase which controls the balance of reactive oxygen species (ROS) in the cell through the inhibition of NDAPH-oxidase dependent ROS. The suppression of *FER* gene significantly decreased ROS accumulation (Duana *et al.* 2010). In conclusion, balancing the ROS in the cells by the suppression of *CrRLK1Ls* expression could be the mechanism for plants to survive under unavoidable environment.

The mutation of LOC_Os06g37750 in LPT123-TC171 rice encodes S-locus-like receptor protein kinase. The S-locus receptor kinase (SRK) is the receptor-like kinase that has been intensive studied about self-incompatibility mechanism. The recognition of self-gamete enhanced the genetic diversity in the population (Shiu and Bleecker 2001).

Moreover, SRK also identified as a negative regulator in biotic defense. The T-DNA insertion mutation of S-locus receptor-like kinase, *cbrlk1*-1, induced the expression of PR protein while *PR1* expression was inhibited in the transgenic plant with overexpression of *CBRLK1* gene (Kim *et al.* 2009).

12. DENN containing protein

Based on SIFT score prediction, the salt-sensitive LPT123 rice contains the point mutation in the genome that leads to the loss of function of LOC_Os03g05200 gene, encoding DENN containing protein, while in the mutant rice line, LPT123-TC171, the region of this gene was reverted to be expressed. The Differentially Expressed in Normal and Neoplastic cell (DENN) containing protein found in all eukaryotes. In mammals, DENN containing proteins control the vesicle trafficking that specifically interacted with Rab proteins and GTPase proteins (Marat *et al.* 2011). However, DENN containing proteins poorly characterized in plants, so DENN containing protein is also a candidate to study its role in stress response.

13. mRNA splicing proteins

The environmental stress is the key factor affecting to the splicing activity of mRNA (Lorkovic´ *et al.* 2000). The deleterious mutation of LOC_Os01g21420 which encodes pre-mRNA-splicing factor SF2 was found in LPT123-TC171 genome. The Ser/Arg protein AtSRp30 which is similar to human pre-mRNA-splicing factor SF2 modulates mRNA splicing and nucleosome assembly. The constitutive expression of AtSRp30 altered the splicing activity of endogenous genes while the null mutant of this gene

resulted in early flowering, bigger rosette leaves and numerous secondary inflorescences (Lopato *et al.* 2006).

In addition, *SR45* is also the member of Ser/Arg proteins and involves in pre-mRNA splicing process. The recent study in *sr45* mutant revealed that the SR45 was the negative regulator of glucose and ABA signaling. The ABA level in *sr45* significantly elevated and regulated three ABA-biosynthesis gene expression. These data provided the evidence in the new role of Ser/Arg protein in abiotic stress response.

14. Uncharacterized protein

LOC_Os01g25920 and LOC_Os02g47560 were recorded as the deleterious mutant genes.

The LOC_Os01g25920 is the revertant mutation in salt-tolerant rice, LPT123-TC171, genome. However, there have been no reports about the function of this DNA-binding protein; it may play some important roles in salt-stress response.

The LOC_Os02g47560 was annotated as the DNA-binding protein that showed the putative expression in rice (http://rice .plantbiology.msu.edu/). Moreover, according to SIFT score prediction, the LOC_Os02g47560 was loss the function in the salt-tolerant rice, LPT123-TC171. Because the literature about its function is still unclear, the LOC_Os02g47560 is good candidate genes that need more characterization study about its activity in stress-response mechanism in further study.

2. *OsNUC1-L* expression quantification

The northern blot analysis was performed to study the response of *OsNUC1* gene expression under salt treatment and to investigate the forms of *OsNUC1* transcripts. The closely genetic plant materials at 14 days old, the salt-sensitive rice, LPT123 and its somaclonal variegated salt-tolerant line, LPT123-TC171 were selected for this study

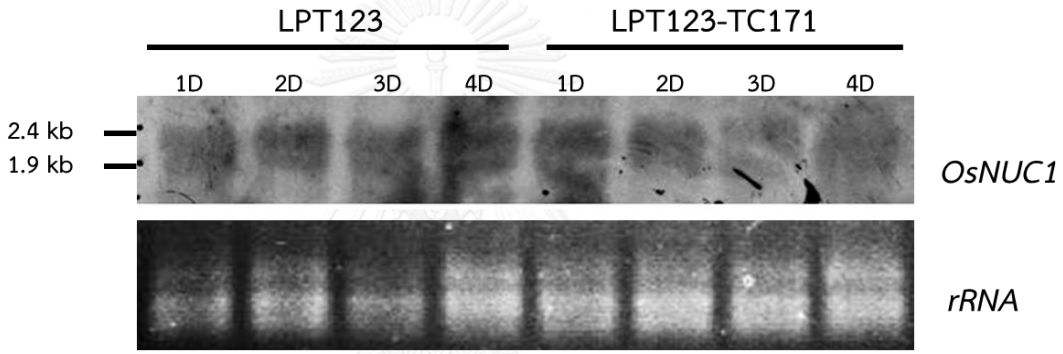


Figure4. 7 The northern blot analysis of LPT123 and LPT123-TC171 rice treated with 85mM NaCl for 1, 2, 3 and 4 days hybridized with *OsNUC1-S* cDNA (GENBANK accession number: AK063918)

Based on northern analysis using *OsNUC1-S* cDNA as a probe, it confirmed that the *OsNUC1* transcripts were present in two forms. The positive bands corresponded to the size of *OsNUC1-S* and *OsNUC1-L* mRNA which were 1.9 and 2.4 kb, respectively. Besides, both forms of *OsNUC1* responded to salt treatment (Figure 4.7).

In LPT123 rice, both forms of *OsNUC1* transcripts slightly increased their expression level. Besides, the highest expression of LPT123 was found after 4 days of treatment. In contrast, the expression of *OsNUC1* in salt-tolerant rice, LPT123-TC171, was rapidly increased in the early stage after stress treatment. The LPT123-TC171 rice showed the strongest signal after a day of salt stress and slightly declined after 3 days of stress (Figure 4.7).

The rapid modification of particular biochemical processes in the cell is the key of stress tolerance (Hasegawa *et al.* 2000). The Pokkali rice rapidly adapted the physiological and biochemical process when faced the saline condition such as photosynthetic recovery, reduced stomatal conductance and maintain water content faster than those IR29 (Kawasaki *et al.* 2001). Likewise, the LPT123-TC171 rapidly induced the expression of *OsNUC1* gene compared 'LPT123' rice, reflecting the different speed of the external stimuli. The more rapidly responses to stress are, the more tolerance level is.

Generally, the expression of *NUC* gene was mostly found in meristematic zone, especially root apical meristem and tightly correlated with mitotic activity of cells (Bogre *et al.* 1996). Moreover, *NUC* expression also responded to various environmental stimuli such as cold (Vashisht *et al.* 2005) and salt stress and ABA (Seki *et al.* 2002, Sripinyowanich *et al.* 2013) suggest *NUC* may play some important role in salt-adaptive mechanism in plants.

3. Transcriptomics of overexpression *OsNUC1-S* in transgenic Arabidopsis

3.1 *OsNUC1-S* altered the expression of gene in ABA metabolism and ABA responses

To characterize the role of *OsNUC1-S* gene, Sripinyowanich and colleagues generated the transgenic Arabidopsis that overexpressed *OsNUC1-S* and study the functions of *OsNUC1-S* in salt-adaptive mechanism. They discovered that the transgenic Arabidopsis showed the higher salt-tolerant ability when compared to the wild-type Arabidopsis. Furthermore, the transgenic plant had the less H_2O_2 content and displayed the longer primary root length (Sripinyowanich *et al.* 2013).

The ABA hormone controls many responses in plant including gene regulation, stomatal opening as well as seed dormancy. ABA involved stress response especially the response to water status such as drought and salinity. To test the response of the overexpressed *OsNUC1-S* transgenic to ABA, seeds of wild-type Arabidopsis and the transgenic with *OsNUC1-S* overexpression were germinated on MS medium or the medium the containing 0.2 μM ABA. All wild-type seeds were successfully germinated after 4 days of culture while the germination of most *35SCaMV::OsNUC1-S* transgenic were highly inhibited by ABA. (Figure 4.8)

The transgenic line with *35SCaMV::OsNUC1-S* construct showed the hypersensitive phenotype to ABA. So, in this study, I also investigated the response of the transgenic *35SCaMV::OSNUC1-S* during salt stress condition at the transcriptomic level. , the wild-type and overexpression *OsNUC1-S* were challenged with 100mM NaCl for 48 hours. The transcriptomic analysis using RNA-Seq method discovered; 84 significantly different genes in control condition and 4595 significantly different genes in stress condition. Moreover, the high-throughput gene expression revealed the expression of genes in ABA biosynthesis process.

In Arabidopsis genome, *9-cis-epoxycarotenoid dioxygenase* appears as a gene family composes of 9 related-sequences (Nambara and Marion-Poll 2005). However, only 5 members, which are *NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9*, locate in plastid (Tan *et al.* 2003). Moreover, reverse and forward-genetic studies also showed that *NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9* involved in ABA biosynthesis process (Luchi *et al.* 2001, Toh *et al.* 2008). The transcripts of *NCED2*, *NCED3* and *NCED5* in transgenic plant were significantly different when compared to the wild type's gene expression. The expression level of *NCED2*, *NCED3* and *NCED5* was 3.61, 3.22 and 21.88 fold higher than wild type respectively (Table4.2).

The transgenic line with *35SCaMV::OsNUC1-S* construct showed the hypersensitive phenotype to ABA and also altered the expression of genes in ABA biosynthesis indicating that the transgenic plants might alter the transcription activity of ABA-inducible genes. Therefore, ABA inducible genes reported by Hoth and colleagues were retrieved from the transcriptomic data and shown in Table4.3 (Hoth *et al.* 2002).

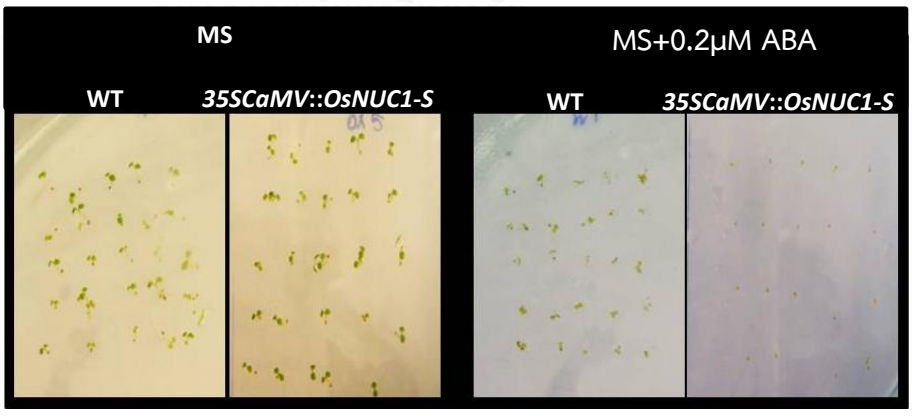


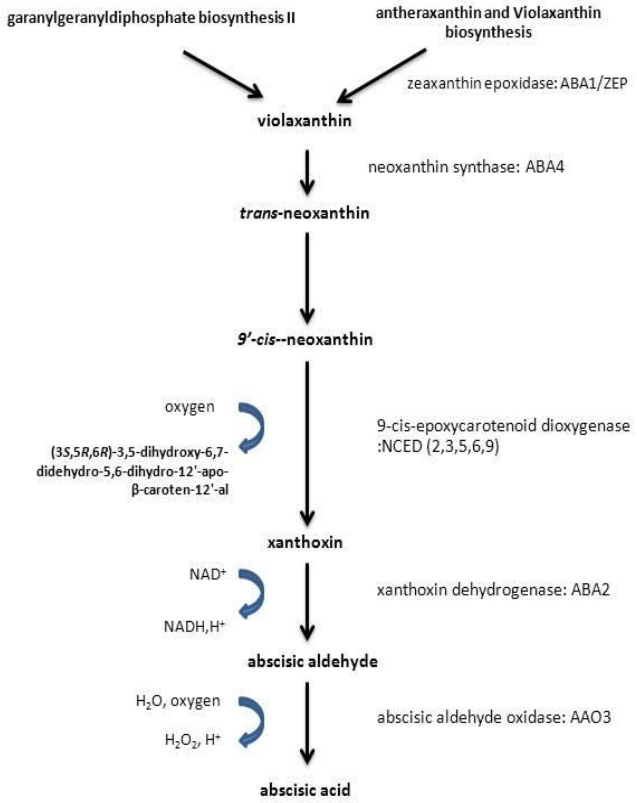
Figure4. 8 Germination of wild type Arabidopsis and the transgenic Arabidopsis with *35SCaMV::OsNUC1-S* on MS media and 0.2 μM ABA supplemented media (4 days after stratification)

As shown in Table4.3, the higher gene expression abundance of ABA-inducible genes was found in transgenic plant with *35SCaMV::OsNUC1-S* construct than in wild-type Arabidopsis. These results suggest that the *OsNUC1-S* gene expression enhances the ABA signaling in the transgenic lines by increasing the production of the key enzyme in ABA biosynthesis during salt stress.

Several studies reported about the association between RNA-binding protein and physical-stresses tolerance especially the response to water status such as drought and salinity, but the mechanism of this response is still unclear (Kim *et al.* 2008). The recent studies of RNA-binding proteins mutants revealed that they had the ABA-sensitive phenotypes such as delayed seed germination, inhibited root growth and increased stomatal response upon the ABA application that insight the correlation of ABA signal transduction and RNA metabolism (Kuhn and Schroeder 2003).

ABA is noted as the plant-stress hormone because it associates many physiological responses under stress such as seed dormancy, stomatal closure as well as the signal transduction pathway for salt stress responses. In the ABA biosynthesis pathway, 9-cis-epoxycarotenoid dioxygenase (NCED) is the enzyme that controls the cleavage step of 9-cis-violaxanthin to xanthoxin that is the rate-limiting step in ABA biosynthesis (Qin and Zeevaart 2002). Overexpressing *AtNCED3* significantly increased the ABA content in transgenic Arabidopsis and also improved the osmotic tolerance by reduction the leave transpiration rate (Luchi *et al.* 2001). Furthermore, Frey and colleagues (Frey *et al.* 2012) also found the *nced3* and *nced5* knock-out mutants, which decreased the ABA content and were susceptible to drought.

It clearly showed that, after salt treatment, the transcripts of *NCED* genes, which were *NCED2*, *NCED3* and *NCED5*, in transgenic Arabidopsis were dramatically increased by 3.61, 3.22 and 21.88 folds higher than those in wild type. Most of ABA-inducible genes were also induced in transgenic line when compared to the wild type. Likewise, seeds of *OsNUC1-S* Arabidopsis showed the hypersensitive response to ABA. These suggested the function of *OsNUC1-S* gene in salt condition that *OsNUC1-S* promoted the osmotic tolerance by positively regulated the genes in ABA production.



Gene (Locus)	Number of reads (after normalization)		Fold change
	Wild type	*35SCaMV::OsNUC1-S*	
ABA1/ZEP (AT5G67030)	686.98	505.18	0.74
ABA4 (AT1G67080)	191.81	218.24	1.14
NCED2 (AT4G18350)	4.51	16.28	**3.61***
NCED3 (AT3G14440)	27.65	88.55	**3.20***
NCED5 (AT1G30100)	0.41	9.04	**21.88***
NCED6 (AT3G24220)	0	0.48	-
NCED9 (AT1G78390)	1.43	2.48	1.73
ABA2 (AT1G52340)	260.36	219.72	0.84
AAO3 (AT2G27150)	171.73	282.14	1.64

Table4. 2 The transcription activity of genes in ABA biosynthesis between transgenic *35SCaMV::OsNUC1-S* and wild-type Arabidopsis. Where; $p < 0.05$ (asterisk)

ABA inducible genes (Locus number)	Normalized read counts (average)		Fold change
	Wild type	*35SCaMV::OsNUC1-S*	
Late embryogenesis abundant protein LEA like (At5g06760)	6.71	1100.51	163.97**
Low-temperature-induced protein 78 (At5g52310)	113.58	3228.4	28.42**
Homeobox-leucine zipper protein (At3g61890)	300.5	1817.86	6.05**
Acid Phosphatase type 5 (At3g17790)	134.29	507.48	3.78**
AtKIN1 (At5g15960)	50.96	591.28	11.60**
Dehydrin RAB18-like protein (At5g66400)	40.96	2055.69	50.18**
Homeodomain transcription factor AtHB-7 (At2g46680)	754.77	2413.14	3.20**
Delta-1-pyrroline 5-carboxylase synthetase, AtP5C1 (At2g39800)	612.24	2652.95	4.33**

Table4. 3 The expression level of known ABA-inducible genes (Hoth *et al.* 2002) between transgenic *35SCaMV::OsNUC1-S* compared to wild-type Arabidopsis. Where; $p < 0.01$ (asterisk)

ABA inducible genes (Locus number)	Normalized read counts (average)		Fold change
	Wild type	*35SCaMV::OsNUC1-S*	
ABA-responsive element-binding protein, AtABF3 (At4g34000)	234.76	706	3.01**
Protein phosphatase 2C, AtABI2 (At5g57050)	82.08	732.35	8.92**
Cold-regulated protein cor15b precursor (At2g42530)	50.42	41.26	0.82
Alcohol dehydrogenase (At1g77120)	249.01	4540.32	18.23**
Putative receptor-like protein kinase RPK1 (At1g69270)	282.87	278.45	0.98
Hypothetical protein, AtCOR47 (At1g20440)	5194.21	9182.63	1.77
Cold-regulated protein COR6.6,KIN2 (At5g15970)	526.14	1660.79	3.16**
Cold-regulated protein cor15a precursor (At2g42540)	132.51	1355.81	10.23**

Table 4.3 (cont.) The expression level of known ABA-inducible genes (Hoth *et al.* 2002) between transgenic *35SCaMV::OsNUC1-S* compared to wild-type Arabidopsis. Where; $p < 0.01$ (asterisk)

ABA inducible genes (Locus number)	Normalized read counts (average)		Fold change
	Wild type	*35SCaMV::OsNUC1-S*	
Calcium binding protein, ATCLO3 (At2g33380)	322.87	6193.21	19.18**
AtABI1 (At4g26080)	813.11	1489.3	1.83**
Protein phosphatase 2C, AtP2C-HA (At1g72770)	495.89	1462.53	2.95**
PIP1B aquaporin (At2g45960)	7577.07	8289.81	1.09
Hypothetical protein, AtERD10 (At1g20450)	2079.69	10648.71	5.12**
ABA-responsive protein-like (At5g13200)	83.96	561.55	6.69**

Table 4.3 (cont.) The expression level of known ABA-inducible genes (Hoth *et al.* 2002) between transgenic *35SCaMV::OsNUC1-S* compared to wild-type Arabidopsis. Where; $p < 0.01$ (asterisk)

4. Characterization of transgenic *35SCaMV*::*OsNUC1-L* Arabidopsis

In rice, two *OsNUC1* full-length cDNA species were submitted to GENBANK database. The *OsNUC1-L*, accession number: AK103446, has three domains that are: acidic N-terminal region, the central domain contains two bipartite RNA-recognition motifs (RRM) and the glycine-arginine rich (GAR) domain at the C-terminal end, while its alternative form, *OsNUC1-S* (accession number: AK063918) has only RNA-binding motifs and the GAR domain.

