FUNCTIONAL CHARACTERIZATION OF RICE MALATE SYNTHASE IN ARABIDOPSIS RESPONDING TO SALT STRESS

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ลักษณะสมบัติเชิงหน้าที่ของมาเลตซินเทสจากข้าวในอะราบิดอปซิสที่ตอบสนองต่อภาวะเครียดจากเกลือ

น.ส.สุภิสรา ธนบัตร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Associate Professor Dr. TEERAPONG BUABOOCHA

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สุภิสรา ธนบัตร : ลักษณะสมบัติเชิงหน้าที่ของมาเลตซินเทสจากข้าวในอะราบิดอปซิสที่ตอบสนองต่อภาวะเครียดจากเกลือ. (FUNCTIONAL CHARACTERIZATION OF RICE MALATE SYNTHASE IN ARABIDOPSIS RESPONDING TO SALT STRESS) อ.ที่ปรึกษาหลัก : รศ. ดร.ธีรพงษ์ บัวบูชา

ภาวะเครียดจากเกลือเป็นปัจจัยหลักอย่างหนึ่งที่จำกัดการเจริญเติบโตและผลผลิตของพืชเศรษฐกิจทั่วโลกโดยเฉพาะข้าว ภาวะเครียดจากเกลือส่งผลต่อพืชโดยทำให้เกิดความเครียดออสโมติกและความเป็นพิษจากไอออน ความเครียดออสโมติกส่งผลให้ความสามารถในการดูดซึมน้ำของพืชลดลง ในขณะที่ความเป็นพิษจากไอออนทำให้เกิดการขัดขวาง การสังเคราะห์แสง การสังเคราะห์โปรตีน และแอกทิวิตีของเอนไซม์ ก่อนหน้านี้ได้มีการศึกษาการแสดงออกของยีนในข้าวขาวดอกมะลิ 105 ภายใต้ภาวะเครียดจากเกลือ พบว่ายีนที่เข้ารหัสมาเลตซินเทสจากข้าว (*OsMS*) ซึ่งเป็นเอนไซม์สำคัญในวัฏจักรไกลออกซิเลต มีการแสดงออกสูงขึ้นภายใต้ภาวะเครียดจากเกลือ ดังนั้น *OsMS* อาจมีหน้าที่เกี่ยวข้องกับกลไกการทนต่อภาวะเครียดจากเกลือ ในงานวิจัยนี้ได้ถ่ายยีน *OsMS* เข้าสู่อะราบิดอปซิสมิวแทนต์ของยีน *Atms* และอะราบิดอปซิส wild type เพื่อสร้างอะราบิดอปซิสรีเวอร์แทนต์ และอะราบิดอปซิสที่มีการแสดงออกเกินปกติของยีน *OsMS* ตามลำดับ เพื่อศึกษาลักษณะสมบัติเชิงหน้าที่ของยีน *OsMS* และเข้าใจถึงความเกี่ยวข้องของยีนดังกล่าวในกลไกการทนต่อภาวะเครียดจากเกลือ ผลการทดลองแสดงว่าอะราบิดอปซิสมิวแทนต์ของยีน *Atms* มีอัตราการงอกต่ำกว่าอะราบิดอปซิส wild type และพบว่าอะราบิดอปซิสรีเวอร์แทนต์จำนวน 2 สายพันธุ์สามารถฟื้นคืนฟีโนไทป์ภายใต้ภาวะเครียดจากเกลือที่ความเข้มข้นสูง สำหรับน้ำหนักสดพบว่าอะราบิ ดอปซิสมิวแทนต์ของยีน *Atms* อายุ 5 วันที่อยู่ภายใต้ภาวะเครียดจากเกลือเป็นระยะเวลา 3 วัน และกลับสู่ภาวะปกติเป็นระยะ 5 วัน มีเปอร์เซ็นต์การลดลงของน้ำหนักสดสูงกว่าอะราบิดอปซิส wild type อะราบิดอปซิสรีเวอร์แทนต์ และอะราบิดอปซิสที่มีการแสดงออกเกินปกติของยีน *OsMS* ภายใต้ภาวะเครียดจากเกลือ ความเขียวของใบ ปริมาณคลอโรฟิลล์ และปริมาณแคโรทีนอยด์ถูกพบว่ามีปริมาณสูงในอะราบิดอปซิสมิวแทนต์ของยีน *Atms* มากกว่าในอะราบิดอปซิส wild type และอะราบิดอปซิสที่มีการแสดงออกเกินปกติของยีน *OsMS* ซึ่งผลการทดลองดังกล่าวสอดคล้องกับปริมาณคลอโรฟิลล์และปริมาณแคโรทีนอยด์ในภาวะเสื่อมตามอายุที่ถูกเหนี่ยวนำด้วยความมืด ภายใต้ภาวะดังกล่าวอะราบิดอปซิสรีเวอร์แทนต์มีปริมาณคลอโรฟิลล์และปริมาณแคโรทีนอยด์ใกล้เคียงกับอะราบิดอปซิส wild type และอะราบิดอปซิสที่มีการแสดงออกเกินปกติของยีน *OsMS* นอกจากนี้ ยีน *SAG12* ซึ่งเป็นยีนที่เกี่ยวข้องกับภาวะเสื่อมตามอายุของพืช มีการแสดงออกในอะราบิดอปซิสมิวแทนต์ของยีน *Atms* ต่ำกว่าอะราบิดอปซิส wild type อะราบิดอปซิสรีเวอร์แทนต์ และอะราบิดอปซิสที่มีการแสดงออกเกินปกติของยีน *OsMS* ดังนั้นเป็นเป็นไปได้ว่ายีน *OsMS* จึงมีบทบาทสำคัญในการเหนี่ยวนำภาวะเสื่อมอายุของใบเพื่อตอบสนองต่อภาวะเครียดจากเกลือ

