TRANSCRIPTOME OF RICE *Oryza sativa* L. OVER-EXPRESSING *OsCam1-1* GENE AND CHARACTERIZATION OF SALT STRESS-RESPONSIVE ISOCITRATE LYASE GENE

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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ทรานสคริปโทมของข้าว *Oryza sativa* L. ที่มีการแสดงออกเกินปกติของยีน *OsCam1-1* และลักษณะสมบัติของยีนไอโซซิเทรตไลเอสที่ตอบสนองต่อภาวะเครียดจากเกลือ

นายวรวัฒน์ ยืนยง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Thesis Title	TRANSCRIPTOME OF RICE *Oryza sativa* L. OVER-EXPRESSING *OsCam1-1* GENE AND CHARACTERIZATION OF SALT STRESS-RESPONSIVE ISOCITRATE LYASE GENE
By	Mr. {Worawat Yuenyong
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วรวัฒน์ ยืนยง : ทรานสคริปโทมของข้าว *Oryza sativa* L. ที่มีการแสดงออกเกินปกติของยีน *OsCam1-1* และลักษณะสมบัติของยีนไอโซซิเทรตไลเอสที่ตอบสนองต่อภาวะเครียดจากเกลือ. (TRANSCRIPTOME OF RICE *Oryza sativa* L. OVER-EXPRESSING *OsCam1-1* GENE AND CHARACTERIZATION OF SALT STRESS-RESPONSIVE ISOCITRATE LYASE GENE) อ.ที่ปรึกษาหลัก : รศ. ดร.ธีรพงษ์ บัวบูชา, อ.ที่ปรึกษาร่วม : ศ. ดร.ลี่เจีย ฉู่

จากการศึกษาก่อนหน้าของยีน *OsCam1-1* แสดงให้เห็นว่าข้าวขาวดอกมะลิ 105 ทรานส์เจนิกที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติสามารถทนต่อภาวะเครียดจากความเค็มได้ดีกว่าข้าวขาวดอกมะลิ 105 ปกติ ดังนั้น เพื่อศึกษาบทบาทของยีน *OsCam1-1* ซึ่งเข้ารหัสโปรตีนคัลมอดุลินในกลไกการตอบสนองต่อภาวะเค็ม การศึกษาทรานสคริปโทมโดยเทคนิค RNA-Seq จึงได้ถูกใช้ในข้าวขาวดอกมะลิ 105 ทรานส์เจนิกที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติภายใต้ภาวะเครียดจากความเค็ม (โซเดียมคลอไรด์ 150 มิลลิโมลาร์) เป็นเวลา 4 ชั่วโมงเทียบกับข้าวปกติ ทำให้พบยีนที่ระดับการแสดงออกเปลี่ยนแปลงที่มีหน้าที่เกี่ยวข้องกับหลายกระบวนการ ได้แก่ การสื่อสารสัญญาณภายในเซลล์ การควบคุมด้วยฮอร์โมน การถอดรหัส เมแทบอลิซึมทุติยภูมิ การตอบสนองต่อภาวะเครียด เมแทบอลิซึมของไขมัน ไกลโคไลซิส วัฏจักรไตรคาร์บอกซิลลิกแอซิด การสังเคราะห์ด้วยแสง และเมแทบอลิซึมของคาร์โบไฮเดรต ในการวัดอัตราการสังเคราะห์ด้วยแสง และการเปิดปากใบ พบว่าในวันที่ 3 และ 5 ภายใต้ภาวะเครียดจากความเค็ม ค่าเหล่านี้ลดลงเนื่องจากผลของการแสดงออกเกินปกติของยีน *OsCam1-1* และพบว่าปริมาณแป้ง และซูโครส ซึ่งเป็นผลิตภัณฑ์ของกระบวนการสังเคราะห์ด้วยแสง ในข้าวทรานสเจนิกมีปริมาณสูงกว่าเมื่อเทียบกับข้าวปกติในวันที่ 3 และ 5 ภายใต้ภาวะเครียดจากความเค็ม จากการศึกษาการแสดงออกของยีนโดยการศึกษาทรานสคริปโทม และ qRT-PCR พบว่ายีนที่เข้ารหัสเอนไซม์ไอโซซิเตรตไลเอส (*ICL*) ซึ่งเป็นเอนไซม์สำคัญในวัฏจักรไกลออกซิเลต มีการแสดงออกสูงขึ้นในข้าวทรานเจนิกภายใต้ภาวะเครียดจากความเค็ม เราตั้งสมมติฐานว่า *ICL* มีหน้าที่เกี่ยวข้องกับกลไกการทนความเครียดจากความเค็มที่ควบคุมด้วย *OsCam1-1* ดังนั้น จึงศึกษาลักษณะสมบัติเชิงหน้าที่ของยีน *OsICL* ด้วยวิธี gene complementation โดยถ่ายยีน *OsICL* เข้าสู่อะราบิดอปซิสมิวแทนต์ของยีน *Aticl* (รีเวอร์แทนต์) และ อะราบิดอปซิสสายพันธุ์ป่า (อะราบิดอปซีสที่มีแสดงออกเกินปกติของยีน *OsICL*) เพื่อเปรียบเทียบการตอบสนองต่อเกลือกับอะราบิดอปซีสสายพันธุ์ป่า และอะราบิดอปซิสมิวแทนต์ของยีน *Aticl* ผลการทดลองแสดงว่าอะราบิดอปซิสมิวแทนต์ *Aticl* มี อัตราการงอก น้ำหนักสด และน้ำหนักแห้ง ต่ำกว่าอะราบิดอปซีสสายพันธุ์ป่า รีเวอร์แทนต์ และอะราบิดอปซีสที่มีแสดงออกเกินปกติของยีน *OsICL* ภายใต้ภาวะเครียดจากเกลือ แสดงให้เห็นว่า *OsICL* เป็นยีนในกลไกทนเค็มในข้าว

สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อนิสิต .....
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Worawat Yuenyong : TRANSCRIPTOME OF RICE *Oryza sativa* L. OVER-EXPRESSING *OsCam1-1* GENE AND CHARACTERIZATION OF SALT STRESS-RESPONSIVE ISOCITRATE LYASE GENE. Advisor: Assoc. Prof. Teerapong Buaboocha, Ph.D. Co-advisor: Prof. Li-Jia Qu, Ph.D.

The previous study of *OsCam1-1* showed that the transgenic KDML105 rice over-expressing *OsCam1-1* was more tolerant to salt stress than the wild type, so to investigate the role of calmodulin encoded by *OsCam1-1* in the salt-responsive mechanism, the transcriptome approach using RNA-seq technique was applied. Transcriptome profile of the transgenic KDML105 rice over-expressing *OsCam1-1* under 4 hr of salt stresss (150 mM NaCl) revealed that several cell processes were affected; signaling, hormone-mediated regulation, transcription, secondary metabolism, stress responses, lipid metabolism, glycolysis, TCA cycle, glyoxylate cycle, photosynthesis, and carbohydrate metabolism. Photosynthesis rate and stomatal conductance at day 3 and 5 of salt stress treatment were repressed by *OsCam1-1* effect. The sucrose and starch contents, the products of photosynthesis, were higher in the transgenic rice at day 3 and 5 of salt stress treatment. According to the gene expression study by transcriptomics and qRT-PCR, the gene encoding a key enzyme in glyoxylate cycle, isocitrate lyase (*ICL*), was enhanced by the effect of *OsCam1-1* over-expression under salt stress. *ICL* was hypothesized to involve in salt tolerance mechanism by *OsCam1-1* regulation. Thus, *OsICL* was functionally characterized using gene complementation approach. *OsICL* was transferred into the *Aticl* Arabidopsis mutant and wild type for generating revertant and *OsICL* overexpression line respectively, in order to compare their salt stress response with wild type and the *Aticl* mutant line. The results showed that the *Aticl* mutant had significantly lower germination rate, fresh weight, and dry weight than wild type, revertant, and *OsICL* over-expression lines under salt stress, suggesting that *OsICL* was a salt tolerance gene in rice.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
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		Co-advisor's Signature .....

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3FL9	transgenic mutant Arabidopsis disrupting isocitrate lyase gene that express rice isocitrate lyase gene driven by 2,138 bp upstream *AtICL* or revertant line number 9
35SCaMV	35S cauliflower mosaic virus promoter
A_{324}	absorbance at 324 nm
A_{340}	absorbance at 340 nm
A_{595}	absorbance at 595 nm
ABA	abscisic acid
AtICL	Arabidopsis isocitrate lyase gene
Aticl	mutant Arabidopsis isocitrate lyase
bp	base pair
°C	degree Celsius
Ca^{2+}	calcium ion
CaM	calmodulin protein
cDNA	complementary deoxyribonucleic acid
C_i	intercellular carbon dioxide concentration
DEG	differentially expressed gene

DEPC	dietyl pyrocarbonate
DNA	deoxyribonucleic acid
E	transpiration
EDTA	ethylenediaminetetraacetic acid
g	gram
g_s	stomatal conductance
GUS	β-glucuronidase gene
GUS	β-glucuronidase protein
hr	hour
ICL	isocitrate lyase protein
IPTG	isopropyl-β-D-thiogalactopyranoside
l	liter
L1	transgenic KDML 105 rice over-expressing *OsCam1-1* line 1
L2	transgenic KDML 105 rice over-expressing *OsCam1-1* line 2
L7	transgenic KDML 105 rice over-expressing *OsCam1-1* line 7
LB medium	Luria Bertani medium
min	minute
mg	milligram
ml	milliliter
mM	millimolar
MS medium	Murashige and Skoog medium

mRNA	messenger ribonucleic acid
NaCl	sodium chloride
ng	nanogram
NOS	nopaline synthase terminator
OsCam1-1	rice calmudulin 1-1 gene
OsICL	rice isocitrate lyase gene
OX*OsICL*/*icl*	transgenic mutant Arabidopsis disrupting isocitrate lyase gene that express rice isocitrate lyase gene driven by 35SCaMV promoter
OX*OsICL*/WT	wild type Arabidopsis that express rice isocitrate lyase gene driven by 35S promoter
PCR	polymerase chain reaction
pH	power of hydrogen ion
P_n	net photosynthesis
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RPKM	read per kilobase of transcript per million mapped reads
rpm	round per minute
RNA	ribonucleic acid
TAE	tris acetate EDTA buffer
TBE	tris borate EDTA buffer
µg	microgram
µl	microliter
µmol	micromole

UTR	untranslated region
v/v	volume by volume
WT	wild type
xg	times gravity
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

Saline soil is a major threat in agriculture, particularly cultivation of rice which is the major cereal crop feeding for around 2.4 billion people in Asia excluding some regions of India, China and Pakistan (FAO, 1998). It has been well known that salinity results in crop yield loss. Therefore, breeding for salinity stress tolerance in crop plants is an important approach that should be given priority. Knowledge of molecular mechanisms for abiotic stress tolerance is necessary for applying in the breeding program (W. Wang, Vinocur, & Altman, 2003).

A pervious report showed that over-expression of rice *calmodulin1-1* (*OsCam1-1*) gene in Khao Dawk Mali 105 (KDML 105) rice resulted in enhanced salt tolerance ability. Looking back to the known functions of calmodulin protein (CaM), it reveals that CaM is a Ca^{2+} binding protein, and Ca^{2+} is involved with cell signaling and recognition of extracellular stimuli. The Ca^{2+}-CaM interaction causes a change of protein conformation resulting in alteration of the protein-protein interaction ability, consequently Ca^{2+}-CaM binds to various protein targets causing a wide range of regulation of cell processes.

Because of the downstream components causing regulation of CaM are so widely spread in the cell, the roles of CaM in conjunction with these components of the cell still have not been completely understood, especially the roles involved in salt stress responses. Although *OsCam1-1* was proven as a gene contributing salt stress tolerance ability to rice, its actual mechanisms have not been well studied. To

comprehend the whole downstream components of *OsCam1-1*, transcriptomics, which is the genome-scale gene expression study was implemented in this research scheme with the expectation to fulfill the knowledge on CaM. In this study, the transcriptome profiling leads to discover a potential salt tolerance gene, isocitrate lyase gene (*OsICL*), which its expression level was enhanced by the effect of *OsCam1-1* overexpression under salt stress, and the gene would be characterized.

1.1 Salinity Stress, A Crucial Agricultural Problem

Salinity, a major plant abiotic constraint, widely spreads in the world as about 20% of the world's cultivated land is affected by salinity (Sumner, 1999). W. X. Wang, Vinocur, Shoseyov, and Altman (2001) have shown that the increase of arable land salinization will have devastating global effects, which 30% of land will be lost within the next 25 years, and up to 50% in year 2050. Primary salinization is a natural process leading to saline soil, namely geochemistry of rock minerals, sedimentation or evaporation concentrating salt in the soil, etc. Interestingly, several arid areas were the land under the ancient seas and they were uplifted by the impact of geologically tectonic forces resulting in the rocks containing minerals and sea salts being presented on the soil, and Na^+, Mg^{2+}, Cl^- and SO_4^{2-} dominating in the saline soil. Additionally, another reason of soil salinity is from human (anthropogenic source) called secondary salinization caused by human-induced practices e.g. salt from irrigation water, livestock waste, fertilizers and sewage as the secondary salinization may increase due to the increase in human activity involving agriculture, irrigation, deforestation and so on (Pitman & Läuchli, 2002). In the saline soil area, about one third of rice yield or economic returns are lost comparing the non-saline soil area nearby (Hall, Greiner, & Yongvanit, 2004). There are 397 million hectares of saline soil area distributed around

the world and the Asia Pacific and Australia region possess highest proportion of the saline soil (Table 1).

Table 1 Worldwide distribution of saline soils and sodic soils area ((taken from (FAO, 2018))

Regions	Total area*	Saline soils*	%	Sodic soils*	%
Africa	1899.1	38.7	2.0	33.5	1.8
Asia and the Pacific and Australia	3107.2	195.1	6.3	248.6	8.0
Europe	2010.8	6.7	0.3	72.7	3.6
Latin America	2038.6	60.5	3.0	50.9	2.5
Near East	1801.9	91.5	5.1	14.1	0.8
North America	1923.7	4.6	0.2	14.5	0.8
Total	12781.3	397.1	3.1%	434.3	3.4%

*Area unit: million hectares

1.2. Rice

Rice is a staple food feeding half of the world population. Approximately 480 million metric tons milled rice is produced from 715 million metric tons paddy rice every year. About 90% of rice in the world was harvested from Asian countries including China, India, Indonesia, Bangladesh, Vietnam, Myanmar, Thailand, the Philippines, Japan, Pakistan, Cambodia, the Republic of Korea, Nepal and Sri Lanka. The non-Asian countries producing rice are Brazil, the United States, Egypt, Madagascar and Nigeria, which together account for around 5% of rice production in the world. It can be observed that China and India are extremely high rice productive countries, may be

due to their large country area, but the major rice productive country are mainly located in Southeast Asia region (Figure 1 A). China and India together account for about 50% of world rice consumption while nearly all countries in Southeast Asian; the Lao People's Democratic Republic, Cambodia, Vietnam, Myanmar, Thailand, Indonesia and the Philippines including nearby country as Bangladesh consume rice more than 300 g/capita/day or over 110 kg/capita/year. Furthermore, at least 50% of the dietary caloric supply of about 520 million poor people in Asia come from rice. Additionally, the high rice consumption countries (>300 g/capita/day) are also found in Latin America and Caribbean; Guyana, Suriname, Cuba, Panama, Costa Rica, Peru, Ecuador, and Nicaragua. In South America, the average rice consumption is around 125 g/capita/day. There are only 12 high rice exporting countries; Thailand, Vietnam, Pakistan, the United States, India, Italy, Uruguay, China, the United Arab Emirates, Benin, Argentina and Brazil (Figure 1 B). The world rice market has preferred volatile and thin grain rice, which account for about 7% of global rice trading or 35 million metric tons. (Muthayya, Sugimoto, Montgomery, & Maberly, 2014).

The cultivated rice consists of two species, *Oryza sativa* L. and *Oryza glarberrima* (Morishima, 1984) among 22 species of wild rice (Table 2). *Oryza sativa* L. is cultivated worldwide while *Oryza glarberrima* is mainly cultivated in Africa (OECD, 1999). Generally, *Oryza sativa* L. consists of 2 main subspecies, indica and japonica, in which each subspecies has many cultivars. KDML 105 is a cultivar in subspecies indica (*Oryza sativa* L. ssp. indica cv. KDML 105), which is the famous Thai fragrance rice

exporting around the world. Mr. Soontorn Seehanoen, staff of Rice Department collected seed from 199 spikes of various rice cultivars in Bangkla district, Chachoengsao province, Thailand in 1950-1951. Then those seeds were applied in pure line selection and cultivated to compare property between cultivars at Koksamrong rice research station, then they were cultivated to compare to the local cultivar in Northern, Northeastern and Central region. Finally, the seed from 105th spike of 199 spike, which is Khao Dawk Mali cultivar was selected, so it has been called Khao Dawk Mali 105 until now (Khonkaen-Rice-Seed-Center, 2018).

A

B

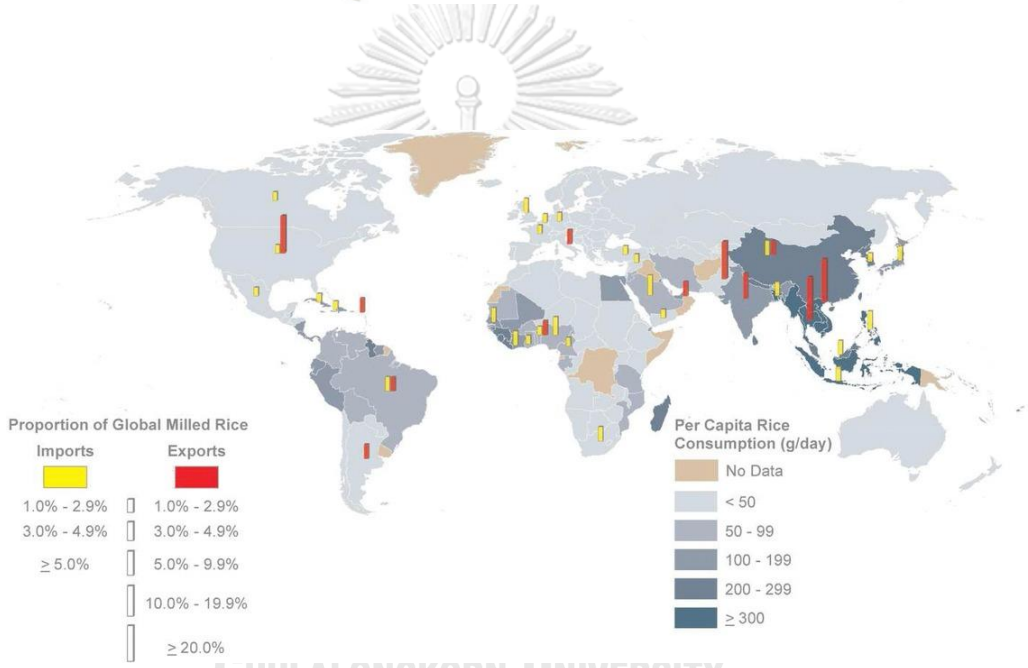


Figure 1 The distribution of milled rice production country in year 2011, B) Export, import and consumption of rice, country by country, for year 2009-2010 (taken from (Muthayya et al., 2014))

Table 2 Species belonging to the genus Oryza (taken from (OECD, 1999))

Species	Number of chromosomes (2n)	Geographical distribution
O. sativa L.	24	Worldwide, Cultivated
O. nivara Sharma et Shastry	24	Asia
O. rufipogon Griff.	24	Asia, Australia, America (Latin, South)
O. glaberrima Steud.	24	Africa, Cultivated
O. barthii A. Chev.	24	Africa
O. longistaminata Chev. et Roehr.	24	Africa
O. meridionalis Ng.	24	Australia
O. officinalis Wall. ex Watt	24	Asia, New Guinea
O. minuta Presl. et Presl.	48	Asia, New Guinea
O. eichingeri Peter	24	Africa, Asia (Sri Lanka)
O. *rhizomatis* Vaughan	24	Asia (Sri Lanka)
O. punctata Kotschy ex Steud.	24,48	Africa
O. latifolia Desv.	48	America (Latin, South)
O. alta Swallen	48	America (South)
O. grandiglumis Prod.	48	America (South)
O. australiensis Domin	24	Australia
O. brachyantha Chev. et Roehr.	24	Africa
O. schlechteri Pilger	48	New Guinea
O. ridleyi Hook. f.	48	Asia, New Guinea
O. longiglumis Jansen	48	New Guinea
O. meyeriana Baill	24	Asia
O. granulata Nees et Arn. ex Watt	24	Asia

1.3. Stress Responsive Mechanism in Plant

Salinity stress is an abiotic stress, which affects plant growth resulting in loss of crop yield, especially rice, which is the most salt sensitive plant comparing to other cereals (Horie, Karahara, & Katsuhara, 2012; Munns & Tester, 2008). Salt stress affects the plant via both osmotic and ionic effects. Osmotic effects result in reduction of water absorption ability which are similar to drought stress effect whereas the ionic stress effects disrupt photosynthesis, protein synthesis, and enzyme activity (Figure 2) (Horie et al., 2012; Läuchli & Grattan, 2007). Kim et al. (2005) reported that when rice seedlings experienced 130 mM NaCl, several growth parameters were significantly decreased at the third day of the treatment. Another report revealed that the grain yield and total chlorophyll content of five local Malaysian rice cultivars were decreased at the salinity level of 8 dS m^{-1} (Hakim et al., 2014).

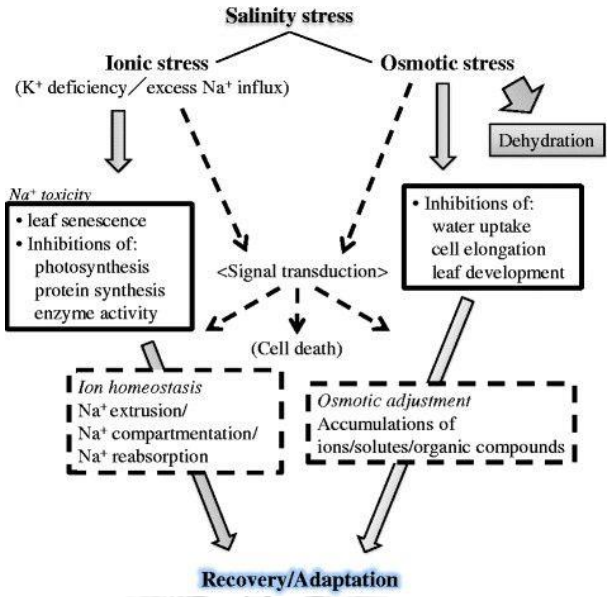


Figure 2 Schematic summary of plant physiological responses to salt stress (taken from Horie et al. (2012))

Plants use calcium signaling to perceive and respond to the environmental stimuli including abiotic stress (Knight, 1999). The calcium-signaling pathway is involved with alteration of Ca^{2+} concentration in the cytosol which results from the release of Ca^{2+} from intracellular organelles or out of cell (Knight, 1999; Tuteja & Mahajan, 2007). Mahajan and Tuteja (2005) and Einspahr and Thompson (1990) suggested that, the membrane receptor recognizes stress signal first, resulting in activation of phospholipase C (PLC), and conversion of PLC hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-trisphosphate (IP3), which mediates the increase of cytoplasmic Ca^{2+} concentration. Consequently, the signal is perceived by Ca^{2+} sensors, which in turn enhance downstream effects via e.g. kinases or phosphatases

that affect the expression of stress responsive genes leading to adaptive physiological responses (Figure 3).

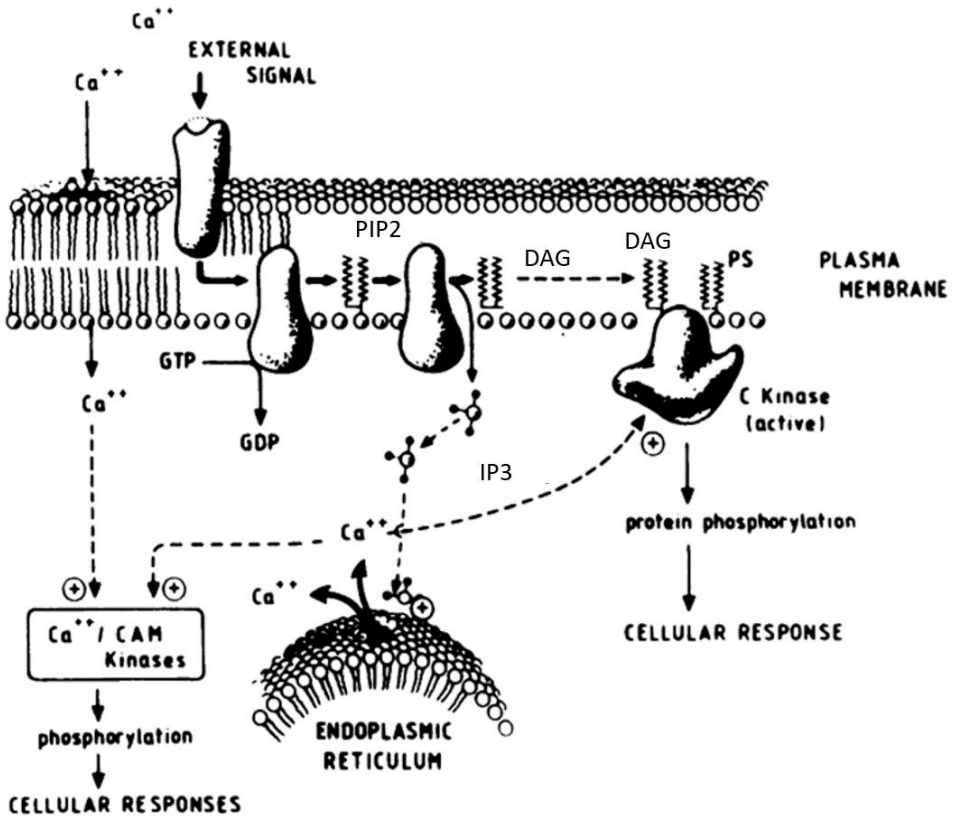


Figure 3 Overview of signaling mechanisms for stress recognition (taken from (Einspahr & Thompson, 1990))

1.4. CaM and Plant Stress Responses

CaM has been well known as a calcium signaling protein that is found in all eukaryotes. CaM is a high-affinity and specificity Ca^{2+} binding protein (Means & Dedman, 1980), due to its EF-hand motifs, which are helix–loop–helix structures that function in Ca^{2+} binding. CaM transduces a calcium signal via binding to Ca^{2+} that results in

exposure of its hydrophobic regions on the surface of the molecule and interaction with their target proteins (Yang & Poovaiah, 2003). Those protein interactions result in regulation of several cell systems (Ranty, Aldon, & Galaud, 2006). There are many evidences indicating that calcium signal and CaM involve with response to abiotic stress in plant including salt stress. Rice consists of 5 *Cam* genes: *OsCam1-1*, *OsCam1-2*, *OsCam1-3*, *OsCam2* and *OsCam3*; and 32 calmodulin-like (*OsCML*) genes (Boonburapong & Buaboocha, 2007). Previous study revealed that expression level of *OsCam1* was highly increased under NaCl, mannitol and wounding treatment, while that of *OsCam2* was not significantly changed under those treatment conditions, and that of *OsCam3* transiently responded to NaCl and wounding treatment (Phean-o-Pas, Punteeranurak, & Buaboocha, 2005). Saeng-ngam, Takpirom, Buaboocha, and Chadchawan (2012) reported that the *AAO* and *NCED3* genes in the ABA biosynthesis pathway, and ABA content were more up-regulated in the transgenic KDML 105 rice over-expressing *OsCam1-1* under salt stress comparing to the wild type and the transgenic control line. In addition, they also found the transgenic rice overexpressing *OsCam1-1* can maintain root and shoot dry weights better, resulting in the transgenic rice exhibiting better tolerance to salt stress than the wild type rice (Figure 4). The transcription factors (TFs) that bind to CaM and Ca^{2+}/CaM were identified in plant, and some of these TFs were verified to play role in stress signaling pathways, but Ca^{2+} and Ca^{2+}/CaM-regulating TFs mechanisms are not nearly completely understood, so further investigation is required (Reddy, Ali, Celesnik, & Day, 2011).

A

B

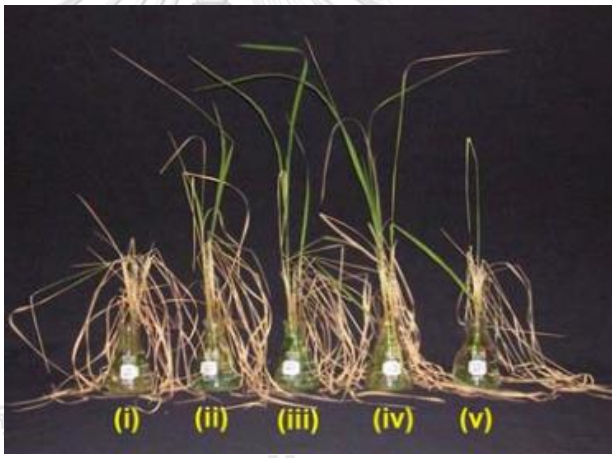


Figure 4 The wild type KDML 105 (i), the tree transgenic KDML 105 rice lines overexpressing *OsCam1-1*; L1 (ii), L2 (iii), L7 (iv) and the vector control line (v), A) under normal condition, B) under salt stress condition, at day 15 of salt stress treatment (0.5% w/v NaCl). (taken from (Saeng-ngam et al., 2012))

OsCaM1 contains 149 amino acid residues (Boonburapong & Buaboocha, 2007). CaM has four conserved Ca^{2+} binding EF-hand motifs found in pairs embedded within two separate globular domains in the C- and N-terminal regions separated by a flexible central helix which, overall, forms the dumbbell-shaped structure (Figure 5 A). In respect of protein-protein interaction, CaM interacts its target by several kinds of interactions. One of these interactions is the relief of autoinhibition which induces rearrangement of conformation displacing autoinhibitory domain and allows full enzyme activity. Another is active site remodeling, which assists in reorganizing disordered loop Ca^{2+}/CaM binding site of CaM target to stabilize the protein structure leading to activation of enzyme activity. This interaction pattern was found in oederma factor e.g. CaM-induced dimerization, which has found in K^+ channel as the C-terminal EF hand binds a domain of K^+ channel linking another K^+ channel domain by binding to the N-terminal EF hand upon the presence of Ca^{2+}. Dimerizatin of the two domains will be induced activating the channel for ion transfer (Figure 5 B) (Yang & Poovaiah, 2003).

A

B

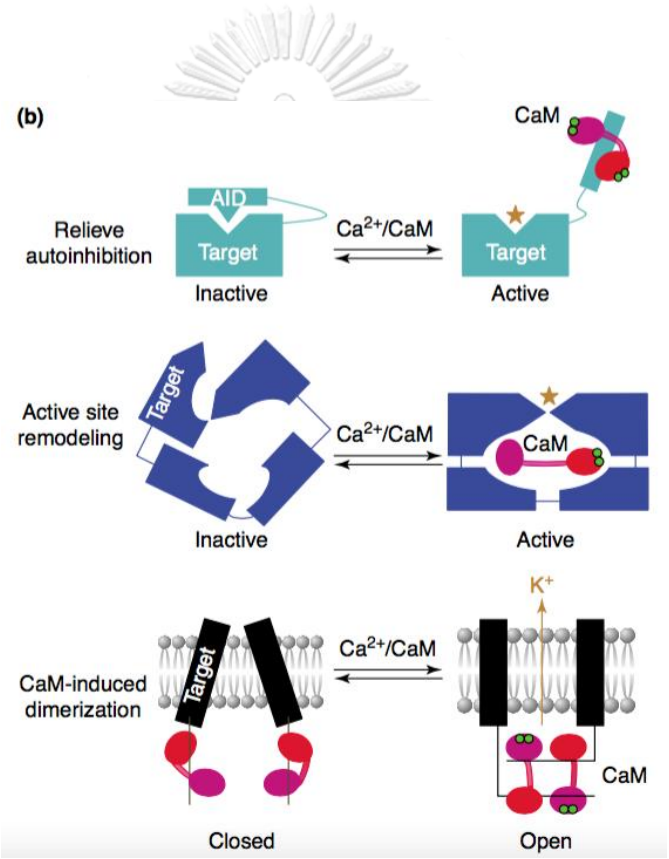


Figure 5 A) Crystal structure of Ca^{2+}-bound CaM, the green balls represent Ca^{2+} ions. B) The kinds of mechanism of CaM activating protein target (taken from (Yang & Poovaiah, 2003))

According to the topic of plant stress responsive mechanism as described in the early part of this thesis, the signification of Ca^{2+} signal pathway in stress recognition and the structure and interaction of CaM as a Ca^{2+} coordinating protein is emphasized. Because CaM and CML can directly and indirectly interact with wide array of proteins or DNA targets, therefore the downstream regulation of CaM and CML is various and highly complicated. Zeng et al. (2015) summarized the schematic cell regulation of CaM and CML in stress responses that pulsation of Ca^{2+} concentration is recognized by cell via interaction of CaM or CML. At high Ca^{2+} concentration, CaM or CML chelates the Ca^{2+}, then Ca^{2+}/CaM or Ca^{2+}/CML binds the targets namely; AtSR1/CAMTA3 leading to plant response to chilling stress; and CAT3, MAPK8 and MKP1 for controlling the homeostasis of reactive oxygen species signals. It was also found that plant light signal response is regulated by CaM7, which acts as a DNA-binding transcription factor. CaMs or CMLs also regulated the transportation process by controlling some protein channels such as AtACA4 or AtNHX1 in response to salt stress. According to this information, it can be suggested that CaMs or CMLs have broad functions in the cell closely coordinating to Ca^{2+} signals and changes in activities of protein targets, which ultimately result in physiological responses (Figure 6).

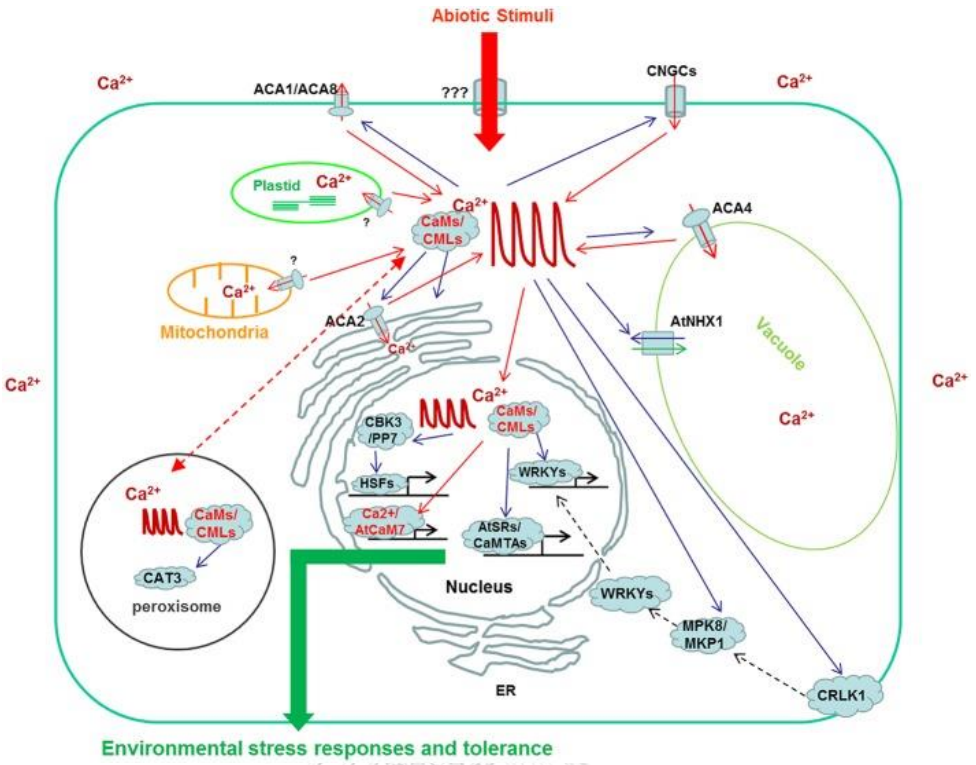


Figure 6 Role of CaMs and CMLs in regulation of various cell processes through Ca^{2+}-signaling responding to abiotic stimuli. The red arrows represent actions modifying Ca^{2+} transients or CaMs/CMLs and the blue arrows represent actions regulated by Ca^{2+}/CaMs or Ca^{2+} (taken from (Zeng et al., 2015))

1.5. Transcriptome

Transcriptome is a genome-wide scale gene expression study. In the area of plant study, transcriptome study can reveal gene expression patterns of plant under different factors: environmental conditions, stages of development, types of organ, etc., which leads to discovery of the genes or processes that respond to those factors. There is a report on transcriptome study in rice flag leaf under heat stress condition, which showed that the genes involved with glycolysis, ubiquitin-proteasome, shikimate, lignin, mevalonic acid metabolisms and heat-shock protein gene were up-regulated, while the genes involved with TCA, carotenoid, dihydroflavonol, anthocyanin and light-reaction in the photosynthesis were down regulated (X. Zhang et al., 2013). Liao et al. (2015) employed RNA-seq technique using Next Generation Sequencing (NGS) to study the transcriptome of heat sensitive and heat tolerant rice in early milky stage under high night temperature. They found that the genes involved with oxidation-reduction, metabolism, transport, and transcript regulation, defend response and photosynthesis were differently expressed between the two rice lines under the stress condition. Oono et al. (2013) also used NGS performing RNA-seq to study transcriptome of wheat (*Triticum aestivum* L.) under phosphate starvation condition. The result showed that IPS1 (Induced by Phosphate Starvation 1) was responsive to phosphate starvation condition. Here, transcriptome profiles of the transgenic KDML 105 rice over-expressing *OsCam1-1* under salt stress will be generated by RNA-seq technique using NGS.