To elucidate the function of *OsNUC1* gene, the transgenic plants that overexpressed *OsNUC1-S* was generated and tested the series of physiological, biochemical and molecular analysis. The overexpressed *OsNUC1-S* transgenics showed the increased in lateral root production, early flowering and especially increased in salt-tolerant capacity (Sripinyowanich *et al.* 2013). This research is focus on the characterization of the overexpression effects of *OsNUC1-L*.

4.1 Transgenic determination

Total of 100 putative transgenic seed (T_0 generation) were screened on sterile half-strength MS media (Murashige and Skoog 1962) that supplemented with 50μg/ml of kanamycin then the resistant seedlings were selected and grew on soil and collected the T_1 generation seed.

To test for the homozygosity, T_1 seeds were tested on half-strength MS (Murashige and Skoog 1962) media, supplemented with 50μg/ml of kanamycin. The segregation ratio for single gene insertion (resistant: sensitive kanamycin seedlings, 3:1) was use to indicate the single locus insertion of the transgenic lines.

Likewise, the transgenic plants were confirmed the foreign gene insertion by using PCR technique. The positive bands were shown on lane number 3, 4, 5, 9, 10 and 12 (Figure 4.9). Moreover, the homozygous transgenic lines were double checked for the existence of the *OsNUC1* overexpression construct by PCR analysis.

Summarily, after PCR and segregation screening, three homozygous transgenic lines 14, line 22 and line 24 were used for the *OsNUC1-L* gene characterization.

Figure4. 9 The PCR amplification of transgenic Arabidopsis using gene specific primer for *OsNUC1-L* gene, where; lane1 = wild type Arabidopsis, lane2 = transgenic line 7, lane3 = transgenic line 6, lanes4 and 5 = transgenic line 14, lane6 = transgenic line 16, lanes7-10 = transgenic line 22, lanes11-12 = transgenic line 24

4.2 Study the transcription activity of *OsNUC1* in transgenic Arabidopsis

The T_2 generation of independent homozygous transgenic, line 14, line 22 and line 24, were verified the transcriptional activity of transgene, *OsNUC1-L*, by using quantitative RT-PCR. To study the gene expression, the transgenic plants and wild type plant were culture on sterile half-strength MS media (Murashige and Skoog 1962) for a week and then planted to soil mixture (peat moss: perlite: vermiculite = 3: 1: 1) for another week. The total RNA was isolated from leaf tissue by using the Plant RNA Reagent (Invitrogen, USA). The quantitative RT-PCR method was described in Appendix B. The *OsNUC1-L* transcriptional level of each line was represented in relative value compared with the wild type Arabidopsis.

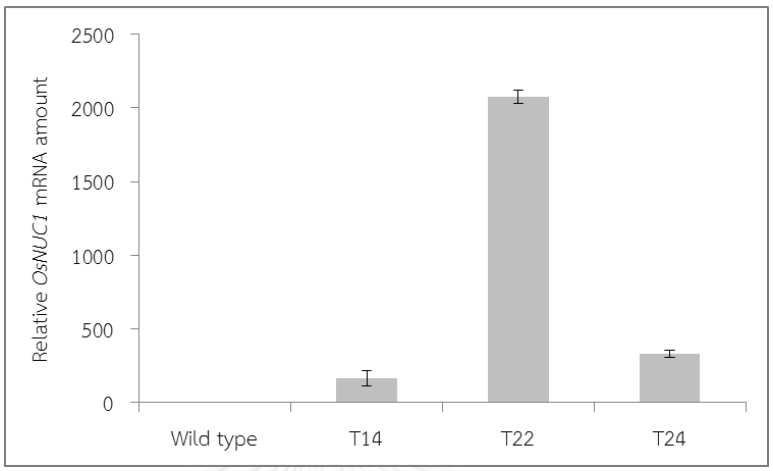


Figure4. 10 The relative level of *OsNUC1-L* transcripts in transgenic Arabidopsis with *35SCaMV::OsNUC1-L* construct (T14, T22 and T24) and Arabidopsis wild type. The relative expression was calculated using Pfaffl's method (Pfaffl 2001). The data presented means±SDs of three replicates and *AtEF-1 alpha* was served as the internal control.

Basically, the expression of the transgene depended on two factors. One factor is the effect of the inserted DNA, and the other factor is the location of foreign inserted DNA in the genome. It is also called 'chromosomal position effect' (al-Shawi *et al.* 1990). The insert region of the transgene may positively or negatively impact to the activity of transgene.

The relative transcription activities of *OsNUC1-L* in transgenic plants were showed in different degree of gene expression. The transgenic line 22 showed the strongest *OsNUC1-L* expression followed with line 24 and line 14 respectively (Figure 4.10). Because the insertion of the foreign DNA is the randomly unpredictable event, the variable expression of three transgenic lines was expected to be resulted from the 'position effect'.

4.2 Study the salt resistance of *35SCaMV::OsNUC1-L* Arabidopsis

4.2.1 Relative growth rate

To study the relative growth rate of transgenic lines with the *OsNUC1-L* overexpression, the five-day-old seedlings of wild-type Arabidopsis ecotype Columbia and three independent transgenic lines were placed on MS media for control condition and the MS media supplemented with 100mM NaCl for salt stress condition.

Under the normal condition, the significant difference in relative growth rate was detected in the overexpression *OsNUC1-L* transgenic line 24 and line 22 at 2-4 day and 4-6 day, respectively (Figure4.11-a). However, under the salt stress condition, no significant difference was found between wild type and all three transgenic plants (Figure4.11-b). Interestingly, when grew on normal MS media, the transgenic lines with *OsNUC1-L* overexpression showed the early flowering character (Figure4.11-c), which is similar to what found in *35SCaMV*::*OsNUC1-S* transgenic (Sripinyowanich *et al.* 2013).

At cell ultrastructure, NUC is mostly found in nucleolus, particularly around fibrillar centers (FC) and dense fibrillar component (DFC) (Tong *et al.* 1997, Pontvianne *et al.* 2007, Stepinski 2012) where is involved in rRNA transcription and pre-ribosomal formation (Medina *et al.* 2010). Yeast and Arabidopsis mutant that lacked function of *NUC* showed overproduction of unprocessed rRNA (Lee *et al.* 1992, Petricka and Nelson 2007, Pontvianne *et al.* 2007). Thus, *NUC* associated in rRNA transcription and maturation as well as ribosome biogenesis.

Moreover, the level of *NUC* expression associates with activity of cell and protein translation (Stepinski 2004). The study about the activity of *NUC* gene expression in alfalfa clearly revealed that *NUC* expression was absent in the stationary phase cells while the expression strongly induced in logarithmically dividing cells (Bogre *et al.* 1996). Likewise, *NUC* also affected the 40S/60S ribosomal subunit ratio. Yeast that defected the *NUC* gene

function dramatically decreased in 40S/60S ribosomal subunit ratio (Kondo and Inouye 1992).

When investigated the response of the transgenic *35SCaMV::OSNUC1-L* during salt stress condition at the transcriptomic level., two-week old wild-type and overexpression *OsNUC1-L* line T22, which showed the strongest expression level of *OsNUC1-L* (Figure 4.10), were challenged with 100mM NaCl for 5 hours by placing the seedlings on the MS media supplemented with or without 100mM NaCl for salt stress or control condition, respectively.

When compared the transcriptome data of wild-type Arabidopsis and the transgenic *35SCaMV::OSNUC1-L* by using DESeq method (Anders and Huber 2010), 1967 significantly different expressed genes were found in control condition. Up to 1281 genes showed the higher level in the transgenic lines and 686 genes were found to be lower. On the other hand, under the salt stress condition, 999 significantly different expressed genes were found. Five hundred and forty five genes were up-regulated and four hundred fifty four genes were down-regulated. (Table 4.4) The gene list was provided in supplemented table E.1 in the appendix E. (CD-ROM)

Condition	Up-regulated genes	Down-regulated genes	Significantly expressed genes
Control condition	1281	686	1967
Salt stress	545	454	999

Table4. 4 The summary view of the significantly expressed genes under normal and salt condition.

Because the analysis of high-throughput gene expression data focus on the sets of genes rather than individual genes, the gene set enrichment analysis was used to determine the group of genes which were dominantly expressed. Besides, the gene set enrichment simplified the complicated form of data to a comprehensible format (Subramanian *et al.* 2005).

Cluego (Bindea *et al.* 2009), the cytoscape plugin, was used to generate and visualize the gene set enrichment result. To start the analysis, the significantly expressed genes were divided into two groups according their regulation; the up-regulated gene and down-regulated genes. Then, they were arranged into the proper format for Cluego. The data were organized according Gene Ontology; Cellular compartment, Biological processes and Molecular function. The overrepresented terms were visualized in the network format that also represented the up- and down- regulated genes by using the red and blue color respectively.

For the cellular compartment, in normal condition, the transgenic *35SCaMV::OSNUC1-L* positively overrepresented gene in extracellular organelle and plasma membrane components while suppressed gene in photosynthesis, external encapsulate structure, vacuole and endoplasmic reticulum under normal condition (Figure 4.12). On the other hand, under salt stress condition, the transgenic *35SCaMV::OSNUC1-L* positively induced gene in ribosome, light-harvesting complex and plasma membrane components (Figure 4.12).

Interestingly, the overexpression of *OSNUC1-L* differently affected to the genes in photosynthesis in control and salt stress condition. As photosynthesis is the important metabolism process in plants and the salt stress condition inhibits the photosynthetic process and leads to crop yield reduction (Chaves *et al.* 2009). For these reasons, the photosynthetic activity of the transgenic line with *35SCaMV::OSNUC1-L* was measured in comparison with wild type in both control and salt conditions. The results are shown in the section 5.2.

Under salt stress condition, no significantly difference was detected in term of growth. However, the transcriptomic data supported that Os*NUC1-L* dominantly induced the expression of ribosome components and genes that involved in ribosome biogenesis (Figure4.12, Table 4.5 and 4.6).

It suggested that Os*NUC1-L* improved growth under the normal condition via the regulation of ribosome production and cell proliferation. While, under saline condition, the Os*NUC1-L* induced the expression of ribosomal component proteins which is controlled the balance of the translation machinery.

For the biological processes, the overexpression of *OsNUC1-L* gene induced genes that response to stress in both control and stress condition (Figure 4.13). However, the genes controlling the secondary metabolite processes were significantly decreased in both normal and stress treatments.

In addition, under normal condition, the overexpressed *OsNUC1-L* transgenic also positively induced transporter genes, membrane docking genes and genes that regulated stomatal movement while the genes that controlled root morphology and endomembrane system were decreased (Figure 4.13). On the other side, under salt stress condition, the transgenic plant was increased genes in auxin metabolism and transporter genes (Figure 4.13).

Lastly, for the molecular function, the overexpressed *OsNUC1-L* transgenic positively increased the genes involving in carbohydrate transmembrane transporter, UDP-glucosyltransferase, kinase, phosphate, metal ion binding, quercetin 3-O-glucosylreansferase and glutathione transferase, while the genes encoding monooxygenase, calcium-dependent phospholipid binding were reduced in control condition. On the otherhand, during salt stress condition, the efflux transmembrane transporter genes and peptide binding protein genes were up-regulated, while the genes encoding monooxygenase, phosphotidyl ethanolamine binding, sugar transmembrane transporter, UDP-glucosyltransferase and carboxylic acid transmembrane transporter were significantly suppressed their expression (Figure 4.14).

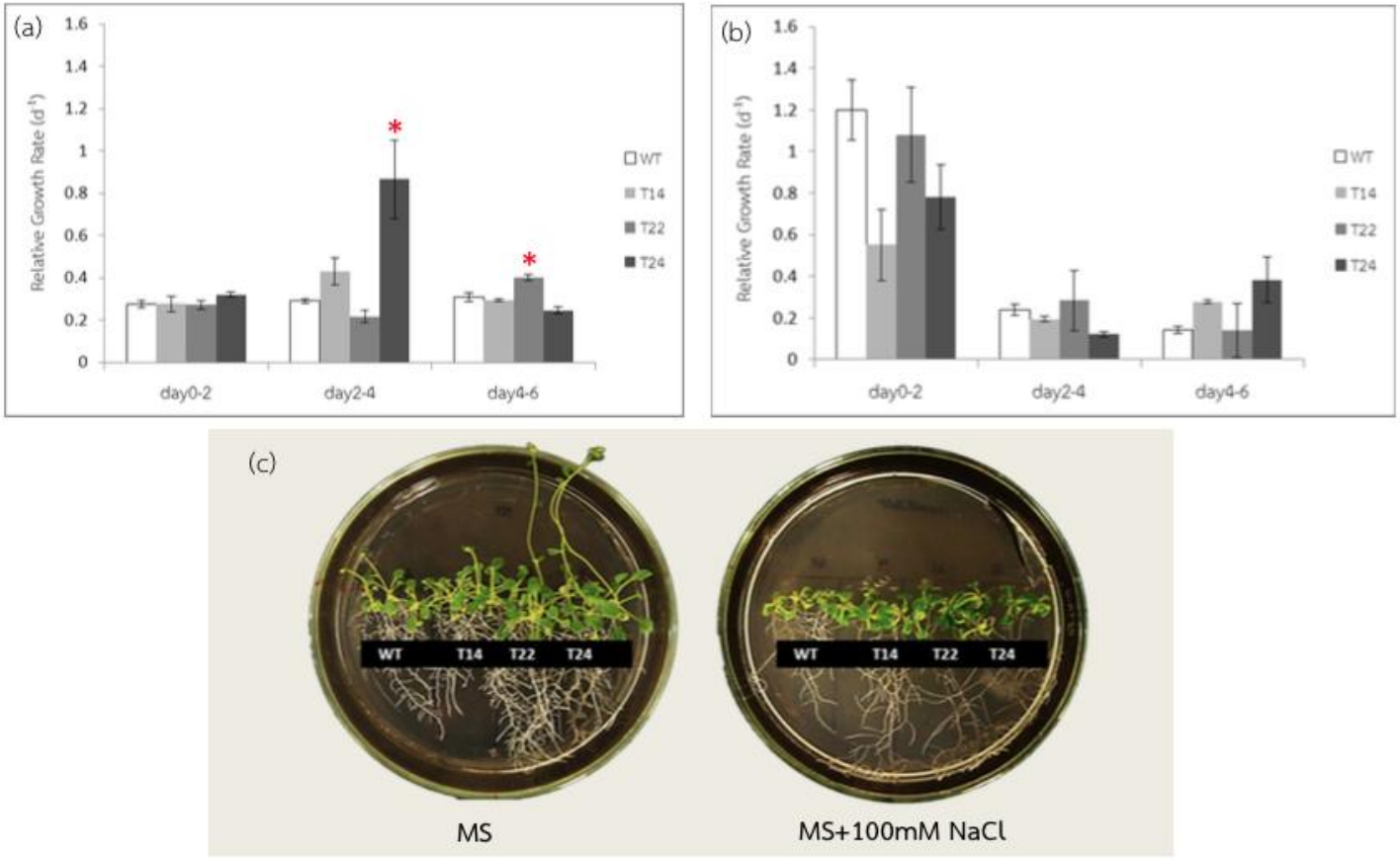


Figure4. 11 Relative growth rate (d^{-1}) of transgenic *35S:OsNUC1-L* Arabidopsis (T14, T22 and T24) grew on MS media for control condition (a) and supplemented with 100mM NaCl (b) for salt stress for 6 days. The data were tested and compared to the wild type, $p<0.05$ (asterisk)