สาขาวิชา	ชีวเคมีและชีววิทยาโมเลกุล	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Supisara Thanabut : FUNCTIONAL CHARACTERIZATION OF RICE MALATE SYNTHASE IN ARABIDOPSIS RESPONDING TO SALT STRESS. Advisor: Assoc. Prof. Dr. TEERAPONG BUABOOCHA

Salt stress is one of the major abiotic stresses that seriously limit plant growth and development and can lead to a significant reduction in crop yield, especially rice. Salt stress affects plants via ionic toxicity and osmotic stress. Osmotic stress results in a reduction of water absorption ability. Ionic toxicity causes Na^+ toxicity, which disrupts photosynthesis, protein synthesis, and enzyme activity. The previous study of KDML105 rice transcriptome profile and qRT-PCR revealed that rice malate synthase (*OsMS*) encoding malate synthase, which is one of the key enzymes in glyoxylate cycle was up-regulated under salt stress, so *OsMS* may involve in salt tolerance mechanisms. In this study, *OsMS* was transferred into *Atms* mutant Arabidopsis and wild type to constructed revertant Arabidopsis and *OsMS*-overexpressing Arabidopsis, respectively, in order to characterize the function of *OsMS* in response to salt stress. The results showed that *Atms* mutant Arabidopsis exhibited the lower germination rate than wild type and two lines of revertant (OX/ms4-2 and OX/ms5-2) were able to revert the phenotype under high salt treatment. For fresh weight, 5-day old *Atms* mutant Arabidopsis treated with salt stress for 3 days and recovered for 5 days had the higher reduction of fresh weight than wild type, revertants (OX/ms4-2, OX/ms5-2, and OX/ms13-3), and *OsMS*-overexpressing lines (OX/WT13). Leaf greenness, chlorophyll, and total carotenoid were found to be higher in *Atms* mutant Arabidopsis than wild type, which consistent with those during dark-induced senescence. Under dark-induced condition, revertant lines exhibited chlorophyll and carotenoid contents closer to those of wild type and OX/WT13. Moreover, expression levels of *SAG12* involving in senescence in the *Atms* mutant were lower than wild type, OX/WT13 and all revertant lines. Taken together, *OsMS* may play important roles in leaf senescence induction in response to salt stress condition.