1.6. Isocitrate Lyase

ICL encodes isocitrate lyase enzyme which is a key enzyme in the glyoxylate cycle that converts isocitrate to glyoxylate and succinate. *ICL* is a single-copy gene in both rice (Lu, Wu, & Han, 2005) and Arabidopsis (Thorneycroft, Sherson, & Smith, 2001). Cooper and Beevers (1969) studied mitochondria and glyoxysomes of castor bean endosperm and found that the glyoxysome consists of more than 85% activity of isocitrate lyase and malate synthase. Previous study showed that the *icl* mutant Arabidopsis seedling slowly grew and could not convert lipid to sugar. Interestingly, when [^{14}C] acetate was fed to the *icl* mutant line, the result showed that the amount of ^{14}C-lebelled sugar in the *icl* mutant line was significantly lower than the wild type, so they suggested that ICL may play role in lipid-sugar conversion in Arabidopsis by using acetyl unit from acetyl-CoA, the product of β-oxidation, which is the lipid degradation pathway, for synthesizing sugar via glyoxylate cycle and gluconeogenesis (Figure 7) (Cornah, Germain, Ward, Beale, & Smith, 2004; P. J. Eastmond et al., 2000).

The hypothesis of this study is that *OsCam1-1* regulates *OsICL*, which may play an important role in starch and sugar accumulation in rice under salt stress condition. Therefore, in order to verify this hypothesis, the sugar and starch contents were determined and compared between the transgenic rice overexpressing *OsCam1-1* and the wild type. In addition, to characterize the functions of *OsICL* whether it involved

with salt stress response, the *OsICL* revertant Arabidopsis was constructed, and exermined compared with the *icl* mutant Arabidopsis under salt stress.

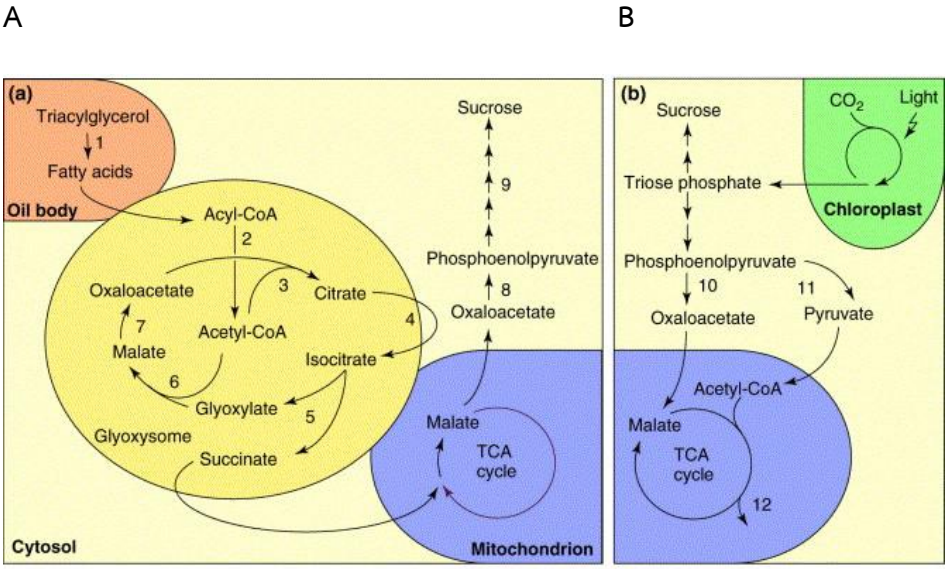


Figure 7 Schematic summary of A) metabolic pathway involved with lipid-sugar conversion containing β-oxidation, glyoxylate cycle, TCA cycle and gluconeogenesis, and B) metabolic of sugar production from photosynthesis, the number indicate as following; 1 triacylglycerol lipase, 2 fatty acid β-oxidation, 3 citrate synthase, 4 aconitase, 5 isocitrate lyase, 6 malate synthase, 7 malate dehydrogenase, 8 phosphoenolpyruvate cayboxykinase, 9 gluconeogenesis, 10 phosphoenolpyruvate carboxylase, 11 pyruvate kinase, 12 biosynthetic pathways (e.g. amino acids, purines, pyrimidines, heme and chlorophyll) (taken from (Peter J. Eastmond & Graham, 2001))

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

Khao Dawk Mali 105 Rice (*Oryza sativa* CV. KDML105) seed

Transgenic Khao Dawk Mali 105 rice over-expressing *OsCam1-1* (L1, L2 and L7) (Saeng-ngam et al., 2012)

Arabidopsis thaliana ecotype Columbia (Col-0)

Aticl (isocitrate lyase) *Arabidopsis thaliana* (GK-008E03) mutant, the mutant line Arabidopsis seed obtained from Nottingham Arabidopsis Stock Centre (UK).

2.1.2. Microorganisms

E. coli DH5 α

E. coli DB 3.1

Agrobacterium tumefaciens GV3101

2.1.3. DNA Vectors and DNA materials

Plasmid pCAMBIA1301

pTZ57R/T, TA cloning plasmid (Thermo Fisher, USA)

pENTR/D-TOPO gateway cloning plasmid (Invitrogen™, USA)

pK2GW7

cDNA Library *OsICL* (LOC_Os07g34520) (AK063353) (KOME clone Number 001-114-C03) (Genetic Resource Center of the National Agriculture and Food Research Organization, Japan)

DNA primer (DNA primers were synthesized by Macrogen, South Korea and Intergrated DNA Technology, USA)

GeneRuler DNA ladder mix (100-10,000 bp) (Thermo Fisher, USA)

GeneRuler 100 bp DNA ladder (100-3,000 bp) (Thermo Fisher, USA)

GeneRuler 1 kb DNA Ladder (250-10,000 bp) (Thermo Fisher, USA)

2.1.4. Enzymes and Test Kits

2XSSoFAST EVAGreen (Bio-Rad, USA)

2X*Taq*-mixed (Vivantis, Malaysia)

5XiScrip (Bio-Rad, USA)

D-Fructose/D-Glucose Assay Kit (Megazyme, Ireland)

DNA polymerase I (Thermo Fisher, USA)

End Repair Enzyme (New England Biolabs, UK)

Fragmentase (New England Biolabs, UK)

Klenow exo- (New England Biolabs, UK)

LR-Clonase II (Invitrogen, USA)

Phusion® High-Fidelity DNA Polymerases (New England Biolabs, UK)

Plant Genomic DNA Mini Kit (Geneaid, Taiwan)

Restriction endonuclease *Eco*RI-HF (New England Biolabs, UK)

Restriction endonuclease *Kpn*I-HF (New England Biolabs, UK)

Restriction endonuclease *Nde*I (New England Biolabs, UK)

Restriction endonuclease *Not*I (New England Biolabs, UK)

Restriction endonuclease *Xba*I (New England Biolabs, UK)

RiboLock RNase Inhibitor (Thermo Fisher, USA)

RNaseH (New England Biolabs, UK)

Superscript III® reverse transcriptase (Invitrogen™, USA)

T4 Ligase (New England Biolabs, UK)

Taq DNA Polymerase (Thermo Fisher, USA)

TIANgel Midi Purification Kit (Tiangen, China)

TIANprep Mini Plasmid Kit (Tiangen, China)

α-amylase (Sigma, USA)

β-amylase (Sigma, USA)

2.1.5. Chemicals

5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-Gal) (Bio Basic Canada Inc., Canada)

6X DNA loading dye (New England Biolabs, UK)

Agarose (Vivantis, Malaysia)

Agar (Himedia, India)

Ammonium nitrate (NH_4NO_3) (Carlo Erbra Reagent, France)

Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$) (Carlo Erbra Reagent, France)

Ampicillin (Bio Basic Canada Inc., Canada)

AMPure (Beckman Coulter, USA)

Boric acid (H_3BO_3) (Carlo Erba Reagent, France)

Bradford 5X (Bio-Rad, USA)

Buffer 2.1 (New England Biolabs, UK)

Calcium chloride ($CaCl_2$) (Carlo Erba Reagent, France)

Chloroform (Carlo Erba Reagent, France)

Citric acid (monohydrate) (Carlo Erba Reagent, France)

Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) (Carlo Erba Reagent, France)

CutSmart buffer (New England Biolabs, UK)

Diethylpyrocarbonate (DEPC) (Sigma, USA)

Dipotassium hydrogen phosphate (K_2HPO_4) (Carlo Erba Reagent, France)

Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$) (Carlo Erba Reagent, France)

D-L isocitric acid ($C_6H_5Na_3O_7$) (Sigma, USA)

Dynabeads® (Ambion™, USA)

Ethylenediaminetetraacetic acid (EDTA) (Carlo Erba Reagent, France)

Ethanol (Carlo Erba Reagent, France)

Gentamycin (Bio Basic Canada Inc., Canada)

Glacial acetic acid (Carlo Erba Reagent, France)

Hydrochloric acid (HCl) (Carlo Erba Reagent, France)

Iron (III) chloride ($FeCl_3 \cdot 6H_2O$) (Carlo Erba Reagent, France)

Isopropanol (Carlo Erba Reagent, France)

Kanamycin (Bio Basic Canada Inc., Canada)

Liquid nitrogen

Magnesium chloride ($MgCl_2$) (Carlo Erba Reagent, France)

Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) (Carlo Erba Reagent, France)

Manganese chloride ($MnCl_2 \cdot 4H_2O$) (Carlo Erba Reagent, France)

Murashige and Skoog medium (PhytoTechnology Laboratories®, USA)

Phenyl hydrazine (Sigma, USA)

Potassium dihydrogen phosphate (KH_2PO_4) (Carlo Erba Reagent, France)

Potassium hydroxide (KOH) (Carlo Erba Reagent, France)

Potassium sulfate (K_2SO_4) (Carlo Erba Reagent, France)

Potato starch (Sigma, USA)

RedSafe™ Nucleic Acid Staining Solution 20,000X (Intron Biotechnology, South Korea)

Rifampicin (Bio Basic Canada Inc., Canada)

Silwet (PhytoTechnology Laboratories®, USA)

Sodium acetate (CH_2COONa) (Carlo Erba Reagent, France)

Sodium chloride (NaCl) (Carlo Erba Reagent, France)

Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$) (Carlo Erba Reagent, France)

Sodium glyoxylate monohydrate ($HC(O)COONa \cdot H_2O$) (Sigma, USA)

Sodium hydroxide (NaOH) (Carlo Erba Reagent, France)

Sodium hypochlorite (NaOCl) (Haiter, Thailand)

Spectinomycin (Bio Basic Canada Inc., Canada)

Sucrose (Carlo Erba Reagent, France)

Tris (Carlo Erba Reagent, France)

TRI-Reagent® (Molecular Research Center, USA)

Tryptone (Himedia, India)

Yeast extract (Himedia, India)

Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Carlo Erba Reagent, France)

2.1.6. Equipments, Glasswares, and Plasticwares

0.2 ml PCR tube

1ml pipette (Gilson, France)

1-10 µl multichannel pipette (Finpipette® Thermo Scientific, USA)

1.5 ml microtube

100 µl pipette (Gilson, France)

2 µl pipette (Rainin, USA)

20 µl pipette (Gilson, France)

25 ml plastic tube

200 µl pipette (Gilson, France)

30-300 µl multichannel pipette (Finpipette® Thermo Scientific, USA)

5-50 µl multichannel pipette (Finpipette® Thermo Scientific, USA)

50 ml plastic tube

96 well plate (Falcon, USA)

Alcohol burner

Autoclave (Sanyo, Japan)

Breaker

CFX96™ Real-Time PCR Detection System (Bio-Rad, USA)

C-MAG HS 7 Magnetic Stirrers (IKA®, USA)

CP224S Competence Analytical Balance, 220 g x 0.1 mg (Sartorius, Germany)

CP423S Precision Balance, 420 g x 0.001 g (Sartorius, Germany)

Disposable plastic pipette tip

FE20 FiveEasy™ Benchtop pH Meter (Mettler Toledo™, USA)

Flasks

Fume Hood

Illumina HiSeq2000 Genome Analyzer (Illumina, USA)

Innova 4000 Benchtop Incubator Shaker (Eppendorf, Germany)

Gas Analyzer LI6400XT Portable Photosynthesis System (Li-Cor, USA)

Gel Documentation (Syngene, UK)

Glass spreader

Growth chamber (Humanlab, South Korea)

Laminar air flow (Cambridge, USA)

Loop

Low profile PCR strip tube and cab (Bio-Rad, USA)

Lyophilizer (Labconco, USA)

Micro-Centrifuge (Hettich, Germany)

MicroPulser™ Electroporator (Bio-Rad, USA)

Mixer Mill MM 400 (Retsch, Germany)

MUPID-exU Horizontal Electrophoresis System (Mupid, Japan)

Petri dish

Refrigerated centrifuge 5418 R (Eppendorf, Germany)

Refrigerated centrifuge 5804 R (Eppendorf, Germany)

Refrigerated centrifuge Sorvall Legend XTR (Thermo Fisher, USA)

Synergy H1 microplate reader (BioTek®, USA)

Thermal Cycler T100™ (Bio-Rad, USA)

UV-gel pad (Thermo Fisher, USA)

UV flat bottom microtiter plate (Greiner, Germany)

Volumetric cylinder

Vortex-Genie 2 (Scientific Industries, Inc., USA)

2.1.7. DNA Sequencing

Plasmid and PCR product DNA were sequenced by 1st BASE Laboratories (Malaysia) and Beijing Ruibo Xingke Biotechnology Co., Ltd. (China).

2.1.8. Planting Materials

Peat moss

Perlite

Vermiculite

2.2. Methods

2.2.1. Transcriptome and Gene Expression Study of The Transgenic KDML105 Rice Overexpressing *OsCam1-1* under Salt Stress

2.2.1.1. Rice Cultivation and Salt-Stress Treatment

The seed of KDML 105 rice and its three transgenic lines overexpressing *OsCam1-1* obtained from Ms. Warintra Takpirom (Saeng-ngam et al., 2012), were soaked in distill water for three days. Then, they were transferred to half-strength Yoshida's solution (Yoshida, Forno, & Cock, 1971) for 1 week in the growth chamber under 25 °C and 16/8 hr light/dark period. Then, the rice seedlings were transferred to full-strength Yoshida's solution and they were moved to natural light at green house in Faculty of Science, Chulalongkorn University for further 2 weeks with completely randomize design (CRD). The 3 weeks old seedlings of the 4 rice lines (wild type, and 3 transgenic lines over-expressing *OsCam1-1*) were divided into two groups, control and stress, as follows;

a. For the experiment of RNA-Seq and qRT-PCR, the plants were transferred to
   - full-strength Yoshida solution for 4 hr or
   - full-strength Yoshida solution containing 150 mM NaCl for 4 hr.

b. For the experiment of gas exchange measurement, and sucrose and starch determination, the plants were transferred to
   - full-strength Yoshida solution for 3 and 5 days or
   - full-strength Yoshida solution containing 150 mM NaCl for 3 and 5 days.

Then the parameters were measured, or the tissue samples were collected by snap freezing in liquid nitrogen for further processes. Every step of plant growing was performed using completely randomized design (CRD) experimental plan.

2.2.1.2. RNA Isolation

Two independent biological replicates of rice leaf blade and leaf sheath samples were ground with liquid nitrogen using chilled mortar and pestle until the samples became fine powder. RNA was extracted using TRI-Reagent® following the manufacturer's protocol based on phenol-chloroform extraction principle. One milliliter of TRI-Reagent® was added to 50-100 mg of ground-leaf samples in a 1.5 ml microtube, then mixed vigorously with 200 µl chloroform using vortex mixer, and incubated at room temperature for 5 min. The mixtures were centrifuged at 12,000xg under 4-25 °C for 15 min. As the mixtures separated into two phrases, the aqueous phase was carefully transferred to a new clean microtube. The equal-to-the-aqueous-mixture volume of isopropanol was added into the transferred aqueous mixtures then they were mixed by inverting the tube for 10-20 times and were centrifuged at 12,000xg under 4-25 °C for 15 min. After the centrifugation, the RNA would be precipitated as

pellet, then the liquid phase was poured out and the pellet was washed using 75% ethanol in diethylpyrocarbonate-treated water (DEPC-water) then the mixtures were centrifuged at 8,000xg under 4-25 °C for 8 min. The ethanol solution was poured out and the pellets were dried at room temperature for 5-10 min, then the dried pellets were dissolved using 50-100 µl DEPC-treated water. The quantity and quality of RNA were then checked by spectrophotometric method and agarose gel electrophoresis, respectively.

2.2.1.3. Library preparation and RNA-seq

RNA-seq was carried out at the Genome Center, University of California Davis, California, US. Firstly, mRNA was purified from the total RNA obtained from 2.2.1.2 for library preparation using Dynabeads®. Then, first strand cDNA was synthesized from the mRNA template using Superscript III® reverse transcriptase. After that, second strand cDNA was synthesized using a combination of RNaseH and DNA polymerase I. To fragmentize the cDNA, fragmentase was used. Next, the cDNA fragments were modified into blunt end fragments using End Repair Enzyme. Klenow exo- was applied for adding deoxyadenosines at the 3' end of the fragments. The ligase was then added to ligate the fragments to adapters, which would benefit in sample identification in the data analysis process as each sample was ligated to different adapters. Then, the ligated fragments were amplified by PCR using Phusion® high-fidelity DNA polymerases with adapter primers, and those PCR products were checked for quality by gel electrophoresis. Every step was followed by purification step using AMPure. The libraries were sequenced by the Illumina HiSeq 2000 Genome Analyzer.

2.2.1.4. RNA-Seq Data Analysis

The RNA-Seq data obtained from the Illumina HiSeq 2000 genome analyzer, were analyzed based on the Linux or MS-DOS operation system. Firstly, The sequence data were demultiplexed by barcode sequences, then the sequences were aligned to the rice genome from MSU annotation database version 7 (Kawahara et al., 2013) using the computer software, POPE (Missirian, Henry, Comai, & Filkov, 2012). The gene expression data of the wild type and the transgenic rice under normal or salt stress condition as well as those under normal and salt stress condition of the wildtype or the transgenic rice were compared using DESeq (Anders & Huber, 2010) in order to identify differentially expressed genes (DEGs). The downstream data analysis including construction of Venn diagram by web-based tool (http://bioinformatics.psb.ugent.be/webtools/Venn/), pathway analysis by MapMan (Thimm et al., 2004), and gene enrichment analysis by agriGO (Z. Du, Zhou, Ling, Zhang, & Su, 2010), were performed.

2.2.1.5. qRT-PCR Verifying Transcriptome Data

The four biological replicates of wild type and the transgenic rice line L1 were grown and RNA was isolated following the steps of plant growth and treatment (2.2.1.1) and RNA isolation (2.2.1.2) as described, respectively. The RNA was treated with RNAse-free DNase I and converted to cDNA using iScript™ cDNA Synthesis Kit. The qRT-PCR was performed using SsoFast™ EvaGreen® Supermixes. The primers shown in table 3 were used in the qRT-PCR. The gene expression level was calculated employing the $2^{-(\Delta\Delta CT)}$ method, and the expression level difference was reported in fold change compared with wild type under control condition.

2.2.1.6. qRT-PCR Investigating Expression of Rice Isocitrate Lyase and Malate Synthase Gene (*OsCIL* and *OsMS*) in Transgenic Rice

The four biological replicates of the wild type and three lines of the transgenic rice overexpressing *OsCam1-1* (L1, L2 and L7) were grown and RNA was isolated following steps of plant growth and treatment (2.2.1.1) and RNA isolation (2.2.1.2) as described, respectively. Then qRT-PCR was performed following step 2.2.1.5, and the DNA primers used are shown in Table 3.

Table 3 Primers useing in qRT-PCR for RNA-seq data verification

Gene	Locus	Forward primer (5'-3')	Reverse primer (5'-3')
EF-1 alpha	LOC_Os03g08010	ATGGTTGTGGAGACCTTC	TCACCTTGGCACCGGTTG
Aconitase	LOC_Os08g09200	CATCCTCCCATACGTCATCC	TGTCTCCTGCGGCTTTATTT
Isocitrate lyase	LOC_Os07g34520	AGAGCAGCAGCCATGTTCTT	CGTGCGTGCTGTAGTTCAGT
Malate synthase	LOC_Os04g40990	CGTACAACCTCATCGTGGTG	CGGAGAAGTTACACGGAGAGA
Aldolase	LOC_Os09g02540	CCATCATCTGCCAGGAGAAC	TTGCAATTTTTGTCGTGTCTG
AP2	LOC_Os03g08470	CTGTGGAGCTTCGACGACTT	ACAAACACAAACACCGCAAT
ERD1	LOC_Os02g32520	GAGCCACCTGAATGAGAAGG	TTATATGCCCGAACGAATCC
Pyruvate decarboxylase	LOC_Os03g18220	AGGACGACACCAGCAAAGAG	GAGGGAATGGACACAAGGAA
Glycosyl hydrolase	LOC_Os04g45290	GCTCAGGTGTGTGTGGTACA	CGACAGCACACATCCTCTGT
Beta amylase	LOC_Os03g22790	ATGGATGATGCCCCCTGT	TTGGGGTACACGTCTCATGT

2.2.1.7. qRT-PCR Investigating Expression of Rice Isocitrate Lyase Gene (*OsCIL*) in Senesced and Young Leaf

The wild type and the three lines of transgenic rice (L1, L2 and L7) were grown following step 2.2.1.1 with four biological replicates. The first leaves (lowest leaf) which represented senesced leaf and the third leaves (the third upward leaf) which represented young leaf were collected, and the RNA isolation following step 2.2.1.2 was performed. Then qRT-PCR following step 2.2.1.5 was carried out.

2.2.2. Biochemical and Physiological Study of Transgenic KDML105 Rice Overexpressing *OsCam1-1* under Salt Stress

2.2.2.1. Sucrose and Starch Determinations

The transgenic and wild type rice were grown following step 2.2.1.1, then the leaf blades were collected for sucrose and starch content assays.

2.2.2.1.1 Sucrose and Starch Isolation

Sucrose extraction protocol was modified from Cowan et al. (2005) whereas starch extraction protocol was modified from Smith and Zeeman (2006). Plant tissues were collected by snap-freezing in liquid nitrogen, then grounding by mixer mill until tissues became fine powder. After that, the tissue powders were lyophilized. They were weighted to determine their dry weight. Four ml of 80% v/v ethanol was added to the prepared sample and the mixture tubes were incubated in the hot water (80 °C) for 10 min. Then, the mixtures were centrifuged at 4,000xg for 15 min and supernatants were transferred to new clean tubes. Four ml of 80% v/v ethanol were added into sample tube again, and this process was repeated 3 times. After that, the

supernatant and pellet were dried by hot air oven at 60 °C for 3-4 days. The dried supernatants were resuspended in 1 ml sterilized distill water, would be used for glucose and sucrose determination. The dried pellets were resuspended in 1-2 ml of 50 mM acetate buffer pH 4.5, then they were heated in boiled water for 15-30 min or until the pellet were broken and homogeneously mixed into the buffer. After that, 5 µl of each α- and β-amylase (50 mg/ml) were added to the resuspended pellets, then the mixtures were incubated under 37 °C for 2 hours, and centrifuged at 4,000xg for 15 min. The supernatant would be used for starch content determination.

2.2.2.1.2. Sucrose and Starch Content Measurements

The D-Fructose/D-Glucose Assay Kit was applied to determine sucrose and starch content base on glucose determination by coupling reaction. The process started with hexokinase (provided by the test kit) converting glucose and ATP to glucose-6-phosphate (G-6-P) and ADP, then glucose-6-phosphate dehydrogenase (provided by the test kit) subsequently converted G-6-P and $NADP^+$ to gluconate-6-phosphate, NADPH and H^+, therefore A_{340}, the NADP absorbed wavelength, was measured (Figure 8). To determine the sucrose and starch content, the crude extract of sugar and starch were treated by enzyme to glucose following step 2.2.2.1.1, then the glucose content was measured following the previously explained steps. For sucrose measurement the crude samples were aliquoted from the glucose sample to be treated by invertase to convert sucrose to be glucose, then the glucose content (in molar unit) of the invertase-treated sample was subtracted by invertase-untreated (glucose sample) to determine the sucrose content. The starch sample was also digested to glucose by α- and β-amylase and the glucose was subsequently

measured, therefore the glucose content of known varied concentrations of potato starch treating with α- and β-amylase was used as standards.

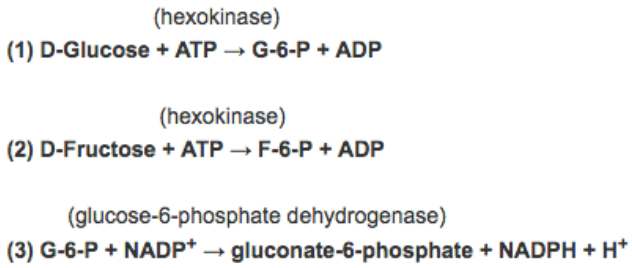


Figure 8 Enzymatic reaction of glucose assay base on D-Fructose/D-Glucose Assay Kit

2.2.2.2. Gas Exchanged Measurement

The three lines of transgenic rice over-expressing *OsCam1-1* (L1, L2 and L7) and the wild type KDML105 rice were grown and treated with salt stress following step 2.2.1.1. Then the photosynthesis rate (P_n), stomatal conductance (g_s), intercellular carbon dioxide (C_i), transpiration rate (E) and FV′/FM′ were measured by gas analyzer, LI6400XT portable photosynthesis system, at days 3 and 5 of the treatment.

2.2.3. Characterization of Salt Responsive Gene, *OsICL*, in *Arabidopsis thaliana*

This experiment part aimed to investigate effect(s) of isocitrate lyase involved in salt responsive mechanism via gene manipulation approach in Arabidopsis, the plant model. The biochemical or physiological characters of wild type and isocitrate lyase (*Aticl*) Arabidopsis mutant under salt stress were compared. Moreover, to consolidate the evidences showing the effect(s) of isocitrate lyase in salt responsive mechanism, the transgenic *Aticl* Arabidopsis mutant expressing rice isocitrate lyase gene (*OsICL*)

(revertant) and the transgenic Arabidopsis overexpressing *OsICL* were constructed. The three recombinant plasmids; *GUS-NOS*-upstream-*AtICL*-*OsICL*-pK2GW7, *OsICL*-pK2GW7 and POsICL-pHGWFS7, were constructed. The *GUS-NOS*-upstream-*AtICL*-*OsICL*-pK2GW7 was designed for mimicking *OsICL* expression level as native *AtICL* in Arabidopsis by placing the 2,138-bp upstream sequence of *AtICL* in front of *OsICL*, and the *GUS-NOS* encoding β-glucuronidase linking with nopaline synthase terminator was placed in front of the upstream *AtICL* fragment to obstruct the 35SCaMV promoter provided by the destination vector. *OsICL*-pK2GW7 was designed for *OsICL* overexpression.

2.2.3.1. Arabidopsis Cultivation and Seed Harvesting

Murashige and Skoog medium (MS) with 1% sucrose is basic medium for growing wild type Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) (Col-0), *Aticl* mutant (GK-008E03), T-DNA insertion Arabidopsis knock-out line for isocitrate lyase gene, and other mutant or transgenic lines. For salt stress condition, NaCl was added to MS medium to a desired molar concentration. Normally, the Arabidopsis seed was decontaminated by quickly rinsing with 75% ethanol and soaking in 2% NaOCl for 10 min before rinsing with sterilized tween80 contained water for 5-8 times, and then transferred to MS medium plate. Each step was performed using aseptic technique in laminar air flow. The seed was sown on plate and was stored in 4 °C refrigerator with dark condition for 2 days, then the plates were moved to the growth chamber or the growth room at 25 °C under 16/8 hr light/dark period for 2 weeks. The plants were transferred to pot containing planting material, peat moss : perlite : vermiculite is 3:1:1 and grown under the same condition while watering every 2-3 days until the plant was matured. The seeds were harvested and stored in 4 °C refrigerator.

2.2.3.2 Mutant *Aticl* Arabidopsis Genotyping

To perform plant genotyping by PCR, the genomic DNA extraction was carried out by grinding fresh plant tissue (around 100 mg) by mixer mill in a 1.5 ml microtube. Then, 300 µl of the plant lysis buffer for crude plant genomic extraction (Appendix A4) was added to the sample and inverted several times to homogenize the sample. Then, 300 µl of 99% isopropanol was added to the sample mixture and inverted several times to homogenize the mixture. The mixture was centrifuged at 12,000xg for 15 min, then the supernatant was discarded, and the pellet was dried at room temperature about 15 min or until the isopropanol was completely evaporated. Then, 50 µl of sterilized distill water was added to the pellet and the mixture was used as PCR template.

The mutant was confirmed by PCR genotyping using three primers, LP (5'GGTAAGAGTCTATCGGGCTTAGTG3'), RP (5'GTTATCTTAAGACCGTCCAAGGTG3') and LB (5'ATAATAACGCTGCGGACATCTACATTTT3'), LP and RP were located on the gene while LB located on the left border of inserted T-DNA in the 4th exon of Arabidopsis *ICL*. The PCR product using the LP and RP primers should be around 911 bp in Arabidopsis without the T-DNA insertion (wild type). While the PCR product using the LB and RP primers should be approximately 500 bp in Arabidopsis containing T-DNA-inserted *AtICL* (*Aticl* mutant) (Figure 9). In the mutant, the inserted T-DNA is sufficiently long to obstruct PCR amplification using the LP and RP primers, furthermore in heterozygous, PCR product of both sizes (911 and ~500 bp) should be expected. In case of heterozygous, cultivation of one more generation to select homozygous is needed to implement the mutant for further experiments.

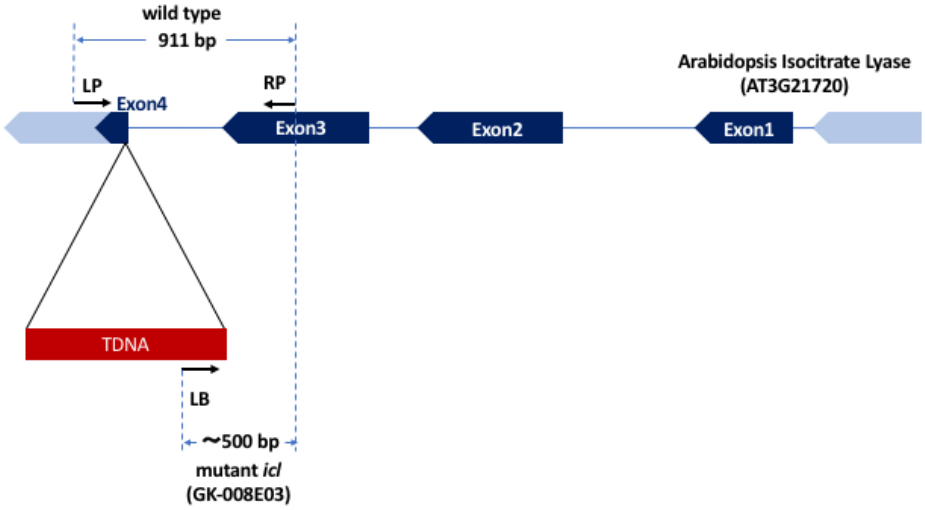


Figure 9 Inserted T-DNA location on the *icl* Arabidopsis mutant (GK-008E03) and the positions of the primers for *icl* Arabidopsis mutant genotyping, LP and RP represent primers locating on the AtICL and LB represents primer locating on the inserted T-DNA.

2.2.3.3. Construction of Recombinant Plasmid for *OsICL* Expression under *AtICL* Promoter

Three DNA fragments; β-glucuronidase and nopaline synthase terminator (*GUS-NOS*) from pCAMBIA1301, 2,138-bp upstream sequence of the Arabidopsis isocitrate lyase gene (*AtICL*) isolated from the Arabidopsis genomic DNA, and the rice isocitrate lyase (*OsICL*) coding sequence from the japonica rice cDNA library (AK063353, KOME clone Number 001-114-C03), were amplified by PCR using DNA primers, which were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), the web base tool for DNA primer design.

First, DNA fragment of the *OsICL* coding sequence form the cDNA library, which was accessorized with *Nde*I restriction site on the upstream of the *OsICL* by primer

design, was amplified by PCR using *Taq* DNA polymerase with *Nde*I_*OsICL*_F and *OsICL*_R primers (Table 4). Then, the PCR product of the *OsICL* coding sequence was purified by TIANgel midi purification kit and inserted in to pTZ57R/T, TA cloning plasmid, using T4 ligase and incubated in 4 °C refrigerator overnight. Next, the ligated mixture was transformed to competent *E. coli* DH5α by electroporation, then 1 ml LB medium was added to the transformants and incubated at 37 °C with shaking for 1 hr. Then, the transformants were transferred to LB agar plate containing 100 µg/ml ampicillin, 40 µg/ml IPTG and 40 µg/ml X-gal, by spread plate technique for blue-white colony screening and the plate was incubated at 37 °C in the incubator overnight. The white single colonies on the plate were picked to perform colony PCR using *Nde*I_*OsICL*_F and T7 promoter (5'TAATACGACTCACTATAGGG3') primers. The positive clone should produce the PCR product size of around 1,700 bp. Then, the positive colonies were grown in LB broth containing 100 µg/ml ampicillin in the incubator shaker at 37 °C with 250 rpm shaking overnight. The transformant *E. coli* cells were harvested by centrifugation at 12,000xg for 1 min, then the plasmids were isolated from the harvested cells using TIANprep mini plasmid kit, which was based on alkaline lysis plasmid extraction. After that, the recombinant plasmids were double-digested by two restriction enzymes, *Nde*I and *Xba*I, with compatible buffer, then the 0.8-1% agarose gel electrophoresis of the digested recombinant plasmids was performed. Next, the digested recombinant plasmid band, which the size should be around 4,600 bp (1,719 bp of *OsICL* + 2,886 bp of pTZ57R/T vector), was quickly observed on the UV-gel pad and the expected band was cut for further purification and cloning.

The 2,138-bp *AtICL* upstream sequence was amplified from Arabidopsis genomic DNA, which was extracted from Arabidopsis leaf tissue following step 2.2.3.2, using Phusion® high-fidelity DNA polymerase with *Xba*I_pATICL_F and pATICL_*Nde*I_R, primers (Table 4) accessorizing the restriction site *Xba*I on the upstream side of the 2,138-bp upstream *AtICL* fragment and *Nde*I was added to the other side of the fragment to link to the *OsICL* fragment. Then, the upstream *AtICL* fragment was purified from the PCR reaction mixture using TIANgel midi purification kit. The 2,138-bp upstream *AtICL* was double-digested by *Xba*I and *Nde*I using compatible buffer and the reaction was cleaned up by TIANgel midi purification kit again. The double-digested 2,138-bp upstream *AtICL* was ligated to the digested *OsICL*-pTZ57R/T fragment by T4 ligase under 16 °C overnight. The competent *E. coli* DH5α was transformed with recombinant plasmids in the ligation mixture by electroporation method. The transformant cells were recovered by adding 1-ml LB medium into the cells and incubated in the incubator shaker at 37 °C for 1 hr, then the transformant cells were spread on LB agar plate containing 100 µg/ml ampicillin and incubated at 37 °C overnight. The single colonies were picked up to perform colony PCR and grown for further experiments. For colony PCR using *Taq* DNA polymerase with T7 promoter and *Xba*I_pATICL_F primer, the positive colony should produce around 3,850-bp PCR product (1,719 bp form *OsICL* coding region + 2,138 bp form *AtICL* upstream sequence). Then, positive colonies were grown in LB broth, and the transformant cells were harvested by centrifugation at 12,000xg for 1 min, then the recombinant plasmids were isolated from the transformant cells using TIANprep mini plasmid kit. The purified

plasmids were double-digested using *Eco*RI and *Xba*I with compatible buffer. The double-digested DNA was separated by agarose gel electrophoresis and observed on the UV-gel pad, then the band with the size around 6,740 bp was cut. The linearized recombinant plasmid DNA of the upstream-*AtICL*-*OsICL*-pTZ57R/T fragment was purified for the next cloning step.

The *GUS-NOS* fragment was amplified from pCAMBIA1301 vector using Phusion® high-fidelity DNA polymerase with *Eco*RI_DTOPO_*GUS-NOS*_F and *GUS-NOS_Xba*I_R primers (Table 4). The PCR product was purifiied, then the *GUS-NOS* fragment, which was 2,304 bp, was double-digested using *Eco*RI and *Xba*I. Then the *GUS-NOS* fragment in the digestion reaction mixture was purified again. The double-digested *GUS-NOS* fragment was ligated to the upstream-*AtICL*-*OsICL*-pTZ57R/T fragment, then competent *E. coli* DH5α was transformed with the ligation mixture by electroporation method. The transformant cells were recovered by adding LB medium to the cells and incubated with shaking at 37 °C for 1 hr. The cells were then spread on LB agar plate containing 100 µg/ml ampicillin and incubated at 37 °C overnight. The single colonies were picked up for colony PCR and grown for further experiments. The colony PCR was carried out using *Taq* DNA polymerase with *Eco*RI_DTOPO_*GUS-NOS*_F and pATICL_*Nde*I_R primers (Table 4). The positive colony should produce around 4,480-bp PCR product (2,304 bp of the *GUS-NOS* fragment + 2,183 bp of the *AtICL* upstream sequence). The recombinant plasmids of *GUS-NOS*-upstream-*AtICL*-*OsICL*-pTZ57R/T were isolated and used as template for PCR amplification. The fragment of *GUS-NOS*-upstream-*AtICL*-*OsICL* was amplified using Phusion® high-fidelity DNA

polymerase with DTOPO_*GUS-NOS*_F and *OsICL*_R primers (Table 4). Agarose gel electrophoresis was performed to check the size of the fragment, which was expected to be around 6,206 bp. The accurate PCR product was purified for further recombinant plasmid construction (Figure 10 A).