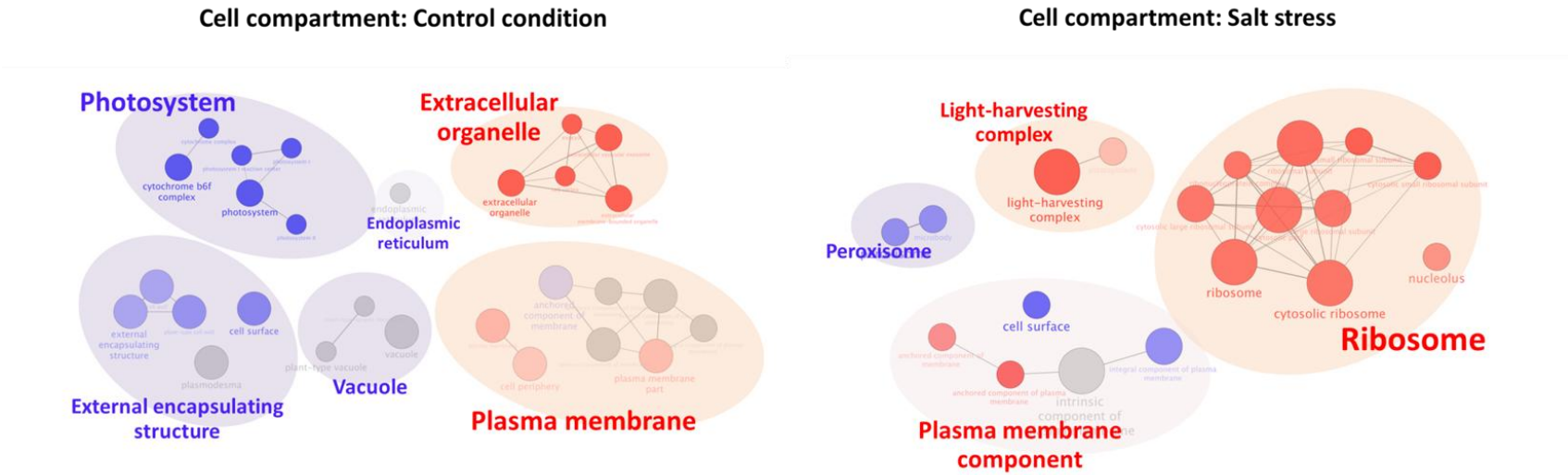


Figure 4.12 The gene set enrichment analysis of of significantly expressed gene in transgenic *35SCaMV::OsNUC1-L* under normal and salt stress. The red color represented the up-regulated gene while the blue color represented the down-regulated genes. The cellular comparment classification created by ClueGo (Bindea *et al.* 2009) with Benjamini and Hochberg correction

Ribosome Biogenesis			
	Locus name	Description	Fold change
Control	AT1G10300	nucleolar GTP-binding protein; K06943 nucleolar GTP-binding protein	2.61
	AT1G67120	protein MIDASIN1; K14572 midasin	1.60
	AT3G06530	U3snoRNP10 and NUC211 domain-containing protein; K14550 U3 small nucleolar RNA-associated protein 10	1.44
	AT4G17640	CKB2; casein kinase II beta chain 2; K03115 casein kinase II subunit beta	0.60
	ATMG00020	rrn5; 5S ribosomal RNA; K01981 large subunit ribosomal RNA, 5S	0.53
	ATMG01390	rrn18; 18S ribosomal RNA; K01979 small subunit ribosomal RNA	0.59
Salt	AT3G55620	emb1624; translation initiation factor IF6; K03264 translation initiation factor 6	1.32
	AT4G25630	FIB2; mediator of RNA polymerase II transcription subunit 36a; K14563 rRNA 2'-O-methyltransferase	1.31

Table 4.5 List of gene in ribosome biosynthesis (KEGG pathway: ath03008) which significantly increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under normal and salt stress

Ribosome-Arabidopsis thaliana (KEGG pathway: ath03010)			
	Locus name	Description	Fold change
Control	AT1G61580	RPL3B; 60S ribosomal protein L3-2; K02925 large subunit ribosomal protein L3e	0.28
	AT3G28500	60S acidic ribosomal protein P2-3; K02943 large subunit ribosomal protein LP2	0.25
	AT5G40040	60S acidic ribosomal protein P2-5; K02943 large subunit ribosomal protein LP2	6.20
	ATMG00020	rrn26; 26S ribosomal RNA protein; K01982 large subunit ribosomal RNA	0.53
	ATMG01390	rrn18; 18S ribosomal RNA; K01979 small subunit ribosomal RNA	0.59
Salt	AT1G15250	60S ribosomal protein L37-1; K02922 large subunit ribosomal protein L37e	1.36
	AT1G16740	ribosomal protein L20; K02887 large subunit ribosomal protein L20	1.31
	AT1G18540	60S ribosomal protein L6-1; K02934 large subunit ribosomal protein L6e	1.28
	AT1G23410	40S ribosomal protein S27a-1; K02977 small subunit ribosomal protein S27Ae	1.53
	AT1G26880	60S ribosomal protein L34-1; K02915 large subunit ribosomal protein L34e	1.32
	AT1G52300	60S ribosomal protein L37-2; K02922 large subunit ribosomal protein L37e	1.28
	AT1G58983	40S ribosomal protein S2-2; K02981 small subunit ribosomal protein S2e	0.00
	AT1G61580	RPL3B; 60S ribosomal protein L3-2; K02925 large subunit ribosomal protein L3e	0.40
	AT1G70600	60S ribosomal protein L27a-3; K02900 large subunit ribosomal protein L27Ae	1.35
	AT2G04390	40S ribosomal protein S17-1; K02962 small subunit ribosomal protein S17e	1.34

Table4. 5 List of ribosome composition (KEGG pathway: ath03010) which significantly increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under normal and salt stress

Ribosome-Arabidopsis thaliana (KEGG pathway: ath03010)			
	Locus name	Description	Fold change
Salt	AT2G19750	40S ribosomal protein S30; K02983 small subunit ribosomal protein S30e	1.32
	AT2G20450	60S ribosomal protein L14-1; K02875 large subunit ribosomal protein L14e	1.32
	AT2G27710	60S acidic ribosomal protein P2-2; K02943 large subunit ribosomal protein LP2	1.29
	AT2G36620	RPL24A; 60S ribosomal protein L24-1; K02896 large subunit ribosomal protein L24e	1.34
	AT2G45710	40S ribosomal protein S27-1; K02978 small subunit ribosomal protein S27e	1.41
	AT3G04770	RPSAb; 40S ribosomal protein Sa-2; K02998 small subunit ribosomal protein SAe	1.35
	AT3G06680	60S ribosomal protein L29-2; K02905 large subunit ribosomal protein L29e	1.53
	AT3G06700	60S ribosomal protein L29-1; K02905 large subunit ribosomal protein L29e	1.26
	AT3G10090	40S ribosomal protein S28-1; K02979 small subunit ribosomal protein S28e	1.53
	AT3G10610	40S ribosomal protein S17-3; K02962 small subunit ribosomal protein S17e	1.30
	AT3G16080	60S ribosomal protein L37-3; K02922 large subunit ribosomal protein L37e	1.37
	AT3G44010	40S ribosomal protein S29; K02980 small subunit ribosomal protein S29e	1.33
	AT3G44590	60S acidic ribosomal protein P2-4; K02943 large subunit ribosomal protein LP2	1.31
	AT3G60245	zinc-binding ribosomal protein family protein; K02921 large subunit ribosomal protein L37Ae	1.39
	AT3G61110	RS27A; 40S ribosomal protein S27-2; K02978 small subunit ribosomal protein S27e	1.42

Table 4.5 (cont.) List of ribosome composition (KEGG pathway: ath03010) which significantly increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under normal and salt stress

Ribosome-Arabidopsis thaliana (KEGG pathway: ath03010)			
	Locus name	Description	Fold change
Salt	AT4G14320	60S ribosomal protein L36a; K02929 large subunit ribosomal protein L44e	1.29
	AT4G15000	60S ribosomal protein L27-3; K02901 large subunit ribosomal protein L27e	1.27
	AT4G18100	60S ribosomal protein L32-1; K02912 large subunit ribosomal protein L32e	1.29
	AT4G29390	40S ribosomal protein S30; K02983 small subunit ribosomal protein S30e	1.30
	AT4G30800	40S ribosomal protein S11-2; K02949 small subunit ribosomal protein S11e	1.40
	AT4G34555	40S ribosomal protein S25-3; K02975 small subunit ribosomal protein S25e	1.32
	AT4G36130	60S ribosomal protein L8-3; K02938 large subunit ribosomal protein L8e	1.27
	AT5G03850	40S ribosomal protein S28-1; K02979 small subunit ribosomal protein S28e	1.35
	AT5G56670	40S ribosomal protein S30; K02983 small subunit ribosomal protein S30e	1.35
	AT5G59850	40S ribosomal protein S15a-1; K02957 small subunit ribosomal protein S15Ae	1.27
	AT5G60670	60S ribosomal protein L12-3; K02870 large subunit ribosomal protein L12e	1.27
	AT5G64140	RPS28; 40S ribosomal protein S28-2; K02979 small subunit ribosomal protein S28e	1.31
	AT5G67510	60S ribosomal protein L26-2; K02898 large subunit ribosomal protein L26e	1.32

Table 4.5 (cont.) List of ribosome composition (KEGG pathway: ath03010) which significantly increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under normal and salt stress

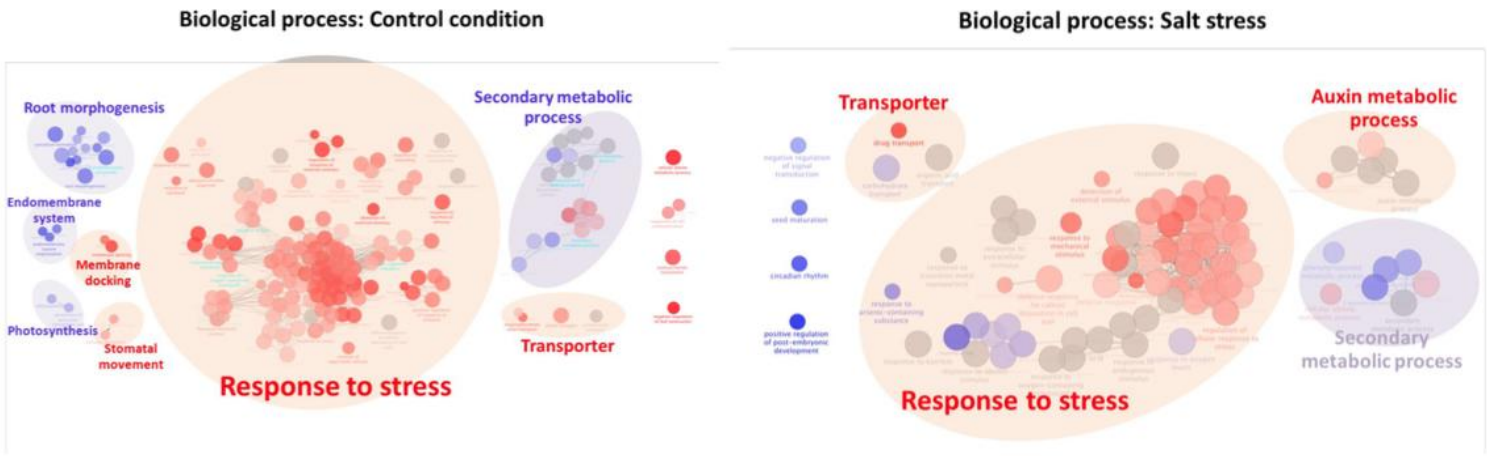


Figure4. 12 The gene set enrichment analysis of of significantly expressed gene in transgenic *35SCaMV::OsNUC1-L* under normal and salt stress. The red color represented the up-regulated gene while the blue color represented the down-regulated genes. The biological process classification created by ClueGo (Bindea *et al.* 2009) with Benjamini and Hochberg correction

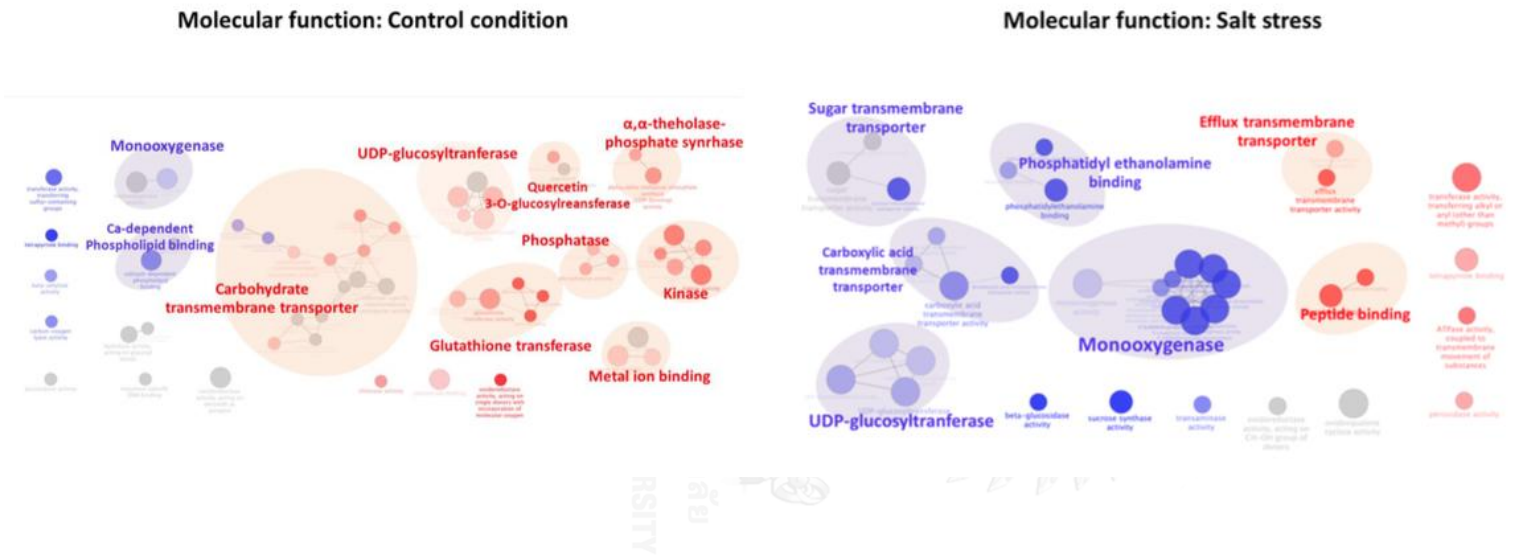


Figure4. 13 The gene set enrichment analysis of of significantly expressed gene in transgenic *35SCaMV::OsNUC1-L* under normal and salt stress. The red color represented the up-regulated gene while the blue color represented the down-regulated genes. The molecular function classification created by ClueGo (Bindea *et al.* 2009) with Benjamini and Hochberg correction

4.3 Primary root growth

To study the response of primary root of the *OsNUC1-L* overexpression transgenic lines, the five-day-old seedlings of wild-type Arabidopsis ecotype Columbia and three independent transgenic lines were vertically grew on half-strength MS media with and without 100mM NaCl added. The primary root length measurement was monitored every day for 10 days and measured using ImageJ program (http://imagej.nih.gov/ij/). Then, the data was compared between wild type and transgenic by using one-way ANOVA method.

After 6 days on the normal MS media, only primary root length of transgenic line 22 significantly dominated when compared to the wild type plant. However, after 10 days, no significantly difference was found between wild type and all *OsNUC1-L* transgenic lines (Figure4.15a). In contrast, when facing salt stress, the wild type plant dramatically decreased in primary root length while all *OsNUC1-L* transgenic lines significantly maintained root growth. (Figure4.15b)

Basically, root growth was inhibited under osmotic stress. The water stress condition inhibited root growth by decreased rate of cell proliferation at the root meristematic zone (Sacks *et al.* 1997). However, all transgenic *35s:OsNUC1* lines significantly developed root growth under stress when compared to the wild type Arabidopsis (Figure4.15b).

In Arabidopsis, The knockout mutant Δ*ATNUC-L1* Arabidopsis was significantly inhibited in root growth when compare to wild-type plant under the normal growth condition (Kojima *et al.* 2007, Petricka and Nelson 2007). The expression of *NUC* gene was mostly found in meristematic zone and tightly correlated with mitotic activity of cells (Bogre *et al.* 1996). In addition, the study in soybean root revealed that the transcriptional activity of *NUC* gene significantly not only coordinated with cell division and ribosome production, but also correlated with cellular metabolism and root growth (Stepinski 2004).

It suggested that *NUC* implicated in normal root development and the constitutive expression of *OsNUC1-L* enhanced root growth in transgenic plants under salt stress condition by transcriptionally induced the group of ribosomal proteins and genes in ribosome production (Figure 4.12 and Table 4.5 and 4.6).