Field of Study:	Biochemistry and Molecular Biology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....

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Supisara Thanabut

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35SCaMV	35S cauliflower mosaic virus promoter
A_{470}	absorbance at 470 nanometers
A_{646}	absorbance at 646 nanometers
A_{663}	absorbance at 663 nanometers
Aticl	mutant Arabidopsis isocitrate lyase
AtMS	Arabidopsis isocitrate lyase gene
Atms	mutant Arabidopsis malate synthase
bp	base pair
°C	degree celcius
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CO_2	carbondioxide
DNA	deoxyribonucleic acid
dS	decisiemens
g	gram
hr	hour
l	liter
L1	transgenic KDML 105 rice over-expressing *OsCam1-1* line 1
LB medium	Luria Bertani medium
m	meter
mg	milligram
min	minute
ml	milliliter

mM	millimolar
MS	malate synthase protein
MS medium	Murashige and Skoog medium
Na^+	sodium ion
NaCl	sodium chloride
NaOCl	sodium hypochlorite
OsCam1-1	rice calmudulin 1-1 gene
OsMS	rice isocitrate lyase gene
OX/*ms*	transgenic mutant Arabidopsis disrupting malate synthase gene that Express rice malate synthase gene driven by 35SCaMV promoter or revertant
OX/*ms*4-2	transgenic mutant Arabidopsis disrupting malate synthase gene that expressrice malate synthase gene driven by 35SCaMV promoter or revertant line 4-2
OX/*ms*5-2	transgenic mutant Arabidopsis disrupting malate synthase gene that Express rice malate synthase gene driven by 35SCaMV promoter or revertant line 5-2
OX/*ms*13-3	transgenic mutant Arabidopsis disrupting malate synthase gene that Express rice malate synthase gene driven by 35SCaMV promoter or revertant line 13-3
OX/WT	wild type Arabidopsis expressing rice malate synthase gene driven by 35SCaMV promoter or overexpression
OX/WT13	wild type Arabidopsis expressing rice malate synthase gene driven by 35SCaMV promoter or overexpression line 13
PCR	polymerase chain reaction

pH	power of hydrogen ion
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RID	refrection index detector
RNA	ribonucleic acid
rpm	round per minute
µg	microgram
µl	microliter
v/v	volume by volume
WT	wild type
w/v	weight by volume
χ^2	chi-square
Xg	times gravity

CHAPTER I
INTRODUCTION

Salt stress is the abiotic stress that limit crop production, especially rice, which is the most widely consumed staple food for human population. Breeding for salt stress tolerance in plants should be given priority because it is one of an important approach. Thereby, functional characterization of salt-responsive gene is one of the necessary knowledge of molecular mechanisms for abiotic stress tolerance and these data can be used for applying in the breeding program.

A previous research revealed that the expression level malate synthase (MS) in rice was enhanced under salt stress. Malate synthase plays a role in converting lipids to sugar and previous report showed that sugar metabolism is the main affected biochemical pathway in plant during salt stress. From this information, MS was hypothesized to involve in salt tolerance mechanism. So, this study aims to investigate the role of malate synthase in response to salt stress using Arabidopsis as a model plant.

1.1 Rice

Rice is the important staple food for over half of the human population. More than 700 million tons rice are produced (Approximately 480 million tons of milled rice) in more than a hundred countries (Muthayya, Sugimoto, Montgomery, & Maberly, 2014). Asia accounts for 90% of global rice production (Figure 1, 2), especially in China and India, the highest rice productive countries (Figure 3). In addition, rice is also harvested from other countries in Asia including Thailand, Indonesia, Bangladesh, Vietnam, Myanmar, Philippines, Cambodia, Japan, Pakistan, Sri Lanka, Nepal and the Republic of Korea.