The *GUS-NOS*-upstream-*AtICL-OsICL* fragment was inserted into pENTR/D-TOPO gateway cloning plasmid employing "CACC" directional cloning. Then *E. coli* DH5α was transformed with the recombinant plasmid by electroporation. The transformed cells were recovered in LB at 37 °C for 1 hr and spread on LB agar plate containing 50 µg/ml kanamycin, then the plate was incubated at 37 °C overnight and the single colonies were picked up to perform colony PCR and grown for harvesting the recombinant plasmid. Colony PCR was carried out using *Taq* DNA polymerase with M13R (5′CAGGAAACAGCTATGAC3′) and *Nde*I_*OsICL*_F primers, and the expected size of PCR product was around 1,700 bp. Then, the positive colonies were grown in LB broth with 50 µg/ml kanamycin at 37 °C overnight, the cells were harvested by centrifugation at 12,000xg for 1 min, and the recombinant plasmids were isolated using the plasmid purification kit.

The fragment of *GUS-NOS*-upstream-*AtICL-OsICL* in pENTR/D-TOPO was exchanged with ccdB fragment in pK2GW7 vector by Gateway cloning employing LR clonase II (Figure 10 B). Then, *E. coli* DH5α was transformed with the recombinant plasmid of *GUS-NOS*-upstream-*AtICL-OsICL*-pK2GW7 by electroporation technique. The transformants were selected on LB agar plate containing 50 µg/ml spectinomycin. Single colonies were picked to grow for further experiments and used to perform

colony PCR using *Taq* DNA polymerase with *Nde*I_*OsICL*_F and 35S_terminator_R (5′CTAGCATGGCCGCGGGATAT3′) primers. The positive clone should produce a PCR product of around 1,700 bp. Then the positive clones were cultured in LB broth with 50 µg/ml spectinomycin, used for recombinant plasmid isolation using plasmid purification kit for further experiments. The nucleotide sequence and PCR checking the inserted fragments were determined in purified recombinant plasmid.

Table 4 DNA primers for construction of the recombinant plasmid expressing OsICL under the control of the 2,138-bp AtICL upstream sequence

Primer Name	Sequence (5' to 3')
*Nde*I_*OsICL*_F	*GTT***CATATG**TCGTCGCCGTTCTCCGTGCCAT
*OsICL*_R	TCACATCCTGGATTTGGCAAGA
*Xba*I_pATICL_F	*A***TCTAGA**CATCATCTTCTATCGGAATCTCA
pATICL_*Nde*I_R	*TGG***CATATG**TTTAACTTTTATAAATTGGAAATG
*Eco*RI_DTOPO_*GUS-NOS*_F	*TAGAT***GAATTC**ACCATGGTAGATCTGAGGGTAAATTTCTAG
*GUS-NOS_Xba*I_R	*T***TCTAGA**ATAATTTATCCTAGTTTGCGCGCTA
DTOPO_*GUS-NOS*_F	CACCATGGTAGATCTGAGGGTAAATTTCTAG

Remark: the bold characters represent restriction site, the italic characters represent the added nucleotide benefitting in binding of restriction enzyme, and the underlined characters represent the sequence benefitting in directional TOPO cloning

A

B

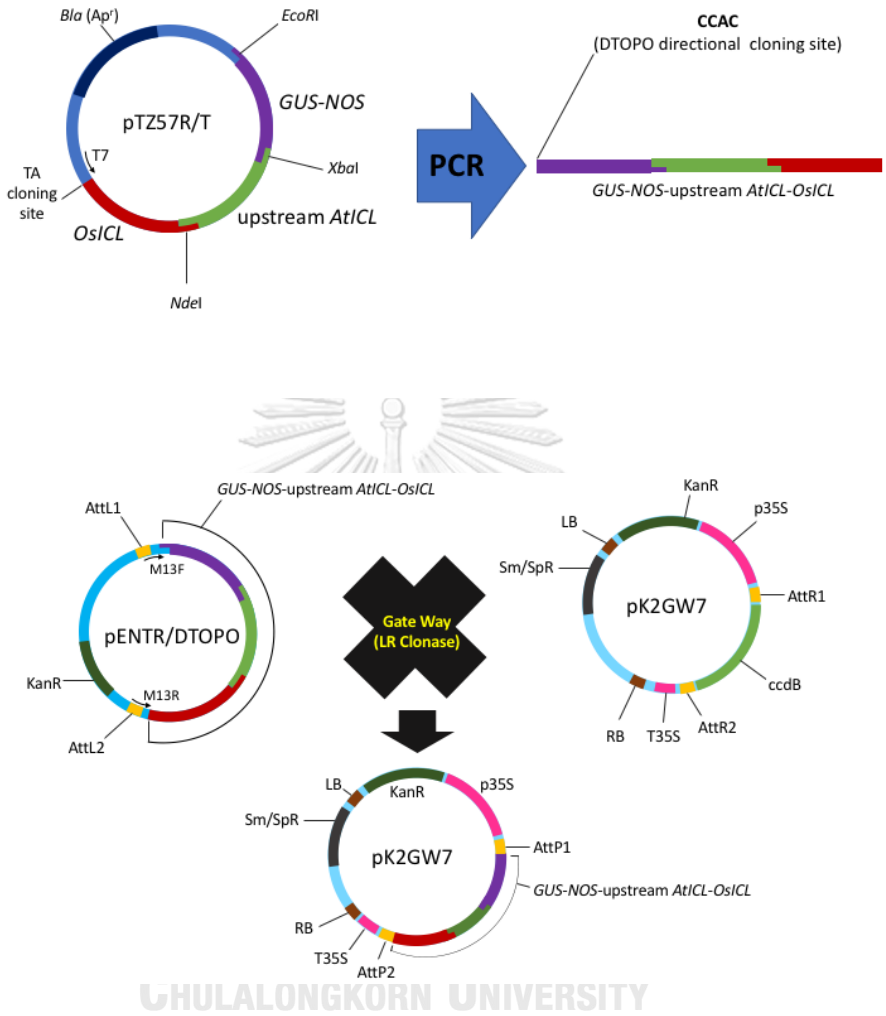


Figure 10 Schematic diagram showing construction steps of the recombinant plasmid for *OsICL* expression under the control of *AtICL* promoter, A) the 3 fragments were inserted into pTZ57R/T employing restriction site cloning strategy, and the target fragments were amplified from the recombinant plasmid *GUS-NOS*-upstream-*AtICL*-*OsICL*-pTZ57R/T. B) The *GUS-NOS*-upstream-*AtICL-OsICL* cassette was directionally inserted into pENTR/DTOPO employing "CACC" site, then it was transferred to pK2GW7, destination vector, by Gateway cloning strategy.

2.2.3.4. Construction of The Recombinant Plasmid for *OsICL* Overexpression

The *OsICL* coding sequence was amplified from cDNA library template using Phusion® high-fidelity DNA polymerases with *OsICL*_DTOPO_F (5´CACC**ATG**TCGTCGCCGTTCTCCGTGCCAT3´) and *OsICL*_R (see Table 4) primers. The cloning strategy was the same to step 2.2.3.3. This plasmid construction steps started with the *OsICL* fragment being directionally inserted into pENTR/DTOPO vector and the recombinant plasmids were introduced to *E. coli* DH5α by electroporation technique. The transformants were selected on the LB agar plate containing 50 μg/ml kanamycin and colony PCR was performed with *Taq* DNA polymerase with *OsICL*_DTOPO_F and M13R primers. The positive clone should produce a PCR product of around 1,700 bp. Then the positive clones were cultured in LB broth with 50 μg/ml kanamycin and the cells were harvested for plasmid isolation. The purified *OsICL*-pENTR/DTOPO recombinant plasmid was used for Gateway cloning using LR clonase II to transfer the *OsICL* fragment into LR cloning site of pK2GW7, destination vector (Figure 11), then the *OsICL*-pK2GW7 recombinant plasmid was transferred to *E. coli* DH5α by electroporation technique. The transformants were selected by LB agar plate containing spectinomycin and the colony PCR was carried out using *Taq* DNA polymerase with *OsICL*_DTOPO_F and 35S_terminator_R primers. The positive clone should produce around 1,700-bp fragment of PCR product. The positive clones, which were cultured in spectinomycin contained LB broth, were harvested for plasmid isolation. Then, nucleotide sequence of the purified recombinant plasmid *OsICL*-pK2GW7, which would be transformed to Arabidopsis, was determined.

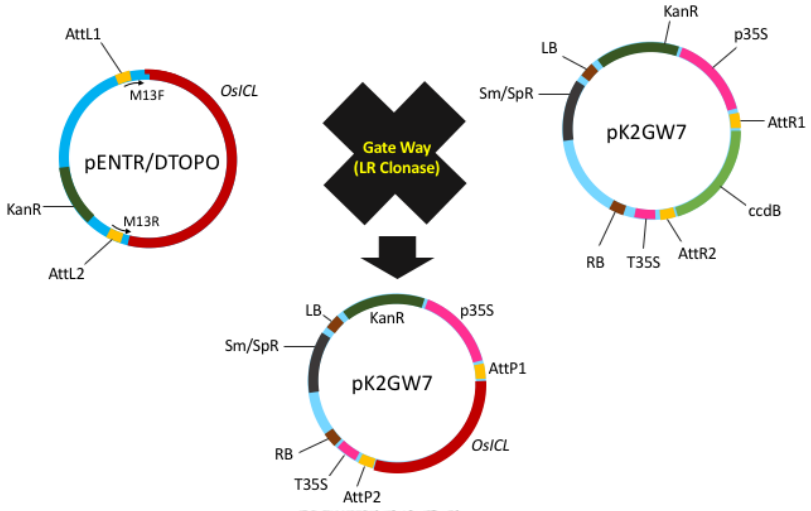


Figure 11 Schematic diagram showing steps of recombinant plasmid construction for *OsICL* overexpression employing directional TOPO and Gateway cloning.

2.2.3.5. *Agrobacterium*-Mediated Transformation with Plasmid Constructs

From steps 2.2.3.3–2.2.3.4, the two recombinant plasmids were obtained, and *Agrobacterium tumefaciens* GV3101 was transformed with each of the purified plasmids by electroporation and the transformants were recovered by incubating in LB broth at 30 °C with shaking for 4 hr. Then, the transformant was selected by spreading on LB agar plate containing 50 µg/ml of spectinomycin, 50 µg/ml of gentamicin and 20 µg/ml of rifampicin and incubated at 30 °C for 48-72 hr. The colony PCR was performed using *Taq* DNA polymerase with appropriate primers of each recombinant plasmid. The positive clones were grown in liquid LB medium with the same antibiotics used in the LB agar plate from the previous step at 30 °C with shaking overnight. The transformant cells were collected, mixed with glycerol (total 10-50% v/v) and kept in the -80 °C freezer as master stock cultures.

The wild type and mutant *Aticl* Arabidopsis were cultured following step 2.2.3.1 until the flowering stage (totally around 3 weeks). The young flowers, which

had not bloomed was the target organ for *Agrobacterium*-mediated transformation technique (floral dipping).

The floral dipping protocol was modified from Clough and Bent (1998). Predictively, around 2 days before the Arabidopsis was ready to be transformed (not over 4 weeks old Arabidopsis), the master stock culture of Agrobacterium carrying the recombinant plasmid was sub-cultured in 20 ml LB broth with the same concentrations to the previous step of rifampicin, gentamicin and spectinomycin, then incubated with shaking at 30 °C overnight and this cultured media would be used as inoculum. The 1% v/v inoculums were added to 400 ml LB medium containing the same antibiotics and incubated with shaking for 18-24 hr. During the time of *Agrobacterium* growing, any bloomed flowers or siliques of the Arabidopsis must be eliminated by cutting to minimize the amount of non-transformant offspring seed. Upon *Agrobacterium* growth within the expected period, the cells were collected by centrifugation at 4,000xg for 15 min at 25 °C. The cell pellet was gently resuspended in 50-100 ml transformation buffer containing MS medium, sucrose and silwet (Appendix A5). Then, the remaining Arabidopsis flowers were soaked in the *Agrobacterium* transformation mixture for 10-15 min, then the soaked Arabidopsis(s) were kept in plastic box containing water (do not soak the Arabidopsis in the water) and the box was closed with opaque material and sealed for maintaining high humidity under dark condition. The transformed Arabidopsis(s) were kept in the box for 24 hr, then they were moved to normal growth condition and let mature until the seeds were ready to harvest. The *Agrobacterium* dipped Arabidopsis was called T1 generation, and their offspring seeds (T2 generation) were separately harvested plant by plant for antibiotic selection.

2.2.3.6. Transgenic Arabidopsis Selection

The harvested transformed Arabidopsis seeds (T2) were grown following step 2.2.3.1 in MS medium containing compatible antibiotic as following;

a) 100 µg/ml of kanamycin for the transgenic Arabidopsis expressing *OsICL*;

- *Aticl* Arabidopsis mutant lines carrying the *GUS-NOS*-upstream-*AtICL-OsICL*-pK2GW7 (3FL) cassette,
- *Aticl* Arabidopsis mutant lines carrying the *OsICL*-pK2GW7 (OX*OsICL/icl*) cassette,
- wild type Arabidopsis lines carrying the *OsICL*-pK2GW7 (OX*OsICL/*WT) cassette

Then, one-week old Arabidopsis plants, which grew well without defect were grown following step 2.2.3.1, and the rosette leaf of 2-3 weeks old Arabidopsis was taken to confirm the existence of the desired inserted DNA by PCR genotyping technique. The PCR was used to confirm that T2 seed (T3 generation) harvested for further experiments was homozygous line.

2.2.3.7. The Transgenic Arabidopsis Genotyping

Genomic DNA from young rosette leaf of the T2 Arabidopsis was isolated using the same protocol as step 2.2.3.2. The genomic DNA was used as template in PCR reaction, which used 2x*Taq*-mixed with OsICL_Seq_M3_F (5′TGTGGCAGTTCATCACGCTC3′) primer, which binding site located on the coding region of *OsICL* and the 35S_Terminator_Seq_R (5′CTAGCATGGCCGCGGGATAT3′) DNA primer. The PCR determined the existence of *OsICL* fragment insertion in the 3FL revertant line, the OX*OsICL/icl* revertant Arabidopsis line and the OX*OsICL/*WT line, which is the

Aticl Arabidopsis mutant expressing *OsICL* under the control of 2,183-bp *AtICL* upstream sequence, the *Aticl* Arabidopsis mutant expressing *OsICL* under the control of 35SCaMV constitutive promoter, and the wild type Arabidopsis expressing *OsICL* under the control of 35SCaMV constitutive promoter, respectively. The PCR product size of those transgenic Arabidopsis expressing *OsICL* was expected to be about 460 bp. The PCR condition of the 2x*Taq*-mixed was 94 ℃ for 2 minutes for first denaturation, 35 cylces of 94 ℃ for 30 seconds, 55 ℃ for 30 seconds, and 72 ℃ for 1 minutes, and the last extension was 72 ℃ for 5 minutes.

2.2.3.8. Homozygous Transgenic Arabidopsis Selection

Survival rate of the T3 seeds was determined by germinating 30-100 seeds from each T2 plant in a compatible antibiotic as following; 3FL, OX*OsICL/icl* and OX*OsICL*/WT lines were grown on MS medium with 100 µg/ml kanamycin. The expected survival rate of hemizygous T3, which has no allelic counterpart or is present as only a single copy instead of the usual two copies in a diploid cell or organism, was 75% or 3:1 of survived : dead, and the χ^2 test was applied for statistical judging of the survival ratio with acceptance of χ^2 test at $p<0.05$.

Then, the T3 seeds exhibiting the accepted survival rate of 3:1 were further grown for harvesting the offspring seeds (T4). The T4 seed was grown on the MS medium with compatible antibiotic again in order to find homozygous lines among the mixture of hemizygous and homozygous plants. Therefore, the survival rate of 100% or 1:0 of survive : dead was expected. However, the survival rate of T4 may be not achieved 100% because some seeds may not germinate due to some environmental

effect, therefore the expected survival rate was set to be >90% with acceptance at $p<0.05$ of χ^2 test.

2.2.3.9. qRT-PCR Determining Gene(s) Expression of *AtICL* and *OsICL* in the Transgenic Arabidopsis

According to step 2.2.3.8, four lines of homozygous transgenic Arabidopsis expressing *OsICL* were constructed; 3FL4, 3FL9, OX*OsICL/icl*, OX*OsICL*/WT. Seeds of the 4 transgenic lines, wild type and mutant *Aticl* Arabidopsis were grown in MS medium containing 120 mM NaCl comparing to the MS medium without NaCl. The growing was performed following steps in 2.2.3.1 with 5 biological replicates. RNA was isolated from the 10-days old Arabidopsis (day 0 was the first day that the Arabidopsis germinating seeds were moved from 4 °C refrigerator to 25 °C growth room) using whole plant following step 2.2.1.2. Then, cDNA synthesis and qRT-PCR amplification were performed following step 2.2.1.5.

The transcript level of *AtICL*, native isocitrate lyase gene in Arabidopsis, was investigated in those six Arabidopsis lines with the pair of primers: AtICL_qRT_F (5′TCTACGTGGCCATCTCAAGC3′) and AtICL_qRT_R (5′TGGCTTGATGGCTTTTGAGC3′).

The *OsICL* transcript level was investigated using the pair of primers: OsICL_CDS_q_F (5′AGAGGGAGGAGAGGAGCAAC3 ′) and OsICL_CDS_q_R (5′CCTGGATTTGGCAAGAACAT3′).

The expression of AtEF1α was determined using the pair of primers: AtEF1α_qRT_F (5′TTCGCTGTTAGGGACATGAGGC3′) and AtEF1α_qRT_R (5′CACCCTTCTTCACTGCAGCCTT3′) benefiting in normalization process of the gene expression of *AtICL* and *OsICL* following gene expression calculation method of

$2^{-\Delta\Delta CT}$. The qRT-PCR condition and amplification efficiency of each gene were shown in Appendix B1.

2.2.3.10. Activity Assay of Isocitrate Lyase

Approximately 50 mg of total plant tissues from the six lines of Arabidopsis; 3FL4, 3FL9, OX*OsICL/icl*, OX*OsICL*/WT, mutant *Aticl* and wild type, which grew under the same condition as step 2.2.3.9 were collected, frozen in liquid nitrogen and grounded by Mixer Mill MM 400 until became a fine powder. The 500 µl of extraction buffer that contained 100 mM potassium phosphate buffer pH 7.6, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT, (Sidari, Mallamaci, & Muscolo, 2008) was added to the grounded sample and mixed by vortex then. The mixture was centrifuged at 12,000xg for 20 min at 4 °C. Then, the supernatant was transferred to a new microtube for using as a crude protein in enzymatic activity assay and the pellet was discarded.

Fifty µl of extracted protein was added into 440 µl of reaction buffer containing 50 mM potassium phosphate buffer pH 6.9, 50 mM $MgCl_2$, 10 mM EDTA and 40 mM phenylhydrazine, which was modified from Cooper and Beevers (1969), then mixed using pipette in 1 ml quart cuvette with 1 cm path length at 30 °C. A_{324} of the reaction mixture was measured by spectrophotometer until it was stable, then 10 µl of 500 mM D-L isocitric acid was added to the mixture and homogeneously mixed. Then A_{324} of the reaction mixture was further measrure for 10 min. The difference of A_{324} between min 10 and min 0 would be used for calculating the amount of glyoxylate production per 10 min employing glyoxylate standard curve (Appendix C3). The principle chemical reaction of isocitrate lyase assay reaction is shown in figure 12, and the equation for calculate isocitrate lyase activity is shown in Figure 13.

After that, the protein content of aliquot crude protein was determined using Bradford reagent in a 96-well plate. A_{595} was measured using microplate reader, and bovine serum albumin (BSA) was used for constructing a standard curve (Appendix C4).

$$\frac{((A_{324}\ \text{min}\ 10) - (A_{324}\ \text{min}\ 0)) \times (\text{coefficient of glyoxylate standard curve})}{(\text{mg protein}) \times (10\ \text{mins})} = \text{nM glyoxylate / mg protein / min}$$

Figure 12 The equation used for calculating activity of isocitrate lyase

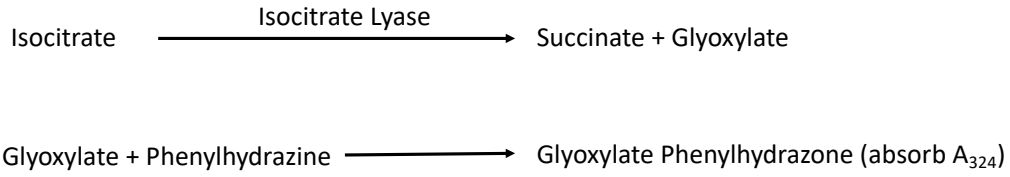


Figure 13 The principle chemical reaction of isocitrate lyase assay reaction

2.2.3.11. Sucrose and Starch Content Determination in Arabidopsis

Sucrose and starch contents were determined in the six Arabidopsis lines; 3FL4, 3FL9, OX*OsICL/icl*, OX*OsICL*/WT, *Aticl* mutant and wild type, which grew under the same condition as in step 2.2.3.9. Whole plants were collected, frozen in liquid nitrogen and grounded by Mixer Mill MM 400 until became a fine powder. Then, sucrose and starch were extracted and their contents were determined following step 2.2.2.1.1 and 2.2.2.1.2 respectively.

2.2.3.12. Arabidopsis Germination Rate Determination

Seeds of the six Arabidopsis lines: 3FL4, 3FL9, OX*OsICL/icl*, OX*OsICL*/WT, *Aticl* mutant and wild type were decontaminated following step 2.2.3.1. Then the seeds were orderly sown onto MS medium with 100, 120, 150, 200 mM NaCl or without NaCl. Then, the Arabidopsis plates were kept in the refrigerator for 2 days. The number of germinated seeds was daily observed for 7 days after the plates were moved to growing condition. For this experiment, we performed 5 biological replicates, which each biological replicate contained 50 seeds of each line. Germination rate was reported as percentage with standard deviation.

2.2.3.13. Arabidopsis Fresh Weight and Dry Weight Determination

Following step 2.2.3.12, the growing periods of Arabidopsis was extended to 10 days, then the 10 biggest Arabidopsis of each biological replicated were weighed to determine the fresh weight. The samples were then baked in 60-70 °C in the hot air oven for 5-7 days. Then, the baked Arabidopsis samples were weighed to determine the dry weight.

2.2.4. Computational and Statistical Medthod

2.2.4.1. Statistic Method for Physiological and Gene Characterization Experiment Data Analysis

The data from real-time RT-PCR, sucrose and starch determination, gas exchange and fluorescence measurements, isocitrate lyase activity, germination rate, fresh weight, and dry weight were compared using analysis of variance (ANOVA), and

then the means were compared with Duncan's multiple range test, with significance accepted at the $p<0.05$ level.

2.2.4.2. Statistical Verification of *OsCam1-1* affected DEGs

Fisher's exact test was used to verify that genes in the known gene set were significantly over-presented in the list of *OsCam1-1* affected DEGs with significance accepted at the $p<0.05$ level.

2.2.4.3. Promoter Analysis of 2,020 bp of *OsICL* Upstream Sequence

The 2,020 bp of *OsICL* upstream sequence including 5´UTR was applied to PLACE, bioinformatics tool for predict plant cis-acting regulatory DNA element, (Higo, Ugawa, Iwamoto, & Korenaga, 1999) in order to finding the transcription factor binding site.

CHAPTER III

RESULTS

3.1. Transcriptome of KDML 105 Rice Over-Expressing *OsCam1-1* under Salt Stress

3.1.1. Sequencing Information of The Rice RNA-Seq Results

CaM, a multifunctional protein, is able to regulate various target protein activities. Genome-wide analysis techniques such as RNA-seq, which provides precise transcript level measurement can expand our view of extent and complexity of eukaryotic transcriptome (Z. Wang, Gerstein, & Snyder, 2009). Transcriptome approach is particularly suitable for identifying the downstream components that are potentially regulated by CaM. According to the previous report, the rice over-expressing *OsCam1-1* showed a significantly higher relative growth rate than its wild type when grown under salt stress (Saeng-ngam et al., 2012). In this study, transcriptome profiling of the 3-week-old rice leaves of the transgenic rice over-expressing *OsCam1-1* and its wild type under normal condition and salt-stress (150 mM NaCl) for 4 hr was conducted. More than 185 million reads from the eight libraries from single-end RNA-Seq by Illumina Hi-Seq 2000 were obtained, with the total read of each library between 22-25 million reads. The reads were processed by POPE (Missirian et al., 2012), giving the total clean read per library of more than 99% of total read. At least 93% of the clean read were mapped to the rice genome reference, Michigan State University rice

annotation project's MSU7 (Kawahara et al., 2013), and less than 11% of clean read are multiple alignment read (Table 5)

Table 5 Read count of comparative transcriptome of transgenic KDML 105 rice and its wild type under normal and salt stress

Sample	Raw Input Read	Clean Read	% Clean Read	Mapped Read	% Mapped Read*	Multiple Alignment Read	% Multiple Alignment Read*
WTNS R1	24,019,397	23,961,896	99.76	22,973,989	95.88	1,952,214	8.15
WTNS R2	22,859,782	22,779,097	99.65	21,709,287	95.30	2,029,217	8.91
WTS R1	23,050,208	22,990,113	99.74	21,979,140	95.60	1,746,186	7.60
WTS R2	23,437,864	23,362,805	99.68	22,119,198	94.68	2,102,270	9.00
L1NS R1	23,259,980	23,234,303	99.89	22,207,203	95.58	2,048,681	8.82
L1NS R2	23,944,162	23,859,454	99.65	22,673,161	95.03	2,110,311	8.84
L1S R1	22,358,313	22,282,400	99.66	21,248,337	95.36	2,332,891	10.47
L1S R2	22,980,550	22,928,854	99.78	21,527,676	93.89	1,739,326	7.59

* The % mapped read and % multiple alignment read were calculated using clean read as denominator

To compare the transcriptome profiles of those rice, differential gene expression analysis of the transcriptome data using DESeq (Anders & Huber, 2010) was carried out. The analysis revealed 12,184 differentially expressed genes (DEGs) ($p<0.05$) in comparison of the wild type rice under normal and that under salt stress condition (WTNSWTS), as 5,842 and 6,342 genes were up-regulated and down-regulated,

respectively. While the comparison of the transgenic rice over-expressing *OsCam1-1* line L1 under normal and that under salt-stress (L1NSL1S) revealed the total of 13,259 DEGs, with 6,434 and 6,825 genes up-regulated and down-regulated, respectively. Furthermore, comparison of the transgenic and the wild type rice under normal condition (WTNSL1NS) revealed 2,022 DEGs, with 892 and 1,130 DEGs found expressed at a higher or lower level in the transgenic rice, respectively. Meanwhile, comparison of the transgenic and wild type rice under salt-stress condition (WTSL1S) showed 1,677 DEGs, with 957 and 720 DEGs found expressed at a higher or lower level in the transgenic rice, respectively (Table 6). The scatterplots of each of the four transcriptome profile comparisons illustrated that salt stress had much more impact on gene expression than the over-expression of *OsCam1-1* (Figure 14). *OsCam1-1* was found highly expressed in the transgenic rice in both normal and stress condition, with the average RPKM of 1,758.67 and 1,644.62, respectively, while the average RPKM of the wild type under normal and stress condition were 91.94 and 97.84 respectively. The expression of *OsCam1-1* in the wild type was not induced at 4 hours after salt stress (150 mM), which agreed well with the previous study. According to gene expression study of Chinpongpanich, Limruengroj, Phean-o-pas, Limpaseni, and Buaboocha (2012), the transcript level *OsCam1-1* determined by qRT-PCR was highly induced at 1 hour after 150 mM NaCl treatment then it sharply decreased after 1 hour. This result validated the over-expression of *OsCam1-1* in the transgenic rice with around 18 folds change of RPKM when compared with the wild type. From differential

transcriptome analysis, the observed gene expression levels were therefore likely affected by the *OsCam1-1* over-expression.

Table 6 Differential gene expression analysis results showing number of significantly different-expressed genes comparing each rice line and/or condition

comparison of rice line and/or condition	total number of DEGs ($p<0.05$)	number of up-regulated DEGs ($p<0.05$)	number of down-regulated DEGs ($p<0.05$)
WTNSWTS	12,184	5,842	6,342
L1NSL1S	13,259	6,434	6,825
WTNSL1NS	2,022	892	1,130
WTSL1S	1,677	957	720

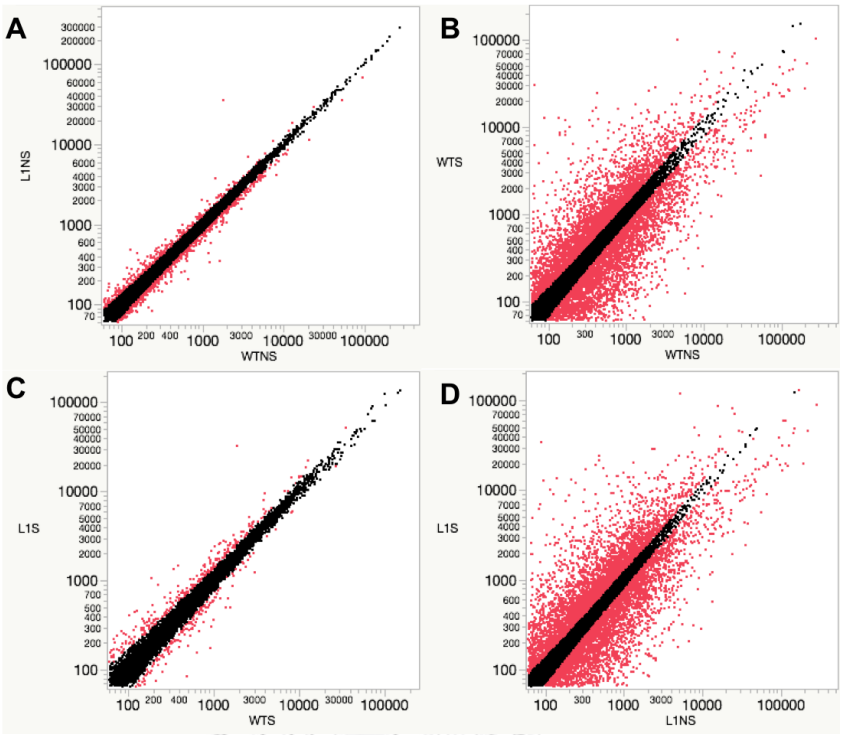


Figure 14 Scatter plot of gene expression of transgenic KDML 105 rice over expressing OsCam1-1 and the wild type KDML 105 under non-stress or salt stress condition (150 mM NaCl, 4 hr). Scatter plot showing significantly differential gene expression (DEG) ($p<.05$) comparing different rice lines and/or conditions; (A) comparison of wild type under non-stress condition (WTNS) and OsCam1-1 over-expression line under non-stress condition (L1NS), (B) wild type under non-stress condition (WTNS) and wild type under stress condition (WTS), (C) wild type under stress condition (WTS) and OsCam1-1 over-expression line under stress condition (L1S), (D) OsCam1-1 over-expression line under non-stress condition (L1NS) and OsCam1-1 over-expression line under stress condition (L1S), X and Y axes represent base mean obtained from the RNA-seq data.

3.1.2. Identification of Salt Stress or *OsCam1-1* Over-Expression Effected DEGs

To categorize the DEGs, Venn diagrams were constructed from the four comparisons. The first Venn diagram was constructed from the salt-responsive DEGs from either wild type (WTNSWTS, blue colored circle) or transgenic rice (L1NSL1S, red colored circle) and the DEGs that were expressed at higher levels in the transgenic rice either under normal (WTNSL1NS_up, green colored circle) or salt stress (WTSL1S_up, yellow colored circle) conditions (Figure 15 A). In contrast, the second diagram was constructed from the salt-responsive DEGs and the DEGs that were expressed at lower levels in transgenic rice either under normal (WTNSL1NS_down, green colored circle) or salt stress (WTSL1S_down, yellow colored circle) conditions (Figure 15 B). According to the Venn diagrams, we identified 1,328 salt-responsive DEGs with higher expression levels in transgenic rice, which will be referred to as HT salt-responsive DEGs (Figure 15 A, red line circle), and 1,431 salt-responsive DEGs with lower expression levels in transgenic rice, which will be referred to as LT salt-responsive DEGs (Figure 15 B, red line circle). For those with unaffected expression levels by salt stress, 290 genes or 200 genes showed higher (Figure 15 A, blue line circle) or lower (Figure 15 B, blue line circle) expression levels in transgenic rice, which will be referred to as HT DEGs or LT DEGs, respectively. In both Venn diagrams, the largest of number DEGs with expression levels that did not differ between transgenic rice and wild type (green circles) were salt-responsive DEGs.

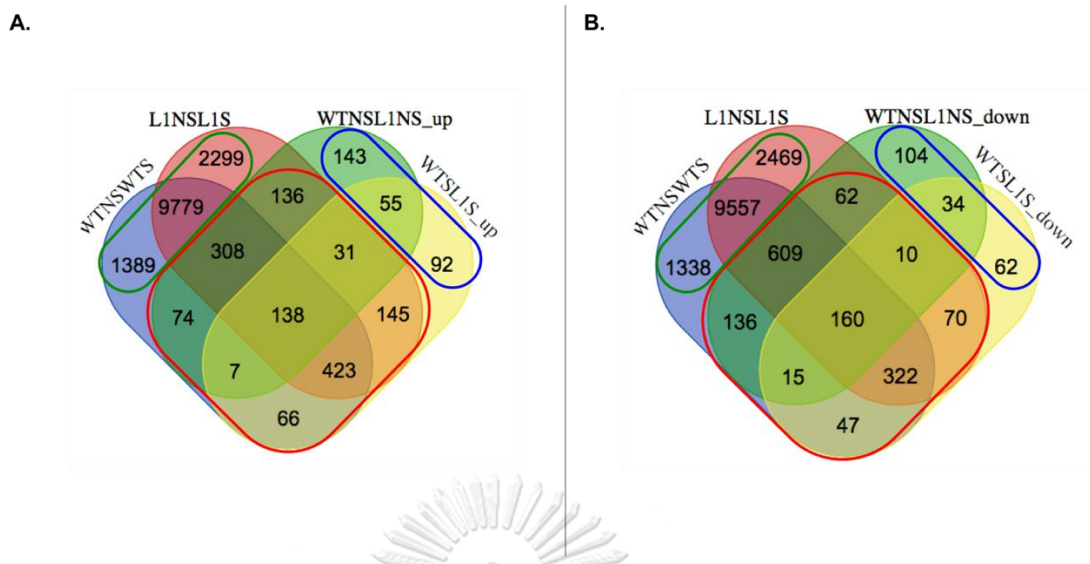
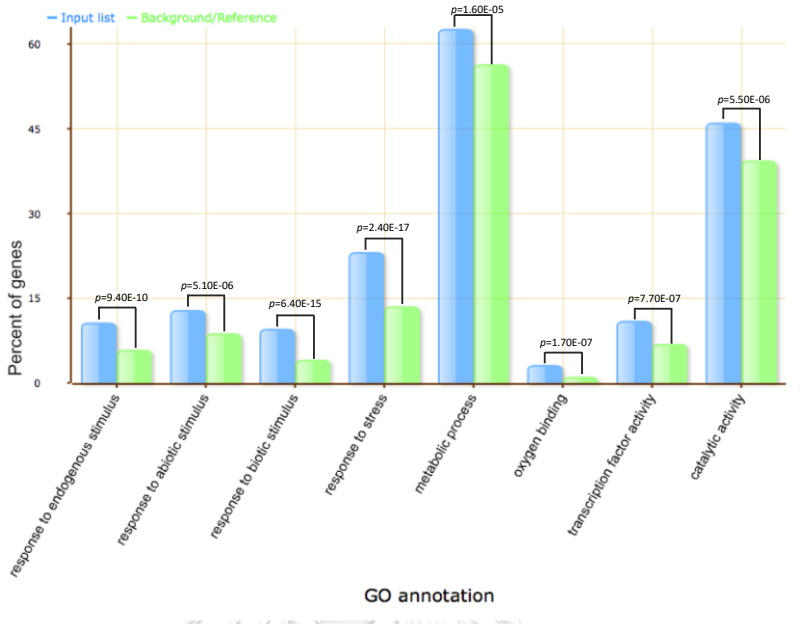


Figure 15 Venn diagram showing (A) the number of significantly ($p<0.05$) HT salt-responsive DEGs (red circle), HT DEGs (blue circle) and salt-responsive DEGs (green circle), while (B) shows the number of LT salt-responsive DEGs (red circle), LT DEGs (blue circle)

3.1.3. Gene Ontology Enrichment Analysis

Gene enrichment analysis was performed using those 1,328 HT salt-responsive DEGs. The results showed that, in terms of biological process, the terms of response to endogenous stimulus (GO:0009719), response to abiotic stimulus (GO:0009628), response to biotic stimulus (GO:0009607), response to stress (GO:0006950) and metabolic process (GO:0008152), were enriched, while for the term of molecular function, the terms of oxygen binding (GO:0019825), transcription factor activity (GO:0003700) and catalytic activity (GO:0003824) were overrepresented (Figure 16 A). For those 1,431 LT salt-responsive DEGs, in terms of biological process, the terms of response to abiotic stimulus (GO:0009628), lipid metabolic process (GO:0006629), secondary metabolic process (GO:0019748), translation (GO:0006412), and photosynthesis (GO:0015979) were enriched, while in terms of the cellular compartment, the enriched terms included the thylakoid (GO:0009579), plastid (GO:0009536), cell wall (GO:0005618), intracellular organelles (GO:0043229), membrane (GO:0016020) and ribosome (GO:0005840), and in terms of molecular function, those of structural molecule activity (GO:0005198) and catalytic activity (GO:0003824) were overrepresented (Figure 16 B). Based on these results, we observed that the set of genes involving photosynthetic process were uniquely allocated in the LT salt-responsive DEGs, while genes involving response to stimuli and metabolic process were distributed in both HT and LT salt-responsive DEGs.