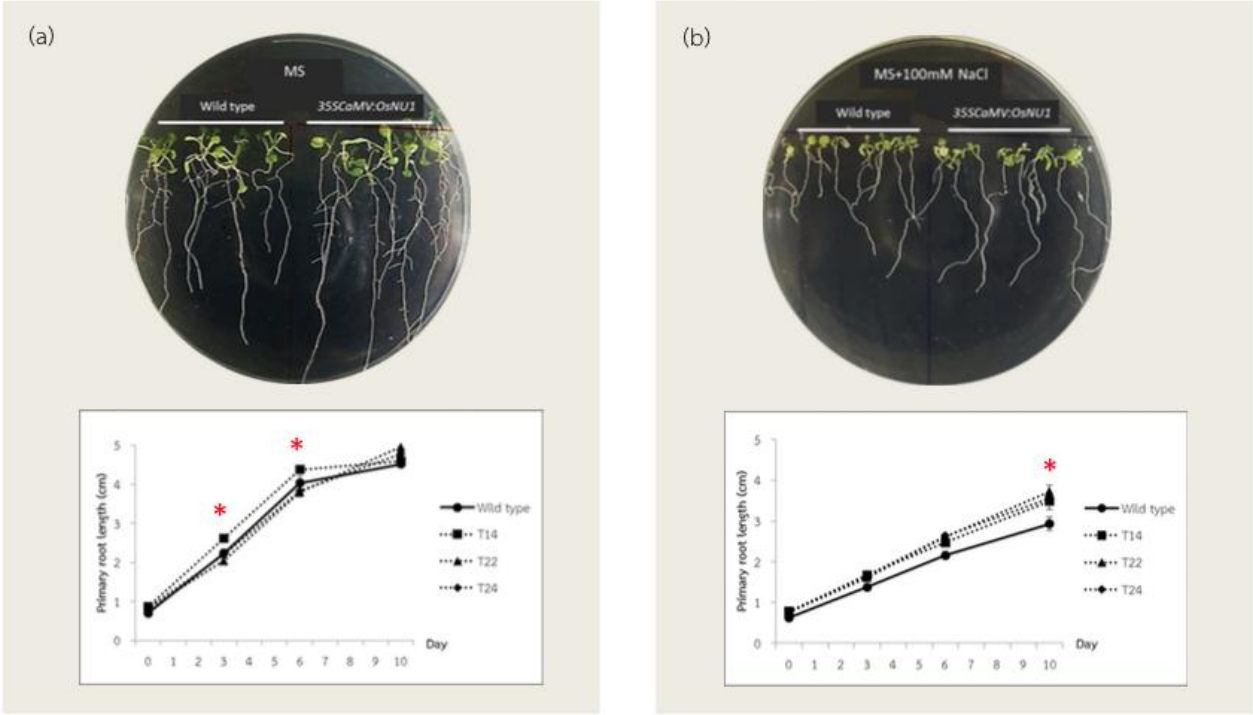


Figure4. 14 The primary root length of transgenic *35S:OsNUC1-L* Arabidopsis (T14, T22 and T24) and wild type Arabidopsis vertically grew on MS media for control condition (a) and supplemented with 100mM NaCl (a) for salt stress for 10 days. The data were tested and compared to the wild type plant, where; $p<0.05$

4. Study the relationship between *OsNUC1-L* and ABA

4.1 Study the response of transgenic Arabidopsis to ABA on seed germination

To examine the effect of ABA on overexpression *OsNUC1-L* transgenic, seeds of wild type and three overexpression *OsNUC1-L* transgenic lines were germinated on MS media supplemented with or without ABA and monitored every day for 9 days. The ABA concentration was varied as 0.2, 0.4 and 0.6μM ABA.

When placed the seeds on 0.2μM ABA for 2 days, the germination of *OsNUC1-L* transgenic (germination percentage=0-9.1%) was dramatically lower than in wild type (germination percentage=44%) (Figure 4.16, 4.17 and 4.18). However, the negative impact on seed germination is dose-dependent effect. The germination of all *OsNUC1-L* transgenic lines was significantly inhibited when grown on the media supplemented with 0.4 and 0.6μM ABA (Figure 4.16, 4.17 and 4.18)

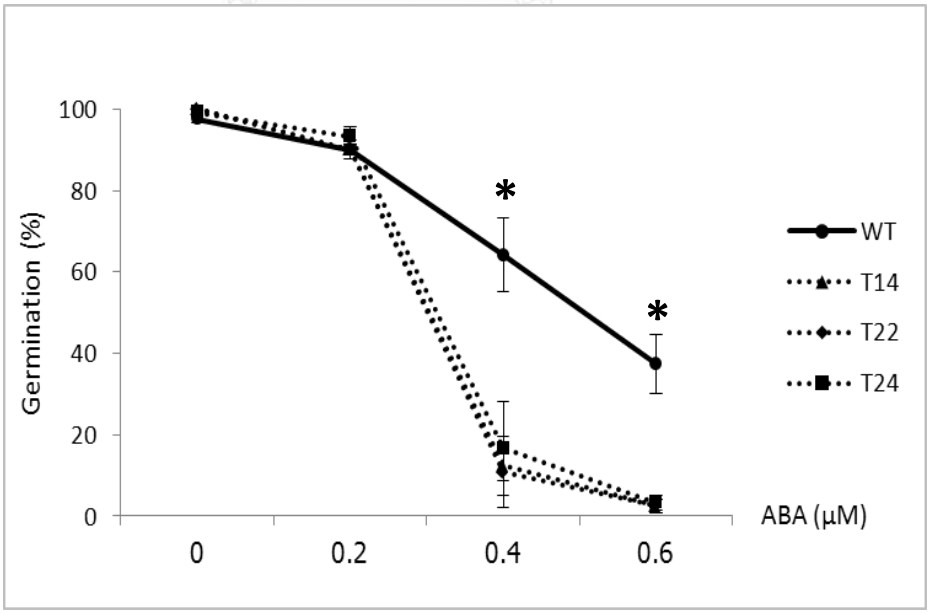


Figure4. 15 Germination of wild-type Arabidopsis and *35SCaMV:: OsNUC1-L* transgenic with on MS media with 0, 0.2, 0.4, 0.6uM ABA added; where; $p<0.05$ (asterisk)

The plant hormone abscisic acid (ABA) involves many plant responses to physical stress, especially the response to water status such as drought and salinity. ABA regulates stomatal closure, plant development, such as seed maturation, germination, seedling growth, and also adaptive responses to osmotic stress (Zhang *et al.* 2006). Generally, ABA negatively affected on seed germination. The recent studies of RNA-binding proteins mutants altered the ABA responsive phenotypes such as delayed seed germination, inhibited root growth and rapid stomatal closure upon the ABA application that led to the correlation of ABA signal transduction and post-transcription RNA processing (Kuhn and Schroeder, 2003).

ABA-hypersensitive 1 (*abh1*) was identified as ABA-hypersensitive mutant. The *ABH1* gene encodes the large subunit of nuclear cap-binding protein complex that was showed to participate in several steps of RNA processing, nuclear export and mRNA decay (Makarov *et al.* 2002). The abh1 mutant showed delay seed germination and rapid stomatal closure and less wilting phenotype during drought stress (Hugouvieux, Kwak and Schroeder, 2001).

Moreover, summarily, the mutation of ribosomal proteins and RNA-binding proteins altered the hormonal response (Kim *et al.* 2010). The knock-out mutant Arabidopsis of another glycine-rich RNA-binding protein, *atgrp7-1*, showed the hypersensitive to ABA and salt stress in germination and root development (Kim *et al.* 2008). Moreover, the *atgrp7-1* was reported about their role in stress response. Cao and colleagues (Cao *et al.* 2006) revealed that the *atgrp7-1* mutant conferred the expression of some ABA-induced genes. The overexpressed *AtGRP7* transgenic increased the survival rate under the freezing stress (Kim *et al.* 2008). In addition, when complement with *OsGRP1* and *OSGRP4* in the *atgrp7* mutant, it clearly showed that it conferred the mRNA export from nucleus to cytosol which defected in the *atgrp7* mutant. It suggested the relationship between RNA metabolism, hormone response and stress adaptation.

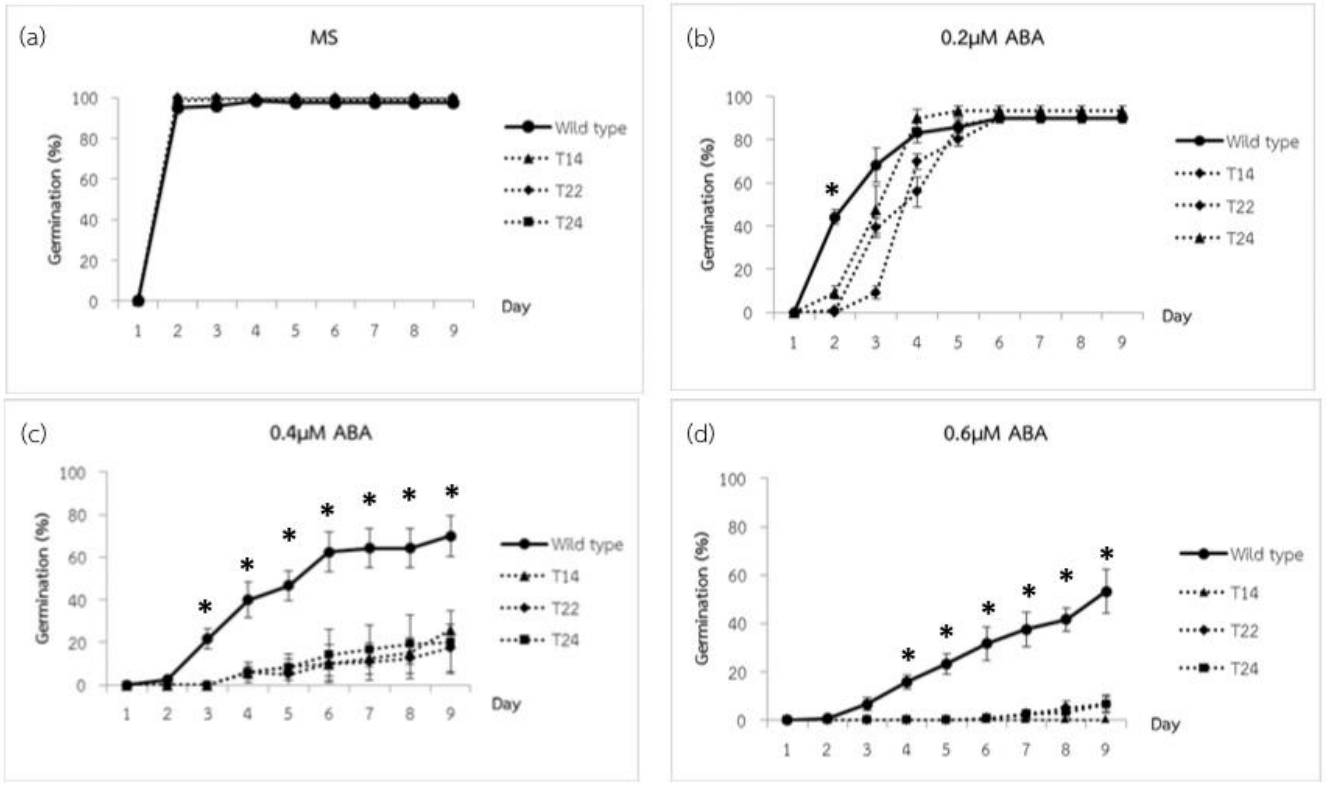


Figure4. 16 The germination percentage of *35SCaMV::OsNUC1-L* (T14, T22, and T24) and wild type Arabidopsis on half-strength MS with the addition of 0µM (a.), 0.2µM (b.), 0.4µM (c.) and 0.6µM ABA (d.). The data were tested and compared to the wild type, where; $p<0.05$ (asterisk)

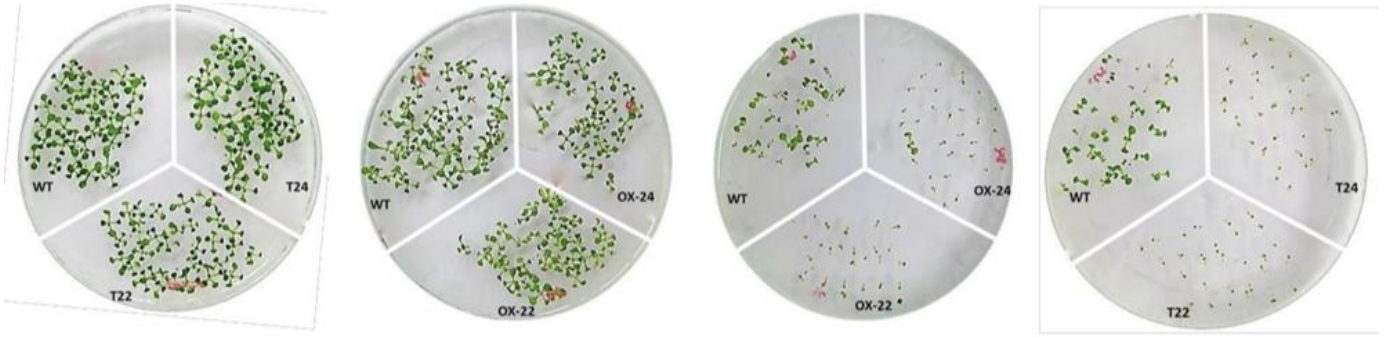


Figure4. 17 The responses of transgenic Arabidopsis to ABA on seed germination. *35SCaMV::OsNUC1-L* (T22, T24) compared with wild-type Arabidopsis on half-strength MS with the addition of 0μM (a.), 0.2μM (b.), 0.4μM (c.) and 0.6μM ABA (d.).

5.2 Overexpressing *OsNUC1-L* gene improved salt-tolerance and enhanced photosynthetic activity by transcriptome modification

To examine the effect of salt stress on photosynthetic activity on overexpression *OsNUC1-L* transgenic lines, the seeds of wild type and three *35SCaMV:: OsNUC1-L* transgenic lines were grown on soil mixture for four weeks under the normal condition at 25°C, 180 µmole photon $m^{-1}s^{-1}$, and 16-hour a day. The net photosynthetic rate, stomatal conductance, and transpiration rate were determined by Gas Analysis System (Li-Cor, LI-6400) with 6400-15 extended reach 1 cm diameter chamber. The gas exchange measurement was performed at 25°C under external light source of 650 µmole photon $m^{-1}s^{-1}$, and the reference carbon dioxide concentration of 350 µmole $m^{-1}s^{-1}$.

Then, the photosynthetic parameters were represented in percentage change and compared using one-way analysis of variance. After irrigation with 100mM NaCl for 24 hours, the net photosynthesis rate in wild type was decreased while it was significantly increased in transgenic plants (Figure4.19-b). The intracellular CO_2 concentration of overexpression *OsNUC1-L* transgenic line was significantly reduced (Figure4.19-c). While the water use efficiency, the ratio of net photosynthesis to the transpiration rate, was significantly increased in transgenic plant (Figure4.20-d). In contrast, no difference was found in stomatal conductivity and transpiration rate (Figure4.19-e and f).

Generally, under salt stress condition, plant growth was limited because the stomata opening were dramatically inhibited in order to reduce the water loss. For these reason, it limited the main substrate of Calvin's cycle. Then, the photosynthetic activity was reduced (Chaves *et al.* 2009).

Moreover, salt stress is the one factor that induced the photo-inhibition (Takahashi and Murata 2008). The photoinhibition was induced by high light intensity. It was resulted from the balance between rate of photodamage and rate of repair to PSII (Allakhverdiev *et al.* 2005). Hypersaline condition negatively affected to the CO_2 fixation by inhibiting the activity of enzyme Rubisco (Solomon *et al.* 1994). Likewise, it also suppressed the protein translation by reducing the ribosome production (Brady *et al.* 1984). At the high salt concentration, it inhibited the repair mechanism of PSII. Allakhverdiev and Murata (2004) revealed that saline treatment reduced D1 protein synthesis. It implied that salt stress negatively affected to the repair process that lead to the photoinhibition.

In contrast, the net photosynthesis in overexpressed *OsNUC1-L* transgenic lines were significantly increased (Figure 4.19-b). Moreover, transcriptomic data revealed that the overexpressed *OsNUC1-L* transgenic line mainly showed the significant enhancement in the genes involving in PSII composition such as PSII light harvesting complex genes (LHCB2.1, LHCB2.2 and LHCB2.3) and PSII reaction center protein (PSBA) (Figure4.12, 4.20 and Table4.7). It suggested that the overexpressed *OsNUC1-L* transgenic lines improved the photosynthetic capacity during salt stress by modified gene expression that assisted the repair process of PSII, which reduced the effect of photodamage.

NUC gene was strongly induced after exposed by light (Tong *et al.* 1997, Reichler *et al.* 2001) indicated the relate function of *NUC* and light (Tong *et al.* 1997). The transcriptomic study revealed that the transgenic lines with *OsNUC1-L* overexpression had the significant enhancement in light harvesting system protein and photosynthetic membrane components during salt stress (Figure4.12, 4.20 and Table4.7). The CAB 2 and 3 (AT1G29910, AT1G29920) and group of photosystem II light harvesting complex (AT2G05070, AT2G05100, AT3G27690, ATCG00020) were dominantly up-regulated (Figure4.12, 4.20 and Table4.7).

In addition, chlorophyll *a/b* binding protein and photosynthetic membrane components also increased during salt stress (Figure4.12, 4.20 and Table4.7). The chlorophyll *a/b* binding proteins (CAB) are the main component in PS and the most abundant protein in plant cell. The T-DNA knockouts of some light-harvesting chlorophyll a/b binding proteins (LHCB) were positively regulated the stomatal opening. Moreover, the mutant with the lack of *LHCBs* function increased the sensitivity to drought stress (Xu *et al.* 2012). It implied that the increase of *LHCB* gene could help plant more tolerant to stress condition.

ABA regulated many abiotic stress responses, particularly osmotic stress by controlling the stomatal opening (Zhang *et al.* 2006). As mention previously, the *35SCaMV::OsNUC1-L* transgenic displayed the hypersensitive character to ABA when tested with the germination analysis (Figure 4.16, 4.17 and 4.18). However, when the whole plants were treated with saline condition, no significance was found in stomatal conductance (Figure4.19e). It reflected the complex regulation of stomatal closure and the different regulation of ABA on seed germination and stomatal opening.