There are two cultivated rice species that important for human nutrition including *Oryza sativa*, which were grown worldwide, and *Oryza glaberrima*, which known as African rice (Manful & Graham-Acquaah, 2016). *Oryza sativa* contains two major subspecies, japonica which is the sticky and short grained rice, and indica which is the non-sticky and long-grained rice, in which each subspecies has many cultivars. In Thailand, Khao Dawk Mali 105 (KDML105) is a very popular rice with aromatic scent. KDML105 is well-grown in Thailand and it makes Thai rice an export product known worldwide. It has been developed from White Dawk Mali rice, which is the native rice

found and known in Bangkhla district, Chachoengsao province in 1950-1951 (OECD, 1999)

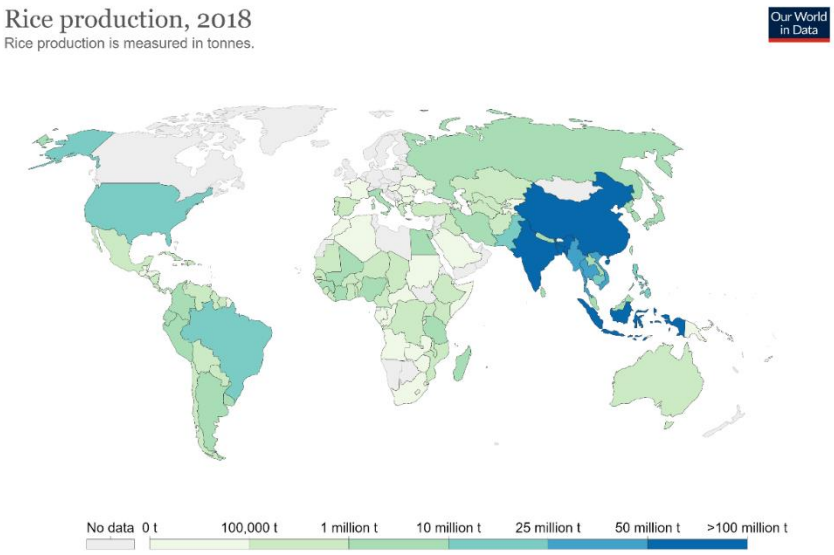


Figure 1 Wolrd rice production in 2018 (Source : https://ourworldindata.org/grapher/rice-production)

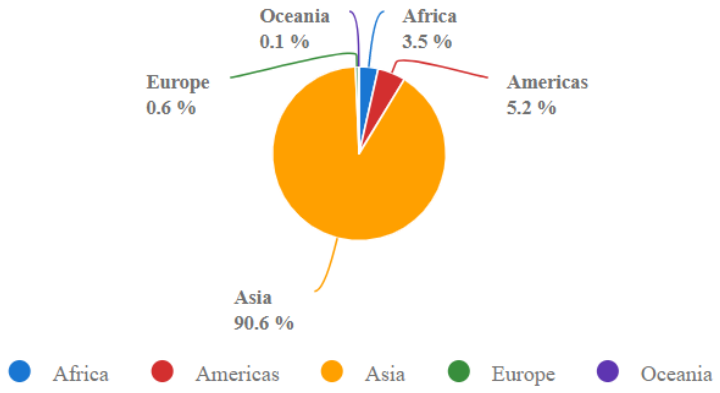


Figure 2 Proportion of rice production in the world (Source : http://www.fao.org/faostat/en/#data/QC/visualize)