A.



B.

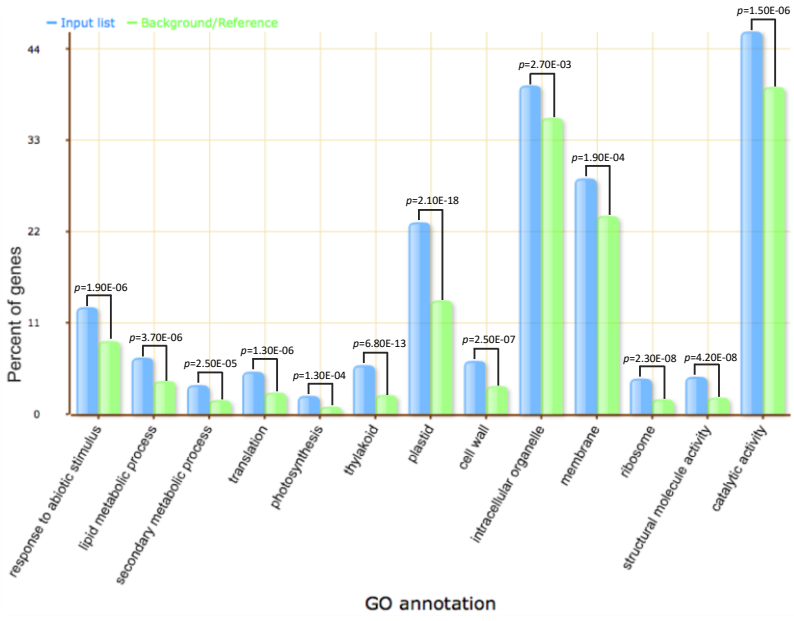


Figure 16 GO enrichment analysis results of (A) the salt-responsive DEGs with higher expression levels in transgenic rice, and (B) the salt-responsive DEGs with lower expression levels in transgenic rice

3.1.4. Functional Identification of *OsCam1-1* Regulated DEGs

When these salt-responsive DEGs were mapped onto metabolic pathways, several genes with consistent changes in their expression levels within certain pathways were observed, including the light reactions and Calvin cycle of the photosynthetic process, sucrose and starch metabolism, and central energy pathways. In Figure 17 and Figure 18, gene expression ratios between the transgenic rice and the wild type rice both under normal and salt stress conditions are presented for each corresponding step of these pathways. Overall, expression levels of 31 out of 33 DEGs in the photosynthetic process (e.g., chlorophyll a/b binding protein, protein subunit in photosystem I and II, ferredoxin, plastoquinone dehydrogenase complex, ribulose-bisphosphate carboxylase, which were repressed by salt stress) were lower in the transgenic rice overexpressing *OsCam1-1* (Figure 17). In Figure 18, the expression levels of several genes involved in sucrose degradation (e.g., LOC_Os03g22120 encoding sucrose synthase, which was highly induced by salt stress; LOC_Os01g22900 and LOC_Os02g33110 encoding invertase, which were repressed by salt stress) were higher in transgenic rice, especially under salt stress, while those of genes in the starch biosynthetic pathway (e.g., LOC_Os08g25734 encoding glucose-1-phosphate adenylyltransferase; LOC_Os02g51070 and Os01g52250 encoding starch synthase, which were repressed by salt stress) were lower in transgenic rice. In addition, genes involved in glycolysis and the TCA cycle (e.g., LOC_Os05g10650 encoding phosphofructokinase; and LOC_Os05g49760 encoding isocitrate dehydrogenase, which

were induced by salt stress) were expressed at higher levels in transgenic rice. Remarkably, three genes in the glyoxylate cycle: LOC_Os08g09200, LOC_Os07g34520, and LOC_Os04g40990 encoding aconitase, isocitrate lyase, and malate synthase, respectively, which were all highly induced by salt stress, were expressed at higher levels in transgenic rice both under normal and salt stress conditions (Figure 18). Moreover, the transcriptome results indicated that other cell processes; signaling, stress responses, hormones, transcription regulation, secondary metabolism, lipid metabolism, glycolysis, tricarboxylic acid cycle, glyoxylate cycle, and starch-sucrose metabolism were also regulated under salt stress condition, and they would be discussed in the discussion part.

Overall, under salt stress, 1,434 salt-responsive genes exhibited different expression levels between the wild type and transgenic rice. Figure 19 shows salt-responsive DEGs that encode potential downstream components of OsCaM1 in salt stress response. These DEGs are involved in several major cellular processes, including signaling and stress responses, hormone-mediate regulation, transcription, secondary metabolism, lipid metabolism, glycolysis, TCA cycle, glyoxylate cycle, photosynthesis, and carbohydrate metabolism. In signaling, the HT salt-responsive DEGs include LOC_Os06g49430, which encodes BWMK1, a rice MAP kinase; LOC_Os02g26720 and LOC_Os10g01480, which encode inositol 1,3,4-trisphosphate 5/6-kinase (IPTK); and LOC_Os04g54200, which encodes diacylglycerol kinase (DGK). Involved in stress response, the transcriptome results showed that the expression of 46 biotic and 19

abiotic stress DEGs was allocated in the HT salt-responsive DEG category. These include a universal stress protein (USP) (LOC_Os07g36600), and a xylanase inhibitor protein gene (OsXIP2) (LOC_Os05g15770). For those involved in hormone-mediated regulation, we have identified three lipoxygenase (LOX) genes (LOC_Os08g39840, LOC_Os12g37350, and LOC_Os03g49380) and three 12-oxo-PDA-reductase (OPR) genes (e.g. LOC_Os06g11210, LOC_Os06g11290, and LOC_Os01g27230), which encode enzymes in the jasmonate (JA) biosynthesis pathway. In addition, the genes encoding key enzymes in the ABA biosynthesis pathway, 9-cis-epoxycarotenoid dioxygenase (NCED) (LOC_Os07g05940) and abscisic aldehyde oxidase (AAO) (LOC_Os07g18120), were identified as HT salt-responsive DEGs, which were up-regulated approximately 1.9-fold and 1.6-fold, respectively in transgenic rice compared with wild type rice under salt stress.

According to the transcriptome results, thirteen APETALA2/ethylene-responsive element binding protein (AP2/EREBP) genes were identified as HT salt-responsive DEGs (e.g. LOC_Os09g35030, LOC_Os09g35010), while five AP2/EREBP genes were identified as LT salt-responsive DEGs. In addition, eight MYB genes were found allocated in the category of HT salt-responsive DEGs (e.g. LOC_Os01g74410) and 16 HT salt-responsive DEGs were WRKY, which is a large TF family that responds to plant stress (e.g. LOC_Os05g27730, LOC_Os02g08440, LOC_Os01g54600, LOC_Os09g25070). In secondary metabolism, 35 DEGs were identified as HT salt-responsive genes such as a

hydroxyphenylpyruvate dioxygenase (HPPD) gene (LOC_Os02g07160) and five laccase genes (e.g. LOC_Os12g15680, LOC_Os01g63180, LOC_Os01g63190).

Functions of several HT salt-responsive DEGs involve in the energy metabolism. These included 30 DEGs in lipid metabolism with examples including three 3-ketoacyl-CoA synthase genes (LOC_Os02g11070, LOC_Os05g49900 and LOC_Os02g56860), four class III lipase genes (LOC_Os01g15000, LOC_Os11g43760, LOC_Os02g43700 and LOC_Os05g49840), and four genes encoding enzymes involving beta oxidation (LOC_Os09g39410, LOC_Os03g07140, LOC_Os08g44360 and LOC_Os05g07090). Several HT salt-responsive DEGs are also involved in carbohydrate metabolism including a fructose bisphosphate aldolase (FBP) gene (LOC_Os09g02540) and a phosphofructokinase (PFK) gene (LOC_Os05g10650) in the glycolysis pathway; and an aconitase gene (LOC_Os08g09200) and an isocitrate dehydrogenase (IDH) gene (LOC_Os05g49760) in the TCA cycle. In addition, two genes encoding key enzymes in the glyoxylate cycle shuttling the TCA cycle pathway, isocitrate lyase (ICL) (LOC_Os07g34520) and malate synthase (MLS) (LOC_Os04g40990) were up-regulated approximately 1.7-fold and 1.5-fold, respectively in transgenic rice compared with wild type rice under salt stress. Additionally, the DEGs included two glucose-6-phosphate transporter genes, LOC_Os07g34006 and LOC_Os07g33954, both with higher expression levels in transgenic rice under salt stress.

Finally, seven DEGs involved in sucrose and starch metabolism were allocated in the category of HT salt-responsive DEGs. Among these DEGs, sucrose synthase (LOC_Os03g22120) was up-regulated in both wild type and transgenic rice under salt stress, with greater up-regulation in transgenic rice. The transcriptome data also revealed three invertase genes (LOC_Os01g22900, LOC_Os11g07440, LOC_Os02g33110) and seven cell wall degradation DEGs, which were expressed at higher levels in transgenic rice including two DEGs encoding polygalacturonase (LOC_Os10g26940 and LOC_Os09g31270).

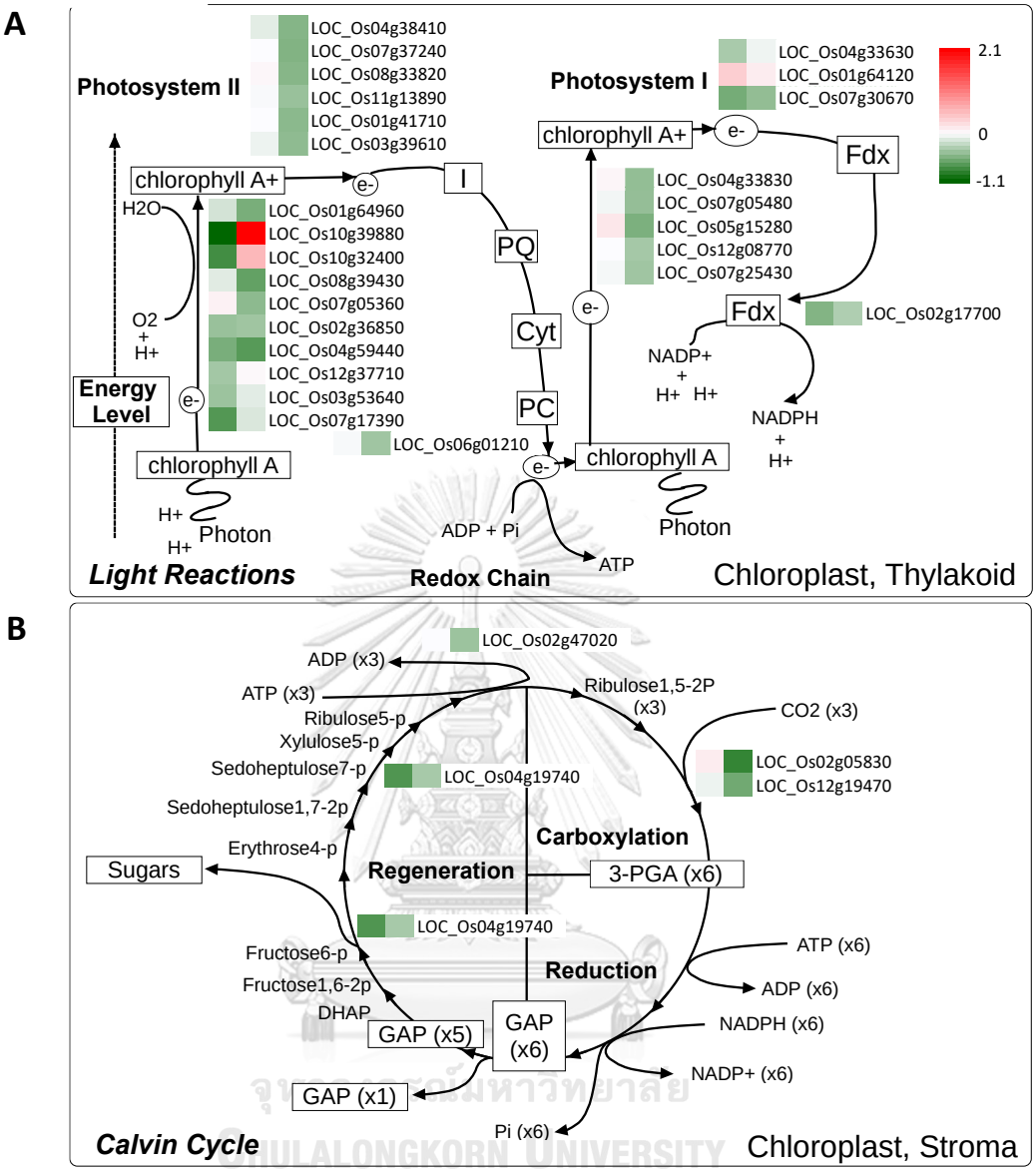


Figure 17 Photosynthetic pathway showing the expression level and role(s) of the genes in the light reaction (A) and Calvin cycle (B). The left box shows the log2-fold change in comparisons of WT and transgenic plants under normal conditions, and the right box represents the log2-fold change under salt stress conditions.

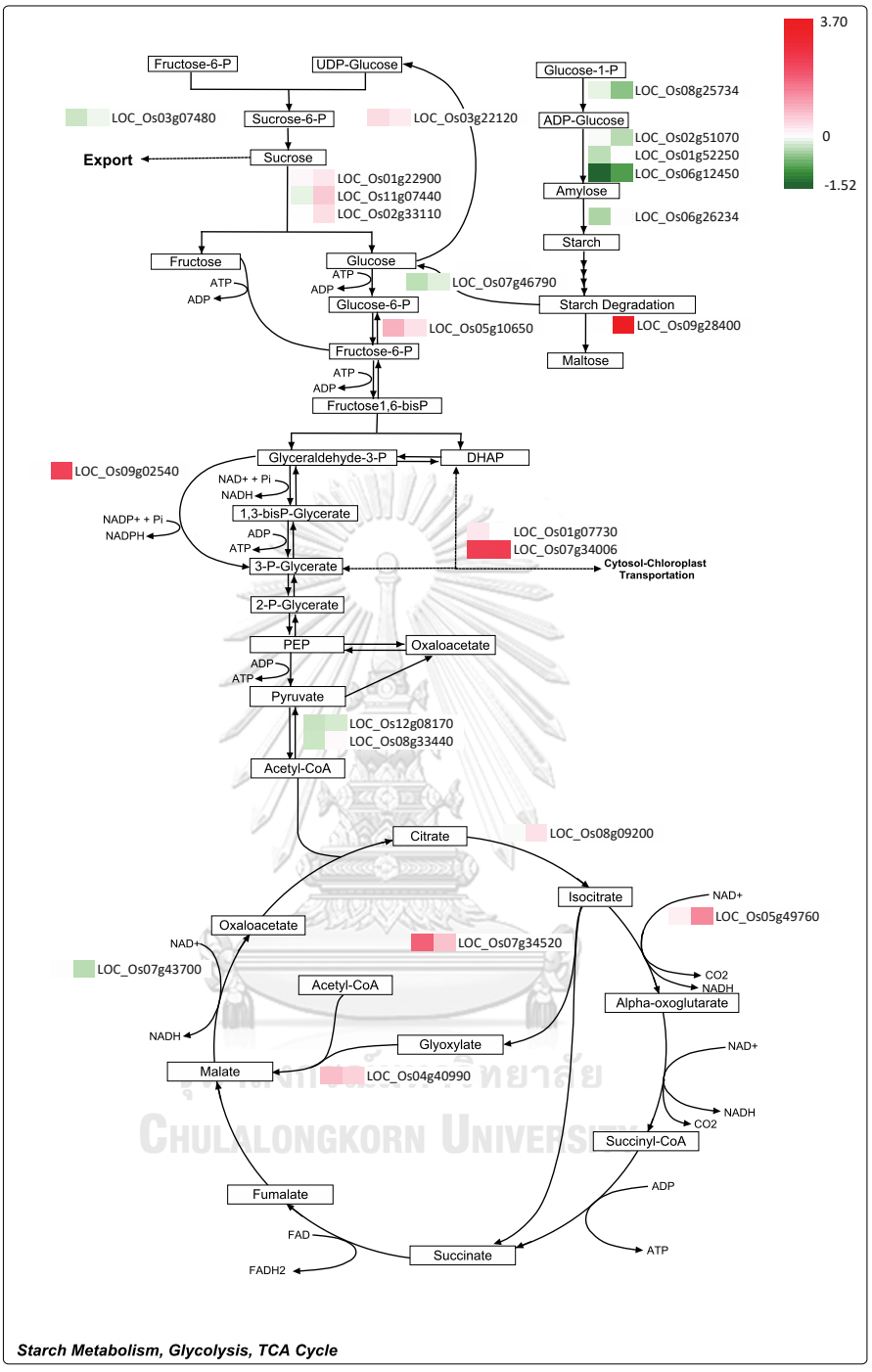


Figure 18 Carbohydrate and energy metabolism pathway consisting of sucrose-starch metabolism, glycolysis and the TCA cycle show the gene expression level and function in metabolism. The left box shows the log2-fold change of comparisons of WT and the transgenic plants under normal conditions, and the right box represents the log2-fold change under salt stress conditions.

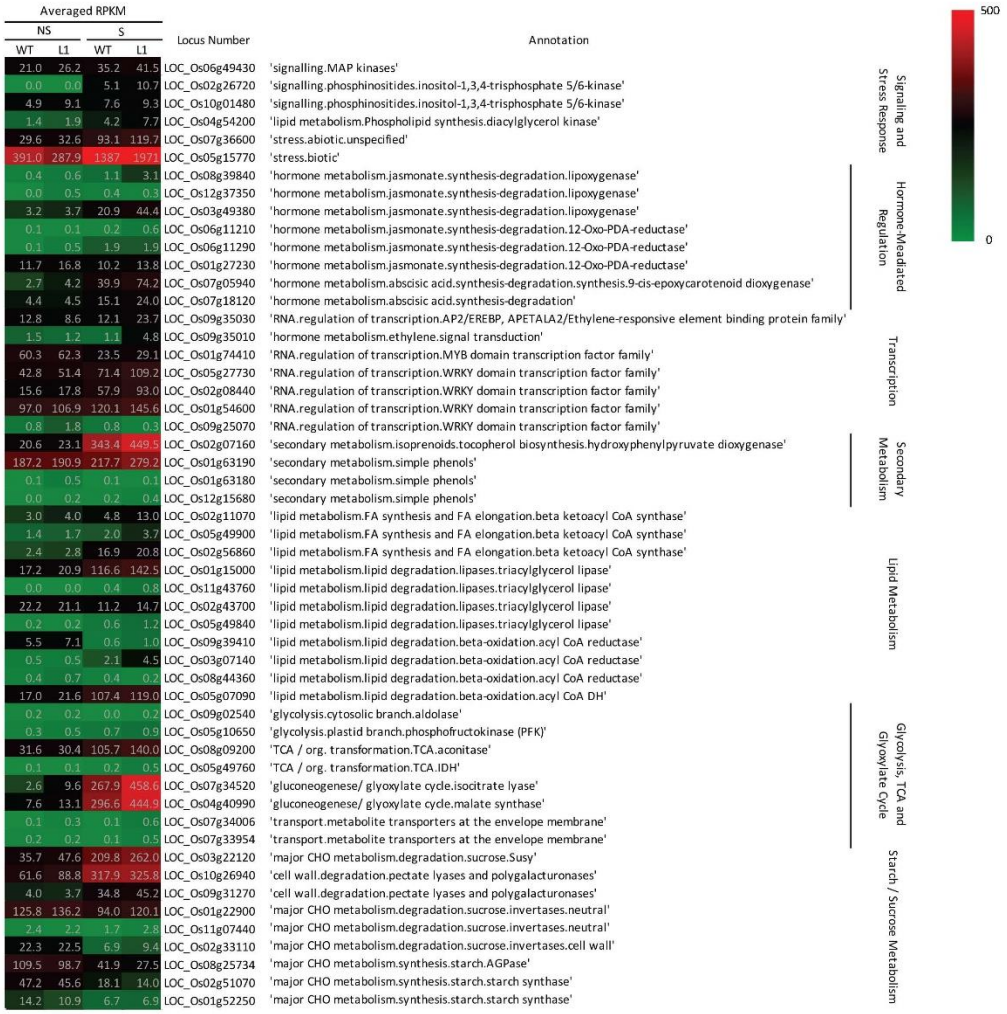


Figure 19 Salt-responsive DEGs that encode potential downstream components of OsCaM1 in salt stress response. Expression levels of each gene by RPKM from wild type and transgenic rice under normal and salt stress conditions were presented as heat map.

3.1.5. qRT-PCR Verification of the Transcriptome Data

To verify the reliability of the transcriptome data, nine salt-responsive genes, β-amylase (LOC_Os03g22790), isocitrate lyase (LOC_Os07g34520), malate synthase (LOC_Os04g40990), aconitase (LOC_Os08g09200), glycosyl hydrolase

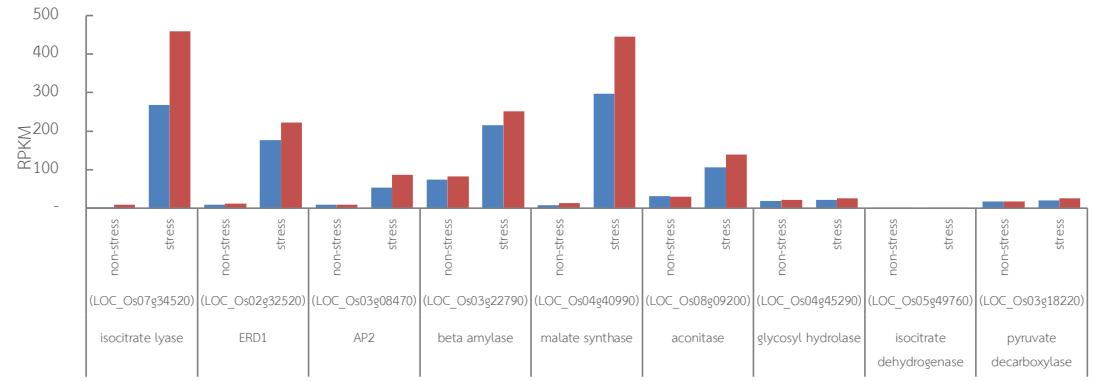
(LOC_Os04g45290), ERD1 (LOC_Os02g32520), AP2 (LOC_Os03g08470), isocitrate dehydrogenase (LOC_Os05g49760) and pyruvate decarboxylase (LOC_Os03g18220), were selected for qRT-PCR. Figure 20 shows the qRT-PCR results for seven genes, which agreed well with the transcriptome data. Compared with wild type, they all exhibited higher levels in transgenic rice, demonstrating a statistically significant difference under salt stress. In contrast, the expression of the other two genes examined by qRT-PCR did not agree well with the transcriptome data, potentially because of their low expression levels.

3.1.6. Statistical Verification of *OsCam1-1* affected DEGs

Overall, by RNA Seq, 3,249 genes were found to be differentially expressed between *OsCam1-1* over-expressing rice and wild type. To confirm the validity of this gene list, a statistical approach was employed using Fisher's exact test to determine the statistical confidence of the data as being true. Of the 55,986 rice genes according to the MSU7 rice genome database (http://rice.plantbiology.msu.edu/analyses_facts.shtml) (Kawahara et al., 2013), 60 rice genes were co-expressed with *OsCam1-1* by co-expression analysis using the web-based tool STRING (https://string-db.org/) (Szklarczyk et al., 2017), which were used as a reference list of known *OsCam1-1*-affected genes. Within this supposed known gene set, 30 genes were found in the list of 3,249 *OsCam1-1*-affected genes based on the RNA-Seq results. By Fisher's exact test, genes in the known gene set were significantly

over-presented in our list of 3,249 *OsCam1-1*-affected genes with a calculated p-value of 1.52 x 10^{-21}.

A



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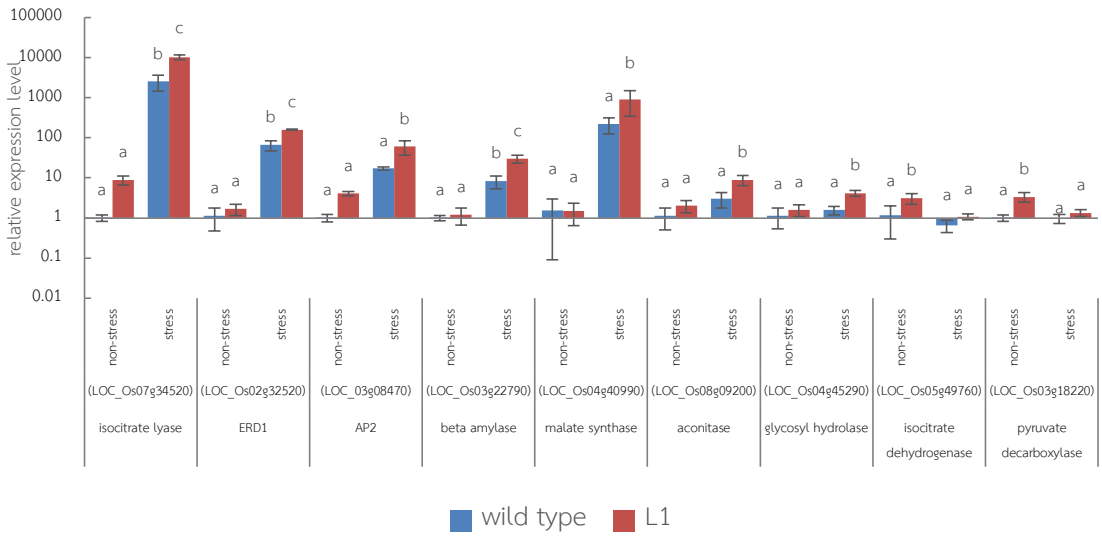


Figure 20 qRT-PCR verification of RNA-Seq. (A) RNA-Seq results with p values from DESeq analysis, (B) qRT-PCR results calculated using the $2^{-(\Delta\Delta CT)}$ method. Data are shown as the mean +1 SD, and are derived from four independent biological replicates. For each gene, means with a different letter are significantly different ($p<0.05$).

3.1.7. Promoter Analysis of 2,020 bp of *OsICL* Upstream Sequence

The 2,020 bp of *OsICL* upstream sequence including predicted 248 bp of 5´UTR was obtained from phytozome database (Goodstein et al., 2012). Then the DNA sequence was analyzed by PLACE (Higo et al., 1999), it was found that 9 OsWRKY71 binding regions located on the upstream 5´UTR of *OsICL* whereas 1 OsWRKY71 binding region located on the 5´UTR. OsWRKY71 was induced by ABA in in aleurone cells (Xie et al., 2005). Furthermore, 2 ABRE, ABA responsive element, were found on the upstream 5´UTR of *OsICL* (Figure 21).

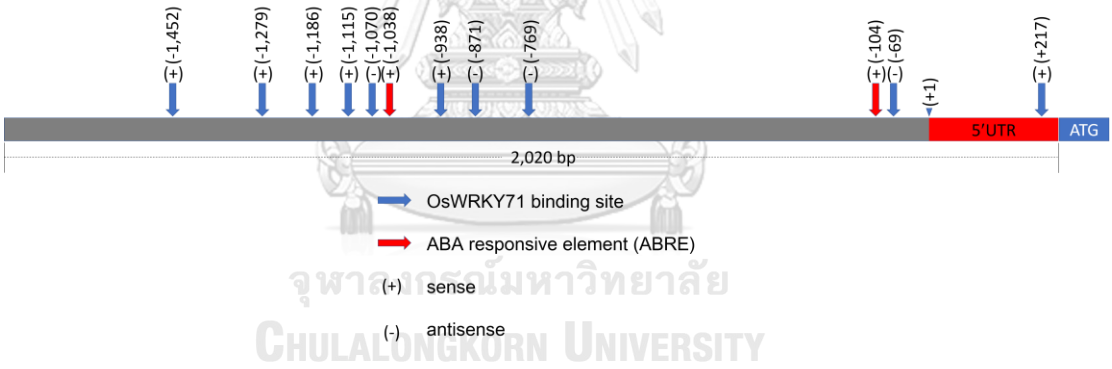


Figure 21 cis-acting element of 2,020 bp of *OsICL* upstream sequence, the number indicating position of the sites on the sequence, and the 2,020 bp of *OsICL* upstream sequence was showed in Appendix F3.

3.2. Biochemical and Physiological of The Transgenic Rice Over-Expressing *OsCam1-1*

3.2.1. Gene Expression of The Three Transgenic Rice Lines Confirming *OsICL* and *OsMS* Were Enhanced by Effect of *OsCam1-1* Over-Expression under Salt Stress

According to the $2^{-(\Delta\Delta CT)}$ method, the expression of *OsICL* or *OsMS* in the wild type rice under non-stress condition was used as base line for their own gene expression comparison. The gene expression level of *OsICL* in either wild type or three lines of the transgenic rice; L1, L2, and L7, under non-stress condition were similar to the base line and was not significantly different among the plants examined. Meanwhile under salt stress condition, *OsICL* expression level in all rice lines were sharply increased and much higher than those under non-stress condition. Furthermore, under stress condition, the *OsICL* expression levels of the three transgenic rice lines were higher than the wild type, which showed salt stress induction at about 500 folds over the base line. L1 and L2 showed statistically significantly higher *OsICL* expression level than wild type with fold-change of 1,400 and 1,300 folds over the base line, respectively, while L7 showed numerically higher expression level than wild type with fold-change of about 800 over the base line.

The expression levels of *OsMS* had similar trend to those of *OsICL* as expression levels of *OsMS* in all rice-lines under non-stress were on the base line level and highly increased under salt stress. The expression of wild type, L1, L2 and L7 under

salt stress increased to about 40, 160, 320, and 110 folds over the base line, respectively.

These gene expression results have shown quite strong evidences that the two key genes in glyoxylate cycle; isocitrate lyase (*OsICL*) and malate synthase (*OsMS*), responded to salt stress and were also enhanced by effect of *OsCam1-1* over-expression (Figure 21).

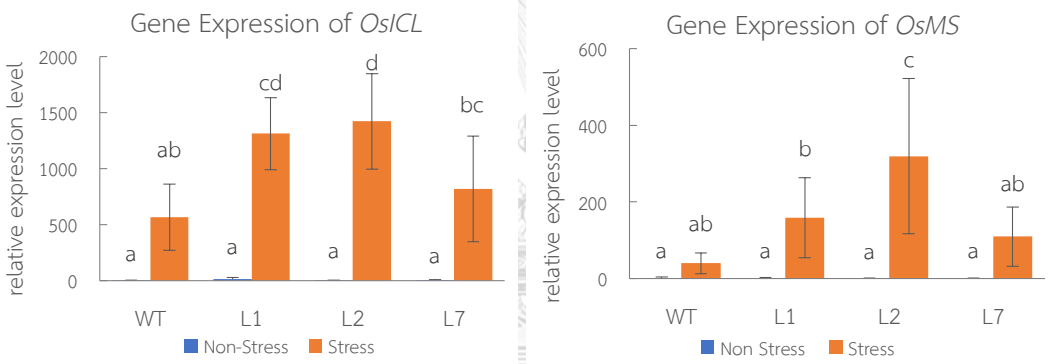


Figure 22 qRT-PCR result of *OsICL* and *OsMS* gene expression levels in three transgenic rice over-expressing *OsCam1-1* (L1, L2, L7) and wild type rice under non-stress and salt stress treatment.

Remark "Non-Stress" is referred to "without salt" and "Stress" is referred to "150 mM NaCl for 4 h"

3.2.2. *OsICL* Expression in Rice Responded to Either Salt Stress or Senescence

Following result 3.2.1, the expression level of *OsICL* of wild type and three transgenic rice lines were determined in leaf of the different age when grown under non-stress or salt stress condition. The results showed that *OsICL* expression was highest in the group of senesced leaf under salt stress with the fold change in the wild type, L1, L2, and L7 of around 340, 390, 435, and 800 folds over the base line of young leaf under non-stress condition. The senesced leaves under non-stress condition also showed high *OsICL* expression level compared to those of young leaf. Under non-stress condition, the fold change over the base line of young leaf in wild type, L1, L2, and L7 were around 108, 150, 170, and 60 folds, respectively. Interestingly, in young leaf, the three lines of the transgenic showed sharply increased expression levels with fold change in L1, L2, and L7 at around 40, 25, and 15 folds over the base line, while wild type did not.

The results revealed *OsICL* also responded senescence and *OsCam1-1* might affected *OsICL* expression in only young leaf, while effect of senescence and salt stress might more impact the *OsICL* expression (Figure 22).

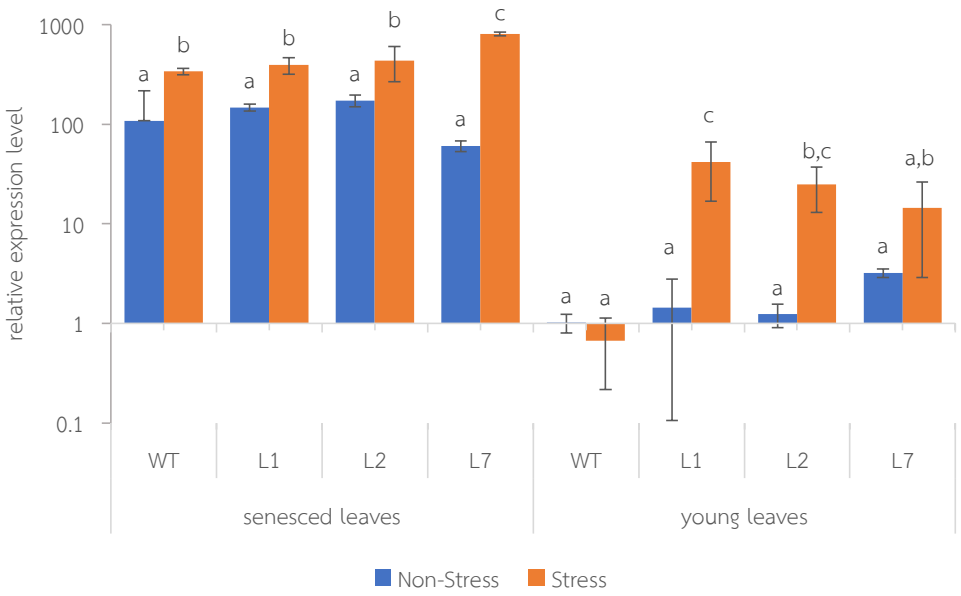


Figure 23 qRT-PCR result of *OsICL* gene expression in young leaves and senesced leaves of three transgenic rice over-expressing *OsCam1-1* (L1, L2, L7) and the wild type rice under non-stress and salt stress treatment, One-way ANOVA with Duncan multiple range test with criteria of $p<0.05$ separately applying for statistical data analysis of senesced leaves and young leaves.

Remark "Non-Stress" is referred to "without salt" and "Stress" is referred to "150 mM NaCl for 4 hr"

3.2.3. Over-Expression of *OsCam1-1* Affected in Decrease of Photosynthesis under Salt Stress

The photosynthesis rate (P_n), stomatal conductance (g_s), intercellular carbon dioxide (C_i) and transpiration rate (E) were examined in the transgenic rice over-expressing *OsCam1-1*. Under salt stress, P_n, g_s and E decreased at both day 3 and day 5, while C_i decreased slightly at day 3 of treatment. Interestingly, transgenic rice had slightly lower P_n values than wild type rice at both day 3 and day 5 and tended to have lower g_s and E values at day 5 of salt stress treatment. In contrast, the C_i measurements did not reveal significant difference between the transgenic and wild type (Figure 23). For FV′/FM′, which reflects the maximum efficiency of photosystem II (Baker, 2008), no change was observed under the given salt stress conditions, and the transgenic rice did not exhibit difference either under normal or salt stress conditions compared with the wild type (Figure 24).