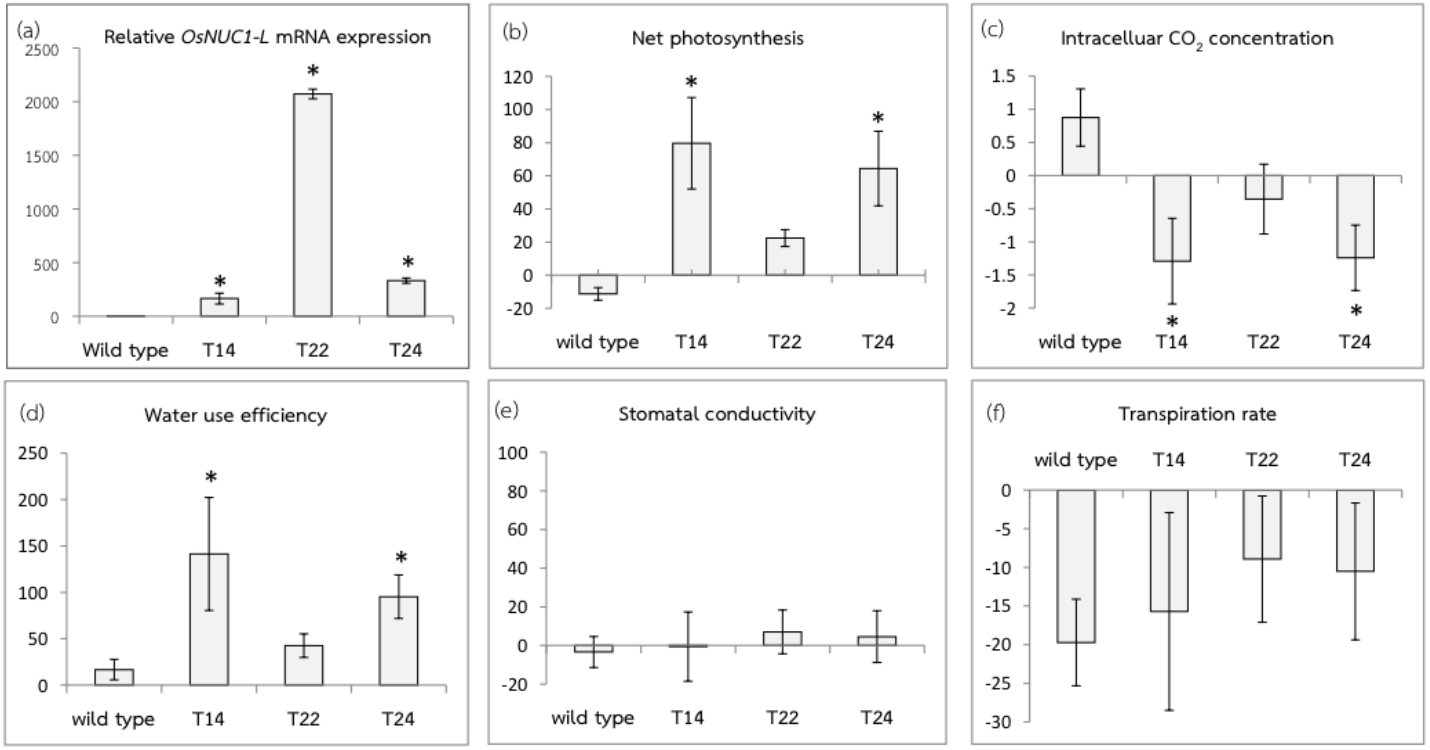


Figure4. 18 The percentage change of photosynthesis parameters after 1 day of salt treatment (a) relative *OsNUC1-L* mRNA expression, (b)net photosynthesis, (c)intracellular CO_2 concentration, (d)water use efficiency, (e)stomatal conductivity and (f) transpiration rate where; $p<0.05$ (asterisk)

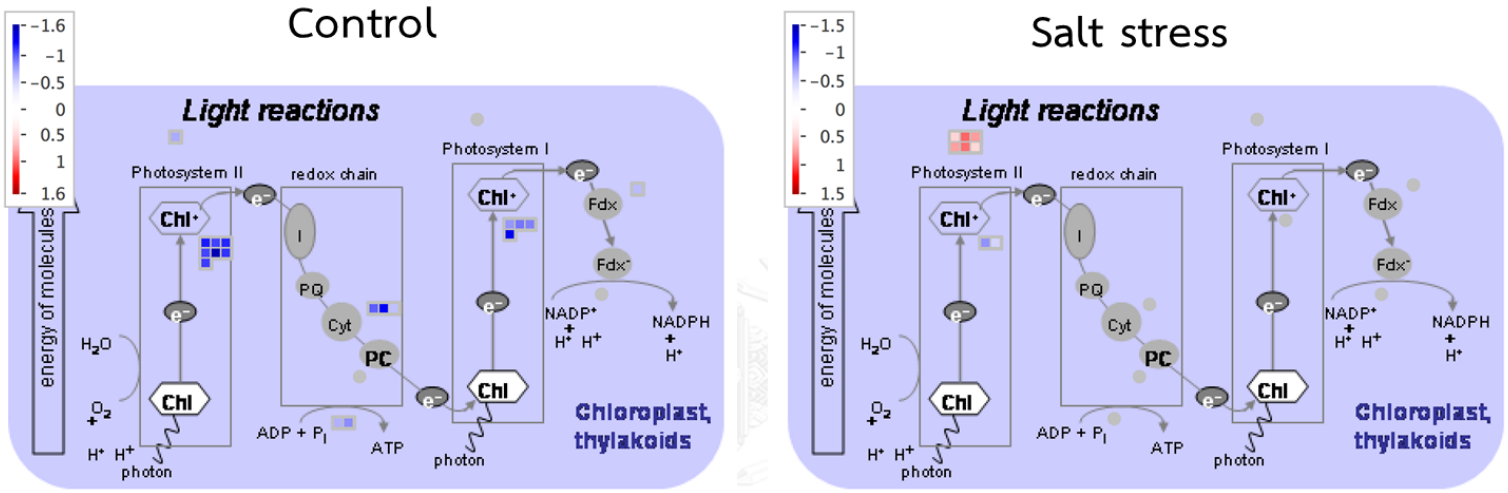


Figure4. 19 The illustration generated by MapMan (Thimm *et al.* 2004) showed that light harvesting system protein and photosynthetic membrane components which significantly decreased in normal condition but increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under salt stress.

Experiment	Location	Locus name	Gene name	Description	Fold Change
Control	Photosystem II	ATCG00020	PSBA	Photosystem II reaction center protein a	0.428
		ATCG00270	PSBD	Photosystem II reaction center protein d	0.464
		ATCG00280	CP43	CP43 subunit of the photosystem II reaction center	0.437
		ATCG00340	PSAB	D1 subunit of photosystem I reaction center	0.566
		ATCG00350	PSAA	psaA protein comprising the reaction center for photosystem I	0.518
		ATCG00630	PSAJ	Subunit J of photosystem I	0.532
		ATCG00680	PSBB	Photosystem II reaction center protein b	0.449
		ATCG00710	PSBH	Photosystem II reaction center protein h	0.457
		ATCG01060	PSAC	PsaC subunit of photosystem I	0.394
	Cytochrome b6f complex	AT5G60920	COB	Glycosylphosphatidylinositol-anchored protein	0.652
		ATCG00540	PETA	Photosynthetic electron transfer a	0.640
		ATCG00720	PETB	Photosynthetic electron transfer b	0.401
		ATCG00730	PETD	Photosynthetic electron transfer d	0.490
		ATMG00160	COX2	Cytochrome c oxidase subunit 2	0.534
		ATMG01360	COX1	Cytochrome c oxidase subunit 1	0.475

Table4. 6 List of light harvesting system protein and photosynthetic membrane components which significantly increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under salt stress

Experiment	Location	Locus number	Gene name	Description	Fold Change
Salt stress	Light-harvesting complex	AT1G29910	CAB3	Chlorophyll a/b binding protein 3	1.403
		AT1G29920	CAB2	Chlorophyll a/b-binding protein 2	1.914
		AT2G05070	LHCB2.2	Photosystem II light harvesting complex gene 2.2	1.682
		AT2G05100	LHCB2.1	Photosystem II light harvesting complex gene 2.1	1.665
		AT3G27690	LHCB2.3	Photosystem II light harvesting complex gene 2.3	1.881
		AT4G31390	ACDO1	ABC1-like kinase related to chlorophyll degradation and oxidative stress 1	0.683
		AT5G54270	LHCB3	Light-harvesting chlorophyll b-binding protein 3	1.390
		ATCG00020	PSBA	Photosystem II reaction center protein A	0.709

Table 4.7 (cont.) List of light harvesting system protein and photosynthetic membrane components which significantly increased in transgenic *35SCaMV::OsNUC1-L* Arabidopsis under salt stress

CHAPTER V

CONCLUSION

1. Genome analysis of salt-tolerant rice

The genetic variation that resulted from *in vitro* propagation was known as somaclonal variation which categorized into 3 levels, chromosome set, chromosome structure and single nucleotide variation. By using the whole genome sequencing, it found that the somaclonal-variegate rice, LPT123-TC171, defected in telomere length, which may be resulted from the oxidative stress during selection process. The mutation rate of somaclonal variation revealed that the pattern of mutation in LPT123-TC171 similar to spontaneous mutation rather than mutation that resulted from mutagenesis. At the nucleotide level, the 23 deleterious mutated genes that contribute to the salt-tolerant character in LPT123-TC171 rice were discovered.

2. Northern blot analysis of *OsNUC1*

The northern blot analysis clearly showed that *OsNUC1* gene presented in two splicing mRNA species which were *OsNUC1-S* and *OsNUC1-L* whose sized were 1.9, 2.4 kb, respectively. Moreover, both splicing forms were up-regulated after treated with 85mM NaCl. In LPT123-TC171, *OsNUC1* rapidly increased after one day of treatment. While, *OsNUC1* in LPT123 rice showed the strongest signal after 4 days of treatment.

3. Transcriptomics of overexpression *OsNUC1-S* transgenic Arabidopsis

The *35SCaMV::OsNUC1-S* transgenics showed the hypersensitive phenotype to ABA and also altered the expression of ABA-inducible genes such as late embryogenesis abundant protein and low-temperature-induced protein 78 by controlling the expression of *NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9* which controlled the rate-limiting step in ABA biosynthesis.

4. Characterization of transgenic *35SCaMV::OsNUC1-L* Arabidopsis

4.1. Study of the salt-stress responses in transgenic Arabidopsis

The primary root length of transgenic lines with *35SCaMV::OsNUC1-L* construct were less inhibited under the saline condition. However, no difference was found in the relative growth rate of both plants during stress. While, under normal condition, the relative growth rate and primary root length of transgenic plants with *35SCaMV::OsNUC1-L* were similar to wild-type plant's.

4.2. Photosynthetic activity

The net photosynthesis and water use efficiency in the overexpressed *OsNUC1-L* transgenic lines were increased when compared to wild-type Arabidopsis during salt-stress condition. In contrast, the intracellular CO_2 concentration in the overexpressed *OsNUC1-L* transgenic significantly declined. However, no significance was detected in stomatal conductivity and transpiration rate between wild type and the transgenic lines.

4.3. The impact of *OsNUC1* overexpression on other gene expression

During normal condition, the transcriptomic analysis showed that the overexpressed *OsNUC1-L* transgenic Arabidopsis negatively enriched the group of genes in photosystem, external encapsulating structure, vacuole and endoplasmic reticulum. However, the genes in extracellular organelle and plasma membrane were positively enriched.

During the stress condition, the overexpressed *OsNUC1-L* transgenic significantly enriched genes in ribosome that include ribosome biosynthesis and ribosome components. Moreover, it induced the genes in plasma membrane component, light-harvesting complex and peroxisome.

4.4. The responses of transgenic Arabidopsis to ABA

The germination test revealed that the transgenic plants with contained *35SCaMV::OsNUC1-L* construct showed the hypersensitive to ABA treatment. When placed the seeds on media supplemented with ABA, the germination percentage of transgenic *35SCaMV::OsNUC1-L* dramatically decreased in the dose-dependent manner.

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Suggestions for further studies

1. Deleterious mutated genes, detected in LPT123-TC171 need to be characterized for their functions on salt-adaptive mechanisms.

2. Transcriptomic comparison between *OsNUC1-S* and *OsNUC1-L* in transgenic Arabidopsis and transgenic rice should be performed.

3. Because the overexpressed *OsNUC1-L* gene dominantly induced genes in plasma membrane composition, the membrane stability of the *OsNUC1-L* overexpressing lines during salt condition should be investigated.

4. The ABA content in the *OsNUC1-L* overexpressing lines under normal and salt condition should be determined.

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APPENDIX

APPENDIX A

Chemical solutions

1. Modified WP no.2 nutrient solution (Vajrabhaya and Vajrabhaya 1991)

Chemicals	Concentration
Macroelements	
KNO_3	580 mg/l
$CaSO_4$	500 mg/l
$MgSO_4 \cdot 7H_2O$	450 mg/l
Triple super phosphate	250 mg/l
$(NH4)_2SO_4$	100mg/l
Microelements	
Na_2EDTA	160mg/l
$FeSO_4 \cdot 7H_2O$	120mg/l
$MnSO4 \cdot H_2O$	15 mg/l
H_3BO_3	5 mg/l
$ZnSO_4 \cdot 7H_2O$	1.5 mg/l
KI	1 mg/l
$Na_2MoO_4 \cdot 2H_2O$	0.1mg/l
$CuSO4 \cdot 5H_2O$	0.05mg/l
$CoCl2 \cdot 6H_2O$	0.05 mg/l

Preparation of 30g/lite $FeSO_4$ stock solution

Na_2EDTA	40g
$FeSO_4 \cdot 7H_2O$	30g

Stir each chemical separately in distilled water with a magnetic stirrer and adjust the final volume to 1 lite.

2. Solutions for DNA and RNA solution

2.1 DNA isolation

2.1.1 Genomic DNA extraction buffer

100 mM Tris pH 8.0

1.4 M NaCl

20 mM EDTA

4% (W/V) CTAB

0.1% (V/V) beta-mercaptoethanol

2.1.2 TE

10 mM Tris pH 8.0

1 mM EDTA

2.2 RNA isolation

2.2.1 10X MOPS

0.2 M MOPS

80 mM sodium acetate

10 mM EDTA

2.2.2 20X SSC

3M NaCl

0.3 M sodium acetate

2.2.3 DEPC-treated TE

10 mM Tris pH8.0

1 mM EDTA

0.1% (V/V) DEPC (diethyl pyrrocarbonate)

2.2.4 RNA extraction buffer

100 mM Tris pH 9.0

100 mM NaCl

20 mM EDTA

1% lauryl sarcosinate 0.1% (V/V)

beta-mercaptoethanol

0.1% (V/V) DEPC (diethyl pyrrocarbonate)

3. Electrophoresis solution

3.1 5XTBE

54g Tris-base

27.5g boric acid

20ml 0.5MEDTA pH8.0

3.2 DNA loading dye

30% glycerol in water

0.25% bromophenol blue

0.25% cylene cyanol

3.3 RNA loading dye for formaldehyde gel (1.5 ml)

80% glycerol

Formamide

Formaldehyde

10X MOPS

DEPC-treated water bromophenol blue (saturated)

4. Solutions for RNA formaldehyde gel

4.1. Formaldehyde gel

Agarose

DEPC-treated water

37% formaldehyde

10XMOPS

4.2. 100X Denhardt's solution

2g bovine serum albumin

2g polyvinyl pyrrolidone

2g ficoll

80ml sterile water

4.3. Hybridization solution

50% Formamide

0.75M Na^+ 20X SSC

1ml 100X Denhart's

23ml water

1ml 20% (W/V) SDS

4.4. Prehybridization solution

50ml Formamide

25ml 20X SSPE

5ml 100X Denhart's

2ml Denatured DNA stock

17ml water

1ml 20% (W/V) SDS

Filled the mixture through a 5μm filter

5. Solutions for molecular cloning

5.1. LB agar medium

1% bacto-tryptone

0.5% bacto-yeast extract

1% NaCl

1.5% LB agar

5.2. LB medium

1% bacto-tryptone

0.5% bacto-yeast extract

1% NaCl

5.3. Solution I

50mM glucose

25mM Tris-HCl

10mM EDTA

5.4. Solution II

0.1N NaOH

1% SDS

5.5. Solution III

5 M Potassium

3M acetate glacial acetic acid

6. Solutions for Arabidopsis transformation

6.1. LB media

1% bacto-tryptone

0.5% bacto-yeast extract

1% NaCl

6.2. Transformation solution for floral dipping method (Clough and Bent 2008)

3% sucrose

0.02% silwet L-77

7. Solutions for genomic DNA library preparation

7.1. Fragmentation:

0.5M EDTA

Bovine serum albumin (BSA)

dsDNA fragmentase buffer

dsDNA fragmentase enzyme

Ultrapure water

7.2. End repair:

10X NEB end repair buffer

NEB end repair enzyme mix

Ultrapure water

7.3. A-base addition:

NEBuffer2

1mM dNTP

NEB Klenow exo-nuclease

7.4. Adapter ligation

0.05 µM premixed adapters

NEB 2X Quick Ligation buffer

NEB Quick Ligation ligase

7.5. Enrichment:

5 µM premixed PE primers

2X Phusion HF master mix

Ultrapure water

8. RNA-Seq library preparation

8.1 mRNA isolation:

Binding Buffer

Dynabeads

Elution buffer

Washing Buffer

8.2 First strand synthesis:

100 mM DTT

5X First Strand buffer

10mM dNTPs

SuperScript III RT

Random hexamers (3µg/µl)

RNase inhibitor/RNaseOUT

8.3 Second strand synthesis:

5X Second strand buffer

Ultrapure water

10 mM dNTPs

DNA polymerase I

RNase H

8.4 Fragmentation:

0.5M EDTA

Bovine serum albumin (BSA)

dsDNA fragmentase buffer

dsDNA fragmentase enzyme

Ultrapure water

8.5 End repair:

10X NEB end repair buffer

NEB end repair enzyme mix

Ultrapure water

8.6 A-base addition:

1mM dNTP

NEB Klenow exo-nuclease

NEBuffer2

8.7 Adapter ligation

0.05 μM premixed adapters

NEB 2X Quick ligation buffer

NEB Quick ligation ligase

8.8 Enrichment:

5 μM premixed PE primers

2X Phusion HF master mix

Ultrapure water

APPENDIX B

Protocols

1. Plant material and growing condition

The original Thai rice, LPT123 rice and its derived salt-resistant lines, LPT123-TC171 were germinated on sand for a week. Then, they were grown in modified WP No.2 nutrient solution (Vajrabhaya and Vajrabhaya, 1991) in the greenhouse with natural light (93–99 μmol photon.$m^{-2}.s^{-1}$) and a relative humidity of between 74% and 81%. The nutrient solution was changed once a week during the experimental period. For the salt treatment, plants were transferred to WP No.2 nutrient solution supplemented with 0.5% NaCl (85mM NaCl)

2. DNA isolation

The plant materials were kept frozen at -80°C. Rice leaf tissues were ground to a fine powder in liquid nitrogen using chilled mortars and pestles. Then, the ground tissues were added into 800μl of hot CTAB buffer (60 °C) and 3μl of beta-mercaptoethanol. After that, the mixture was inverted severaltimes to mix and incubated at 60°C for 30 minutes, and inverted gently every 10 minutes. Afterward, the mixture was left at room temperature for 5 minutes. Then, 500μl of chloroform: isoamylalcohol (24:1) was added and mixed gently for 5 minutes. The mixture was centrifuged at 14,000rpm for 10 minutes at 4°C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. Genomic DNA was precipitated by the addition of 0.1 volumes of 3M NaOAc and 0.6 volumes of isopropanol. The suspension was mixed gently and allowed to stand for 30 minutes on ice. Then, the pellet was collected by centrifugation at 14,000rpm for 10 minutes at 4°C. The pellet was allowed to dry in room temperature for 10-15 minutes. After that, the genomic DNA was resuspended with 100μl of TE buffer.