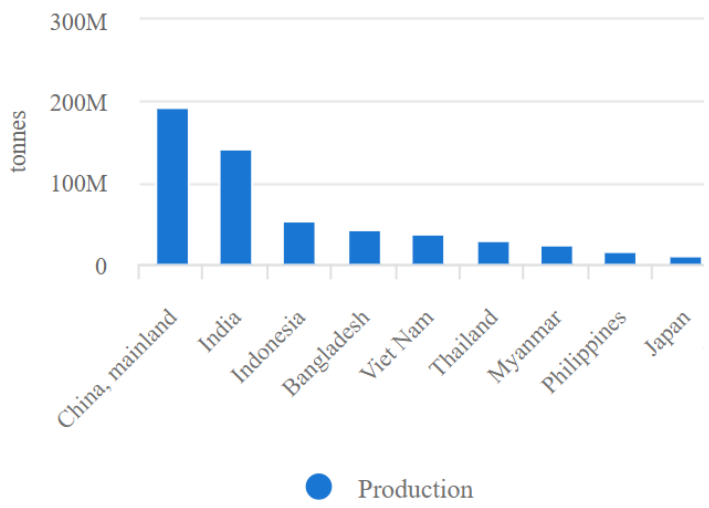


Figure 3 Rice production in Asia (Source : http://www.fao.org/faostat/en/#data/QC/visualize)

1.2 Salt stress and stress response in plants

Salt stress is one of the major abiotic stresses affecting crop production globally. The previous report revealed that salt stress widely spreads as about 20% of the world's cultivated land (Shrivastava & Kumar, 2015). Salt stress cause the osmotic stress, which results in a reduction of water absorption ability, cell elongation, leaf development, and ionic toxicity due to high Na^+ concentration affecting on P, K, Zn, Fe, Ca, and Mn in crop (García, Lucena, Romera, Alcántara, & Pérez-Vicente, 2010; Jung, Shin, & Schachtman, 2009; Rahman et al., 2017). Ionic toxicity also disrupts many processes in plants including photosynthesis, protein synthesis, and enzyme activity (Figure 4) (Horie, Karahara, & Katsuhara, 2012; Läuchli & Grattan, 2007).

From previous study, Hakim et al. (2014) treated eight rice varieties including IR20, Pokkali, MR33, MR52, MR211, MR219, MR232, and BRR1 with different level of salt stress, 4, 8, and 12 dS m^{-1} and determined the chlorophyll content and grain yield. They found that the total chlorophyll content and grain yield of eight rice varieties were decreased and were mostly influenced by the salinity level of 12 dS m^{-1} (Table 1, 2, and 3). In addition, Zahra (2011) have shown that the germination percentage of maize was a linear decrease due to the application of different levels (0, 60, 120, 180 and 240 mM) of applied sodium chloride (NaCl) salt (Figure 5) (Zahra, 2011).

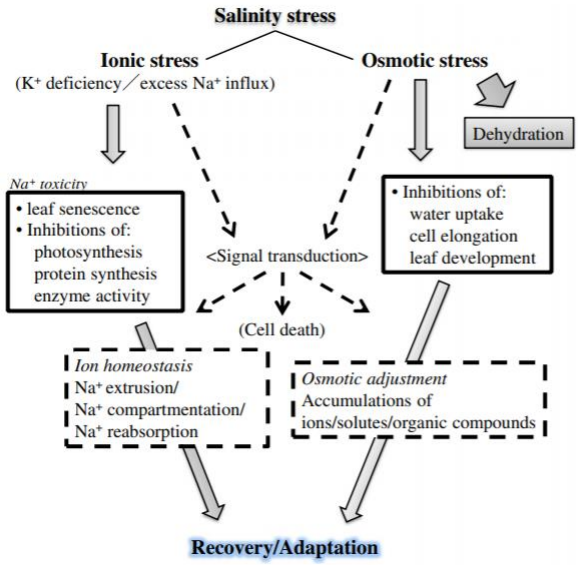


Figure 4 The effects of salinity stress and correspondind response of plants (Taken from (Horie et al., 2012))

Table 1 Effect of salinity on chlorophyll-a content of eight rice varieties (Taken from (Hakim et al., 2014))