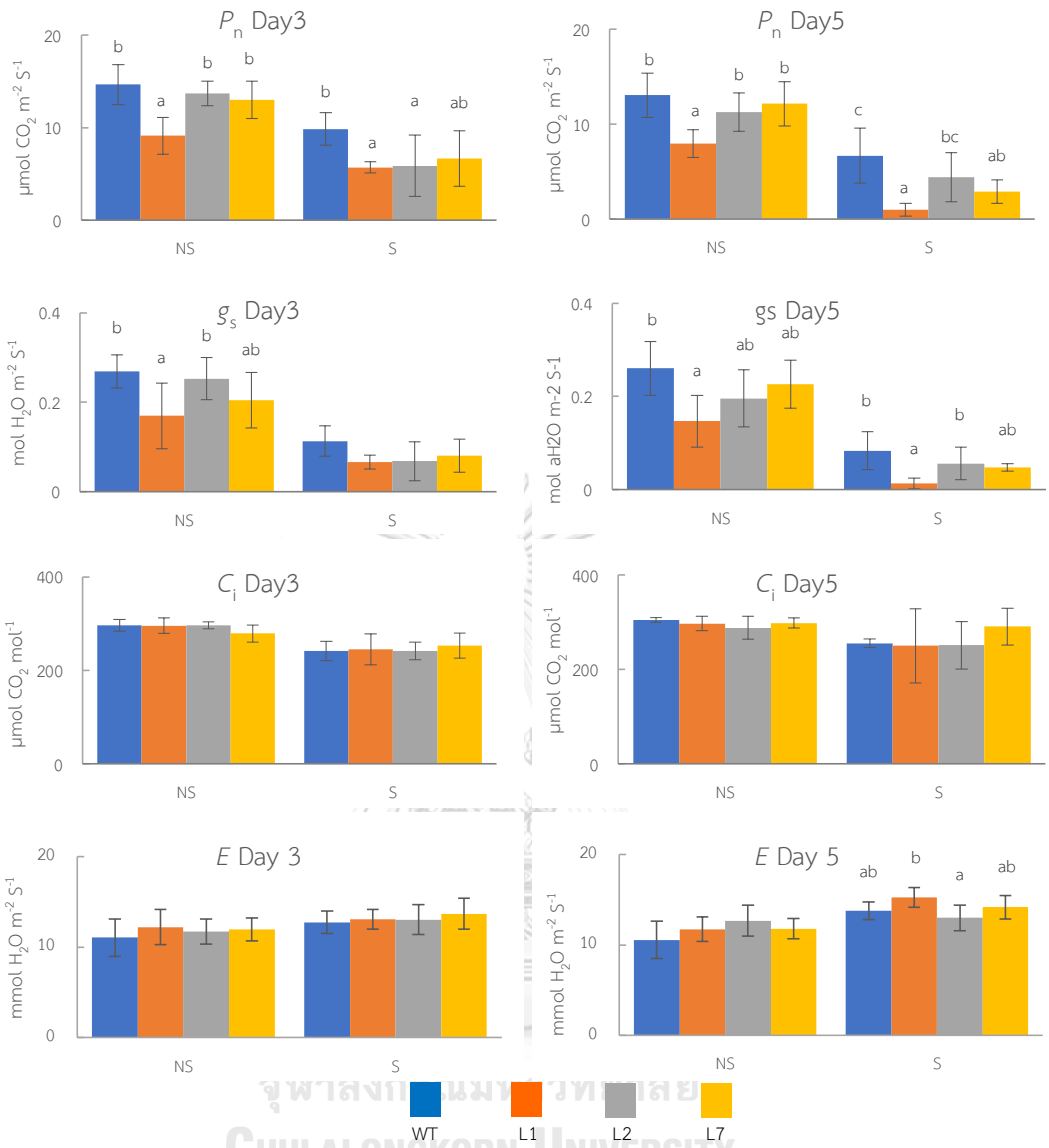


Figure 24 Gas exchange measurements in the leaves of three lines of transgenic rice over-expressing *OsCam1-1* (L1, L2, L7) comparing wild type (WT) under non-stress condition and 150 mM NaCl salt stress treatment from five independent biological replicates. The parameters consist of photosynthesis rate (P_n) at day 3 and day 5, stomatal conductance (g_s) at day 3 and day 5, intercellular carbon dioxide (C_i) at day 3 and day 5, and transpiration rate (E) at day 3 and day 5 of salt stress treatment.

Remark "NS" is referred to "Non-Stress" and "S" is referred to "Stress at 150 mM NaCl".

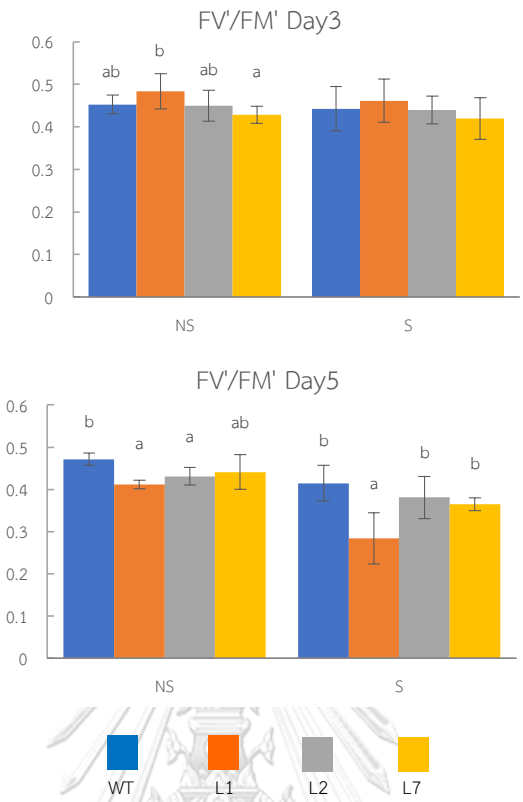


Figure 25 FV'/FM' in the leaves of three lines of transgenic rice over-expressing *OsCam1-1* (L1, L2, L7) comparing wild type (WT) under non-stress condition and 150 mM NaCl salt stress treatment from five independent biological replicates at day 3 and day 5 of salt stress treatment.

Remark "NS" is referred to "Non-Stress" and "S" is referred to "Stress at 150 mM NaCl".

3.2.4. Rice Over-Expressing *OsCam1-1* Exhibited Higher Sucrose and Starch Contents under Salt Stress

The previous report (Saeng-ngam et al., 2012) showed that *OsCam1-1*-over-expression lines showed a significantly higher relative growth rate than wild type when grown under salt stress. Based on the genes identified herein, among which several were involved in central energy pathways, sucrose and starch levels were determined in the three independent lines (L1, L2, L7) under normal and salt stress (150 mM NaCl) conditions at day 3 and 5 after treatment. Salt stress led to a significant reduction of the starch level and slightly decreased sucrose levels in both wild type and transgenic rice lines. Noticeably, at day 3, the transgenic lines could maintain the sucrose and starch levels better than the wild type under salt stress conditions. At day 5, the trends observed for sucrose and starch levels in transgenic rice under salt stress conditions were similar to those in wild type (Figure 25).

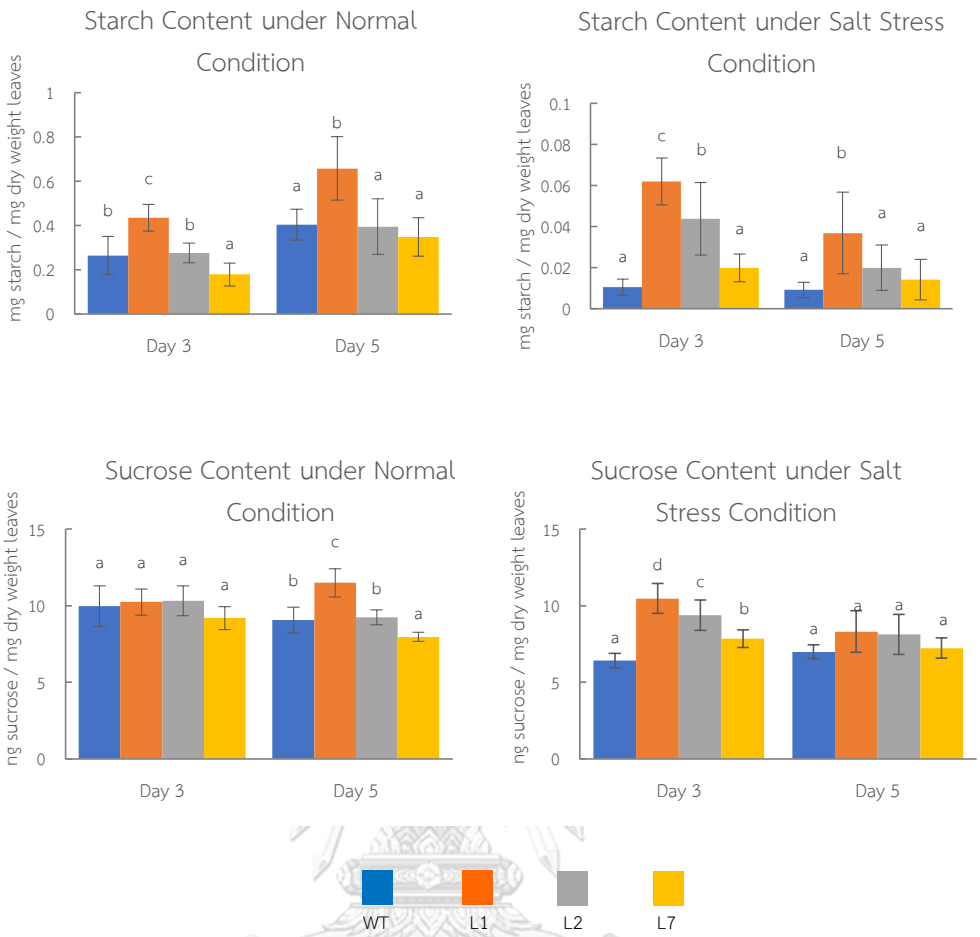


Figure 26 Starch and sucrose contents in the three lines of transgenic rice over-expressing *OsCam1-1* (L1, L2, L7) comparing wild type (WT) at days 3 and 5 exposed to 150 mM NaCl salt stress treatment from five independent biological replicates. The charts showing starch content under normal conditions, starch content under salt stress conditions, sucrose content under normal conditions, and sucrose content under salt stress conditions.

3.3. Functional Characterization of *OsICL* in Arabidopsis

To characterize the roles of *OsICL* in plant salt responsive mechanism, plant genetic engineering approach was applied employing *Arabidopsis thaliana*, the plant model. *OsICL*, which was as a salt responsive gene as evidenced by the previous gene expression study results and potentially regulated by CaM in the rice, was transferred back to the mutant *Aticl* Arabidopsis (revertant) and their biochemical or physiological characters were compared between wild type, mutant, revertant, and overexpression lines.

3.3.1. Plasmid Construction for Revertant *Aticl* Mutant Arabidopsis Expressing *OsICL*

According to topic 2.2.3.3 in the chapter of materials and methods, the cassette of *GUS-NOS*-upstream-*AtICL-OsICL* was successfully inserted into the LR cloning site of pK2GW7 vector. PCR amplification was carried out to confirm the existence of the cassette using 3 pairs of DNA primers that are specific to each fragment; *GUS-NOS* using 35S_Promoter_F (5'CTATCCTTCGCAAGACCCTTC3') and *GUS-NOS_Xba*I_R primiers, *AtICL* upstream sequence using *Xba*I_pATICL_F and pATICL_*Nde*I_R primers, and *OsICL* coding sequence using *Nde*I_*OsICL*_F and 35S_terminator_R primers (see primer sequences at the materials and methods chapter, topic 2.2.3.3). The size of the *GUS-NOS*-upstream-*AtICL-OsICL* cassette is totally 6,161 bp, which contains 2,304 bp of *GUS-NOS*, 2,138 bp of *AtICL* upstream sequence, and 1,719 bp of *OsICL* coding sequence. The PCR product size of each fragment analyzed by agarose gel electrophoresis was

as expected (Figure 26). The constructed *GUS-NOS*-upstream-*AtICL*-*OsICL*-pK2GW7 recombinant plasmid was desired to express *OsICL* under regulation of the *AtICL* upstream sequence to mimic native Arabidopsis expression patterns of *ICL* in Arabidopsis host with the 35SCaMV terminator provided by the vector. The *GUS-NOS* was placed in front of the *AtICL* upstream sequence to obstruct the 35SCaMV promoter provided by the vector. Therefore, the GUS would be constitutively expressed under 35SCaMV promoter with NOS terminator benefitting as transgenic reporting gene, which can be tracked by GUS staining. Furthermore, the full sequence of *GUS-NOS*-upstream-*AtICL-OsICL* cassette was determined (Appendix F1).

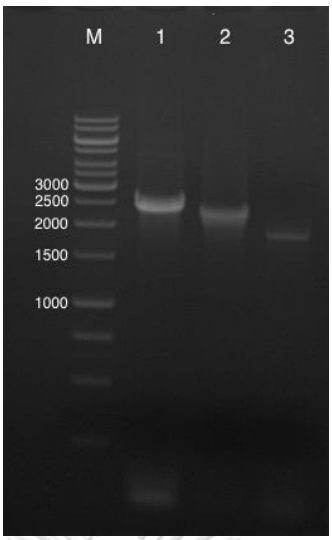


Figure 27 PCR amplification verifing the insertion of *GUS-NOS*, *AtICL* upstream sequence, and *OsICL* coding sequence in the recombinant plasmid *GUS-NOS*-upstream-*AtICL*-*OsCIL*-pK2GW7. The PCR products were analyzed by agarose gel electrophoresis using TAE buffer with 1% agarose gel under 100 mV for 30 min.

Remark

M : DNA marker (bp)

1 : PCR product of the cloned *GUS-NOS* with size of around 2,300 pb

2 : PCR product of the cloned *AtICL* upstream sequence with size of around 2,100 bp

3 : PCR product of the cloned *OsICL* coding sequence with size of around 1,700 bp

3.3.2. Plasmid Construction for Arabidopsis Over-Expressing *OsICL*

Following topic 2.2.3.4 in the chapter of materials and methods, *OsICL* coding sequence was successfully inserted into pK2GW7, therefore *OsICL* should be over-expressed under the control of 35SCaMV promoter with 35SCaMV terminator provided

by pK2GW7. The inserted *OsICL* fragment was confirmed by PCR using 35S_Promoter_F and *OsICL*_R primers. The result by agarose gel electrophoresis has shown that the PCR product was around 1,700 bp, which agreed with the expected size (Figure 27). Nucleotide sequence of *OsICL* in pK2GW7 was determined by DNA sequencing (Appendix F2).

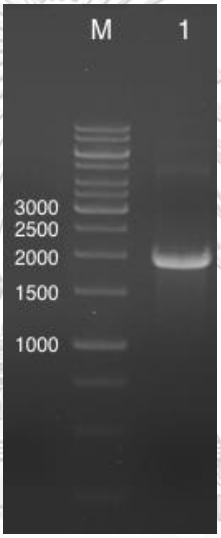


Figure 28 PCR amplification verifying the insertion of *OsICL* coding sequence in the recombinant plasmid *OsCIL*-pK2GW7. The PCR product was analyzed by agarose gel electrophoresis using TAE buffer with 1% agarose gel under 100 mV for 30 min.

Remark

M : DNA marker (bp)

1 : PCR product of the cloned *OsICL* coding sequence with size of around 1,700 bp

3.3.3. Transgenic Arabidopsis Expressing *OsICL* Genotyping

According to the steps 2.2.3.1-2.2.3.8 of the materials and methods chapter, the 4 homozygous transgenic Arabidopsis lines expressing *OsICL* were obtained as follows;

- 3FL4 representing *Aticl* Arabidopsis mutant expressing *OsICL* driven by the *AtICL* upstream sequence (line number 4 obtained from homozygous selection),
- 3FL9 representing *Aticl* Arabidopsis mutant expressing *OsICL* driven by the *AtICL* upstream sequence (line number 9 obtained from homozygous selection),
- OX*OsICL*/*icl* representing *Aticl* Arabidopsis mutant expressing *OsICL* driven by 35SCaMV promoter, and
- OX*OsICL*/WT representing wild type Arabidopsis expressing *OsICL* driven by 35SCaMV promoter.

The results of χ^2 test for homozygous selection were shown in Appendix E. The PCR confirming transgenic Arabidopsis expressing *OsICL* was performed using two sets of primers, specific for background genotype and for the inserted *OsICL*. The background genotyping of the mutant *Aticl* or wild type Arabidopsis was performed following step 2.2.3.2 and the agarose gel electrophoresis result has shown that PCR product size of wild type and OX*OsICL*/WT is around 900 bp, while the PCR product

size of mutant *Aticl,* 3FL4, 3FL9, and OX*OsICL/icl* is around 500 bp (Figure 28 A). For confirmation of the inserted *OsICL*, the process following step 2.2.3.7 was performed, and the agarose gel electrophoresis has shown that wild type and mutant *Aticl* had no PCR product while 3FL4, 3FL9, OX*OsICL/icl*, and OX*OsICL/*WT produced PCR product of around 460 bp (Figure 28 B).

A B

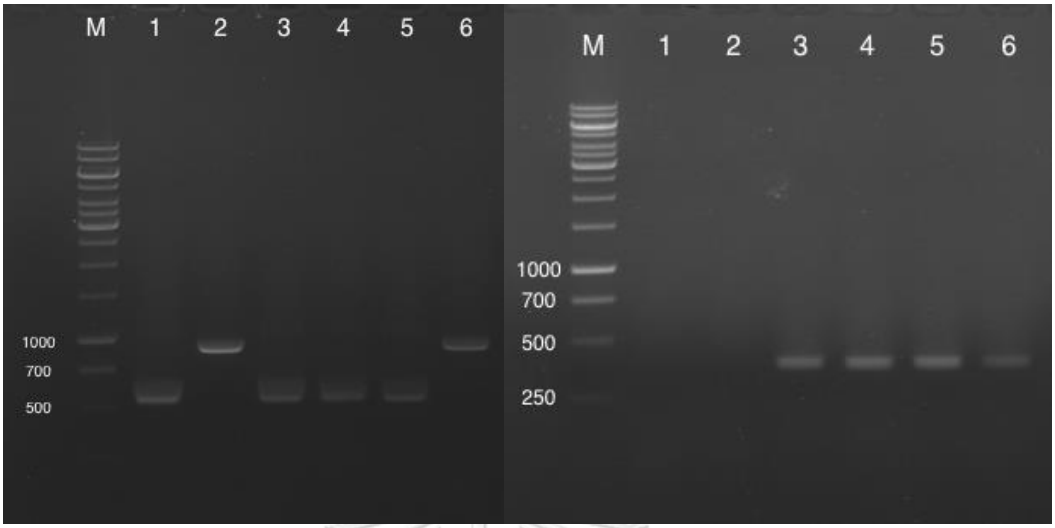


Figure 29 PCR genotyping of the transgenic Arabidopsis, (A) background genotyping, (B) inserted *OsICL* genotyping. PCR products were analyzed by agarose gel electrophoresis using TAE buffer with 1% agarose gel under 100 mV for 30 min

Remark

M : DNA Marker (bp)

1 : *Aticl* mutant Arabidopsis

2 : wild type Arabidopsis

3 : 3FL4

4 : 3FL9

5 : OX*OsICL*/*icl*

6 : OX*OsICL*/WT

3.3.4. Real-Time RT-PCR Determining Expression of *OsICL* and *AtICL* in Transgenic Arabidopsis Expressing *OsICL*

The real time RT-PCR of 5 biological replicates of 10-days old seedlings of the six Arabidopsis lines was performed following step 2.2.3.9. Because of the wild type

and *Aticl* Arabidopsis mutant did not express *OsICL*. Therefore, this experiment used the $2^{-(\Delta CT)}$ method, which use CT of target gene subtracting by CT of EF1α, for gene expression calculation. The results showed that *OsICL* expression was not detected in the wild type and mutant *Aticl* Arabidopsis either controlled non-stress or salt stress condition because they have no inserted *OsICL* gene. For the four lines of the transgenic Arabidopsis, the OX*OsICL*/WT line exhibited outstandingly high expression level in both control and salt stress conditions. Moreover, under salt stress the OX*OsICL*/WT had significantly higher *OsICL* expression than under controlled non-stress condition (Figure 29 A). For the other three transgenic Arabidopsis lines, 3FL4, 3FL9, and OX*OsICL/icl*, which the background was the *Aticl* mutant exhibited lower expression levels of *OsICL*. The OX*OsICL/icl* had higher *OsICL* expression level than the 3FL4 and 3FL9 lines because *OsICL* was driven by 35SCaMV promoter in OX*OsICL/icl* while the expression of *OsICL* in 3FL4 and 3FL9 were at similar levels. The trend of *OsICL* expression levels in 3FL4, 3FL9, and OX*OsICL/icl* was similar as under salt stress condition, the expression level was slightly lower under salt stress condition (Figure 29 B). In addition, expression of *AtICL*, native isocitrate lyase gene in Arabidopsis, was also exammined, and the result showed that expression of *AtICL* was detected in only wild type and OX*OsICL*/WT under salt stress (Figure 29 C).

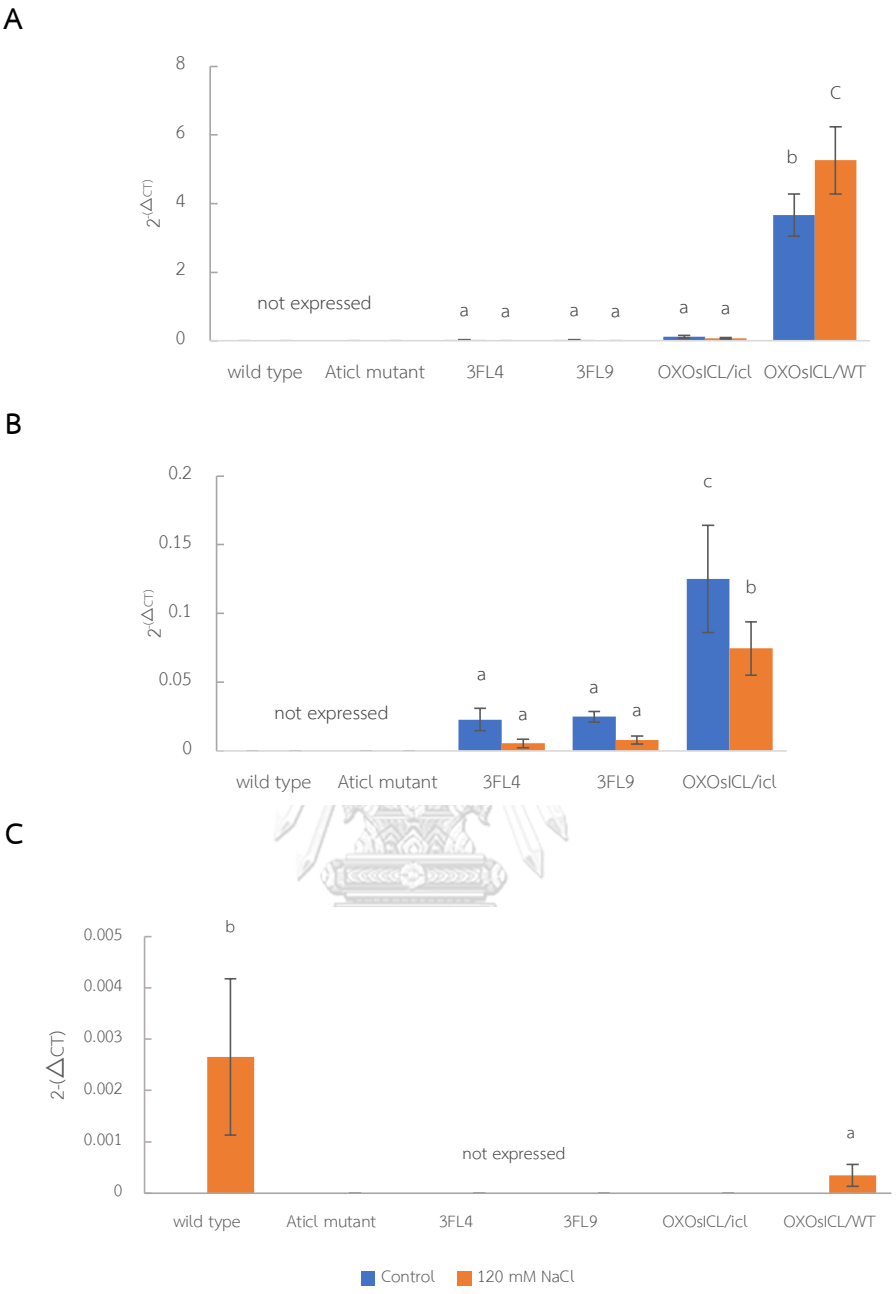
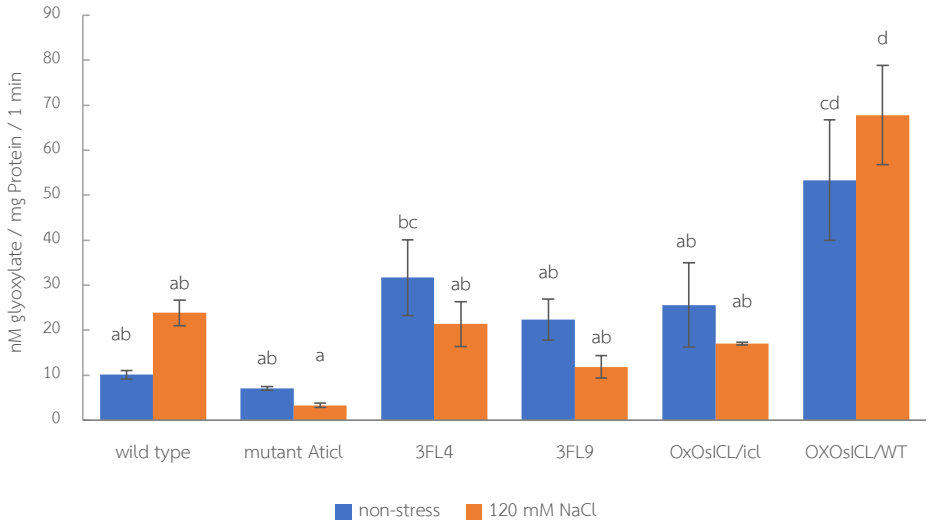


Figure 30 Real-time RT-PCR showing expression levels *OsICL* and *AtICL* in the six lines of Arabidopsis. One-way ANOVA with Duncan multiple range test ($p<0.05$) was used to analyze (A) *OsICL* expression of all Arabidopsis lines, (B) *OsICL* expression excluding OX*OsICL*/WT, and (C) *AtICL* expression of all Arabidopsis lines. The error bars represent SD and different letters indicate statistically significant difference.

3.3.5. Isocitrate Lyase Enzyme Activity of The Transgenic Arabidopsis Expressing *OsICL*

The isocitrate lyase activity was determined following step 2.2.3.10 in the three biological replication and the result showed that the OX*OsICL*/WT had highest isocitrate lyase activity and the salt stress had higher enzyme activity than the wild type without statistical significance. For the wild type, 3FL4, 3FL9, and OX*OsICL/icl* had similar level of the enzyme activity. Meanwhile, the mutant *Aticl*, which should not have the enzyme activity, showed the lowest level enzyme activity, because of the crude protein was applied to assay the enzyme activity therefore some interferences might be occurred. Interestingly, the Arabidopsis line which has wild type background, wild type and OX*OsICL*/WT, contributed higher level isocitrate lyase activity under salt stress than non-stress condition (Figure 30).

A



B

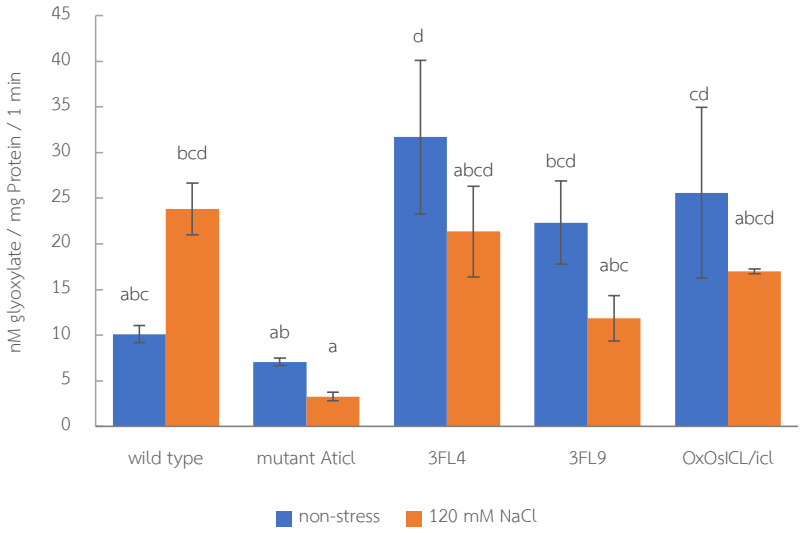
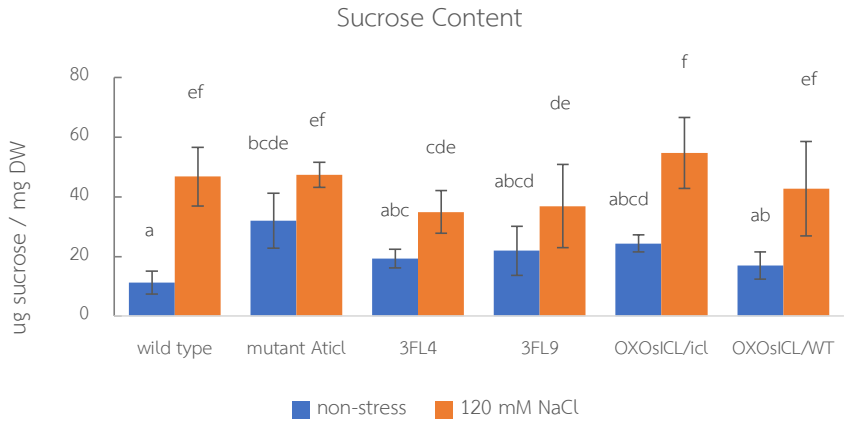


Figure 31 Isocitrate lyase activity of the six Arabidopsis lines. One-way ANOVA with Duncan multiple range test ($p<0.05$) using in the isocitrate lyase activity data analysis, (A) including OX*OsICL*/WT, (B) excluding OX*OsICL*/WT, the error bars represent SD and different letters indicate statistically significant difference.

3.3.6. Sucrose and Starch Content of The Transgenic Arabidopsis Expressing *OsICL*

Sucrose and starch contents were determined following step 2.2.3.11 and the results show that all Arabidopsis lines had similar sucrose contents. Under salt stress, the sucrose contents were higher than under normal condition. For starch content, no relation to either salt stress condition or existence of the isocitrate lyase gene was observed (Figure 31).

A



B

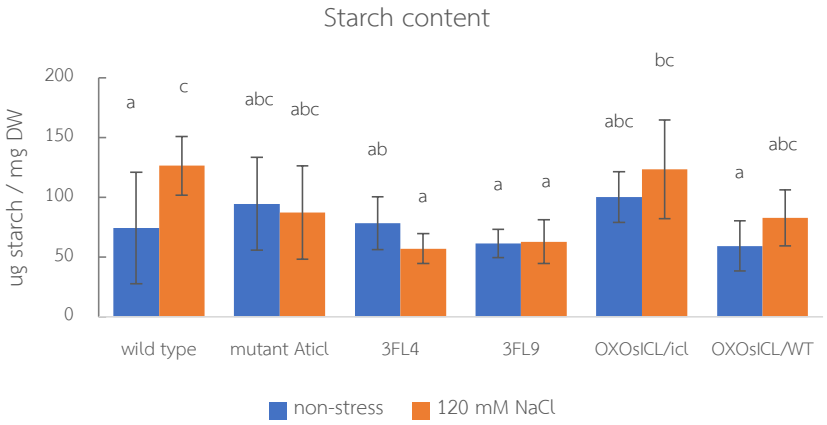


Figure 32 Sucorse and starch contents of the six Arabidopsis lines. One-way ANOVA with Duncan multiple range test ($p<0.05$) was used to analyze the data. The error bars represent SD and different letters indicate statistically significant difference.

3.3.7. Germination Rate of The Transgenic Arabidopsis Expressing *OsICL*

Germination rate of the transgenic Arabidopsis was determined following step 2.2.3.12 and shown in percentage. Under control condition, all Arabidopsis lines almost completely germinated after 1 day. At 120 mM NaCl the wild type, 3FL4, and 3FL9 reached around 95% germination at day 4, OX*OsICL*/WT and OX*OsICL/icl* reached around 95% germination at day 6, while the mutant line had around 92% germination at day 7. At 150 mM NaCl the wild type, 3FL4, and 3FL9 reached over 80% germination at day 4, OX*OsICL*/WT and OX*OsICL/icl* reached over 80% germinated at day 6, while the mutant line has around 83% germination at day 7. Furthermore at day 6 and day 7, the *Aticl* mutant has significantly lower germination rate than the other Arabidopsis lines. At 200 mM NaCl the wild type, 3FL4, and 3FL9 reached over 60% germination at day 5, OX*OsICL*/WT and OX*OsICL/icl* reached over 60% germinated at day 6, and the mutant line has around 62% germinated at day 7 while other lines reach over 70% germination on that day. Collectively, the *Aticl* Arabidopsis mutant line had lower germination rate than the other Arabidopsis lines under salt stress, which was more obvious at higher NaCl concentrations. Moreover, the two transgenic Arabidopsis expressing *OsICL* lines, which were desired to express *OsICL* under the control of 35SCaMV promoter, OX*OsICL*/WT and OX*OsICL/icl*, exhibited lower germination rate than the wild type, 3FL4, and 3FL9 (Figure 32).

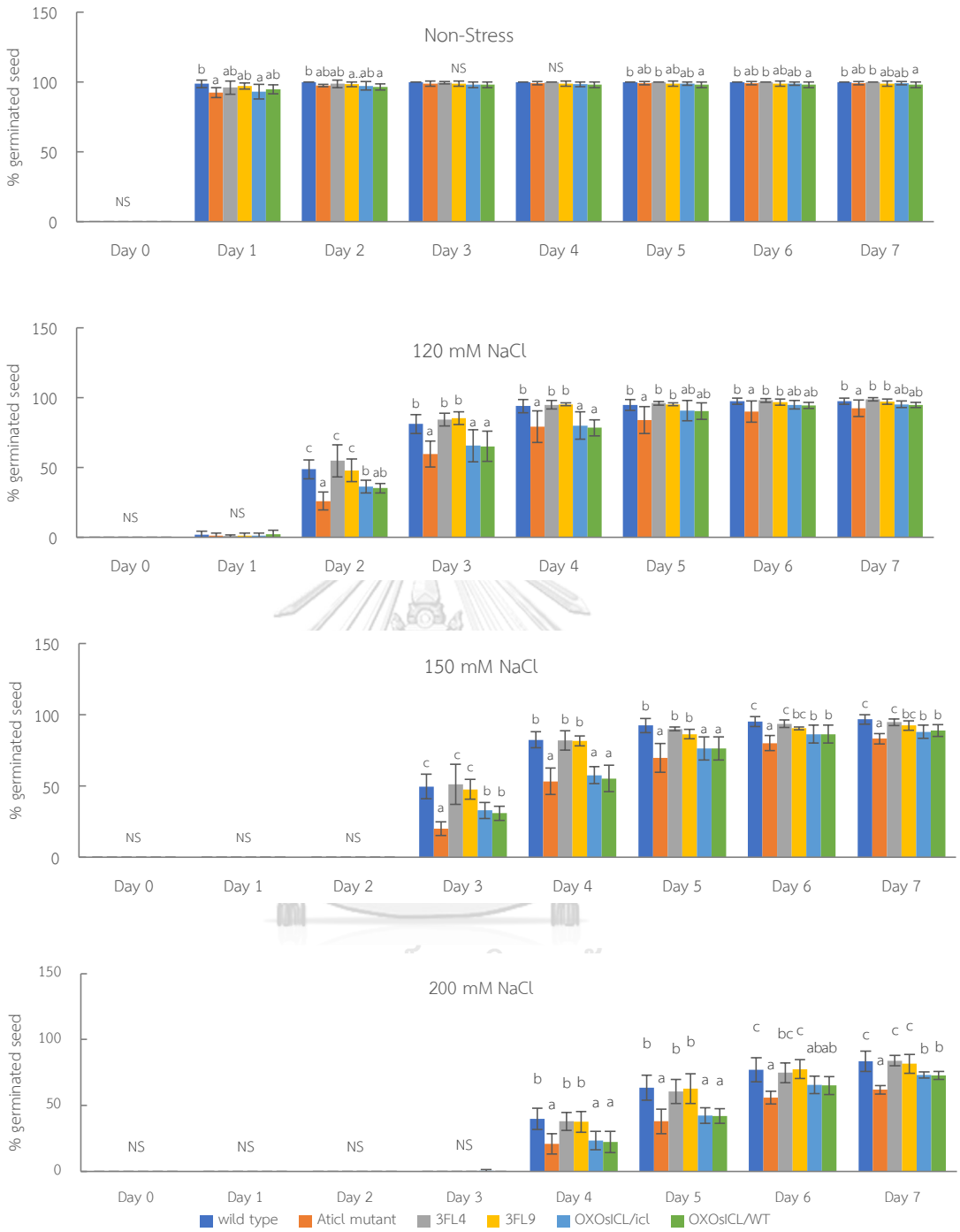
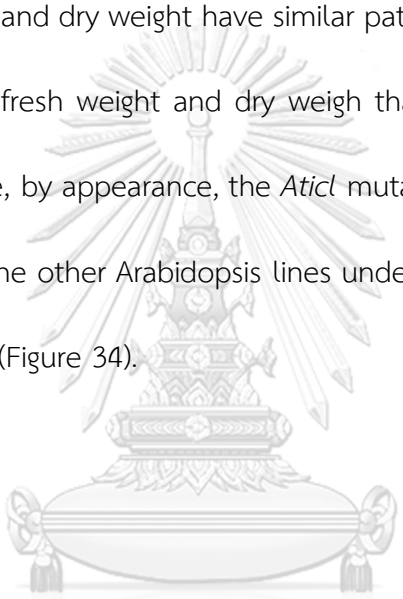


Figure 33 Germination rate of five biological replicates of the six Arabidopsis lines (N=50), One-way ANOVA with Duncan multiple range test ($p<0.05$) was used to analyze percentage of germination in each day separately. The error bars represent SD and different letters indicate statistically significant difference.

3.3.8. Growth of Transgenic Arabidopsis Expressing *OsICL*

Fresh weight and dry weight of the six Arabidopsis lines were determined following step 2.2.3.13. The result has shown that under non-stress condition the fresh weight and dry weight of the six Arabidopsis lines were not significantly different. Under salt-stress, fresh weight and dry weight have similar pattern, in which the *Aticl* mutant had significantly lower fresh weight and dry weigh than the other Arabidopsis lines (Figure 33). Furthermore, by appearance, the *Aticl* mutant also showed growth defect more frequently than the other Arabidopsis lines under salt stress, particularly under high salt concentration (Figure 34).