3. RNA isolation

Total RNA was extracted by the standard hot-phenol method (Thikart *et al.* 2005). *Oryza sativa* L. tissues were ground to fine powder in liquid nitrogen using chilled mortars and pestles. The plant material was kept frozen until the addition of 500µl of hot extraction buffer (80°C) (phenol, 0.1M LiCl, 100mM Tris-HCl (pH 8.0), 10mM EDTA and 1% SDS). After that, the mixtures were homogenized by vortex for 30 seconds. Consequentially, 250µl of phenol: chloroform: isoamyalcohol (25:24:1) was added and mixed gently. The mixture was centrifuged at 14,000 rpm for 5 minutes at 4°C. The upper aqueous phase was transferred to a fresh microcentrifuge tube. RNA was precipitated by addition of two volumes of absolute ethanol and stored at -20°C for 30 minutes. The mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. After centrifugation, the pellet was washed with 80% ethanol and briefly air-dried at room temperature. The pellet was dissolved in 160µl of DEPC-treated TE buffer. RNA solution was added with 40µl of 10M LiCl and stored overnight at -20°C. The mixture was left at room temperature for 5-10 minutes and centrifuged at 14,000 rpm for 20 minutes at 4°C. After centrifugation, the pellet was dissolved in 20 µl DEPC-treated TE buffer. The quality of RNA was performed by agarose electrophoresis.

4. Plasmid DNA isolation

The selected clones of each construct were grown in 10 ml of LB broth (1% (W/V) tryptone, 0.5% (W/V) yeast extract, and 1% (W/V) NaCl) containing 100µg/ml of amplicilin and incubated overnight at 37°C with shaking at 250 rpm. The cells were spun with a microcentrifuge at 8,000 rpm for 7 minutes. The cells were resuspended in 100µl of Lysis buffer (Solution I: 50mM of Glucose, 25mM of Tris-HCl and 10mM of EDTA) and mixed by vortex. Then 200µl of Alkaline-SDS solution (Solution II: 0.2N NaOH and 1%SDS) was added and allowed to stand on ice for 10 minutes. After that 300

µl of high salt solution (Solution III: 3M of potassium acetate) was added to the mixture. The suspension was mixed gently and allowed to stand for 10 minutes on ice. The insoluble salt-genomic DNA precipitate was then removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh microcentrifuge tube and the nucleic acid was precipitated by adding 2 volumes of isopropanol. The sample was mixed thoroughly and immediately centrifuged for 30 minutes to collect the precipitated DNA. The pellet was resuspended in 50 µl of sterile water and the suspension was gently mixed. 10µl of 3M sodium acetate, pH 7 and 300µl of cold absolute ethanol was added to the mixture. The mixture was mixed and chilled on 4°C overnight. The DNA was collected by centrifuging at 14,000 rpm for 20 minutes at 4°C. The pellet was rinsed with 300µl of 70% ethanol and allowed to dry for 10-15 minutes. The plasmid DNA was resuspended with 50µl of sterile water. The concentration was estimated by comparing with DNA marker.

5. RNA formaldehyde gel and northern blot hybridization

Place dry membrane in a Pyrex baking dish. Rehydrate by pouring 500ml of boiling Tris/HCl pH8.0 over the filter. Allow the solution to cool to room temperature for 15-20 minutes. During this time, prepare and warm prehybridization solution to desired temperature. Seal the filter on 3 slides into a plastic bag. Use a heavy “Zip-lock” to seal with the heat sealer. Added prehybridization solution removed the bubbles and sealed the bag. This will make the sealed bag bulge somewhat, and allowed the filter to “slosh” around. Incubate the membrane in a polyethylene box half-filled with water at the desired temperature for 4-24 hours with shaking. Weigh the plastic box down in the shaking water bath with two lead weights. This is particularly important to insure temperature equilibration in the box, and to prevent contaminating the water bath with ^{32}P, should the heat-sealed bag leak during

hybridization. When prehybridization is complete, warm 2 ml of solution to the desired hybridization temperature for each mini gel-sized filter to be probed. Prepare ^{32}P-oligolabeled DNA: 20ng of DNA/ml of hybridization solution in water, incubated in boiling water bath for 5 minutes. Open the filter-containing bag at one corner and discard the prehybridization solution. Add the hybridization solution, using a Pasture pipet. Discard the pipet in the radioactive trash. Carefully remove air bubbles and heat-seal the open corner of the bag. Incubate the membrane as before for at least 16 hours in the shaking water bath. Monitor area in which the solution change was made. When hybridization wascomplete, discard hybridization solution and move the filter to a polyethylene box that contains 2XSSPE and 0.1%SDS. Cover the box and shake for 15minutes at the room temperature and repeat the wash twice. Wash the membrane at least 2X 20minutes in 0.1XSSPE and 0.1%SDS at 55-60°C. After the final wash, wrap the membrane in plastic wrap and place tin X-ray cassette.

6. Genomic DNA library preparation for Genome Analyzer

6.1 DNA Fragmentation

6.1.1 Perform the reaction as

x µl DNA

5 µl NEB fragmentase buffer

0.5 µl BSA

x µl water

48 µl total volume

6.1.2 Incubate 5 minutes on ice.

6.1.3 Add 2µl NEB fragmentase.

6.1.4 Incubate at 37°C for 30 minutes, and then add 5 µl of 0.5 M EDTA to stop the reaction.

6.2 Clean up using AMPure

6.2.1 Clean following standard AMPure protocol by added AMPure in a ratio of 1.8:1 (AMPure: sample).

6.2.2 Let sit on bench for 5 minutes.

6.2.3 Put tube on magnet, and let sit for 2 minutes.

6.2.4 Pipette off supernatant and discard.

6.2.5 Add 200 µl of fresh 70% ethanol, and let sit for 30 seconds, then pipette off and discard wash. Repeat once for a total of 2 washes.

6.2.6 Let beads dry at room temperature for 10 minutes.

6.2.7 Add 20 µl EB, and put back on magnet for elution.

6.2.8 Pipette cleaned sample into a new tube and discard tube with beads.

6.3 End repair

6.3.1 Perform the reaction as

20 µl DNA

5 µl NEB enzyme mix

10 µl NEB 10X end repair module buffer

65 µl water

6.3.2 Incubate 30 minutes at room temperature.

6.3.3 Clean following standard AMPure protocol, elute in 34 µl EB.

6.4 A-base addition

6.4.1 Perform the reaction

34 µl DNA

5 µl NEBuffer 2

10 µl 1 mM dATP

1 µl NEB Klenow exo-nuclease

6.4.2 Incubate 30 minutes at 37°C.

6.4.3 Clean following standard AMPure protocol, elute in 12 µl EB.

6.5 Ligation of adapters

6.5.1 Perform reaction as

12 µl DNA

1 µl 0.5 µM premixed PE adapters

15 µl NEB 2X quick ligation buffer

2 µl NEB quick ligation ligase

6.5.2 Incubate 15 minutes at room temperature.

6.5.3 Add 70 µl EB, mix and spin down, then add 80 µl AMPure. Follow normal protocol for binding and washing, and elute in 30µl EB.

6.6 Enrichment

6.6.1 Perform reaction as

14 µl DNA

1 µl 5 µM premixed PE primers

15 µl Phusion 2X HF master mix

6.6.2 Perform a PCR condition as

PCR: 98°C 0:30 min

(x14 cycles of 98 °C:0:10min, 65 °C: 0:30min, 72°C:0:30)

72°C 5:00 minutes

4°C hold

6.6.3 Testing amplification by run 3 µl of PCR product on a 1.5% TBE agarose gel.

6.6.4 Clean following “magic” AMPure protocol. Elute in 20 µl EB.

7. RNA-Seq library preparation for Genome Analyzer

7.1 Dynabeads for mRNA isolation

7.1.1 Adjust the volume of your total RNA (10-25 µg) to 25 µl with elution buffer (10 mM Tris-HCl, pH 7.5).

7.1.2 Heat at 65°C for 2 minutes, then place on ice.

7.1.3 Transfer 50 µl of well-resuspended Dynabeads to a different tube. Place the tube on the magnet for 30 seconds.

7.1.4 Pipette off the buffer, and add 25 µl of Binding Buffer to the beads off the magnet.

7.1.5 Put the tube back on the magnet and discard the supernatant.

7.1.6 Add 25 µl of Binding Buffer to the Dynabeads off the magnet.

7.1.7 Add the 25 µl of total RNA from step 2, and mix thoroughly. Rotate on a roller or mixer for 3-5 minutes at room temperature to allow mRNA to bind.

7.1.8 Place tube on the magnet until the solution is clear. Discard the supernatant.

7.1.9 Remove the tube from the magnet and wash the beads twice with 50 µl Washing Buffer B. Remove all of the supernatant between each washing step.

7.1.10 Add 9.5 µl of elution buffer (10 mM Tris-HCl, pH 7.5) to the beads.

7.1.11 Heat to 65°C for 2 minutes, and place the tube immediately on the magnet.

7.1.12 Transfer eluted mRNA to a new 200 µl tube.

7.2 First strand synthesis

7.2.1 Add to the 9.5 µl of mRNA in the PCR tube and mix with 1.5 µl of random hexamers (3 µg/µl)

7.2.2 Incubate sample in PCR machine at 65C for 5 minutes, then place tube on ice.

7.2.3 Set PCR machine to 25°C.

7.2.4 Mix the following reagents in the order listed in a separate 200ul tube per sample:

4 µl 5X First Strand Buffer

2 µl 100 mM DTT

1 µl 10mM dNTP Mix

1 µl RNAseOUT

1 µl SuperscriptIII

7.2.5 Add this 9 µl mixture to the tube with 11 µl containing your mRNA, and mix well (20 µl total).

7.2.6 Incubate in PCR machine under following program:

25°C 5 minutes

50°C 60 minutes

70°C 15 minutes

4°C hold

7.2.7 Place tube on ice.

7.3 Second strand synthesis

7.3.1 Add the following components to the first strand reaction (20 µl) on ice:

49 µl Nuclease-free Water

20 µl 5X Second Strand Buffer

5 µl 10 mM dNTP Mix

7.3.2 Mix well and incubate on ice for 5 minutes.

7.3.3 Add the following reagents and mix well but gently:

5 µl DNA polymerase I (50 units)

1 µl RNase H (1.6 units)

7.4.4 Incubate at 16°C for 2.5 hours.

7.4 Clean up using AMPure

7.4.1 Add AMPure in a ratio of 1.8:1 (AMPure:sample) and mix well by pipetting. Let sit on bench for 5 minutes.

7.4.2 Put tube on magnet, and let sit for 2 minutes (or until solution is clear). Pipette off supernatant and discard.

7.4.3 Add 200µl of fresh 70% ethanol, and let sit for 30 seconds, then pipette off and discard wash.

7.4.4 Repeat once for a total of 2 washes.

7.4.5 Let beads dry at room temperature for 10 minutes.

7.4.6 Add 10 µl EB, and put back on magnet for elution.

7.4.7 Pipette sample into a new tube and discard tube with beads.

7.5 Fragment using dsDNA Fragmentase.

7.5.1 Perform the reaction as

10µl DNA

2µl dsDNA Fragmentase buffer

0.5µl BSA

6.5µl water

7.5.2 Incubate 5 minutes on ice.

7.5.3 Add 1µl dsDNA Fragmentase enzyme, and mix.

7.5.4 Incubate 30 minutes at 37°C.

7.5.5 Add 5µl of 0.5M EDTA to kill the reaction after 30 minutes and mix.

7.5.6 Clean following standard AMPure protocol, elute in 25 µl EB.

7.6 End repair

7.6.1 Perform the reaction as

25 µl DNA

5 µl NEB enzyme mix

10 µl NEB 10X end repair module buffer

60µl water

7.6.2 Incubate 30 minutes at room temperature.

7.6.3 Clean following standard AMPure protocol, elute in 34 µl EB

7.7 A-base addition

7.7.1 Perform the reaction as

34 µl DNA

5 µl NEBuffer 2

10 µl 1 mM dATP

1µl NEB Klenow exo-nuclease

7.7.2 Incubate 30 minutes at 37°C.

7.7.3 Clean following standard AMPure protocol, elute in 34 µl EB.

7.8 Ligation of adapters

7.8.1 Perform the reaction as

12 µl DNA

1 µl 0.05 µM premixed PE adapters

15 µl NEB 2X quick ligation buffer

1µl NEB quick ligation ligase

7.8.2 Incubate 15 minutes at room temperature.

7.8.3 Clean up using "Magic" AMPure by add 70 µl EB (to reach 100 µl), mix and spin down, then add 80 µl AMPure. Follow normal protocol, and elute in 20 µl EB.

7.9 Enrichment

7.9.1 Perform the reaction as

10 µl DNA

2 µl 5 µM premixed PE primers

15 µl Phusion 2X HF master mix

3µl water

7.9.2 Perform the PCR reaction as

98°C 0:30minutes

(x14 cycles of 98°C:0:10minutes, 65°C:0:30minutes and 72°C:0:30minutes)

72°C 5:00 min

4°C hold

7.9.3 Testing amplification by run 3 µl of PCR product on 1.5% gel to confirm enrichment product.

7.9.4 Clean following “Magic” AMPure protocol from step 12 by adding 73 µl EB (to reach 100 µl), mixing, and then adding 80 µl AMPure. Elute in 20 µl EB.