Varities	Salinity levels (dS m^{-1})			
	0	4	8	12
IR20	5.85^{a} (100)	4.15^{ab} (71)	2.30^{cd} (39)	1.37^{c} (23)
Pokkali	5.80^{a} (100)	4.65^{a} (81)	3.02^{a} (52)	2.07^{a} (36)
MR33	4.88^{c} (100)	3.44^{cd} (71)	2.42^{cd} (50)	1.52^{bc} (31)
MR52	4.83^{c} (100)	3.19^{d} (66)	2.61^{bc} (54)	1.68^{b} (35)
MR211	5.10^{bc} (100)	4.18^{ab} (82)	2.92^{ab} (57)	2.09^{a} (41)
MR219	5.61^{ab} (100)	4.04^{ab} (72)	2.15^{d} (38)	1.25^{c} (22)
MR232	4.90^{c} (100)	4.13^{ab} (85)	2.95^{ab} (60)	2.07^{a} (42)
BRRI dhan29	5.66^{a} (100)	4.00^{bc} (71)	2.23^{d} (40)	1.27^{c} (23)

Means within columns with the same letters are not significantly different (LSD, $p \leq 0.05$).

Values within parenthesis indicate percent relative to the control.

Table 2 Effect of salinity on chlorophyll-b content of eight rice varieties (Taken from (Hakim et al., 2014))

Varities	Salinity levels (dS m^{-1})			
	0	4	8	12
IR20	10.49^{a} (100)	8.53^{abc} (81)	6.24^{c} (59)	3.85^{d} (37)
Pokkali	9.78^{abc} (100)	9.11^{ab} (93)	7.87^{a} (77)	5.93^{a} (61)
MR33	8.85^{bc} (100)	8.59^{abc} (97)	7.08^{b} (80)	5.04^{b} (57)
MR52	8.26^{c} (100)	7.28^{b} (89)	6.01^{c} (73)	4.05^{b}c (49)
MR211	9.85^{ab} (100)	9.44^{a} (96)	8.52^{a} (87)	5.96^{a} (61)
MR219	10.28^{ab} (100)	8.98^{abc} (88)	6.41^{bc} (62)	3.81^{d} (36)
MR232	9.64^{ab} (100)	9.24^{a} (96)	7.95^{ab} (83)	6.12^{a} (64)
BRRI dhan29	9.55^{abc} (100)	7.54^{b} (79)	6.0^{c} (63)	3.73^{d} (39)

Means within columns with the same letters are not significantly different (LSD, $p \leq 0.05$).

Values within parenthesis indicate percent relative to the control.

Table 3 Effect of salinity on the grain yield of eight rice varieties (Taken from (Hakim et al., 2014))

Varities	Salinity levels (dS m^{-1})			
	0 (control)	4	8	12
IR20	14.65^{cd}	5.24^{d} (36)	0.0^{e}	0.0^{c}
Pokkali	11.93^{d}	0.29^{c} (86)	7.05^{c} (60)	3.31^{b} (28)
MR33	19.44^{ab}	12.37^{bc} (64)	5.64^{cd} (29)	0.0^{c}
MR52	18.59^{ab}	13.36^{b} (72)	5.73^{cd} (31)	0.0^{c}
MR211	18.85^{ab}	17.00^{a} (90)	10.36^{b} (55)	4.83^{a} (26)
MR219	21.48^{a}	10.00^{c} (47)	4.67^{d} (22)	0.0^{c}
MR232	20.10^{ab}	18.07^{a} (90)	12.42^{a} (62)	5.08^{a} (25)
BRRI dhan29	17.26^{bc}	6.72^{d} (59)	0.0^{e}	0.0^{c}

Means within columns with the same letters are not significantly different (LSD, $p \leq 0.05$).

Values within parenthesis indicate percent relative to the control.