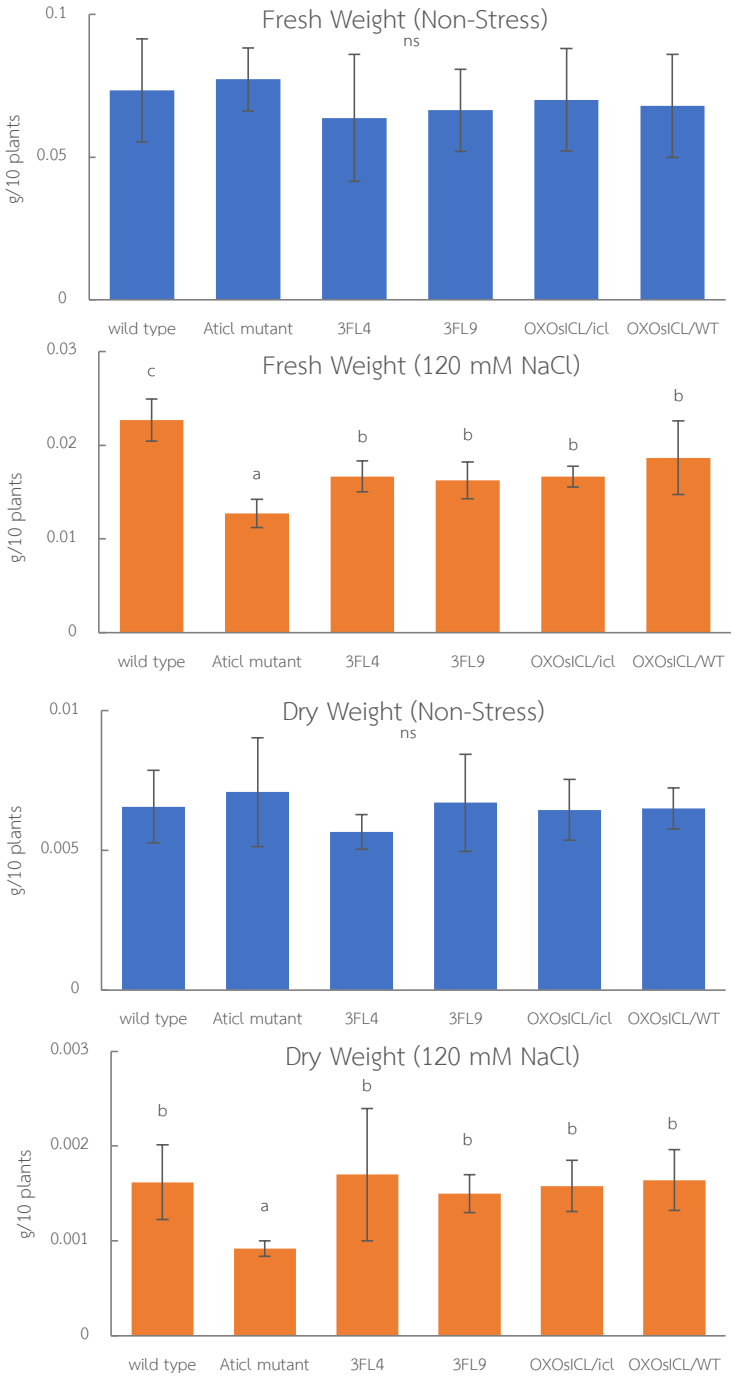


Figure 34 Fresh weight and dry weight of five biological replicates of the six Arabidopsis lines (N=10), One-way ANOVA with Duncan multiple range test ($p<0.05$) was used to analyze the fresh weight and dry weight data. The error bars represent SD and different letters indicate statistically significant difference.

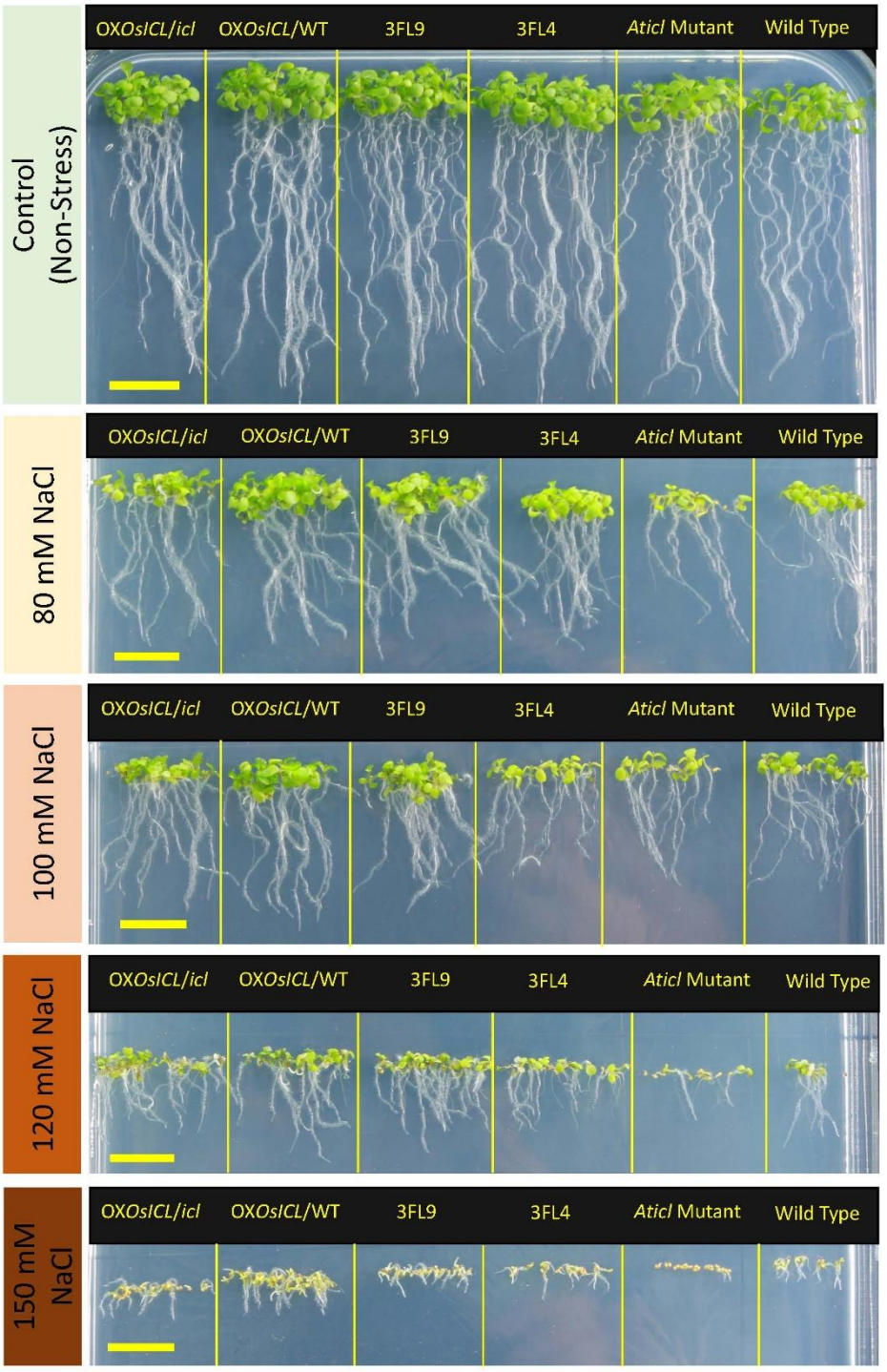


Figure 35 The six Arabidopsis lines growing in MS medium containing varied NaCl concentration at day 10 of growing. The scale bars represent 1 cm.

CHAPTER IV

DISCUSSIONS

4.1. Transcriptome of Transgenic Rice Over-Expressing *OsCam1-1* under Salt Stress

Based on the transcriptomics analysis, *OsCam1-1* over-expression affects genes in several cellular processes, potentially contributing to rice salt tolerance. As the expression level of *OsCam1-1* in transgenic rice was much higher than that in wild type, even under normal conditions, numerous DEGs exhibited altered expression levels in the transgenic rice (WTNSL1NS, green colored circles in Figure 15), suggesting that their functions likely confer advantages to plants in coping with future salt stress. Approximately 80% of these genes were salt-responsive, indicating a likely effect on the salt stress response of *OsCam1-1* over-expression. Under salt stress, an additional 1,434 salt-responsive genes exhibited different expression levels between the transgenic and wild type rice. Overall, approximately 18.4% of the salt-responsive genes obtained in this transcriptome data were differentially expressed between the transgenic and wild type rice (Table 6), which will be further discussed in detail. However, it should be noted that DEGs with expression levels that remained unchanged under salt stress, categorized as HT DEGs and LT DEGs, might also contribute to the salt tolerance of transgenic rice. However, the high-fold-change-DEGs were mainly identified as unknown proteins. Nonetheless, some known genes, which were annotated in the genome database, were found to be involved in salt stress or at least in abiotic stress responses, including ankyrin repeat containing protein (LOC_Os01g09384), hexose carrier protein (LOC_Os11g38160), ATPase/hydrogen-translocating pyrophosphatase (LOC_Os01g23580), UDP glucosyltransferase

(LOC_Os01g49240), C3HC4-type RING finger (LOC_Os10g32760), cleavage and polyadenylation specificity factor 100 kDa subunit (LOC_Os02g06940).

The functions of the GO-enriched DEGs, with expression levels that were impacted by *OsCam1-1* under salt stress, should be discussed. These proteins function in several major cellular processes, including signaling, hormones, transcription factors, secondary metabolism, stress responses, lipid metabolism, glycolysis, TCA cycle, glyoxylate cycle, photosynthesis, and carbohydrate metabolism.

4.1.1. Signaling

The mitogen-activated protein kinase (MAPK/MPK) cascade is a highly conserved central regulator of diverse cellular processes (Fujita et al., 2006). CaM plays role in the MAPK/MPK cascade by binding to mitogen-activated protein kinase (MPK) and/or mitogen-activated protein kinase phosphatase (MKP) (Katou et al., 2007; Takahashi, Mizoguchi, Yoshida, Ichimura, & Shinozaki, 2011). *BWMK1* (LOC_Os06g49430), a rice MAPK, which was categorized in the HT salt-responsive DEG category, could phosphorylate the OsEREBP1 transcription factor for binding to the GCC box element (AGCCGCC), which is a basic component of several pathogenesis-related gene promoters (Cheong et al., 2003). Our results also revealed 2 HT salt-responsive DEGs, LOC_Os02g26720 and LOC_Os10g01480, which encode inositol 1,3,4-trisphosphate 5/6-kinase (IPTK). IPTK phosphorylates inositol 1,3,4-trisphosphate to form inositol 1,3,4,5-tetrakisphosphate and inositol 1,3,4,6, tetrakisphosphate, which are ultimately converted to inositol hexaphosphate (IP6) and play roles in plant growth and development (Tang, Tan, & Xue, 2013). In rice, the T-DNA mutant of an IPTK gene showed reduced osmolyte accumulation and growth under drought conditions, and some genes involved in osmotic adjustment and reactive oxygen species scavenging

were down-regulated. In addition, over-expression of DSM3 (OsITPK2) resulted in a decrease in inositol trisphosphate (IP3), and the phenotypes were similar to the mutant under salt and drought stress conditions. These findings suggested that DSM3 might play a role in fine-tune balancing the inositol phosphate level when plants are exposed to stress or during development (H. Du et al., 2011). The diacylglycerol kinase gene (*DGK*) (LOC_Os04g54200) was identified as an HT salt-responsive DEG. *DGK* catalyzes the conversion of diacylglycerol (DAG) to phosphatidic acid (PA) (Arisz, Testerink, & Munnik, 2009), and PA plays a role in the stress signaling pathway, including the MAPK/MPK cascade (Canonne, Froidure-Nicolas, & Rivas, 2011). A report showed that the expression of *OsBIDK1* encoding rice DGK was induced by benzothiadiazole and fungal infection. Moreover, transgenic tobacco constitutively expressing *OsBIDK1* was more tolerant to plant pathogenic virus and fungi (Weidong, Jie, Huijuan, & Fengming, 2008). These findings suggest that several genes in the signaling process might be enhanced by *OsCam1-1* under salt stress.

4.1.2. Hormone-Mediated Regulation

Plant hormones play a crucial role in acclimation to abiotic stress and regulate the growth and development and often alter gene expression (Peleg & Blumwald, 2011; Sah, Reddy, & Li, 2016). Our study revealed that the expression of several genes involved with hormones were changed due to the impact of *OsCam1-1* over-expression under salt stress. Among the HT salt-responsive DEGs were three LOXs (lipoxygenase): LOC_Os08g39840 and LOC_Os12g37350, a homolog of *AtLOX2*, and LOC_Os03g49380, a homolog of *AtLOX5*, which encode the enzyme in the early step of the jasmonate (JA) biosynthesis pathway (Feussner & Wasternack, 2002). JA plays a role in the physiological response in plants under biotic and abiotic stress (Ahmad et

al., 2016; Kazan, 2015). An earlier report has shown that the absence of *AtLox2* expression results in no change under normal conditions, but JA accumulation induced by wounding is absent and the expression of vsp, a wound-JA-induced gene, is also suppressed (Bell, Creelman, & Mullet, 1995). In addition, three OPR (12-oxo-PDA-reductase) genes, LOC_Os06g11210, LOC_Os06g11290 and LOC_Os01g27230, encoding JA precursor-catalyzed enzyme that catalyzes the cis-12-oxophytodienoic acid (OPDA) reduction reaction (Sobajima et al., 2003), were identified as HT salt-responsive DEGs. These findings suggest that the JA content might be enhanced by the over-expression of *OsCam1-1* under salt stress by enhancing the production of enzymes in the JA biosynthesis pathway.

The expression of genes participating in ABA metabolism was altered by the influence of *OsCam1-1* over-expression under salt stress. Our results showed that the gene encoding 9-cis-epoxycarotenoid dioxygenase (*NCED*), LOC_Os07g05940, was identified as an HT salt-responsive DEG and up-regulated approximately 1.9 folds in transgenic compared with wild type rice under salt stress. Furthermore, the gene encoding abscisic aldehyde oxidase (AAO), LOC_Os07g18120, was up-regulated approximately 1.6 folds. *NCED* and *AAO* are two key enzymes in the ABA biosynthesis pathway (Eiji & Annie, 2005). ABA biosynthesis is activated by abiotic stress through Ca^{2+} signaling and the phosphorylation cascade (L. Xiong & Zhu, 2003). Our previous report has shown that the expression levels of *NCED* and *AAO*, and ABA content are enhanced in transgenic rice over-expressing *OsCam1-1* under salt stress in comparison to wild type (Saeng-ngam et al., 2012).

Collectively, the transcriptome indicates that *OsCam1-1* over-expression likely has effects across biotic and abiotic stresses via plant hormonal regulation through JA

and ABA. It has been suggested that biotic-abiotic stress crosstalk may occur via the MAPK/MPK cascade to regulate the plant hormone response to stress (CristinaRodriguez, Petersen, & Mundy, 2010).

4.1.3. Transcription

Transcription factors (TFs) play roles as master regulators controlling clusters of genes (Nakashima, Ito, & Yamaguchi-Shinozaki, 2009) in the plant regulation of the stress response (Singh, Foley, & Oñate-Sánchez, 2002). According to the transcriptome results, thirteen APETALA2/ethylene-responsive element binding protein (AP2/EREBP) genes were identified as HT salt-responsive DEGs, while five AP2/EREBP genes were identified as LT salt-responsive DEGs. AP2/EREBP is in a large gene family of TFs that function in plant growth, primary and secondary metabolism, and response to hormones and environmental stimuli (Guo, Huang, Duan, & Wang, 2017; Licausi, Ohme-Takagi, & Perata, 2013). LOC_Os09g35030 and LOC_Os09g35010 were identified as OsDREB1A and OsDREB1B, respectively (Nakano, Suzuki, Fujimura, & Shinshi, 2006), and a previous report has shown that OsDREB1A-over-expressing transgenic Arabidopsis exhibit induced expression of target stress-inducible genes of Arabidopsis DREB1A and increased tolerance to drought, high salt and freezing stress, as compared with wild type (Dubouzet et al., 2003). Our results revealed LOC_Os09g35030 as an HT salt-responsive DEG.

MYB is an important gene family of TFs, and several Arabidopsis MYB genes respond to hormone(s) or stress (Yanhui et al., 2006). This study showed that 8 MYB genes were allocated in the category of HT salt-responsive DEGs, and 1 MYB gene was identified as an HT DEG. A previous report has shown that over-expression of *OsMYB48-1* (LOC_Os01g74410), which is a member of those 8 DEGs, resulted in enhanced salt

and drought tolerance in rice. Furthermore, *OsMYB48-1* also controlled ABA biosynthesis by regulating the expression of *OsNCED4* and *OsNCED5* in response to drought stress (H. Xiong et al., 2014).

WRKY is a large TF family that responds to plant stress by regulating the plant hormone signal transduction pathway and is also involved in the biosynthesis of carbohydrate and secondary metabolites, senescence, and development (Jiang et al., 2017). The transcriptome data showed that the expression of 16 WRKY DEGs was HT salt-responsive. Among these WRKY genes, some were identified and characterized, including OsWRKY53 (LOC_Os05g27730), OsWRKY71 (LOC_Os02g08440), *OsWRKY13* (LOC_Os01g54600) and *OsWRKY62* (LOC_Os09g25070). According to several reports, these WRKY genes are involved in the biotic stress response. The evidence shows that *OsWRKY53* can bind to mitogen-activated protein kinases, *OsMPK3* and *OsMPK6*, and inhibit their activity, resulting in a reduction of JA, jasmonoyl-isoleucine and ethylene production and causing a suppression of herbivore defense ability (Hu et al., 2015). The expression of *OsWRKY71* was induced by salicylic acid (SA), JA, and 1-aminocyclo-propane-1-carboxylic acid (ACC). Over-expression of *OsWRKY71* affected the induction of *OsNPR1* and *OsPR1b* expression, which are defense signaling genes, resulting in an enhancement of bacterial plant pathogen resistance (X. Liu, Bai, Wang, & Chu, 2007). WRKY13 has been shown to regulate crosstalk between abiotic and biotic stress by suppressing the SNAC1 and WRKY45-1 genes, which are involved in drought and bacterial infection, by binding to W-like-type cis-elements on their gene promoters (Xiao et al., 2013). *OsWRKY62*, which was down-regulated by effect of *OsCam1-1* over-expression and salt stress, was found in two splicing forms, short and full-length forms. Over-expression of the full-length form of *OsWRKY62* resulted in the suppression of

blast fungus resistance. In contrast, the knockout *Oswrky62* line showed an enhanced defense-related gene expression level and accumulation of phytoalexins (J. Liu et al., 2016).

Based on the transcriptome profiles, *OsCam1-1* over-expression clearly affected the expression of transcription factors that are well-known to regulate both biotic and abiotic stress responses. Therefore, *OsCam1-1* likely functions through the activity of these transcription factors in mediating biotic-abiotic crosstalk regulation via diverse mechanisms, particularly the MAPK cascade. According to our transcriptomics data analysis, plant hormones might mediate the regulation of these TFs, leading to the downstream acclimated phenotypes in response to diverse stresses.

4.1.4. Secondary Metabolism

Secondary metabolites play important roles in acclimating the plant to the environment and stress conditions (Akula & Ravishankar, 2011). According to the transcriptome results, 35 DEGs involved in secondary metabolism were members of the HT salt-responsive DEGs. A hydroxyphenylpyruvate dioxygenase (HPPD) (LOC_Os02g07160), which participates in the first committed reaction in the vitamin E biosynthesis pathway (Ajjawi & Shintani, 2004), was highly expressed and enhanced by the effect of either *OsCam1-1* over-expression or salt stress. Previous evidence has shown that the expression of HPPD responded to oxidative stress in barley leaf because it was induced by senescence, methyl jasmonate, ethylene, hydrogen peroxide and herbicide; paraquat and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (Falk, Krauß, Dähnhardt, & Krupinska, 2002). Furthermore, five laccase genes were also categorized as HT salt-responsive DEGs, as LOC_Os01g63190, which was identified as LAC7, was highly expressed. A report on the expression of two rice laccase genes

(LOC_Os01g63180 and LOC_Os12g15680) in yeast cells suggested that the laccases played roles in atrazine and isoproturon herbicide detoxification (Huang, Lu, Zhang, Luo, & Yang, 2016). In Arabidopsis, *atlac1* and *atlac2* mutants exhibited deficient root elongation under polyethylene glycol (PEG) treatment, while the *atlac8* mutant showed early flowering and the atlac15 mutant showed abnormal seed color. In addition, the evidence revealed that the expression level of *AtLAC2* was enhanced by salt and PEG treatment (Cai et al., 2006).

4.1.5. Stress Response

The transcriptome results showed that the expression of 46 biotic and 19 abiotic stress DEGs was allocated in the HT salt-responsive DEG category. Interestingly, a cupin domain gene (LOC_Os08g08970), identified as an abiotic stress gene, was highly expressed and affected by both *OsCam1-1* over-expression and salt stress. Cupin-domain protein is a member of a protein superfamily that possesses diverse functions (Dunwell, Purvis, & Khuri, 2004). *AtPirin1*, an Arabidopsis cupin-domain protein, plays a role in early seedling development and seed germination regulation by binding to the α-subunit of G protein (Lapik & Kaufman, 2003). A universal stress protein (USP) (LOC_Os07g36600) was highly expressed and induced by *OsCam1-1* over-expression and salt stress. Evidence has shown that the expression of tomato USP (*spUSP*) is induced by drought, salt, oxidative stress and ABA, and over-expression of *spUSP* improves tomato drought tolerance via interactions with annexin, leading to the accumulation of ABA (Loukehaich et al., 2012). LOC_Os05g15770, which encodes xylanase inhibitor protein (*OsXIP2*), was highly expressed and induced by salt stress and *OsCam1-1* over-expression. A previous report has shown that *OsXIP* can be induced by methyl jasmonate and wounding, so it was suggested that *OsXIP* may play

a role in pathogen defense (Tokunaga & Esaka, 2007). As many *OsCam1-1* and/or salt stress affecting DEGs involve both biotic and abiotic stresses, *OsCam1-1* may be a component that mediates the crosstalk of biotic stress and abiotic stress responses.

4.1.6. Lipid Metabolism

The transcriptome results revealed that the HT salt-responsive DEG category included 30 DEGs in lipid metabolism. There were three 3-ketoacyl-CoA synthase genes, LOC_Os02g11070, LOC_Os05g49900 and LOC_Os02g56860, with enriched GO terms. Previous evidence has shown that 3-ketoacyl-CoA synthase plays a role in wax biosynthesis. The *kcs1–1* mutant exhibited reduced wax content, a thin seedling stem and low moisture sensitivity (Todd, Post-Beittenmiller, & Jaworski, 1999). Another report has shown that the expression of *KCS20* and *KCS2/DAISY*, two other Arabidopsis 3-ketoacyl-CoA synthase genes, is induced by salt, ABA and drought conditions and that these genes play roles in cuticular wax and suberin biosynthesis in root (S.-B. Lee et al., 2009). Additionally, four class III lipase DEGs, LOC_Os01g15000, LOC_Os11g43760, LOC_Os02g43700 and LOC_Os05g49840, and four DEGs encoding enzymes involving beta oxidation, LOC_Os09g39410, LOC_Os03g07140, LOC_Os08g44360 and LOC_Os05g07090, were also identified as GO-enriched HT salt-responsive DEGs. Previous evidence has shown that Arabidopsis genes encoding class III triacylglycerol lipase are involved in many processes. At4g16070, which is orthologous to LOC_Os11g43760, was predicted to be a gene involved in stress or the Ca^{2+} signaling pathway. At4g16820, which is orthologous to LOC_Os02g43700 and At4g18550 (an ortholog of LOC_Os05g49840), are involved in seed germination, senescence or the stress response. At1g02660, which is orthologous to LOC_Os01g15000, is involved in the plant defense response signaling pathway (Li et al., 2012). LOC_Os05g07090

encodes acyl-CoA dehydrogenase, and an early report showed that the enzyme functions in mitochondrial β-oxidation in maze root tip under glucose starvation conditions (Dieuaide, Couée, Pradet, & Raymond, 1993). Based on these results, the activity of *OsCam1-1* might affect lipid metabolism and possibly be linked to energy metabolism during salt stress.

4.1.7. Glycolysis, TCA and Glyoxylate Cycle

Glycolysis and TCA cycle are essential in the respiratory pathway to generate energy (Fernie, Carrari, & Sweetlove, 2004). The fructose bisphosphate aldolase (FBP) gene (LOC_Os09g02540) and phosphofructokinase (PFK) gene (LOC_Os05g10650) in the glycolysis pathway were identified herein as HT salt-responsive DEGs. Earlier comparative proteomic reports comparing salt-sensitive and salt-tolerant rice strains showed that the expression of FBP was induced by salt stress in a salt-sensitive rice cultivar (D.-G. Lee et al., 2011) and in either salt-sensitive or salt-tolerant strains of barley (Rasoulnia, Bihamta, Peyghambari, Alizadeh, & Rahnama, 2011), while the activity of PFK was increased under NaCl treatment along with that of pyruvate kinase and phosphoenolpyruvate carboxylase, resulting in an increase in respiratory O_2 uptake and drastic changes in the levels of glycolytic metabolites in Bruguiera sexangula cell cultures (Suzuki, Hashioka, Mimura, & Ashihara, 2005). The aconitase gene (LOC_Os08g09200), which encodes the enzyme that isomerizes citrate to isocitrate in the early step of the TCA cycle, was also in the HT salt-responsive DEG category. A previous study has shown that in addition to its other function as an RNA binding protein, aconitase mediates resistance to oxidative stress in plants (Moeder, del Pozo, Navarre, Martin, & Klessig, 2007). The isocitrate dehydrogenase (IDH) gene (LOC_Os05g49760), which encodes the enzyme that catalyzes the oxidative

decarboxylation of isocitrate in the TCA cycle, was also categorized as an HT salt-responsive DEG. Previous evidence has demonstrated a high homology of IDH genes among rice, Arabidopsis and poplar and that over-expression of maize IDH in Arabidopsis enhances salt tolerance in Arabidopsis (Y. Liu, Shi, Song, Wang, & Li, 2010). Two genes encoding key enzymes in the glyoxylate cycle shuttling the TCA cycle pathway, isocitrate lyase (ICL) (LOC_Os07g34520) and malate synthase (MLS) (LOC_Os04g40990), were expressed at higher levels in transgenic rice under salt stress. Early reports suggested that ICL and MLS play a role in converting lipids to sugar using an acetyl unit from β-oxidation to generate the substrate of gluconeogenesis, and this process is important during the post-germination stage in Arabidopsis (Cornah et al., 2004; P. J. Eastmond et al., 2000). Additionally, the DEGs included two glucose-6-phosphate transporter genes, LOC_Os07g34006 with higher expression levels in transgenic rice under salt stress and LOC_Os07g33954 with higher expression levels under normal conditions. A previous study has demonstrated that the transcript level of glucose-6-phosphate/phosphate translocator (GPT2) in Arabidopsis correlates with the sugar level in leaf (Kunz et al., 2010), and another study has suggested that GPT2 functions as a plastid anti-porter transporting glucose-6-phosphate into the plastid to support starch biosynthesis (Kammerer et al., 1998). Recently, a proteomic study has found that cucumber seed germination is enhanced by melatonin under high salt conditions via regulated energy metabolism and the up-regulation of proteins involved in glycolysis, the TCA cycle and the glyoxylate cycle (N. Zhang et al., 2017). The transcriptome results herein illustrated possible changes in the cellular respiratory pathway in transgenic rice under salt stress. These lines of evidence suggest that *OsCam1-1* may confer salt tolerance by regulating central energy metabolism.

4.1.8. Starch and Sucrose Metabolism

Salt stress inhibited the activity of granule-bound starch synthase (GSSB) and suppressed the expression of GSSBI and GSSBII, resulting in a decrease in starch content in rice leaf (Chen, Chen, & Wang, 2008). An earlier report has shown that Pokkali, the standard salt-tolerant rice, shows significantly higher starch concentration under salt stress than KDML 105, which was identified as a salt-sensitive cultivar (Pattanagul & Thitisaksakul, 2008). The transgenic KDML105 rice examined herein exhibited significant decrease in starch levels, but to a lesser extent than the wild type, while it showed improved maintenance of sucrose levels under salt stress, which probably reflected the higher salt tolerance ability. The transcriptome results revealed that several sucrose and starch degradation genes were up-regulated. Seven DEGs involved in sucrose and starch metabolism were allocated in the category of HT salt-responsive DEGs. Among these DEGs, sucrose synthase (LOC_Os03g22120) was up-regulated in both wild type and transgenic rice under salt stress, with greater up-regulation in transgenic rice. An early report revealed that sucrose synthase plays a role in starch synthesis by generating ADP-glucose or UDP-glucose through the cleavage of sucrose, which can be used for starch polymerization. Moreover, the findings showed that sucrose synthase activity correlated with starch and ADP-glucose accumulation in developing barley seed (Baroja-Fernández et al., 2003) and that transgenic potato plants with a disrupted sucrose synthase gene were defective in starch accumulation (Zrenner, Salanoubat, Willmitzer, & Sonnewald, 1995). However, the transcriptome results herein showed that lower expression levels of several genes in the starch biosynthetic pathways in transgenic compared with wild type rice. These findings suggested that starch metabolism in higher plant might be regulated by several

mechanisms: post-translational modifications such as redox modulation and protein phosphorylation, or allosteric modulation by metabolites, which is related to the metabolic flux (Tetlow, Morell, & Emes, 2004).

Three invertase genes, encoding a sucrose-digesting enzyme, were identified as HT salt-responsive DEGs. A double mutant of two isoforms of Arabidopsis neutral invertase genes, *inv1/inv2*, has been shown to exhibit severe growth defects, and therefore the authors suggested that cytosolic invertase may play role in supplying sucrose to Arabidopsis non-photosynthetic cells (Barratt et al., 2009). The transcriptome data also revealed 7 cell wall degradation DEGs, which were expressed at higher levels in transgenic rice. Moreover, two of those DEGs encoding polygalacturonase (LOC_Os10g26940 and LOC_Os09g31270) showed very high expression levels in transgenic rice.

Together with the altered expression levels of these genes, our results for increased starch and sucrose levels in transgenic rice suggest that starch and sucrose metabolism are likely downstream components that are regulated by *OsCam1-1* under salt stress. The down-regulation of photosynthetic genes due to the impact of *OsCam1-1* and up-regulation of genes involved in lipid metabolism suggests that transgenic rice may balance carbon and energy metabolism under salt stress by obtaining monosaccharide units through the mobilization of lipids, which might be converted to sugar via the glyoxylate cycle and gluconeogenesis, as in previous discussions and/or the cell wall, which is a large carbon reservoir in the cell.

4.2. *OsICL* Expression and Physiological Study of Transgenic Rice Over-Expressing *OsCam1-1*

According to the transcriptome study, *OsICL* (LOC_Os07g34520) encoding isocitrate lyase gene was identified as a salt responsive gene and was enhanced by *OsCam1-1.* Therefore, to confirm the induction of *OsICL* expression by salt stress and *OsCam1-1* overexpression, the expression levels of *OsICL* were investigated in the three lines of transgenic rice over-expressing *OsCam1-1*. The higher *OsICL* expression levels found in the three transgenic rice lines than the wild type provides a strong evidence that *OsCam1-1* directly or indirectly regulates *OsICL* expression under salt stress. Furthermore, gene expression of *OsMS* encoding malate synthase was parallelly studied and the gene expression had similar pattern to the *OsICL*. *OsMS* also responded to salt stress and was enhanced by *OsCam1-1* over-expression (Figure 21). It has been well known that isocitrate lyase and malate synthase are the key enzymes in glyoxylate cycle. Isocitrate lyase reversibly cleaves isocitrate to succinate and glyoxylate then malate synthase uses glyoxylate and acetyl-CoA as substrates to produce malate (Carpenter & Beevers, 1959). Therefore, the glyoxylate cycle is a shuttle pathway of TCA cycle. Some evidences have shown the glyoxylate cycle plays role in sugar synthesis in plant by converting acetyl unit to sugar via gluconeogenesis (Cooper & Beevers, 1969; Cornah et al., 2004; P. J. Eastmond et al., 2000). Moreover, the evidences show that isocitrate lyase and malate synthase involved in carbohydrate synthesis supporting our results of sucrose and starch content that the transgenic rice, which had higher *OsICL* expression exhibited higher sucrose and starch levels than the wild type under salt stress (Figure 25).

Moreover, the result of photosynthesis rate and stomatal conductance, which have well known as the plant process for carbohydrate production using CO_2 as substrate. The transgenic rice had lower photosynthetic rate than the wild type under salt stress (Figure 23) while their sucrose and starch contents were higher. The contradiction of photosynthetic rate and carbohydrate content reveal that the higher contents of sucrose and starch in the transgenic rice did not come from higher performance of photosynthesis, thus one possibility may be that the higher sucrose and starch contents in the transgenic rice are resulted from the effects of *OsICL* and *OsMS* under regulation of OsCaM1.

Following the principle of transportation in plant, plants absorb water and ions from root through the xylem to the shoot such as leaf and the force lifting the water from the root to the shoot occurs by water evaporation at the leaf through stoma (Molz, 1981). Therefore, reduction of photosynthetic rate affected by the over-expression of *OsCam1-1* may result from the decrease of water evaporation at the leaf, which might be beneficial in salt tolerance by maintaining water in the plant and reducing salt absorption by the root.

OsICL responded to senescence as the expression level of *OsICL* was much higher during senescence when compared to that of young leaf (Figure 22). The senesced leaf exhibited high induction of *OsICL* expression by salt stress resulting very high level of *OsICL* expression in the senesced leaf. However, only young leaf that the expression of *OsICL* responded to the effect of *OsCam1-1* over-expression was observed. Upon salt stress, only the transgenic rice overexpressing *OsCam1-1* exhibited induction of *OsICL* expression in young leaf.

Several reports have shown evidences of isocitrate lyase function involving plant senescence, activity of isocitrate lyase and malate synthase was induced by senescence and darkness in barley leaf and some lipid was lost, therefore it was suggested that the lipid might be used in gluconeogenesis via the glyoxylate cycle (Gut & Matile, 1988). Another report has shown that activity of isocitrate lyase, malate synthase and β-oxidation was induce by dark senescence in rice and wheat leaf (Pistelli, De Bellis, & Alpi, 1991). Previous report found that H_2O_2 played role as a shared signal molecule in both salt-induced and developmental leaf senescence, however regulatory systems that control the interplay between developmental program and environmental stress are still not clear (Allu, Soja, Wu, Szymanski, & Balazadeh, 2014).

The early report revealed that the transgenic rice over-expressing *OsCam1-1* exhibited induction of ABA content under salt stress and salt tolerance (Saeng-ngam et al., 2012). According to 2,020 *OsICL* upstream sequence revealed that there are 2 ABRE, ABA responsive element on the position of -104 and -1038 upstream 5′UTR of *OsICL*, and 9 OsWRKY71 binding sites on the upstream 5′UTR of *OsICL* and 1 OsWRKY71 binding site on the 5′UTR of *OsICL*. Moreover, our transcriptome study revealed *OsWRKY71* (LOC_Os02g08440) was induced by effect of salt stress and enhanced by *OsCam1-1* over-expression (Figure 19). A report showed that the *OsWRKY71* was induced by ABA in rice aleurone cells (Xie et al., 2005). These evidences indicated that *OsICL* was possibly induced by *OsCam1-1* via ABA by ABA induced *OsWRKY71*, then *OsICL* was induce under *OsWRKY71* regulation (Figure 36). Due to isocitrate lyase located on the conjunction of various metabolism, therefore the specific metabolite

affecting isocitrate lyase which play role in salt stress tolerance should be further identified.