APPENDIX C

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os01g05960	1	2841588	C	T	LPT123	Synonymous coding	3	Turquoise
LOC_Os01g19694	1	11172951	C	T	LPT123	Synonymous coding	3	Turquoise
LOC_Os01g25920	1	14703101	A	C	LPT123	Stop gained	0	Turquoise
LOC_Os01g40280	1	22743310	A	C	LPT123	Intron	4	Turquoise
LOC_Os01g54890	1	31567601	T	C	LPT123	Intron	4	Turquoise
LOC_Os01g71820	1	41597530	C	G	LPT123	Intron	4	Blue
LOC_Os02g14500	2	7995421	C	T	LPT123	Non synonymous coding	0.9172	Turquoise
LOC_Os02g32520	2	19248699	G	A	LPT123	Non synonymous coding	0.949	Turquoise
LOC_Os03g05200	3	2521112	G	A	LPT123	Non synonymous coding	0.036	Turquoise
LOC_Os03g11550	3	5983864	C	T	LPT123	Synonymous coding	3	Turquoise
LOC_Os03g20870	3	11815700	G	A	LPT123	Intron	4	Turquoise

TableC. 1 List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os03g25750	3	14727600	G	T	LPT123	5'UTR	4	Turquoise
LOC_Os03g25750	3	14727654	T	G	LPT123	Non synonymous coding	0.06	Turquoise
LOC_Os03g62750	3	35508865	C	T	LPT123	Non synonymous coding	0.1714	Turquoise
LOC_Os04g40070	4	23675066	A	T	LPT123	Synonymous coding	3	Turquoise
LOC_Os04g59420	4	35142201	A	T	LPT123	5'UTR	4	Turquoise
LOC_Os05g07420	5	3958957	A	G	LPT123	Synonymous coding	3	Turquoise
LOC_Os05g14220	5	7973054	C	T	LPT123	3'UTR	4	Turquoise
LOC_Os05g41420	5	24192211	A	G	LPT123	Synonymous coding	3	Blue
LOC_Os05g49770	5	28492285	C	T	LPT123	Non synonymous coding	0.1707	Turquoise
LOC_Os06g06400	6	2987728	C	T	LPT123	Non synonymous coding	0.2938	Blue
LOC_Os06g07040	6	3345018	G	A	LPT123	Intron	4	Blue
LOC_Os06g13720	6	7602343	G	A	LPT123	Intron	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os06g37750	6	22353382	C	A	LPT123	Non synonymous coding	1	Turquoise
LOC_Os06g44470	6	26858405	G	A	LPT123	Synonymous coding	3	Turquoise
LOC_Os06g45100	6	27276338	C	T	LPT123	Non synonymous coding	0.0519	Turquoise
LOC_Os06g50950	6	30838721	C	T	LPT123	Non synonymous coding	0.2	Turquoise
LOC_Os07g23570	7	13324833	C	T	LPT123	Synonymous coding	3	Blue
LOC_Os07g23570	7	13324860	G	A	LPT123	Synonymous coding	3	Blue
LOC_Os07g34520	7	20690523	C	T	LPT123	Synonymous coding	3	Turquoise
LOC_Os07g49480	7	29634270	T	C	LPT123	Non synonymous coding	1	Turquoise
LOC_Os07g49480	7	29635740	C	T	LPT123	Non synonymous coding	0.3087	Turquoise
LOC_Os08g14850	8	8943424	T	C	LPT123	Non synonymous coding	0.2902	Blue
LOC_Os08g14850	8	8943811	G	A	LPT123	Non synonymous coding	0.5848	Blue
LOC_Os08g14850	8	8943829	G	A	LPT123	Non synonymous coding	0.8258	Blue

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os08g14850	8	8943838	T	C	LPT123	Non synonymous coding	0.8331	Blue
LOC_Os09g31040	9	18671417	C	T	LPT123	5'UTR	4	Turquoise
LOC_Os10g13810	10	7516163	A	G	LPT123	Synonymous coding	3	Turquoise
LOC_Os10g20470	10	10231911	G	A	LPT123	Intron	4	Turquoise
LOC_Os11g06100	11	2901382	A	G	LPT123	Non synonymous coding	1	Blue
LOC_Os11g06130	11	2906095	A	G	LPT123	Non synonymous coding	0.1746	Turquoise
LOC_Os11g10760	11	5906285	T	C	LPT123	Non synonymous coding	0.1629	Turquoise
LOC_Os11g12650	11	7121785	G	A	LPT123	Synonymous coding	3	Turquoise
LOC_Os11g26750	11	14857139	T	C	LPT123	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28204876	C	G	LPT123	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205419	C	T	LPT123	Synonymous coding	3	Turquoise
LOC_Os11g47520	11	28207277	A	C	LPT123	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os01g03710	1	1536267	G	A	LPT171	Non synonymous coding	0.4068	Turquoise
LOC_Os01g09900	1	5149204	G	A	LPT171	Non synonymous coding	0.0961	Blue
LOC_Os01g11730	1	6342045	G	A	LPT171	Non synonymous coding	0.6113	Turquoise
LOC_Os01g11730	1	6342125	C	T	LPT171	Intron	4	Turquoise
LOC_Os01g12820	1	7094978	A	G	LPT171	Non synonymous coding	0.8803	Turquoise
LOC_Os01g16170	1	9135937	T	A	LPT171	Intron	4	Blue
LOC_Os01g16170	1	9136420	T	A	LPT171	Non synonymous coding	0.309	Blue
LOC_Os01g16170	1	9136485	G	T	LPT171	Non synonymous coding	0.186	Blue
LOC_Os01g19694	1	11166532	A	T	LPT171	Intron	4	Turquoise
LOC_Os01g20830	1	11610177	G	C	LPT171	Intron	4	Turquoise
LOC_Os01g21420	1	11955883	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g21420	1	11955900	T	C	LPT171	Non synonymous coding	0	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os01g21420	1	11955948	C	T	LPT171	Non synonymous coding	0.0222	Turquoise
LOC_Os01g21420	1	11955956	C	T	LPT171	Intron	4	Turquoise
LOC_Os01g21420	1	11955997	G	A	LPT171	Intron	4	Turquoise
LOC_Os01g22980	1	12915445	T	G	LPT171	Intron	4	Turquoise
LOC_Os01g25920	1	14701801	A	C	LPT171	3'UTR	4	Turquoise
LOC_Os01g25920	1	14702980	A	C	LPT171	Non synonymous coding	0.0647	Turquoise
LOC_Os01g25920	1	14703016	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g25920	1	14703022	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g25920	1	14703027	T	C	LPT171	Non synonymous coding	0.1967	Turquoise
LOC_Os01g25920	1	14703071	G	A	LPT171	Non synonymous coding	0.2644	Turquoise
LOC_Os01g27390	1	15291606	C	T	LPT171	Intron	4	Turquoise
LOC_Os01g27390	1	15291620	C	A	LPT171	Intron	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os01g47050	1	26873110	A	C	LPT171	Non synonymous coding	0.3521	Turquoise
LOC_Os01g47400	1	27083820	G	A	LPT171	Intron	4	Turquoise
LOC_Os01g50616	1	29069375	A	T	LPT171	Intron	4	Turquoise
LOC_Os01g50616	1	29069656	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g50700	1	29122484	A	G	LPT171	Non synonymous coding	0.525	Turquoise
LOC_Os01g53090	1	30517609	A	G	LPT171	Intron	4	Turquoise
LOC_Os01g53220	1	30581935	T	C	LPT171	Intron	4	Turquoise
LOC_Os01g54030	1	31076162	T	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g54030	1	31076478	A	G	LPT171	Intron	4	Turquoise
LOC_Os01g54890	1	31567601	T	C	LPT171	Intron	4	Turquoise
LOC_Os01g57420	1	33189859	A	C	LPT171	Intron	4	Turquoise
LOC_Os01g57450	1	33197823	A	G	LPT171	3'UTR	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os01g57740	1	33393069	G	A	LPT171	Non synonymous coding	0.7475	Turquoise
LOC_Os01g58194	1	33649412	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g59100	1	34143086	T	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g59100	1	34143156	A	G	LPT171	Non synonymous coding	0.0692	Turquoise
LOC_Os01g66890	1	38843502	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g66890	1	38843531	C	A	LPT171	Non synonymous coding	0.4883	Turquoise
LOC_Os01g66890	1	38843663	G	A	LPT171	Non synonymous coding	0.381	Turquoise
LOC_Os01g66890	1	38843679	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os01g67010	1	38915283	A	G	LPT171	Non synonymous coding	0.7058	Blue
LOC_Os01g69010	1	40094061	G	A	LPT171	Non synonymous coding	0.0603	Turquoise
LOC_Os01g69010	1	40094229	A	G	LPT171	Intron	4	Turquoise
LOC_Os01g71810	1	41591370	A	G	LPT171	Non synonymous coding	0.4626	Blue

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os01g71820	1	41596929	G	T	LPT171	Non synonymous coding	0.288	Blue
LOC_Os02g02120	2	627900	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g02120	2	628385	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g02120	2	628395	G	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g04640	2	2078153	T	G	LPT171	5'UTR	4	Blue
LOC_Os02g04780	2	2192398	A	T	LPT171	Intron	4	Turquoise
LOC_Os02g11870	2	6149710	T	A	LPT171	Non synonymous coding	3.5	Turquoise
LOC_Os02g14500	2	7995301	T	G	LPT171	Intron	4	Turquoise
LOC_Os02g14500	2	7995347	A	G	LPT171	Intron	4	Turquoise
LOC_Os02g14500	2	7995421	C	T	LPT171	Non synonymous coding	0.9172	Turquoise
LOC_Os02g14500	2	7995516	A	G	LPT171	Non synonymous coding	0.4033	Turquoise
LOC_Os02g14500	2	7996484	A	G	LPT171	Non synonymous coding	0.3018	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os02g14500	2	7996491	C	T	LPT171	Non synonymous coding	0.4402	Turquoise
LOC_Os02g20970	2	12398485	C	A	LPT171	Non synonymous coding	1	Turquoise
LOC_Os02g20970	2	12398906	C	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g20970	2	12399147	A	G	LPT171	Non synonymous coding	0.6462	Turquoise
LOC_Os02g20970	2	12399603	T	A	LPT171	Non synonymous coding	0.2705	Turquoise
LOC_Os02g20970	2	12402074	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g30100	2	17869680	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g30910	2	18450419	A	G	LPT171	Intron	4	Turquoise
LOC_Os02g32520	2	19248757	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g47560	2	29054960	A	C	LPT171	Non synonymous coding	0.7797	Turquoise
LOC_Os02g47560	2	29056041	G	A	LPT171	Non synonymous coding	0.0193	Turquoise
LOC_Os02g47560	2	29056088	G	A	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os02g47560	2	29056208	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g47560	2	29056268	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g47560	2	29056319	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g50340	2	30734480	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g51110	2	31262948	G	A	LPT171	Intron	4	Blue
LOC_Os02g51930	2	31801648	T	G	LPT171	Non synonymous coding	0.3323	Turquoise
LOC_Os02g52780	2	32273974	C	T	LPT171	Intron	4	Turquoise
LOC_Os02g54240	2	33243999	A	G	LPT171	Non synonymous coding	0.1661	Turquoise
LOC_Os02g54240	2	33244034	G	A	LPT171	Non synonymous coding	0.3174	Turquoise
LOC_Os02g54240	2	33245156	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os02g56370	2	34504658	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g56370	2	34504706	A	G	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os02g56370	2	34506123	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g56370	2	34506135	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os02g56370	2	34506530	A	G	LPT171	Non synonymous coding	0.1828	Turquoise
LOC_Os03g05200	3	2521016	T	C	LPT171	Non synonymous coding	0.5346	Turquoise
LOC_Os03g05200	3	2521017	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g10110	3	5117767	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g10110	3	5117770	A	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g11550	3	5983727	G	A	LPT171	Intron	4	Turquoise
LOC_Os03g11550	3	5983864	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g11900	3	6237185	A	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g16920	3	9410875	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os03g16920	3	9410948	A	G	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os03g16920	3	9412304	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g16920	3	9414783	G	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g18490	3	10365994	G	A	LPT171	Non synonymous coding	0.2697	Turquoise
LOC_Os03g22200	3	12729550	A	C	LPT171	Intron	4	Turquoise
LOC_Os03g26870	3	15331623	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os03g27920	3	16031978	A	C	LPT171	Intron	4	Turquoise
LOC_Os03g43590	3	24351310	A	G	LPT171	Non synonymous coding	0.7465	Turquoise
LOC_Os03g51350	3	29380585	C	T	LPT171	Intron	4	Turquoise
LOC_Os03g62750	3	35508862	C	T	LPT171	Non synonymous coding	0.2322	Turquoise
LOC_Os04g01470	4	316822	C	A	LPT171	Intron	4	Turquoise
LOC_Os04g12960	4	7149246	G	A	LPT171	Non synonymous coding	0.0915	Turquoise
LOC_Os04g12960	4	7150241	T	C	LPT171	Non synonymous coding	0.0996	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os04g12960	4	7150500	C	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g28780	4	16874483	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g32480	4	19322760	A	*1A	LPT171	Frameshift-deletion	0	Turquoise
LOC_Os04g33200	4	19913150	G	T	LPT171	Intron	4	Turquoise
LOC_Os04g33200	4	19913193	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g33200	4	19913916	A	G	LPT171	Intron	4	Turquoise
LOC_Os04g33920	4	20371843	C	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g33920	4	20372183	G	A	LPT171	Intron	4	Turquoise
LOC_Os04g33920	4	20372256	C	T	LPT171	3'UTR	4	Turquoise
LOC_Os04g33920	4	20372273	T	C	LPT171	3'UTR	4	Turquoise
LOC_Os04g39350	4	23237570	C	G	LPT171	Non synonymous coding	0.7787	Turquoise
LOC_Os04g39350	4	23238095	T	G	LPT171	5'UTR	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os04g40070	4	23674165	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g40070	4	23674230	T	C	LPT171	Non synonymous coding	0.1024	Turquoise
LOC_Os04g40070	4	23674677	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os04g40070	4	23674715	C	T	LPT171	Intron	4	Turquoise
LOC_Os04g40070	4	23674722	T	C	LPT171	Intron	4	Turquoise
LOC_Os04g40070	4	23675005	C	T	LPT171	Intron	4	Turquoise
LOC_Os04g40070	4	23675016	T	G	LPT171	Intron	4	Turquoise
LOC_Os04g40070	4	23675066	A	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g40070	4	23675228	T	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g40070	4	23675405	T	G	LPT171	Intron	4	Turquoise
LOC_Os04g40070	4	23675425	T	A	LPT171	Intron	4	Turquoise
LOC_Os04g40470	4	23864051	C	A	LPT171	3'UTR	4	Blue

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os04g40470	4	23864096	C	T	LPT171	Synonymous coding	3	Blue
LOC_Os04g40470	4	23864101	T	C	LPT171	Non synonymous coding	0.1268	Blue
LOC_Os04g40470	4	23864142	G	A	LPT171	Non synonymous coding	0.0881	Blue
LOC_Os04g43200	4	25379582	G	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g43290	4	25424057	A	C	LPT171	Intron	4	Turquoise
LOC_Os04g43290	4	25424111	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g43290	4	25424913	T	C	LPT171	Intron	4	Turquoise
LOC_Os04g43290	4	25425197	C	T	LPT171	Intron	4	Turquoise
LOC_Os04g43290	4	25425210	G	T	LPT171	Intron	4	Turquoise
LOC_Os04g47700	4	28117653	G	A	LPT171	Non synonymous coding	0.2215	Turquoise
LOC_Os04g47700	4	28117675	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os04g50770	4	29845187	G	T	LPT171	Intron	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os04g57750	4	34212556	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os04g59420	4	35142983	C	G	LPT171	Non synonymous coding	0.6292	Turquoise
LOC_Os04g59420	4	35143067	T	C	LPT171	3'UTR	4	Turquoise
LOC_Os04g59540	4	35230156	G	T	LPT171	Non synonymous coding	0.6338	Turquoise
LOC_Os04g59540	4	35231743	G	A	LPT171	Non synonymous coding	0.5901	Turquoise
LOC_Os04g59540	4	35232486	G	A	LPT171	Non synonymous coding	0.5734	Turquoise
LOC_Os05g07420	5	3957406	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os05g07420	5	3958427	G	A	LPT171	Non synonymous coding	0.1064	Turquoise
LOC_Os05g07420	5	3958957	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os05g14220	5	7973125	T	C	LPT171	Non synonymous coding	0.0028	Turquoise
LOC_Os05g14220	5	7973173	C	T	LPT171	Non synonymous coding	0.5707	Turquoise
LOC_Os05g14220	5	7974228	C	A	LPT171	Non synonymous coding	0.2322	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os05g14220	5	7974291	T	C	LPT171	5'UTR	4	Turquoise
LOC_Os05g14220	5	7974302	C	T	LPT171	5'UTR	4	Turquoise
LOC_Os05g24770	5	14301413	T	A	LPT171	Intron	4	Turquoise
LOC_Os05g25040	5	14432269	G	A	LPT171	Non synonymous coding	0.5651	Turquoise
LOC_Os05g25040	5	14433403	C	T	LPT171	Intron	4	Turquoise
LOC_Os05g31020	5	17957997	T	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os05g34730	5	20538760	C	G	LPT171	Non synonymous coding	0.2826	Blue
LOC_Os05g34730	5	20538773	G	A	LPT171	Non synonymous coding	0.0274	Blue
LOC_Os05g38270	5	22381030	A	G	LPT171	Intron	4	Turquoise
LOC_Os05g39720	5	23248349	C	T	LPT171	Synonymous coding	3	Blue
LOC_Os05g41490	5	24230117	A	T	LPT171	Non synonymous coding	1	Turquoise
LOC_Os05g42270	5	24664240	A	G	LPT171	Non synonymous coding	1	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os05g43910	5	25482267	G	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os05g43910	5	25482276	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os05g44570	5	25866529	C	T	LPT171	Stop gained	0	Blue
LOC_Os05g44570	5	25866530	C	A	LPT171	Non synonymous coding	1	Blue
LOC_Os05g44570	5	25868048	C	T	LPT171	Intron	4	Blue
LOC_Os05g44570	5	25868254	T	C	LPT171	Non synonymous coding	0.5603	Blue
LOC_Os05g44770	5	25962335	T	G	LPT171	Non synonymous coding	1	Blue
LOC_Os05g48700	5	27848897	T	C	LPT171	Intron	4	Turquoise
LOC_Os05g48810	5	27911108	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os05g49730	5	28461662	T	A	LPT171	Intron	4	Turquoise
LOC_Os05g49770	5	28491617	A	G	LPT171	Intron	4	Turquoise
LOC_Os05g50260	5	28740100	A	G	LPT171	Non synonymous coding	1	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os05g51390	5	29408597	C	T	LPT171	Intron	4	Blue
LOC_Os06g05470	6	2471937	A	T	LPT171	Non synonymous coding	0.1464	Turquoise
LOC_Os06g05470	6	2471942	T	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g06230	6	2883406	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g06400	6	2984679	T	C	LPT171	Non synonymous coding	0.6626	Blue
LOC_Os06g06400	6	2985714	C	A	LPT171	Non synonymous coding	0.1178	Blue
LOC_Os06g06400	6	2987728	C	T	LPT171	Non synonymous coding	0.2938	Blue
LOC_Os06g07040	6	3344788	G	A	LPT171	Synonymous coding	3	Blue
LOC_Os06g08280	6	4009171	A	G	LPT171	Non synonymous coding	0.502	Turquoise
LOC_Os06g08280	6	4009213	T	A	LPT171	Non synonymous coding	0.643	Turquoise
LOC_Os06g09560	6	4867983	A	G	LPT171	Non synonymous coding	0.6346	Turquoise
LOC_Os06g21570	6	12441642	A	G	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os06g21910	6	12664905	T	A	LPT171	Non synonymous coding	0.105	Turquoise
LOC_Os06g36850	6	21709605	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g37750	6	22351520	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g37750	6	22351583	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g37750	6	22352192	A	T	LPT171	Non synonymous coding	0.0457	Turquoise
LOC_Os06g37750	6	22352215	C	T	LPT171	Non synonymous coding	0.2803	Turquoise
LOC_Os06g37750	6	22352221	T	C	LPT171	Non synonymous coding	0.1506	Turquoise
LOC_Os06g37750	6	22353426	G	A	LPT171	Non synonymous coding	0.3984	Turquoise
LOC_Os06g39960	6	23772566	T	C	LPT171	Intron	4	Turquoise
LOC_Os06g43020	6	25859237	T	G	LPT171	Non synonymous coding	0.2514	Turquoise
LOC_Os06g44010	6	26510666	G	A	LPT171	Non synonymous coding	0	Blue
LOC_Os06g44470	6	26858380	C	T	LPT171	Non synonymous coding	0.3995	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os06g45100	6	27276237	T	C	LPT171	Intron	4	Turquoise
LOC_Os06g45100	6	27276338	C	T	LPT171	Non synonymous coding	0.0519	Turquoise
LOC_Os06g47270	6	28652032	G	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g50400	6	30519945	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os06g50950	6	30837404	G	A	LPT171	Non synonymous coding	1	Turquoise
LOC_Os06g50950	6	30838721	C	T	LPT171	Non synonymous coding	0.2	Turquoise
LOC_Os07g05365	7	2456025	G	A	LPT171	Non synonymous coding	0.5851	Turquoise
LOC_Os07g05370	7	2458475	C	T	LPT171	Non synonymous coding	0.0046	Turquoise
LOC_Os07g10190	7	5481404	A	G	LPT171	Non synonymous coding	1	Turquoise
LOC_Os07g10190	7	5481413	T	C	LPT171	Non synonymous coding	0.2739	Turquoise
LOC_Os07g18874	7	11181530	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os07g23570	7	13323728	C	T	LPT171	Stop gained	0	Blue