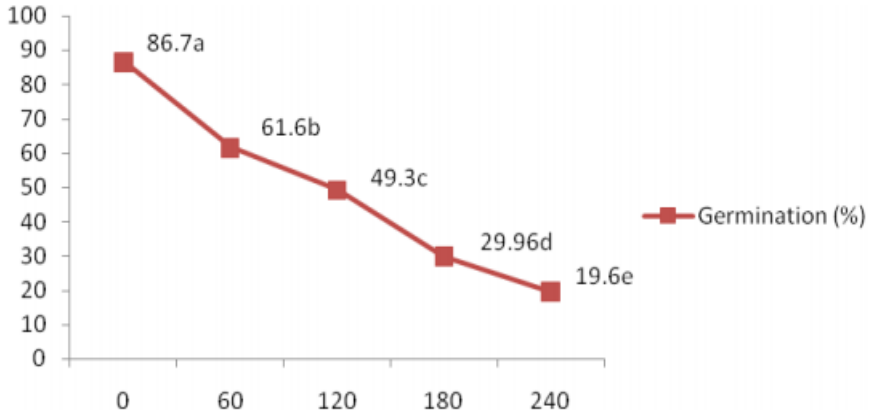


Figure 5 Germination percentage of maize under different salinity stress levels (Taken from (Zahra, 2011))

Under environmental stress, plant has many ways to response to salt stress such as physiological responses, molecular responses, and biochemical responses (Figure 6) (Mbarki et al., 2018). For physiological responses, salt stress induces various physiological responses such as decrease of germination rates, loss of turgor pressure, decrease of leaf water potential, decrease of internal CO_2 concentration, decreased in stomatal conductance, photosynthesis slow down, and lower growth rates. Moradi and Ismail investigated the physiological response to salt stress in different rice cultivars contrasting in salt stress tolerance during vegetative and reproductive stages. The results revealed that CO_2 fixation, stomatal conductance (g_s) and transpiration decreased in salt sensitive cultivar, compared to salt tolerant cultivars. In addition, salt tolerant cultivars had more responsive stomata that tended to close faster than salt sensitive cultivar (Moradi & Ismail, 2007). In another study, the researcher demonstrated the effect of salt stress on internal water relations in bean plants. The results reported that the osmotic potential decreased with the increase in concentrations of stress (Abdul Qados, 2011).

For molecular responses, salt stress induced various molecular responses of plant such as stress-induced gene expression, activation of abscisic acid (ABA) responsive gene, and production of proteins (Mbarki et al., 2018). Saeng-ngam, Takpirom, Buaboocha, and Chadchawan (2012) studied the involvement of the calmodulin gene, *OsCam1*-1, which is salt-inducible gene, in ABA biosynthesis during salt stress. They revealed that ABA levels were increase in KDML105 rice overexpressing of *OsCam1*-1 gene leading. In another study, Zhang, Long, Huang, and Xia (2020)

analyzed the gene expression in rice under salt stress. They found that *OsNAC5* gene expression was correlated with the concentration of NaCl. In addition, they found that *OsNAC45* gene regulates the expression of gene that involve in ABA-dependent signal pathway, *OsDREB1F* gene.

For biochemical alteration to response the stress, plant increases the different categories of several compatible organic solutes and ammonium compounds, including proline, betaine, glycine betaine, trehalose, polyols, soluble sugars, pipecolate betaine and hydroxy proline betaine (Abd El-Samad & Shaddad, 2010; Abdel Latef & Miransari, 2014) for adjust the osmotic potential, and stabilization of proteins and enzymes (Abdel Latef & Chaoxing, 2014). In addition, salt stress causes the decrease of rubisco activity, and the increased accumulation of antioxidative enzymes such as superoxide dismutase (SOD), catalases (CAT), ascorbate peroxidase (APX), peroxidase (POD), guaiacol peroxidase (GPX), glutathione reductase (GR), which can help mitigate the damage caused by the reactive oxygen species (ROS) (Mbarki et al., 2018). Kumar et al. (2021) determined the concentration of osmolytes and antioxidant molecules in salt tolerant water dropwort (*Oenanthe javanica*) compared to salt sensitive cultivar. They found that the proline concentration, protein content in roots, and glutathione (GSH) content were increased with the increasing NaCl treatment, and were found higher in salt tolerant cultivar compared to salt sensitive cultivar. Azevedo Neto, Prisco, Enéas-Filho, Abreu, and Gomes-Filho (2006) studied the effects of salt stress on antioxidative enzymes in maize. They revealed that SOD, APX, GPX, and GR activities in salt-treated maize were increased when compared to the controls. The enzyme activities was higher in the salt tolerant maize than in the salt sensitive maize. Moreover, salt stress had no effect on CAT activity in the salt tolerant maize while it was decreased in salt sensitive maize. They suggested that the maintenance and/or increase of antioxidative enzymes activities may be an important biochemical trait for salt tolerance mechanism. In another study, Ren and co-workers Ren, Lyle, Jiang, and Penumala (2016) generated the transgenic Arabidopsis expressing *GmST1*, soybean salt tolerance 1, and examined H_2O_2 production during salt stress. They revealed that the *GmST1*-overexpressed Arabidopsis decreased ROS under salt stress.