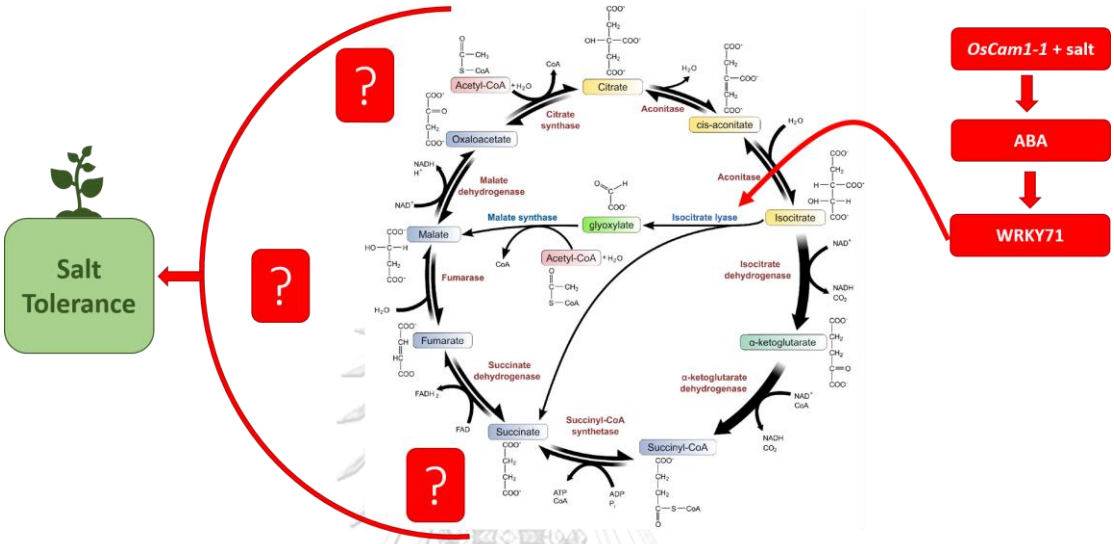


Figure 36 The possible salt-responsive regulatory mechanism of *OsICL* involving *OsCam1-1*

4.3. Characterization of *OsICL* as Salt Tolerance Gene in Arabidopsis

Isocitrate lyase gene is a single copy gene in both rice and Arabidopsis genomes (Lu et al., 2005). The shared identity of DNA coding sequence and amino acid sequence between rice and Arabidopsis is 66.07% and 72.50%, respectively. To contribute stronger evidence that the *OsICL* plays role in plant salt stress response, gene complementation approach was performed. The revertant Arabidopsis expressing *OsICL*; 3FL4 and 3FL9, which is the *Aticl* mutant expressing *OsICL* driven by the *AtICL* promoter (2,138 bp upstream *AtICL*) to mimic the expression level of *OsICL* to the same level as that of the native *AtICL*, was constructed. On another hand, the

overexpression construct of *OsICL* driven by the 35SCaMV constitutive promoter was used to transform both *Aticl* mutant (resulting in Ox*OsICL/icl*), and wild type (resulting in Ox*OsICL*/WT). The 4 homozygous transgenic Arabidopsis lines were successfully constructed and the existence of *OsICL* in the genome was confirmed by PCR genotyping, gene expression, and enzymatic activity assay. Following real time RT-PCR results of *AtICL*, only Arabidopsis with wild type background (wild type and Ox*OsICL*/WT) under salt stress condition had expression of *AtICL* while Arabidopsis with *Aticl* mutant background had no expression of *AtICL* as expected. For *OsICL* expression analysis, the wild type and *AtICL* mutant had no expression while the 3FL4 and 3 FL9 had lower expression level of *OsICL* than the other two lines (Ox*OsICL/icl* and Ox*OsICL*/WT) as expected because of the presence of different promoters. The Ox*OsICL/icl* had higher expression level of *OsICL* than the 3FL4 and 3FL9 because of the strong constitutive 35SCaMV promoter. Interestingly, the over-expression line Ox*OsICL*/WT had outstandingly high *OsICL* expression level. For the isocitrate lyase activity, the results show that the activity of isocitrate lyase more or less agreed with gene expression data at the transcript level. The Ox*OsICL/icl* had similar isocitrate lyase activity level to 3FL4 and 3FL9 while the *Aticl* mutant had lowest activity with not significant to 3FL4, 3FL9 and Ox*OsICL/icl* under salt stress, and wild type under non-stress. Unfortunately, 10-day old Arabidopsis seedlings of all 6 lines showed no difference in the sucrose and starch content. Therefore, the suggestion that isocitrate lyase is related to gluconeogenesis and sugar synthesis (Cornah et al., 2004; P. J. Eastmond et al., 2000; Peter J. Eastmond & Graham, 2001) was not supported by the sucrose and starch result. It might because of the 10-days-old seedling Arabidopsis was not at an appropriate stage to observe the difference in content of these

carbohydrates. Another possible reason is that sucrose in the MS medium was abundant, so the sucrose might be over-supplied to the plant resulting in the effect of isocitrate lyase on the carbohydrate content being obscured.

For the growth parameters, germination rate, fresh weight, and dry weight were affected by isocitrate lyase activity. The germination rate of *AtICL* mutant, which had lowest activity of isocitrate lyase was more sensitive to salt stress than wild type, revertant line (3FL4, 3FL9, and Ox*OsICL/icl*), and *OsICL* overexpression line (Ox*OsICL*/WT), particularly under 150 and 200 mM NaCl. Moreover, the fresh weight and dry weight of 10-days-old Arabidopsis has shown that the mutant had significantly lower growth under salt stress than the wild type, revertant, and *OsICL* overexpression line.

Following the result of gene expression level of *OsICL* in senesced rice leaf, it was found that senescence was affected to the expression of *OsICL*. Some report revealed the isocitrate gene was responded to senescence, Gut and Matile (1988) found that isocitrate lyase was induced in senesced barley leaves, whereas McLaughlin and Smith (1994) suggested that isocitrate lyase, which was induced by acetate under dark condition, may affected by acetate enhancing senescence-like process. Interestingly, a report showed that Arabidopsis calmodulin1 induced ABA response and senescence (Dai, Lee, Lee, Nam, & Kwak, 2018). The evidences suggested that there are salt-stress and senescence crosstalk in plant response.

Because of isocitrate lyase is located near the junction of several metabolic pathways, therefore the growth of Arabidopsis under salt stress might be affected by isocitrate lyase via other metabolisms. A report has shown that glyoxylate, which is a product of photorespiratory pathway and also the isocitrate lyase product, was toxic

to Rubisco activities and Calvin–Benson cycle and it is detoxified by converting to glycine and serine (Dellero, Jossier, Schmitz, Maurino, & Hodges, 2016). Therefore, further information is needed to indicate the role of plant isocitrate lyase in the salt responsive mechanism.

CHAPTER V

CONCLUSIONS

1. Comparative transcriptome profiling identified 1,434 salt responsive *OsCam1-1* affected DEGs involving in several cellular processes including signaling and stress response, hormone-mediated regulation, transcription, secondary metabolism, lipid metabolism, glycolysis, TCA and glyoxylate cycle, carbohydrate metabolism, and photosynthesis.

2. *OsICL* and *OsMS* expression responded to salt stress and *OsCam1-1* overexpression and *OsICL* expression was induced by senescence while in young leaf, only the transgenic rice overexpressing *OsCam1-1* exhibited induction by salt stress.

3. The overexpression of *OsCam1-1* in transgenic rice resulted in reduction in photosynthesis and increase in starch and sucrose content in seedlings grown under salt stress.

4. *OsICL* possibly affected by salt-stress and OsCam1-1 via ABA through regulation of OsWRKY71 transcription factor, the ABA responsive gene.

5. *OsICL* was confirmed as a salt tolerance gene in transgenic Arabidopsis as its expression in wild type and *AtICL* mutant conferred the transgenic Arabidopsis higher germination rate and growth under salt stress.

Suggestion for Further Study

The function in salt stress tolerance mechanism of *OsICL* should be further investigated in *Osicl* mutant, *OsICL* revertant, and *OsICL* overexpression rice line via mutant *Osicl* and/or *OsICL* over-expression rice line. Furthermore, to prove the hypothesis that *OsCam1-1* may contribute rice salt tolerance via enhance expression of *OsICL*, calmodulin gene should be expressed in *icl* mutant Arabidopsis and wild type Arabidopsis and the parameter involving salt tolerance should be investigated. Moreover, the role of OsWRKY71 in *OsICL* regulation should be confirmed.

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APPENDICES

Appendix A

Solution Formulas

A1. Yoshida's Solution (Stock Solution)

Nutritional Element	Reagent (AR grade)	Preparation (g/10 liters of distilled water)	
N	NH_4NO_3	914	
P	$NaH_2PO_4 \cdot 2H_2O$*	403	
K	K_2SO_4*	714	
Ca	$CaCl_2$*	886	
Mg	$MgSO_4 \cdot 7H_2O$*	3240	
Mn	$MnCl_2 \cdot 4H_2O$	15.0	separately
Mo	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.74	dissolve and mix
B	H_3BO_3	9.34	with 500 ml
Zn	$ZnSO_4 \cdot 7H_2O$	0.35	concentrated
Cu	$CuSO_4 \cdot 5H_2O$	0.31	H_2SO_4 then make
Fe	$FeCl_3 \cdot 6H_2O$*	77.0	the volume up to
	Citric acid(monohydrate)	119	10 liters with DI water

use 5 ml of each stock solution to prepare 4 liters Yoshida's solution

*Remark; in case of lacking some chemical, it can be use substituted chemical as following

$Na_2HPO_4 \cdot H_2O$	356/10 l	instead for	$NaH_2PO_4 \cdot 2H_2O$
$CaCl_2 \cdot 2H_2O$	1174/10 l	instead for	$CaCl_2$
$MgSO_4 \cdot H_2O$	1820/10 l	instead for	$MgSO_4 \cdot 7H_2O$
KNO_3	615/10 l	instead for	K_2SO_4
$FeSO_4 \cdot 4H_2O$	74.8/10 l	instead for	$FeCl_3 \cdot 6H_2O$
$FeSO_4 \cdot 7H_2O$	91.8/10 l	instead for	$FeCl_3 \cdot 6H_2O$

A2. 50X tris acetate EDTA buffer (TAE)

Tris base		242	g
Glacial acetic acid		57.1	ml
$Na_2EDTA{\cdot}2H_2O$		37.2	g
Distill water	up to	1	l

pH is should be around 8.5

A3. 10X tris boric EDTA buffer (TBE)

Tris base		108	g
Boric acid		55	g
500 mM EDTA, pH 8.0		40	ml
Distill water	up to	1	l

pH is should be around 8.3

A4. Plant lysis buffer for crude plant genomic extraction (for Arabidopsis genotyping)

1 M Tris-HCl buffer pH 7.5		200	ml
500 mM EDTA		50	ml
5 M NaCl		50	ml
1% W/V SDS		50	ml
Distill Water	up to	1	l

Sterile by autoclave at 121 ℃ for 15 min before use

A5. Transformation buffer for Arabidopsis floral dipping

Murashige & Skoog medium	0.22	g
Sucrose	5	g
Silwet	20	µl
Distill Water	100	ml

A6. DNAse reaction muxture eliminating contaminated DNA form RNA

Total RNA		10	µg
10x DNAse buffer		3	µl
RNAse inhibitor (Ribolock)		0.5	µl
DNAse I RNAse free		1	µl
DEPC treated water	up to	30	µl

A7. Reverse transcription reaction mixture

5x iScript reverse transcription supermix		4	µl
DNAse treated RNA		1	µg
Nuclease-free water	up to	20	µl

A8. qRT-PCR reaction mixture

2X SsoFast™ EvaGreen® Supermixes	5	µl
1,000 nM Forward primer	0.5	µl
1,000 nM Reverse primer	0.5	µl
cDNA template	1	µl
Sterile distill water	3	µl

A9. Extraction buffer for plant isocitrate lyase acitvity assay

1M Potassium Phosphate Buffer pH 7.6		5	ml
1M $MgCl_2$		500	µl
500mM EDTA		100	µl
1M DTT		50	µl
Sterile distill water	up to	50	ml

A10. Reaction buffer for plant isocitrate lyase assay

1M Potassium Phosphate Buffer pH 6.9		2.5	ml
1M $MgCl_2$		2.5	ml
500mM EDTA		1	ml
Phenylhydrazine		200	µl
500mM DL-Isocitric acid		1	ml
Sterile Distill Water	up to	50	ml

Appendix B

qRT-PCR Condition and Efficiency

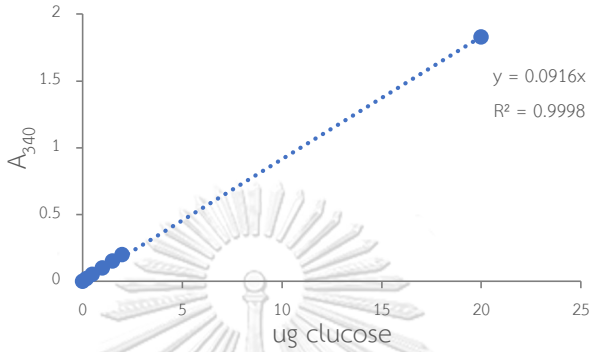
B1. Efficiency and PCR Condition of qRT-PCR

Gene	Locus Number	Efficiency	R^2	Tm	PCR Product Size (bp)
Rice					
OsEF1-α	LOC_Os03g08010	94.9	0.977	55.0	218
aconitase	LOC_Os08g09200	98.6	0.977	59.6	219
isocitrate lyase	LOC_Os07g34520	90.3	0.990	59.6	150
malate synthase	LOC_Os04g40990	94.1	0.982	59.6	183
AP2	LOC_Os03g08470	93.6	0.990	59.6	186
ERD1	LOC_Os02g32520	96.8	0.990	59.6	188
glycosyl hydrolase	LOC_Os04g45290	93.2	0.966	59.6	157
β-amylase	LOC_Os03g22790	93.7	0.971	59.6	154
isocitrate dehydrogenase	LOC_Os05g49760	92.0	0.975	56.9	190
pyruvate decarboxylase	LOC_Os03g18220	98.8	0.988	59.6	168
Arabidopsis					
AtEF1-α	AT1G07930	105.6	0.955	56.6	109
Isocitrate lyase (*AtICL*)	AT3G21720	100.7	0.955	61.5	83
Isocitrate lyase (*OsICL*) (inserted OsICL CDS)	LOC_Os07g34520	107.5	0.976	56.6	212

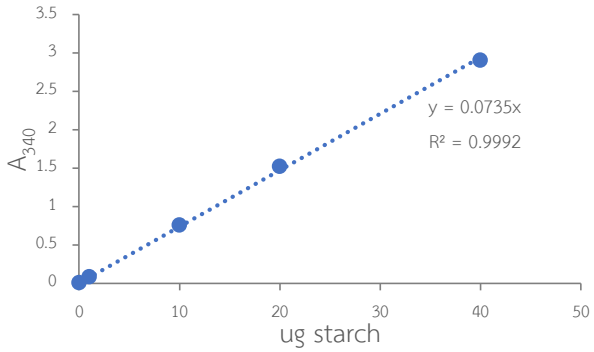
Appendix C

Standard Curves

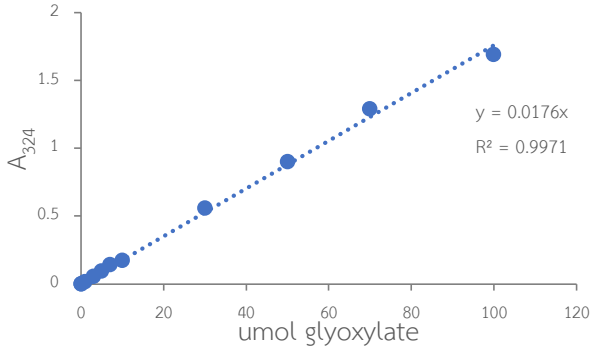
C1. Standard curve of glucose content for determining the rice sucrose content



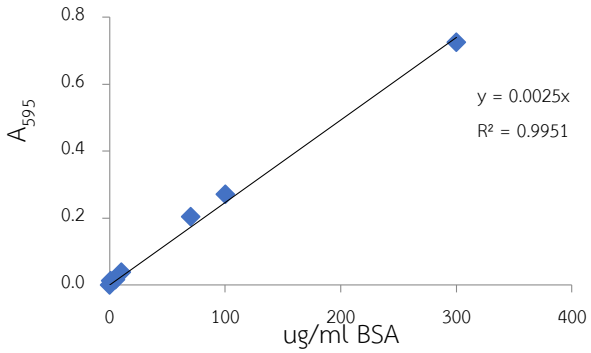
C2. Standard curve of starch content for determining the rice starch content



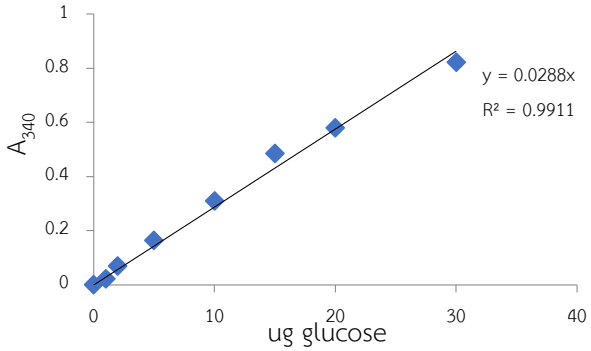
C3. Standard curve of glyoxylate content for isocitrate lyase activity assay



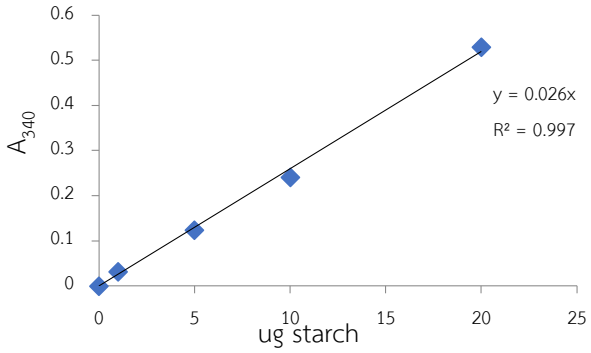
C4. Standard curve of Bradford assay (Microplate)



C5. Standard curve of glucose content for determining the Arabidopsis sucrose content



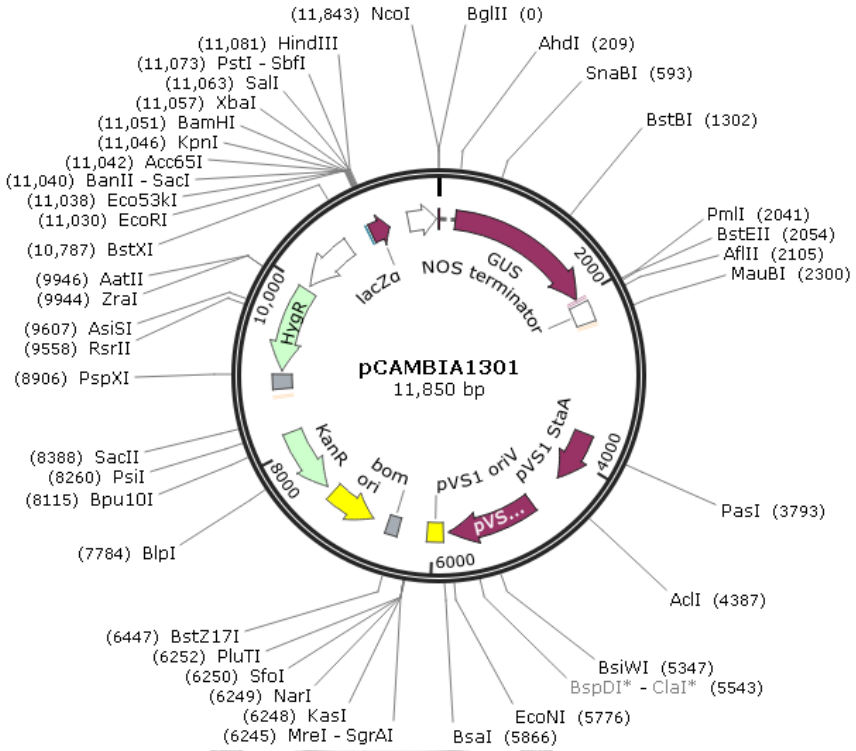
C6. Standard curve of starch content for determining the Arabidopsis starch content



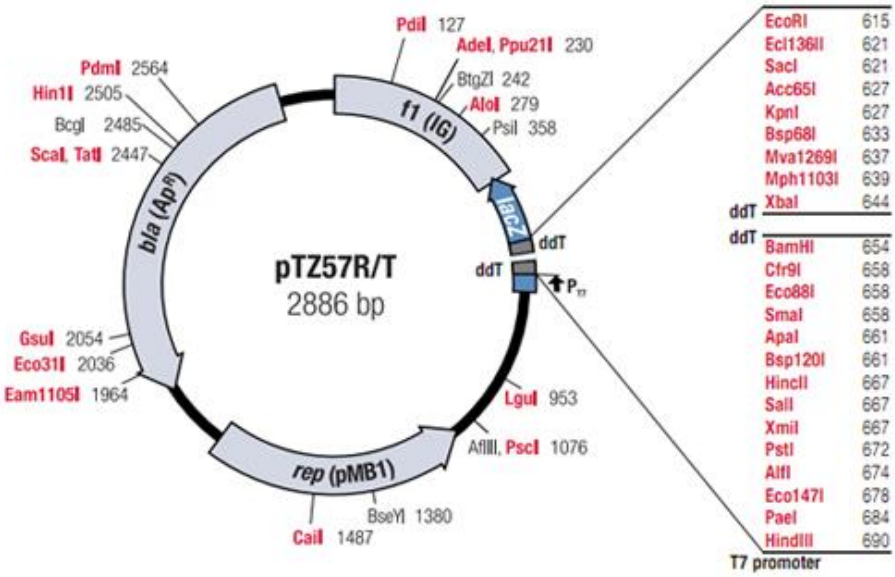
Appendix D

Vectors

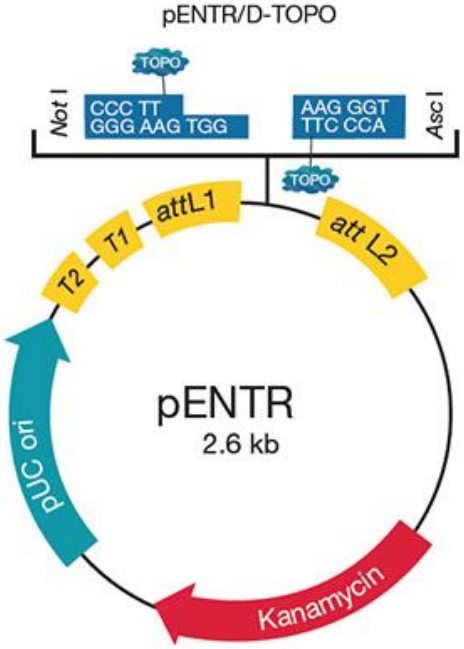
D1. pCAMBIA1301



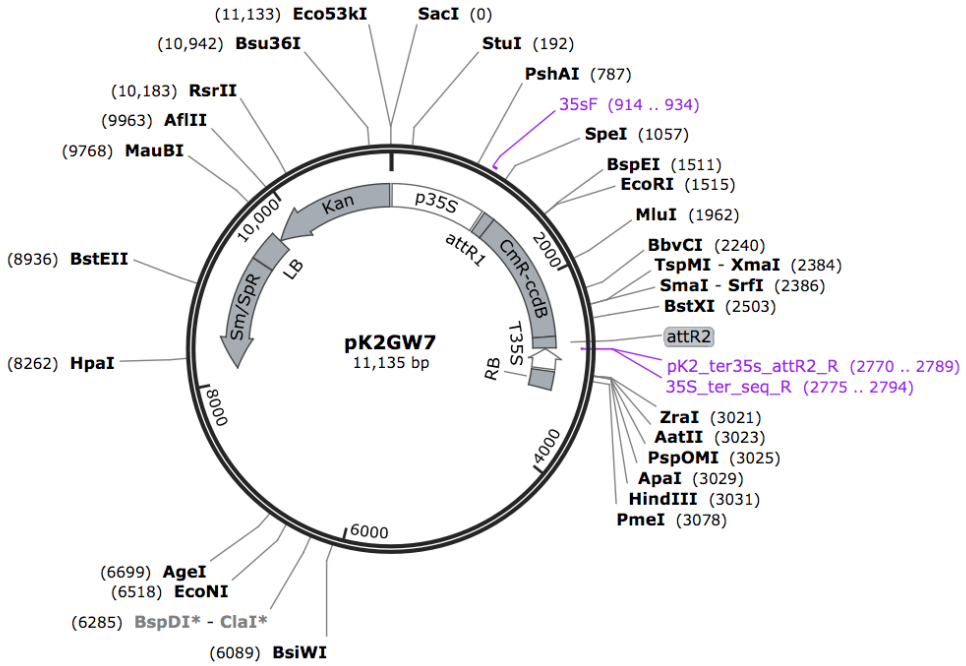
D2. pTZ57R/T



D3. pENTR/D-TOPO



D4. pK2GW7



Appendix E

Transgenic Arabidopsis Homozygous Selection

E1. Hemizygous selection of T2 revertants constructed from the *Aticl* Arabidopsis mutant by expressing *OsICL* under the control of the *AtICL* promoter (2,138 bp upstream sequence of *AtICL*)

Line	Total Seed	Observe Germinated	Expected Germinated	Chi- Germinated	Observe Non- Germinated	Expected Non- Germinated	Chi-Non- Germinated	SUM-Chi*	Status
3FL-1	400	305	300.0	0.083	95	100.0	0.250	0.333	Hemi
3FL-2	401	376	300.8	18.828	25	100.3	56.484	75.313	Non-Hemi
3FL-3	300	200	225.0	2.778	100	75.0	8.333	11.111	Non-Hemi
3FL-4	200	141	150.0	0.540	59	50.0	1.620	2.160	Hemi
3FL-5	240	162	180.0	1.800	78	60.0	5.400	7.200	Non-Hemi
3FL-6	210	182	157.5	3.811	28	52.5	11.433	15.244	Non-Hemi
3FL-7	206	175	154.5	2.720	31	51.5	8.160	10.880	Non-Hemi
3FL-8	213	142	159.8	1.972	71	53.3	5.917	7.889	Non-Hemi
3FL-9	208	152	156.0	0.103	56	52.0	0.308	0.410	Hemi
3FL-10	208	176	156.0	2.564	32	52.0	7.692	10.256	Non-Hemi
3FL-12	136	99	102.0	0.088	37	34.0	0.265	0.353	Hemi
3FL-14	220	128	165.0	8.297	92	55.0	24.891	33.188	Non-Hemi
3FL-15	221	152	165.8	1.141	69	55.3	3.422	4.563	Non-Hemi

*Remark: SUM-Chi < 3.84 indicating that Chi-Square test follow the criteria at significant level $p < .05$

Hemi = Hemizygous, Non-Hemi = Non-Hemizygous

E2. Hemizygous selection of T2 revertants constructed from the *Aticl* Arabidopsis mutant by over-expressing *OsICL* under the control of CaMV 35SCaMV promoter

Line	Total Seed	Observe Germinated	Expected Germinated	Chi-Germinated	Observe Non-Germinated	Expected Non-Germinated	Chi-Non-Germinated	SUM-Chi*	Status
OX*OsICL*/*icl*-3	214	150	160.5	0.687	64	53.5	2.061	2.748	Hemi
OX*OsICL*/*icl*-4	215	176	161.3	1.349	39	53.8	4.048	5.397	Non-Hemi
OX*OsICL*/*icl*-5	202	170	151.5	2.259	32	50.5	6.777	9.036	Non-Hemi
OX*OsICL*/*icl*-6	210	149	157.5	0.459	61	52.5	1.376	1.835	Hemi

*Remark: SUM-Chi < 3.84 indicating that Chi-Square test follow the criteria at significant level $p < .05$

Hemi = Hemizygous, Non-Hemi = Non-Hemizygous

E4. Hemizygous selection of T2 transgenic Arabidopsis over-expressing *OsICL* under the control of CaMV 35SCaMV promoter

Line	Total Seed	Observe Germinated	Expected Germinated	Chi-Germinated	Observe Non-Germinated	Expected Non-Germinated	Chi-Non-Germinated	SUM-Chi*	Status
OX*OsICL*/WT-1	201	120	150.8	6.272	81	50.3	18.817	25.090	Non-Hemi
OX*OsICL*/WT-2	205	85	153.8	30.742	120	51.3	92.226	122.968	Non-Hemi
OX*OsICL*/WT-3	210	114	157.5	12.014	96	52.5	36.043	48.057	Non-Hemi
OX*OsICL*/WT-4	124	112	93	3.882	12	31	11.645	15.527	Non-Hemi
OX*OsICL*/WT-5	230	154	172.5	1.984	76	57.5	5.952	7.936	Non-Hemi
OX*OsICL*/WT-6	105	40	78.8	19.068	65	26.3	57.202	76.270	Non-Hemi
OX*OsICL*/WT-7	170	139	127.5	1.037	31	42.5	3.112	4.149	Non-Hemi
OX*OsICL*/WT-8	210	126	157.5	6.300	84	52.5	18.900	25.200	Non-Hemi
OX*OsICL*/WT-9	160	140	120	3.333	20	40	10.000	13.333	Non-Hemi
OX*OsICL*/WT-10	210	144	157.5	1.157	66	52.5	3.471	4.629	Non-Hemi
OX*OsICL*/WT-11	101	74	75.8	0.040	27	25.3	0.121	0.162	Hemi

*Remark: SUM-Chi < 3.84 indicating that Chi-Square test follow the criteria at significant level $p < .05$

Hemi = Hemizygous, Non-Hemi = Non-Hemizygous

E5. Homozygous Selection of T3 Revertants constructred from the A*ticl* Arabidopsis Mutant by Expressing *OsICL* under *AtICL* promoter (2,138 bp upstream of *AtICL*)

Line	Total Seed	Germinated Seed	Expected 90% of Germinated Seed	Status
3FL-1-29	54	28	48.6	Non-Homozygous
3FL-1-30	61	40	54.9	Non-Homozygous
3FL-4-1	30	27	27	Non-Homozygous
3FL-4-5	34	32	30.6	Homozygous
3FL-4-7	33	33	29.7	Homozygous
3FL-4-11	36	36	32.4	Homozygous
3FL-4-13	33	23	29.7	Non-Homozygous
3FL-4-14	40	28	36	Non-Homozygous
3FL-4-15	43	38	38.7	Non-Homozygous
3FL-4-16	30	26	27	Non-Homozygous
3FL-4-19	33	26	29.7	Non-Homozygous
3FL-4-48	30	27	27	Non-Homozygous
3FL-4-49	40	39	36	Homozygous
3FL-4-52	34	34	30.6	Homozygous
3FL-4-53	32	23	28.8	Non-Homozygous
3FL-4-55	27	27	24.3	Homozygous
3FL-4-64	36	22	32.4	Non-Homozygous
3FL-4-77	34	21	30.6	Non-Homozygous
3FL-9-1	60	36	54	Non-Homozygous
3FL-9-2	50	15	45	Non-Homozygous
3FL-9-3	50	27	45	Non-Homozygous
3FL-9-4	50	32	45	Non-Homozygous
3FL-9-6	50	17	45	Non-Homozygous
3FL-9-7	51	25	45.9	Non-Homozygous
3FL-9-8	44	25	39.6	Non-Homozygous
3FL-9-11	50	40	45	Non-Homozygous
3FL-9-12	50	32	45	Non-Homozygous
3FL-9-13	53	17	47.7	Non-Homozygous
3FL-9-14	54	20	48.6	Non-Homozygous
3FL-9-17	52	38	46.8	Non-Homozygous
3FL-9-22	64	54	57.6	Non-Homozygous
3FL-9-25	38	35	34.2	Homozygous
3FL-9-30	55	48	49.5	Non-Homozygous
3FL-9-33	42	30	37.8	Non-Homozygous
3FL-9-57	40	20	36	Non-Homozygous

E6. Homozygous selection of T3 revertant constructed from the *Aticl* Arabidopsis mutant by over-expressing *OsICL* under the control of CaMV 35SCaMV promoter

Line	Total Seed	Germinated seed	Expected 90% of Germinated Seed	Status
OX*OsICL*/*icl*-3-12	49	45	44.1	Homozygous
OX*OsICL*/*icl*-3-13	39	24	35.1	Non-Homozygous
OX*OsICL*/*icl*-3-18	32	22	28.8	Non-Homozygous
OX*OsICL*/*icl*-3-2	29	21	26.1	Non-Homozygous
OX*OsICL*/*icl*-3-20	35	25	31.5	Non-Homozygous
OX*OsICL*/*icl*-3-24	42	27	37.8	Non-Homozygous
OX*OsICL*/*icl*-3-26	46	40	41.4	Non-Homozygous
OX*OsICL*/*icl*-3-3	51	40	45.9	Non-Homozygous
OX*OsICL*/*icl*-3-5	50	47	45	Homozygous
OX*OsICL*/*icl*-3-6	64	61	57.6	Homozygous
OX*OsICL*/*icl*-3-7	39	31	35.1	Non-Homozygous
OX*OsICL*/*icl*-3-9	53	48	47.7	Homozygous
OX*OsICL*/*icl*-6-2	61	50	54.9	Non-Homozygous
OX*OsICL*/*icl*-6-5	47	33	42.3	Non-Homozygous
OX*OsICL*/*icl*-6-9	54	40	48.6	Non-Homozygous
OX*OsICL*/*icl*-6-12	50	29	45	Non-Homozygous
OX*OsICL*/*icl*-6-15	40	28	36	Non-Homozygous
OX*OsICL*/*icl*-6-16	55	36	49.5	Non-Homozygous
OX*OsICL*/*icl*-6-17	55	43	49.5	Non-Homozygous
OX*OsICL*/*icl*-6-19	73	46	65.7	Non-Homozygous
OX*OsICL*/*icl*-6-23	97	68	87.3	Non-Homozygous
OX*OsICL*/*icl*-6-25	60	45	54	Non-Homozygous
OX*OsICL*/*icl*-6-29	52	30	46.8	Non-Homozygous
OX*OsICL*/*icl*-6-31	85	45	76.5	Non-Homozygous
OX*OsICL*/*icl*-6-39	61	47	54.9	Non-Homozygous

E7. Homozygous selection of T3 transgenic Arabidopsis over-expressing *OsICL* under the control of 35SCaMV promoter

Line	Total Seed	Germinated Seed	Expected 90% of Germinated Seed	Status
OX*OsICL*/WT-11-5	55	42	49.5	Non-Homozygous
OX*OsICL*/WT-11-6	64	61	57.6	Homozygous
OX*OsICL*/WT-11-7	55	39	49.5	Non-Homozygous
OX*OsICL*/WT-11-8	50	45	45	Non-Homozygous
OX*OsICL*/WT-11-11	51	40	45.9	Non-Homozygous
OX*OsICL*/WT-11-12	50	50	45	Homozygous
OX*OsICL*/WT-11-13	46	43	41.4	Homozygous
OX*OsICL*/WT-11-14	50	41	45	Non-Homozygous
OX*OsICL*/WT-11-15	54	53	48.6	Homozygous
OX*OsICL*/WT-11-16	53	53	47.7	Homozygous
OX*OsICL*/WT-11-17	45	44	40.5	Homozygous
OX*OsICL*/WT-11-18	41	30	36.9	Non-Homozygous
OX*OsICL*/WT-11-19	47	39	42.3	Non-Homozygous
OX*OsICL*/WT-11-20	41	35	36.9	Non-Homozygous
OX*OsICL*/WT-11-21	47	38	42.3	Non-Homozygous
OX*OsICL*/WT-11-23	42	37	37.8	Non-Homozygous
OX*OsICL*/WT-11-24	50	47	45	Homozygous
OX*OsICL*/WT-11-29	49	47	44.1	Homozygous
OX*OsICL*/WT-11-31	52	39	46.8	Non-Homozygous
OX*OsICL*/WT-11-32	47	43	42.3	Homozygous
OX*OsICL*/WT-11-36	45	43	40.5	Homozygous
OX*OsICL*/WT-11-42	47	36	42.3	Non-Homozygous
OX*OsICL*/WT-11-43	50	49	45	Homozygous
OX*OsICL*/WT-11-44	48	32	43.2	Non-Homozygous
OX*OsICL*/WT-11-45	46	35	41.4	Non-Homozygous
OX*OsICL*/WT-11-46	44	35	39.6	Non-Homozygous