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os07g23570	7	13323781	C	T	LPT171	Synonymous coding	3	Blue
LOC_Os07g23570	7	13323815	C	T	LPT171	Non synonymous coding	0.0302	Blue
LOC_Os07g23570	7	13323841	G	A	LPT171	Synonymous coding	3	Blue
LOC_Os07g23570	7	13323847	T	C	LPT171	Synonymous coding	3	Blue
LOC_Os07g23570	7	13324350	G	A	LPT171	Synonymous coding	3	Blue
LOC_Os07g23570	7	13324363	G	A	LPT171	Non synonymous coding	0.2732	Blue
LOC_Os07g23570	7	13324372	G	T	LPT171	Non synonymous coding	0.015	Blue
LOC_Os07g23570	7	13324567	T	C	LPT171	Synonymous coding	3	Blue
LOC_Os07g23570	7	13324578	G	T	LPT171	Non synonymous coding	0	Blue
LOC_Os07g23570	7	13324725	C	A	LPT171	Synonymous coding	3	Blue
LOC_Os07g24000	7	13606909	T	G	LPT171	5'UTR	4	Turquoise
LOC_Os07g26900	7	15580884	G	A	LPT171	Intron	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os07g26900	7	15580935	G	*2GT	LPT171	Frameshift-deletion	0	Turquoise
LOC_Os07g26900	7	15581948	T	C	LPT171	Intron	4	Turquoise
LOC_Os07g37730	7	22620042	G	A	LPT171	Non synonymous coding	0.852	Turquoise
LOC_Os07g40250	7	24127744	T	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os07g42280	7	25299171	G	A	LPT171	Intron	4	Turquoise
LOC_Os07g42994	7	25752637	T	G	LPT171	3'UTR	4	Turquoise
LOC_Os07g42994	7	25753493	G	A	LPT171	Intron	4	Turquoise
LOC_Os07g43960	7	26270272	T	C	LPT171	Non synonymous coding	0.4097	Turquoise
LOC_Os07g47670	7	28483353	A	G	LPT171	Start_gained	4	Turquoise
LOC_Os07g47670	7	28483493	G	A	LPT171	Intron	4	Turquoise
LOC_Os07g48830	7	29220499	C	G	LPT171	Non synonymous coding	0.1331	Turquoise
LOC_Os07g48830	7	29220500	C	T	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os07g49480	7	29634270	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os07g49480	7	29635175	A	G	LPT171	Non synonymous coding	1	Turquoise
LOC_Os07g49480	7	29635964	T	C	LPT171	Non synonymous coding	0.399	Turquoise
LOC_Os07g49480	7	29636686	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g04340	8	2129261	T	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g04340	8	2129468	T	C	LPT171	3'UTR	4	Turquoise
LOC_Os08g04630	8	2312617	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g14850	8	8939879	G	A	LPT171	Non synonymous coding	0.6392	Blue
LOC_Os08g14850	8	8943389	T	G	LPT171	Non synonymous coding	1	Blue
LOC_Os08g14850	8	8943424	T	C	LPT171	Non synonymous coding	0.2902	Blue
LOC_Os08g14850	8	8943640	C	A	LPT171	Non synonymous coding	0.6753	Blue
LOC_Os08g14850	8	8950290	T	C	LPT171	Synonymous coding	3	Blue

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os08g14850	8	8950365	G	C	LPT171	Non synonymous coding	0.3992	Blue
LOC_Os08g14850	8	8950664	T	A	LPT171	Non synonymous coding	0.521	Blue
LOC_Os08g32060	8	19872978	T	C	LPT171	Non synonymous coding	0.0342	Turquoise
LOC_Os08g32060	8	19872997	T	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g35600	8	22413918	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g37210	8	23500634	A	G	LPT171	Synonymous coding	3	Blue
LOC_Os08g37210	8	23500637	G	T	LPT171	Non synonymous coding	0.4899	Blue
LOC_Os08g37210	8	23500643	G	C	LPT171	Synonymous coding	3	Blue
LOC_Os08g37210	8	23501525	T	G	LPT171	Non synonymous coding	0.4	Blue
LOC_Os08g37210	8	23501716	A	T	LPT171	Non synonymous coding	1	Blue
LOC_Os08g37210	8	23501720	G	T	LPT171	Synonymous coding	3	Blue
LOC_Os08g38990	8	24644726	G	A	LPT171	Non synonymous coding	0.001	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os08g39694	8	25131016	C	A	LPT171	Non synonymous coding	0.0014	Turquoise
LOC_Os08g39840	8	25215233	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g43120	8	27273232	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os08g43120	8	27273429	G	A	LPT171	Intron	4	Turquoise
LOC_Os09g16090	9	9824609	C	A	LPT171	3'UTR	4	Blue
LOC_Os09g16090	9	9824628	A	T	LPT171	3'UTR	4	Blue
LOC_Os09g16090	9	9824638	T	C	LPT171	Non synonymous coding	0.991	Blue
LOC_Os09g16090	9	9824688	A	G	LPT171	Non synonymous coding	0.5877	Blue
LOC_Os09g16090	9	9824752	C	T	LPT171	Non synonymous coding	0.1405	Blue
LOC_Os09g16090	9	9824767	C	T	LPT171	Non synonymous coding	0.5788	Blue
LOC_Os09g16090	9	9824768	C	T	LPT171	Synonymous coding	3	Blue
LOC_Os09g16090	9	9824798	C	A	LPT171	Synonymous coding	3	Blue

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os09g16090	9	9824903	G	C	LPT171	Non synonymous coding	1	Blue
LOC_Os09g20284	9	12167039	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os09g20284	9	12167055	T	A	LPT171	Non synonymous coding	0.018	Turquoise
LOC_Os09g20284	9	12167064	T	C	LPT171	Non synonymous coding	0.3757	Turquoise
LOC_Os09g20284	9	12171945	G	A	LPT171	Intron	4	Turquoise
LOC_Os09g20284	9	12172091	A	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os09g20284	9	12173782	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os09g20480	9	12348034	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os09g24860	9	14840271	T	A	LPT171	Non synonymous coding	0.4339	Turquoise
LOC_Os09g24860	9	14840300	G	A	LPT171	Non synonymous coding	0.2705	Turquoise
LOC_Os09g31031	9	18669893	G	A	LPT171	Intron	4	Blue
LOC_Os09g34160	9	20163425	T	G	LPT171	Non synonymous coding	0.4944	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os09g34160	9	20163636	A	C	LPT171	Non synonymous coding	0.4985	Turquoise
LOC_Os09g39910	9	22883692	C	T	LPT171	Non synonymous coding	0.2017	Turquoise
LOC_Os09g39910	9	22885420	A	G	LPT171	Non synonymous coding	0.2318	Turquoise
LOC_Os10g01470	10	285494	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os10g04020	10	1838627	T	C	LPT171	Non synonymous coding	0.0181	Blue
LOC_Os10g23050	10	11946293	C	T	LPT171	3'UTR	4	Turquoise
LOC_Os10g36500	10	19460146	T	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os10g37230	10	19844721	G	T	LPT171	Non synonymous coding	0.4923	Turquoise
LOC_Os10g37660	10	20085839	T	C	LPT171	Intron	4	Turquoise
LOC_Os10g37670	10	20090777	T	C	LPT171	Intron	4	Turquoise
LOC_Os10g38820	10	20589484	A	T	LPT171	Non synonymous coding	0.8934	Turquoise
LOC_Os10g38820	10	20589608	A	C	LPT171	Non synonymous coding	0.8748	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os10g38940	10	20674093	G	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os10g40480	10	21603428	C	G	LPT171	Non synonymous coding	1	Turquoise
LOC_Os10g40480	10	21603565	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os11g03290	11	1223549	C	T	LPT171	Intron	4	Turquoise
LOC_Os11g09020	11	4795521	C	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g09020	11	4795893	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g09020	11	4795965	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g09020	11	4795983	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g09020	11	4796037	G	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g09020	11	4796516	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g09020	11	4796690	C	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g10760	11	5905182	G	A	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os11g10760	11	5906285	T	C	LPT171	Non synonymous coding	0.1629	Turquoise
LOC_Os11g10760	11	5907464	G	A	LPT171	Stop gained	0	Turquoise
LOC_Os11g10770	11	5912958	A	G	LPT171	Stop_lost	1.25	Turquoise
LOC_Os11g10770	11	5913689	A	C	LPT171	Non synonymous coding	0.3801	Turquoise
LOC_Os11g14900	11	8375141	A	G	LPT171	Synonymous coding	3	Blue
LOC_Os11g14900	11	8375263	T	C	LPT171	Non synonymous coding	0.3939	Blue
LOC_Os11g14900	11	8375276	G	A	LPT171	Synonymous coding	3	Blue
LOC_Os11g17380	11	9687192	G	T	LPT171	Intron	4	Turquoise
LOC_Os11g17380	11	9687329	C	T	LPT171	Non synonymous coding	0.1243	Turquoise
LOC_Os11g17380	11	9687345	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g17380	11	9689524	G	A	LPT171	Non synonymous coding	0	Turquoise
LOC_Os11g17380	11	9689529	A	G	LPT171	Non synonymous coding	0	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os11g17380	11	9689553	G	A	LPT171	Non synonymous coding	0	Turquoise
LOC_Os11g17380	11	9689563	G	A	LPT171	Non synonymous coding	0	Turquoise
LOC_Os11g26750	11	14857007	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g26780	11	14872913	G	C	LPT171	Non synonymous coding	0.5569	Turquoise
LOC_Os11g26780	11	14872967	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g27400	11	15301094	A	T	LPT171	5'UTR	4	Turquoise
LOC_Os11g27400	11	15301160	C	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g29870	11	16889321	A	G	LPT171	3'UTR	4	Turquoise
LOC_Os11g30500	11	17263020	G	A	LPT171	5'UTR	4	Turquoise
LOC_Os11g31550	11	17971411	T	C	LPT171	Intron	4	Blue
LOC_Os11g41710	11	24574797	G	T	LPT171	5'UTR	4	Turquoise
LOC_Os11g41710	11	24576279	A	G	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os11g45740	11	27199184	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199187	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199269	T	C	LPT171	Non synonymous coding	0.4203	Turquoise
LOC_Os11g45740	11	27199273	A	C	LPT171	Non synonymous coding	0.1721	Turquoise
LOC_Os11g45740	11	27199459	C	G	LPT171	Non synonymous coding	0.1181	Turquoise
LOC_Os11g45740	11	27199498	G	T	LPT171	Non synonymous coding	0	Turquoise
LOC_Os11g45740	11	27199502	A	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199511	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199523	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199562	T	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199630	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g45740	11	27199634	T	C	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os11g47510	11	28204708	G	C	LPT171	Non synonymous coding	0.2563	Turquoise
LOC_Os11g47510	11	28204930	A	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205011	G	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205033	C	G	LPT171	Non synonymous coding	1	Turquoise
LOC_Os11g47510	11	28205038	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205041	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205065	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205138	T	C	LPT171	Non synonymous coding	0.1212	Turquoise
LOC_Os11g47510	11	28205461	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os11g47510	11	28205499	T	C	LPT171	Non synonymous coding	1	Turquoise
LOC_Os11g47510	11	28205505	C	A	LPT171	Non synonymous coding	0.5396	Turquoise
LOC_Os11g47510	11	28205537	G	A	LPT171	5'UTR	4	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os11g47510	11	28205545	G	A	LPT171	5'UTR	4	Turquoise
LOC_Os11g47510	11	28205577	T	C	LPT171	5'UTR	4	Turquoise
LOC_Os11g47510	11	28205587	A	G	LPT171	Start_gained	4	Turquoise
LOC_Os11g47520	11	28207167	A	G	LPT171	Non synonymous coding	0.3697	Turquoise
LOC_Os11g47520	11	28207171	C	T	LPT171	Non synonymous coding	1	Turquoise
LOC_Os11g47520	11	28207221	G	A	LPT171	Non synonymous coding	0.2497	Turquoise
LOC_Os11g47520	11	28207226	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os12g08700	12	4414666	A	C	LPT171	Synonymous coding	3	Turquoise
LOC_Os12g14440	12	8237014	G	A	LPT171	Non synonymous coding	1	Turquoise
LOC_Os12g14440	12	8237018	A	C	LPT171	Non synonymous coding	0.3476	Turquoise
LOC_Os12g14440	12	8237044	G	T	LPT171	Non synonymous coding	0.09	Turquoise
LOC_Os12g14440	12	8238274	A	G	LPT171	Non synonymous coding	0.2954	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

Locus name	Chrom.	Position	Reference	Mutation	Library	Location	SIFT score	Module
LOC_Os12g29400	12	17457680	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os12g37690	12	23102078	A	G	LPT171	Synonymous coding	3	Turquoise
LOC_Os12g37690	12	23102237	G	A	LPT171	Synonymous coding	3	Turquoise
LOC_Os12g37690	12	23102255	C	T	LPT171	Synonymous coding	3	Turquoise
LOC_Os12g37690	12	23103299	C	A	LPT171	Intron	4	Turquoise
LOC_Os12g42220	12	26161374	A	G	LPT171	3'UTR	4	Turquoise
LOC_Os12g42220	12	26162142	G	A	LPT171	Synonymous coding	3	Turquoise

Table C.1 (cont.) List of salt/drought responsive loci with mutation

APPENDIX D

Total read and coverage

The sequencing coverage was calculated by

$$C = \frac{N * L}{G}$$

Where;

C= coverage

N= number of reads

L= average read length

and G= haploid genome size

Library	Replicate	Unique	Aligned	Unique	Total reads	Coverage
LPT123	1	7295698	8224755	88.704	9952399	2.37
	2	2776256	2867145	96.83	3623988	0.86
	3	2674491	2774176	96.407	3470117	0.83
LPT123-TC171	1	8377489	9012407	92.955	11062405	2.63
	2	9459576	10308509	91.765	12763202	3.04
	3	2024037	2099238	96.418	2497798	0.59

TableD. 1 Total read and coverage of LPT123 and LPT123-TC171 genomic DNA libraries

Library	Experiment	Replicate	Unique	Aligned	Unique	Total reads	Coverage
Wild-type Arabidopsis	Control	1	3656623	6538735	55.922	8524336	2.03
		2	3656623	6538735	55.922	8524336	2.03
	Salt stress	1	6461737	14442418	44.741	19033707	4.53
		2	8000704	20816571	38.434	26885057	6.40
35SCaMV::OsNUC1-S	Control	1	3104199	6080820	51.049	8352259	1.99
		2	4586014	11443671	40.075	15128398	3.60
	Salt stress	1	5647396	12711403	44.428	16938713	4.03
		2	2719416	6431127	42.285	8979645	2.14

TableD. 2 Total read and coverage of wild-type Arabidopsis and *35SCaMV::OsNUC1-S* RNA-Seq libraries

VITA

Miss Thanikarn Udomchalothorn was born on February 10, 1982 in Surat Thani. After she finished high school in 1999 from Surat Pitaya School, Surat Thani, she was enrolled in Department of Biology, Faculty of Science, Prince of Songkla University and graduated with the first class honor of the degree of Bachelor of Science. In 2004, she continued her Master of Science in Botany at Department of Botany, Faculty of Science, Chulalongkorn University. In 2008, she has gotten the scholarship from ARC Center of Excellent in Plant Energy Biology, University of Western Australia, Australia to be a part of research 'Organic acid metabolism and Metabolomics'. In 2009, she was enrolled in Ph.D. program in Biological Science program (Cell and Molecular Biology), Chulalongkorn University. Since 1999, she has been supported by Development and Promotion of Science and Technology Talents Project (DPST), the Institute for the Promotion of Teaching Science and Technology (IPST).