Appendix F

DNA Sequences

F1. DNA sequence of GUS-NOS-upstream-*AtICL-OsICL*

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ref      ----------ATGGTAGATCTGAGGGTAAATTTCTAGTTTTTCTCCTTCATTTTCT
         46
3FL      TAGATGAATTCACCATGGTAGATCTGAGGGTAAATTTCTAGTTTTTCTCCTTCATTTTCT
         60
                  *******************************************

ref      TGGTTAGGACCCTTTTCTCTTTTTATTTTTTTGAGCTTTGATCTTTCTTTAAACTGATCT
         106
3FL      TGGTTAGGACCCTTTTCTCTTTTTATTTTTTTGAGCTTTGATCTTTCTTTAAACTGATCT
         120
         ************************************************************

ref      ATTTTTTAATTGATTGGTTATGGTGTAAATATTACATAGCTTTAACTGATAATCTGATTA
         166
3FL      ATTTTTTAATTGATTGGTTATGGTGTAAATATTACATAGCTTTAACTGATAATCTGATTA
         180
         ************************************************************

ref      CTTTATTTCGTGTGTCTATGATGATGATGATAGTTACAGAACCGACGACTCGTCCGTCCT
         226
3FL      CTTTATTTCGTGTGTCTATGATGATGATGATAGTTACAGAACCGACGACTCGTCCGTCCT
         240
         ************************************************************

ref      GTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGAT
         286
3FL      GTAGAAACCCCAACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGAT
         300
         ************************************************************

ref      CGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCA
         346
3FL      CGCGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAGCCGGGCA
         360
         ************************************************************

ref      ATTGCTGTGCCAGGCAGTTTTAACGATCAGTTCGCCGATGCAGATATTCGTAATTATGCG
         406
3FL      ATTGCTGTGCCAGGCAGTTTTAACGATCAGTTCGCCGATGCAGATATTCGTAATTATGCG
         420
         ************************************************************

ref      GGCAACGTCTGGTATCAGCGCGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATC
         466
3FL      GGCAACGTCTGGTATCAGCGCGAAGTCTTTATACCGAAAGGTTGGGCAGGCCAGCGTATC
         480
         ************************************************************

ref      GTGCTGCGTTTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTG
         526
3FL      GTGCTGCGTTTCGATGCGGTCACTCATTACGGCAAAGTGTGGGTCAATAATCAGGAAGTG
         540
         ************************************************************
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ref      ATGGAGCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCC
         586
3FL      ATGGAGCATCAGGGCGGCTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCC
         600
         ************************************************************

ref      GGGAAAAGTGTACGTATCACCGTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCCG
         646
3FL      GGGAAAAGTGTACGTATCACCGTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCCG
         660
         ************************************************************

ref      CCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTC
         706
3FL      CCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTTCCATGATTTC
         720
         ************************************************************

ref      TTTAACTATGCCGGAATCCATCGCAGCGTAATGCTCTACACCACGCCGAACACCTGGGTG
         766
3FL      TTTAACTATGCCGGAATCCATCGCAGCGTAATGCTCTACACCACGCCGAACACCTGGGTG
         780
         ************************************************************

ref      GACGATATCACCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCGTCTGTTGACTGG
         826
3FL      GACGATATCACCGTGGTGACGCATGTCGCGCAAGACTGTAACCACGCGTCTGTTGACTGG
         840
         ************************************************************

ref      CAGGTGGTGGCCAATGGTGATGTCAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTT
         886
3FL      CAGGTGGTGGCCAATGGTGATGTCAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTT
         900
         ************************************************************

ref      GCAACTGGACAAGGCACTAGCGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCG
         946
3FL      GCAACTGGACAAGGCACTAACGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCG
         960
         ******************* ****************************************

ref      GGTGAAGGTTATCTCTATGAACTCGAAGTCACAGCCAAAAGCCAGACAGAGTCTGATATC
         1006
3FL      GGTGAAGGTTATCTCTATGAACTGTGCGTCACAGCCAAAAGCCAGACAGAGTGTGATATC
         1020
         ************************    ************************ *******

ref      TACCCGCTTCGCGTCGGCATCCGGTCAGTGGCAGTGAAGGGCCAACAGTTCCTGATTAAC
         1066
3FL      TACCCGCTTCGCGTCGGCATCCGGTCAGTGGCAGTGAAGGGCGAACAGTTCCTGATTAAC
         1080
         ****************************************** ****************

ref      CACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATGAAGATGCGGACTTACGTGGCAAA
         1126
3FL      CACAAACCGTTCTACTTTACTGGCTTTGGTCGTCATGAAGATGCGGACTTGCGTGGCAAA
         1140
         ************************************************* *********
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ref      GGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAAC
        1186
3FL      GGATTCGATAACGTGCTGATGGTGCACGACCACGCATTAATGGACTGGATTGGGGCCAAC
        1200
         ************************************************************

ref      TCCTACCGTACCTCGCATTACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACAT
        1246
3FL      TCCTACCGTACCTCGCATTACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACAT
        1260
         ************************************************************

ref      GGCATCGTGGTGATTGATGAAACTGCTGCTGTCGGCTTTCAGCTGTCTTTAGGCATTGGT
        1306
3FL      GGCATCGTGGTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGT
        1320
         ************************************ * ** ***************

ref      TTCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAACGGGGAAACT
        1366
3FL      TTCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAACGGGGAAACT
        1380
         ************************************************************

ref      CAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGC
        1426
3FL      CAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGTGACAAAAACCACCCAAGC
        1440
         ************************************************************

ref      GTGGTGATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGGTGCACGGGAATAT
        1486
3FL      GTGGTGATGTGGAGTATTGCCAACGAACCGGATACCCGTCCGCAAGGTGCACGGGAATAT
        1500
         ************************************************************

ref      TTCGCGCCACTGGCGGAAGCAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTC
        1546
3FL      TTCGCGCCACTGGCGGAAGCAACGCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTC
        1560
         ************************************************************

ref      AATGTAATGTTCTGCGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGC
        1606
3FL      AATGTAATGTTCTGCGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGC
        1620
         ************************************************************

ref      CTGAACCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTA
        1666
3FL      CTGAACCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAAGGTA
        1680
         ************************************************************

ref      CTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATTATCATCACCGAA
        1726
3FL      CTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGATTATCATCACCGAA
        1740
         ************************************************************
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ref      TACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAG
        1786
3FL      TACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTACACCGACATGTGGAGTGAAGAG
        1800
         ************************************************************

ref      TATCAGTGTGCATGGCTGGATATGTATCACCGCGTCTTTGATCGCGTCAGCGCCGTCGTC
        1846
3FL      TATCAGTGTGCATGGCTGGATATGTATCACCGCGTCTTTGATCGCGTCAGCGCCGTCGTC
        1860
         ************************************************************

ref      GGTGAACAGGTATGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGC
        1906
3FL      GGTGAACAGGTATGGAATTTCGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGC
        1920
         ************************************************************

ref      GGTAACAAGAAAGGGATCTTCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTG
        1966
3FL      GGTAACAAGAAAGGGATCTTCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTG
        1980
         ************************************************************

ref      CAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAGGCAAACAAGCT
        2026
3FL      CAAAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAGGCAAACAAGCT
        2040
         ************************************************************

ref      AGCCACCACCACCACCACCACGTGTGAATTACAGGTGACCAGCTCGAATTTCCCCGATCG
        2086
3FL      AGCCACCACCACCACCACCACGTGTGAATTACAGGTGACCAGCTCGAATTTCCCCGATCG
        2100
         ************************************************************

ref      TTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGAT
        2146
3FL      TTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGAT
        2160
         ************************************************************

ref      TATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGAC
        2206
3FL      TATCATATAATTTCTGTTGAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGAC
        2220
         ************************************************************

ref      GTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGAT
        2266
3FL      GTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAATTATACATTTAATACGCGAT
        2280
         ************************************************************

ref      AGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATC------ATCATCTTCTATCGG
        2320
3FL      AGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATTCTAGACATCATCTTCTATCGG
        2340
         ************************************       ***************
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ref      AATCTCAAACAACAAGTTCGAAAATAAATTATCTGTTTAAGGTGACGTCATTCCCGCCAC
        2380
3FL      AATCTCAAACAACAAGTTCGAAAATAAATTATCTGTTTAAGGTGACGTCATTCCCGCCAC
        2400
         ************************************************************

ref      GTTCTTGTAGCTTCCTGATTTCATCCACGAGCTAAGCAAGTAGCATCCTCTTTTGTCCGT
        2440
3FL      GTTCTTGTAGCTTCCTGATTTCATCCACGAGCTAAGCAAGTAGCATCCTCTTTTGTCCGT
        2460
         ************************************************************

ref      TTTCTCGTGACAACTTTACTACCTATACATATACATATACAATCACCGTATTAATATGCT
        2500
3FL      TTTCTCGTGACAACTTTACTACCTATACATATACATATACAATCACCGTATTAATATGCT
        2520
         ************************************************************

ref      TTGAATCGTCTAATCACACATTATTTGCAGTAATATATTGGTATAAACATCATTTCTTGT
        2560
3FL      TTGAATCGTCTAATCACACATTATTTGCAGTAATATATTGGTATAAACATCATTTCTTGT
        2580
         ************************************************************

ref      TTGAGCCTAGGTTTGTGGTTTATAATGTTTCATATATGGATCGGATTGCAATTTTAGTTT
        2620
3FL      TTGAGCCTAGGTTTGTGGTTTATAATGTTTCATATATGGATCGGATTGCAATTTTAGTTT
        2640
         ************************************************************

ref      AATTTTCTATTTGTGTTCAGGTTTGTCACACAGATTAGTTTAAAACTAAATTAGGTGAAC
        2680
3FL      AATTTTCTATTTGTGTTCAGGTTTGTCACACAGATTAGTTTAAAACTAAATTAGGTGAAC
        2700
         ************************************************************

ref      ATATATACCATCGTATTATGAACCATCTGTTTTAGGTTCCAACTTCCAACAGCAATATCG
        2740
3FL      ATATATACCATCGTATTATGAACCATCTGTTTTAGGTTCCAACTTCCAACAGCAATATCG
        2760
         ************************************************************

ref      TTCTCTTTTTGCATGTGGACGTACGTAGACAACATGACGTTTGTGAATCACGTAAATTTG
        2800
3FL      TTCTCTTTTTGCATGTGGACGTACGTAGACAACATGACGTTTGTGAATCACGTAAATTTG
        2820
         ************************************************************

ref      TGTATGTTTTGTGTGTGTCTATTTGTTTCTTACAACGGTAATAGTAGATAGTTTAAAAAG
        2860
3FL      TGTATGTTTTGTGTGTGTCTATTTGTTTCTTACAACGGTAATAGTAGATAGTTTAAAAAG
        2880
         ************************************************************

ref      ATAATTTATACGTTATGTGAGAAATTTTTTAATATATGATCATGTAATTATTTTATTTGT
        2920
3FL      ATAATTTATACGTTATGTGAGAAATTTTTTAATATATGATCATGTAATTATTTTATTTGT
        2940
         ************************************************************
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ref      TTTTTTCATAATTCCTTTTCTTTTAATTATTTTATTTGTATTTAATTGATTGATATACTA
         2980
3FL      TTTTTTCATAATTCCTTTTCTTTTAATTATTTTATTTGTATTTAATTGATTGATATACTA
         3000
         ************************************************************

ref      CAAAAAATTCACACTCAAAAGAAAACATTCAATTTATTAATATTTAAATAGACTACTAGC
         3040
3FL      CAAAAAATTCACACTCAAAAGAAAACATTCAATTTATTAATATTTAAATAGACTACTAGC
         3060
         ************************************************************

ref      ATACTAGTAGCAGCACACTGATTTGGGAAAAAAATAAACATTGTTTTGGATTATGGACCT
         3100
3FL      ATACTAGTAGCAGCACACTGATTTGGGAAAAAAATAAACATTGTTTTGGATTATGGACCT
         3120
         ************************************************************

ref      ATAAAAACTATTTTTAATTGTATTTTATTATAAATGATGATTTTATGATTCTACAAAAAC
         3160
3FL      ATAAAAACTATTTTTAATTGTATTTTATTATAAATGATGATTTTATGATTCTACAAAAAC
         3180
         ************************************************************

ref      TATTCAATAACAAACAATTCTACATACACCATACATGTTTATGCTTAATGGTTCTATTAA
         3220
3FL      TATTCAATAACAAACAATTCTACATACACCATACATGTTTATGCTTAATGGTTCTATTAA
         3240
         ************************************************************

ref      TTATGATGGAAAACTTTAATTATGATATACAAAACATGATGAAAAGTTATAAAGCATGCA
         3280
3FL      TTATGATGGAAAACTTTAATTATGATATACAAAACATGATGAAAAGTTATAAAGCATGCA
         3300
         ************************************************************

ref      CCTTGCTGACAATCTCTAATAAATTTGTTTACACTAATCAAAAATAATTCCCATTGTGTG
         3340
3FL      CCTTGCTGACAATCTCTAATAAATTTGTTTACACTAATCAAAAATAATTCCCATTGTGTG
         3360
         ************************************************************

ref      GGAATCAAATTCCATCCATAGATGTAAAGTTTTCGTAAACCAAAAA-AAAAAACGCTTCC
         3399
3FL      GGAATCAAATTCCATCCATAGATGTAAAGTTTTCGTAAACCAAAAAAAAAAAACGCTTCC
         3420
         ******************************************** *************

ref      GAGAAAAACAACAACAAAATGTTTAGGCTCAAAAGAAAACAAGATCAAATAAAAGAAGAA
         3459
3FL      GAGAAAAACAACAACAAAATGTTTAGGCTCAAAAGAAAACAAGATCAAATAAAAGAAGAA
         3480
         ************************************************************

ref      GGAAACCGACAGCAACAAAATGGAAAGATAGGATTGCCAAGTCGTATCCAATCTCAATCT
         3519
3FL      GGAAACCGACAGCAACAAAATGGAAAGATAGGATTGCCAAGTCGTATCCAATCTCAATCT
         3540
         ************************************************************
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ref      GCGACTCAATTCAAAGCAAGATAGGGTTTATCTATATTCCTCGGGAAACAAACTGTTTAT
        3579
3FL      GCGACTCAATTCAAAGCAAGATAGGGTTTATCTATATTCCTCGGGAAACAAACTGTTTAT
        3600
         ************************************************************

ref      TTTAAAATTTATTCAGTGTTCAAATACGATTTTAAAACTTTACCCACATTAATATATAAT
        3639
3FL      TTTAAAATTTATTCAGTGTTCAAATACGATTTTAAAACTTTACCCACATTAATATATAAT
        3660
         ************************************************************

ref      CACCAAATAAAATACATAGTTGACAGAAAAAATGACGATACGTAAAATTTTAAATACATT
        3699
3FL      CACCAAATAAAATACATAGTTGACAGAAAAAATGACGATACGTAAAATTTTAAATACATT
        3720
         ************************************************************

ref      TCCTTGTAAAAGTTATTTCACTTTGAAGTTGCTGATAAATTGTGTATGAATGATTGAATA
        3759
3FL      TCCTTGTAAAAGTTATTTCACTTTGAAGTTGCTGATAAATTGTGTATGAATGATTGAATA
        3780
         ************************************************************

ref      TGTATGATAAGTTAAATGTATAGCAATTGTTTCAAGTAAAATGTATAATGCTCTTACTCT
        3819
3FL      TGTATGATAAGTTAAATGTATAGCAATTGTTTCAAGTAAAATGTATAATGCTCTTACTCT
        3840
         ************************************************************

ref      TAAGTATAATGTCCATCAATAGTTGCAGATTATGTCACAATTGATCTACATTTTGAGGCT
        3879
3FL      TAAGTATAATGTCCATCAATAGTTGCAGATTATGTCACAATTGATCTACATTTTGAGGCT
        3900
         ************************************************************

ref      TTAGTGTTCAAACGTTTGAGATTGGTAGGATGATACATAAAAAATTGGTGTTCTTTCTTA
        3939
3FL      TTAGTGTTCAAACGTTTGAGATTGGTAGGATGATACATAAAAAATTGGTGTTCTTTCTTA
        3960
         ************************************************************

ref      ATTGCACCAGCTTCCTTTCTATTAGAGCGAGGCTACCTAGCCTATATTATACACCCATTG
        3999
3FL      ATTGCACCAGCTTCCTTTCTATTAGAGCGAGGCTACCTAGCCTATATTATACACCCATTG
        4020
         ************************************************************

ref      GAAAGAATCTACGATTATTTCTTAAACCCGATTCATTATATGGCTAAAATCCTTGTGCAA
        4059
3FL      GAAAGAATCTACGATTATTTCTTAAACCCGATTCATTATATGGCTAAAATCCTTGTGCAA
        4080
         ************************************************************

ref      CTCTTTAATTGTTCGAACCATTCTATATACATCACATTTTTTCTTAATGACATCAAATGA
        4119
3FL      CTCTTTAATTGTTCGAACCATTCTATATACATCACATTTTTTCTTAATGACATCAAATGA
        4140
         ************************************************************
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ref      ACGAGACAATGAAGATGTCAAATGGTAATTCGTTTCTGCCATAATGCCCTCATACGTGTC
         4179
3FL      ACGAGACAATGAAGATGTCAAATGGTAATTCGTTTCTGCCATAATGCCCTCATACGTGTC
         4200
         ************************************************************

ref      ACAATCATTTACCTTCCGAAAAAAATATAATTTTAGTCCAACTTGGAAAAAAGATATATA
         4239
3FL      ACAATCATTTACCTTCCGAAAAAAATATAATTTTAGTCCAACTTGGAAAAAAGATATATA
         4260
         ************************************************************

ref      CATCAGCACAAGTAGTTTCTTTTTGATCAGAAATAAATTTTAAAATATTTTAATCTAATA
         4299
3FL      CATCAGCACAAGTAGTTTCTTTTTGATCAGAAATAAATTTTAAAATATTTTAATCTAATA
         4320
         ************************************************************

ref      CTTCATAAGAAAGCAATATTTGCCAATGCAGAAATTAGTATATAAGGAGATGATAGGGAA
         4359
3FL      CTTCATAAGAAAGCAATATTTGCCAATGCAGAAATTAGTATATAAGGAGATGATAGGGAA
         4380
         ************************************************************

ref      GAAGAGCAAATATGCTTGAAGCTTCTCTAATTTCATAGCTCCTAACAAAGCAATATCATT
         4419
3FL      GAAGAGCAAATATGCTTGAAGCTTCTCTAATTTCATAGCTCCTAACAAAGCAATATCATT
         4440
         ************************************************************

ref      TCCAATTTATAAAAGTTAAAGCCATGTCGTCGCCGTTCTCCGTGCCATCTCTGATCATGG
         4479
3FL      TCCAATTTATAAAAGTTAAACATATGTCGTCACCGTTCTCCGTGCCATCTCTGATCATGG
         4500
         ******************   ******** ****************************

ref      AGGAGGAAGGGCGGTTCGAGGCGGAGGTGGCGGAGGTGGAGGCGTGGTGGGGAACGGACA
         4539
3FL      AGGAGGAAGGGCGGTTCGAGGCGGAGGTGGCGGAGGTGGAGGCGTGGTGGGGAACGGACA
         4560
         ************************************************************

ref      GGTTCCGGCTCACCAAGCGCCCCTACACGGCGCGCGACGTCGCGCTCCTCCGCGGCACGC
         4599
3FL      GGTTCCGGCTCACCAAGCGCCCCTACACGGCGCGCGACGTCGCGCTCCTCCGCGGCACGC
         4620
         ************************************************************

ref      TCCGGCAGAGCTACGCCTCCGGCGACATGGCCAAGAAGCTGTGGCGCACGCTCAGGGCGC
         4659
3FL      TCCGGCAGAGCTACGCCTCCGGCGACATGGCCAAGAAGCTGTGGCGCACGCTCAGGGCGC
         4680
         ************************************************************

ref      ACCAGGCCAACGGCACGGCGTCGCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTCGCCA
         4719
3FL      ACCAGGCCAACGGCACGGCGTCGCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTCGCCA
         4740
         ************************************************************
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ref      TGATGGCGAAGCACCTCGACACCGTCTACGTCTCCGGATGGCAGTGCTCGTCGACGCACA
         4779
3FL      TGATGGCGAAGCACCTCGACACCGTCTACGTCTCCGGATGGCAGTGCTCGTCGACGCACA
         4800
         ************************************************************

ref      CCTCGACGAACGAGCCGGGCCCCGACCTCGCCGACTACCCCTACGACACCGTCCCCAACA
         4839
3FL      CCTCGACGAACGAGCCGGGCCCCGACCTCGCCGACTACCCCTACGACACCGTCCCCAACA
         4860
         ************************************************************

ref      AGGTCGAGCACCTCTTCTTCGCCCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCA
         4899
3FL      AGGTCGAGCACCTCTTCTTCGCCCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCA
         4920
         ************************************************************

ref      TGTCGATGTCCAGGGCGGAGCGCGCGCACGAGCCGTACGTGGATTATCTTAAGCCCATCA
         4959
3FL      TGTCGATGTCCAGGGCGGAGCGCGCGCACGAGCCGTACGTGGATTATCTTAAGCCCATCA
         4980
         ************************************************************

ref      TCGCCGACGGCGACACCGGGTTCGGCGGCGCCACGGCCACCGTCAAGCTGTGCAAGCTGT
         5019
3FL      TCGCCGACGGCGACACCGGGTTCGGCGGCGCCACGGCCACCGTCAAGCTGTGCAAGCTGT
         5040
         ************************************************************

ref      TCGTCGAGCGCGGGGCGGCCGGGGTTCACCTCGAGGACCAGTCGTCGGTGACCAAGAAGT
         5079
3FL      TCGTCGAGCGCGGGGCGGCCGGGGTTCACCTCGAGGACCAGTCGTCGGTGACCAAGAAGT
         5100
         ************************************************************

ref      GCGGGCACATGGCGGGGAAGGTGCTCGTCGCCGTCTCCGAGCATGTCAACCGCCTCGTCG
         5139
3FL      GCGGGCACATGGCGGGGAAGGTGCTCATCGCCGTCTCCGAGCATGTCAACCGCCTCGTCG
         5160
         ************************** *********************************

ref      CCGCGCGGCTCCAGTTCGACATCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACG
         5199
3FL      CCGCGCGGCTCCAGTTCGACATCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACG
         5220
         ************************************************************

ref      CCGTCGCCGCCACGCTGATCCAGACCAACGTCGACGCGCGCGACCACCAGTTCATCCTCG
         5259
3FL      CCGTCGCCGCCACGCTGATCCAGACCAACGTCGACGCGCGCGACCACCAGTTCATCCTCG
         5280
         ************************************************************

ref      GCGCGACCAACCCGCGCCTCAGGAACCGGAGCCTCGCCGCCGTCCTCTCCGACGCCATGT
         5319
3FL      GCGCGACCAACCCGCGCCTCAGGAACCGGAGCCTCGCCGCCGTCCTCTCCGACGCCATGT
         5340
         ************************************************************
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ref      CGGCGGGCAAGAACGGCAGGGAGCTCCAGGCCATCGAGGACGAGTGGCTCGCCACGGCGC
        5379
3FL      CGGCGGGCAAGAACGGCAGGGAGCTCCAGGCCATCGAGGACGAGTGGCTCGCCACGGCGC
        5400
         ************************************************************

ref      AGCTCAAGACCTTCTCTGACTGCGTCAGGGACGCCATCGCGAGCCTCAACGCCACCGACG
        5439
3FL      AGCTCAAGACCTTCTCTGACTGCGTCAGGGACGCCATCGCGAGCCTCAACGCCACCGACG
        5460
         ************************************************************

ref      CCGACAAGCAGCGCAAGCTCCAGGAGTGGAGCGCCGCCACCAGCCACGACAAGTGCGTGC
        5499
3FL      CCGACAAGCAGCGCAAGCTCCAGGAGTGGAGCGCCGCCACCAGCCACGACAAGTGCGTGC
        5520
         ************************************************************

ref      CCCTCGAGCAGGCGCGCGACATCGCCGCGGGCCTCGGCGTCACATCCCTGTTCTGGGACT
        5559
3FL      CCCTCGAGCAGGCGCGCGACATCGCCGCGGGCCTCGGCGTCACATCCCTGTTCTGGGACT
        5580
         ************************************************************

ref      GGGACCTGCCGAGAACACGGGAGGGGTTCTACCGCTTCCGCGGCTCCGTCGCGGCGGCCG
        5619
3FL      GGGACCTGCCGAGAACACGGGAGGGGTTCTACCGCTTCCGCGGCTCCGTCGCGGCGGCCG
        5640
         ************************************************************

ref      TCGTCCGCGGCCGCGCGTTCGCGCCGCACGCCGACGTGCTCTGGATGGAGACGTCGAGCC
        5679
3FL      TCGTCCGCGGCCGCGCGTTCGCGCCGCACGCCGACGTGCTCTGGATGGAGACGTCGAGCC
        5700
         ************************************************************

ref      CCAACATCGCCGAGTGCACGGCGTTCGCCGAGGGCGTGAGGGCGGCGAGCCCGGGCGCGA
        5739
3FL      CCAACATCGCCGAGTGCACGGCGTTCGCCGAGGGCGTGAGGGCGGCGAGCCCGGGCGCGA
        5760
         ************************************************************

ref      TGCTGGCGTACAACCTCTCGCCGTCCTTCAACTGGGACGCCTCCGGCATGACGGACGCCG
        5799
3FL      TGCTGGCGTACAACCTCTCGCCGTCCTTCAACTGGGACGCCTCCGGCATGACGGACGCCG
        5820
         ************************************************************

ref      ACATGTCGGAGTTCATCCCGCGCGTGGCGAGGCTGGGGTACGTGTGGCAGTTCATCACGC
        5859
3FL      ACATGTCGGAGTTCATCCCGCGCGTGGCGAGGCTGGGGTACGTGTGGCAGTTCATCACGC
        5880
         ************************************************************

ref      TCGCCGGGTTCCACGCCGACGCGCTCGTCACGGACACGTTCGCCCGCGACTTCGCGCGGC
        5919
3FL      TCGCCGGGTTCCACGCCGACGCGCTCGTCACGGACACGTTCGCCCGCGACTTCGCGCGGC
        5940
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ref       GCGGCATGCTGGCGTACGTGGAGAGGATCCAGAGGGAGGAGAGGAGCAACGGCGTGGAGA
          5979
3FL       GCGGCATGCTGGCGTACGTGGAGAGGATCCAGAGGGAGGAGAGGAGCAACGGCGTGGAGA
          6000
          ************************************************************

ref       CGCTGCAGCACCAGAAGTGGTCGGGCGCCAACTTCTACGACAGGGTGCTCAAGACCGTGC
          6039
3FL       CGCTGCAGCACCAGAAGTGGTCGGGCGCCAACTTCTACGACAGGGTGCTCAAGACCGTGC
          6060
          ************************************************************

ref       AGGGTGGCATCTCCTCTACCGCCGCCATGGGCAAAGGAGTTACTGAAGAGCAGTTCAAGG
          6099
3FL       AGGGTGGCATCTCCTCTACCGCCGCCATGAGCAAAGGAGTTACTGAAGAGCAGTTCAAGG
          6120
          *************************** ****************************

ref       GCTCATGGACAGGGCCTGGAAGTGAGAGCAGCAGCCATGTTCTTGCCAAATCCAGGATGT
          6159
3FL       GCTCATGGACAGGGCCTGGAAGTGAGAGCAGCAGCCATGTTCTTGCCAAATCCAGGATGT
          6180
          ************************************************************

ref       GA        6161
3FL       G-        6181
          *
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= *Eco*RI site, = TOPO cloning site, = *Xba*I site = *Nde*I site

F2. DNA sequence of *OsICL*

```
ref
----ATGTCGTCGCCGTTCTCCGTGCCATCTCTGATCATGGAGGAGGAAGGGCGGTTCGA 56
OsICL
CACCATGTCGTCACCGTTCTCCGTGCCATCTCTGATCATGGAGGAGGAAGGGCGGTTCGA 60

    ******** **********************************************

ref
GGCGGAGGTGGCGGAGGTGGAGGCGTGGTGGGGAACGGACAGGTTCCGGCTCACCAAGCG 116
OsICL
GGCGGAGGTGGCGGAGGTGGAGGCGTGGTGGGGAACGGACAGGTTCCGGCTCACCAAGCG 120

************************************************************

ref
CCCCTACACGGCGCGCGACGTCGCGCTCCTCCGCGGCACGCTCCGGCAGAGCTACGCCTC 176
OsICL
CCCCTACACGGCGCGCGACGTCGCGCTCCTCCGCGGCACGCTCCGGCAGAGCTACGCCTC 180

************************************************************

ref
CGGCGACATGGCCAAGAAGCTGTGGCGCACGCTCAGGGCGCACCAGGCCAACGGCACGGC 236
OsICL
CGGCGACATGGCCAAGAAGCTGTGGCGCACGCTCAGGGCGCACCAGGCCAACGGCACGGC 240

************************************************************

ref
GTCGCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTCGCCATGATGGCGAAGCACCTCGA 296
OsICL
GTCGCGCACCTTCGGCGCGCTCGACCCCGTCCAGGTCGCCATGATGGCGAAGCACCTCGA 300

************************************************************

ref
CACCGTCTACGTCTCCGGATGGCAGTGCTCGTCGACGCACACCTCGACGAACGAGCCGGG 356
OsICL
CACCGTCTACGTCTCCGGATGGCAGTGCTCGTCGACGCACACCTCGACGAACGAGCCGGG 360

************************************************************

ref
CCCCGACCTCGCCGACTACCCCTACGACACCGTCCCCAACAAGGTCGAGCACCTCTTCTT 416
OsICL
CCCCGACCTCGCCGACTACCCCTACGACACCGTCCCCAACAAGGTCGAGCACCTCTTCTT 420

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ref
CGCCCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCATGTCGATGTCCAGGGCGGA 476
OsICL
CGCCCAGCTCTACCACGACCGCAAGCAGCGGGAGGCGCGCATGTCGATGTCCAGGGCGGA 480

************************************************************

ref
GCGCGCGCACGAGCCGTACGTGGATTATCTTAAGCCCATCATCGCCGACGGCGACACCGG 536
OsICL
GCGCGCGCACGAGCCGTACGTGGATTATCTTAAGCCCATCATCGCCGACGGCGACACCGG 540

************************************************************

ref
GTTCGGCGGCGCCACGGCCACCGTCAAGCTGTGCAAGCTGTTCGTCGAGCGCGGGGCGGC 596
OsICL
GTTCGGCGGCGCCACGGCCACCGTCAAGCTGTGCAAGCTGTTCGTCGAGCGCGGGGCGGC 600

************************************************************

ref
CGGGGTTCACCTCGAGGACCAGTCGTCGGTGACCAAGAAGTGCGGGCACATGGCGGGGAA 656
OsICL
CGGGGTTCACCTCGAGGACCAGTCGTCGGTGACCAAGAAGTGCGGGCACATGGCGGGGAA 660

************************************************************

ref
GGTGCTCGTCGCCGTCTCCGAGCATGTCAACCGCCTCGTCGCCGCGCGGCTCCAGTTCGA 716
OsICL
GGTGCTCATCGCCGTCTCCGAGCATGTCAACCGCCTCGTCGCCGCGCGGCTCCAGTTCGA 720

******* **************************************************

ref
CATCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACGCCGTCGCCGCCACGCTGAT 776
OsICL
CATCATGGGCGTCGAGACCGTCCTCGTCGCGCGCACCGACGCCGTCGCCGCCACGCTGAT 780

************************************************************

ref
CCAGACCAACGTCGACGCGCGCGACCACCAGTTCATCCTCGGCGCGACCAACCCGCGCCT 836
OsICL
CCAGACCAACGTCGACGCGCGCGACCACCAGTTCATCCTCGGCGCGACCAACCCGCGCCT 840

************************************************************

ref
CAGGAACCGGAGCCTCGCCGCCGTCCTCTCCGACGCCATGTCGGCGGGCAAGAACGGCAG 896
OsICL
CAGGAACCGGAGCCTCGCCGCCGTCCTCTCCGACGCCATGTCGGCGGGCAAGAACGGCAG 900

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ref
GGAGCTCCAGGCCATCGAGGACGAGTGGCTCGCCACGGCGCAGCTCAAGACCTTCTCTGA 956
OsICL
GGAGCTCCAGGCCATCGAGGACGAGTGGCTCGCCACGGCGCAGCTCAAGACCTTCTCTGA 960

************************************************************

ref
CTGCGTCAGGGACGCCATCGCGAGCCTCAACGCCACCGACGCCGACAAGCAGCGCAAGCT 1016
OsICL
CTGCGTCAGGGACGCCATCGCGAGCCTCAACGCCACCGACGCCGACAAGCAGCGCAAGCT 1020

************************************************************

ref
CCAGGAGTGGAGCGCCGCCACCAGCCACGACAAGTGCGTGCCCCTCGAGCAGGCGCGCGA 1076
OsICL
CCAGGAGTGGAGCGCCGCCACCAGCCACGACAAGTGCGTGCCCCTCGAGCAGGCGCGCGA 1080

************************************************************

ref
CATCGCCGCGGGCCTCGGCGTCACATCCCTGTTCTGGGACTGGGACCTGCCGAGAACACG 1136
OsICL
CATCGCCGCGGGCCTCGGCGTCACATCCCTGTTCTGGGACTGGGACCTGCCGAGAACACG 1140

************************************************************

ref
GGAGGGGTTCTACCGCTTCCGCGGCTCCGTCGCGGCGGCCGTCGTCCGCGGCCGCGCGTT 1196
OsICL
GGAGGGGTTCTACCGCTTCCGCGGCTCCGTCGCGGCGGCCGTCGTCCGCGGCCGCGCGTT 1200

************************************************************

ref
CGCGCCGCACGCCGACGTGCTCTGGATGGAGACGTCGAGCCCCAACATCGCCGAGTGCAC 1256
OsICL
CGCGCCGCACGCCGACGTGCTCTGGATGGAGACGTCGAGCCCCAACATCGCCGAGTGCAC 1260

************************************************************

ref
GGCGTTCGCCGAGGGCGTGAGGGCGGCGAGCCCGGGCGCGATGCTGGCGTACAACCTCTC 1316
OsICL
GGCGTTCGCCGAGGGCGTGAGGGCGGCGAGCCCGGGCGCGATGCTGGCGTACAACCTCTC 1320

************************************************************

ref
GCCGTCCTTCAACTGGGACGCCTCCGGCATGACGGACGCCGACATGTCGGAGTTCATCCC 1376
OsICL
GCCGTCCTTCAACTGGGACGCCTCCGGCATGACGGACGCCGACATGTCGGAGTTCATCCC 1380

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ref
GCGCGTGGCGAGGCTGGGGTACGTGTGGCAGTTCATCACGCTCGCCGGGTTCCACGCCGA 1436
OsICL
GCGCGTGGCGAGGCTGGGGTACGTGTGGCAGTTCATCACGCTCGCCGGGTTCCACGCCGA 1440

************************************************************

ref
CGCGCTCGTCACGGACACGTTCGCCCGCGACTTCGCGCGGCGCGGCATGCTGGCGTACGT 1496
OsICL
CGCGCTCGTCACGGACACGTTCGCCCGCGACTTCGCGCGGCGCGGCATGCTGGCGTACGT 1500

************************************************************

ref
GGAGAGGATCCAGAGGGAGGAGAGGAGCAACGGCGTGGAGACGCTGCAGCACCAGAAGTG 1556
OsICL
GGAGAGGATCCAGAGGGAGGAGAGGAGCAACGGCGTGGAGACGCTGCAGCACCAGAAGTG 1560

************************************************************

ref
GTCGGGCGCCAACTTCTACGACAGGGTGCTCAAGACCGTGCAGGGTGGCATCTCCTCTAC 1616
OsICL
GTCGGGCGCCAACTTCTACGACAGGGTGCTCAAGACCGTGCAGGGTGGCATCTCCTCTAC 1620

************************************************************

ref
CGCCGCCATGGGCAAAGGAGTTACTGAAGAGCAGTTCAAGGGCTCATGGACAGGGCCTGG 1676
OsICL
CGCCGCCATGAGCAAAGGAGTTACTGAAGAGCAGTTCAAGGGCTCATGGACAGGGCCTGG 1680

********** **************************************************

ref         AAGTGAGAGCAGCAGCCATGTTCTTGCCAAATCCAGGATGTGA        1719
OsICL       AAGTGAGAGCAGCAGCCATGTTCTTGCCAAATCCAGGATGTG-        1722
            ****************************************
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, ___ = TOPO cloning site

F3 2,020 bp of *OsICL* Upstream Sequence

AGGGCTTGAGAGGTTGGTTGATGTATCTGCATATAGTTAATGCAATAAAGCTGTGATGCCCCTCCCTAT
CCGTGTTCATCTACTATATGATTGTGTGCATGAGATCGTCGTCTTCTACTCCGGATCAGCGAATAGGCC
ACTAGTTATTTAACAATTATGTCATCTTCGCAAACTTTTGGAGAAGATAATTTGTTTTGAAACGAGAAC
TACGACGAGCCGAACATCATCCTCTGCCTCCATCAAACCCTGCCTCACATGAGCACCCACCAGTGCCTA
TAGCAGGTTGGCACGAGGAGCTGATCTGAGCTCCCATCTGAACCTGACAGCGAGACGGGCATCGAAGCT
GTTGCAGAATTCTGAAGGATCAACAACAAGCAAGCGCATGCTGCACGCATGCATGTTTCTTCTCGATGT
AAGGCCGCGCCGCCTCCCTTGTCCCTGGCGATGGCGATTCCGGCTTTTCTCCCGTTCACCGCTGTTGGG
GCGCCTGCGCTGACGGCGACCGCCTAGCGCGCGCGCAGCCGCAGCCTCGATCTTCTTGTGGTCTTTGAT
GCAGCAACAAGAAGCTACGGCACTCTCCTCTGCGTGACGCCACCGTACGTTTCGTTGGAAACGATCGGA
ACGGAACGGTGCCGTGTGCGGGAGAAACGGTTAGGATGACAGCAGCAACAGCCAGGCGCCAGCGAGCGA
GCGAGCGAGCCTACTGATGTGGACGATAAACACCAGAGGAACGTACGTCCCCGTTCCATCCGTCGCAGC
CCATTCGGCCAATCCTTATCCGGTCGTTAGAGTAGTTGATAGGTGTTCCGGCCGCCGGCCCAATCCTAC
CTGGTTTGACTCACGCAGTCATGGGCCTAATTGCAGCCCTTTGGTTCCAACTTACAAATTGCAATATTT
TACAACTGTTTGCATGTTTCTATTTTTTTAATAATAGAACTCATTCTGATTTTGCTTTTGAAATAAAAA
GATACAACCAATTATTATAGAGGTTGTATGATGAACGACTGTAAATTTTACAATCACAATTTATTAGAA
AACAAGAAATTTACTAAGAATACCACCTTTAAAGTATAGACTATTTTTATCTATGGTTGGCGAGAATAT
GCATAACCATTTTTTTTCTCTAACGGCTTACAAGTTCTATATAGTTCTTGTCTTAAACCGAAAACAGAA
TCTCCAAAATCAACAATTAATTATAGTTTCGATTGAATTTCACACACATCACTATAGGCGGGTAACGTC
AAACCGAAAAGAATAACACCATAGAGCAACAGAGCTGTAAATCAATGATTCGCTCAAATCTTGCTAACG
TTGTAGCCATCAAAACGACGCTAAGGACGCCCCTGAACTCTGGAATCTGAACTCGAGCGGTTTTCTCAT
GGCTCTCAGTGCCGGTGTGGGATCGTGGTTGTGTGTGTGATTGCAGAAAAAAAAAAATCCTATCCATTA
TCCATACGTTTTTCTCCACATCACTCGCCGGCCGGTCAGTGTTCGCAGTTTGCTTTGCTCAGAACTAAT
CGGAGGAGAATAAACATGTACGCAACCGATGCGAGCTTATGTGCAACCAGAGCTAACCACAGCTACCTT
CCAAAACGCGCCCGATCCAACTCTTGTCCTCGCGCGAAACCGATCAAAATCCCGGACGCCACCGCCGGC
TCTCGCGTACGTACGTGGCGACCATAACTCGCCGTTGCAGCCATCTCACTGCAGTGTACCCTGCAACAT
GCCAATTAACCTCCAAGATAAGCATCCATCGATCCACCGCTGGATCAACCGCAGCGCGCGAGAGATCGA
TCAACGTACGTAACGCCATGCACCGTAGCCACCAAGTTCCATGCATGCACCGATGCACGAGTGATCGAG
TGAGTGATCTGATCGAGTAATAAGTTGCAGGTTTCTGGACGCCATCGCTCGGTATAAATACGGCGTTTT
CTGCAGGCGAAGCAGCCAAGGCATGCATGCACATTCGCTCTAGCCTTCCTCATCGTTGACAGTGCGTGG
CAGTGTACTCTTGGTTATCATG

OsWRKY71 binding site (sense)

OsWRKY71 binding site (antisense)

ABBRE site

5´UTR

start codon

VITA

NAME	Worawat Yuenyong
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