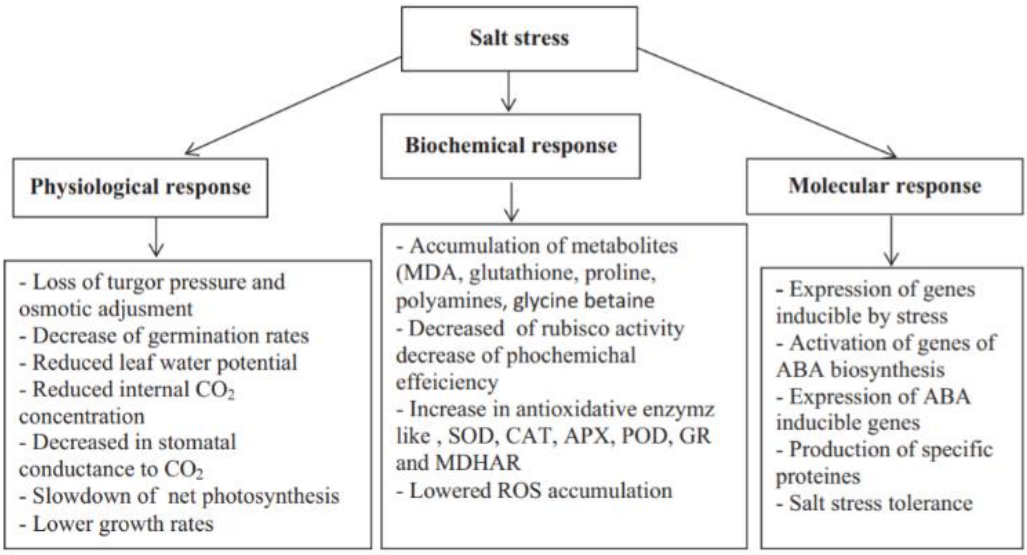


Figure 6 Plant physiological, biochemical, and molecular response to salt stress (Taken from (Mbarki et al., 2018))

1.3 Malate synthase

Malate synthase (*MS*) is a single-copy gene in Arabidopsis (Thorneycroft, Sherson, & Smith, 2001) encoding malate synthase (EC 4.1.3.2), which is the key enzyme in glyoxylate cycle, a variation of the tricarboxylic acid cycle (TCA), and plays a role in converting lipid to sugar by using acetyl CoA from β-oxidation to produce the substrate for gluconeogenesis (Figure 7) (Dunn, Ramírez-Trujillo, & Hernández-Lucas, 2009). Yuenyong, Chinpongpanich, Comai, Chadchawan, and Buaboocha (2018) studied the gene expression of Khao Dawk Mali105 (KDML105) rice by transcriptome analysis, they found that *MS* (LOC_Os04g40990) was up-regulated under salt stress (Figure 8). They verified the reliability of the transcriptome data by qRT-PCR, in which the results showed higher levels of *MS* under salt stress, demonstrating a statistically significant difference under this condition (Figure 9). They also demonstrated that *MS* was up-regulated in the transgenic rice overexpressing *OsCam1-1*, calmodulin gene, under salt stress when compared with the wild type (Figure 8 and 9).

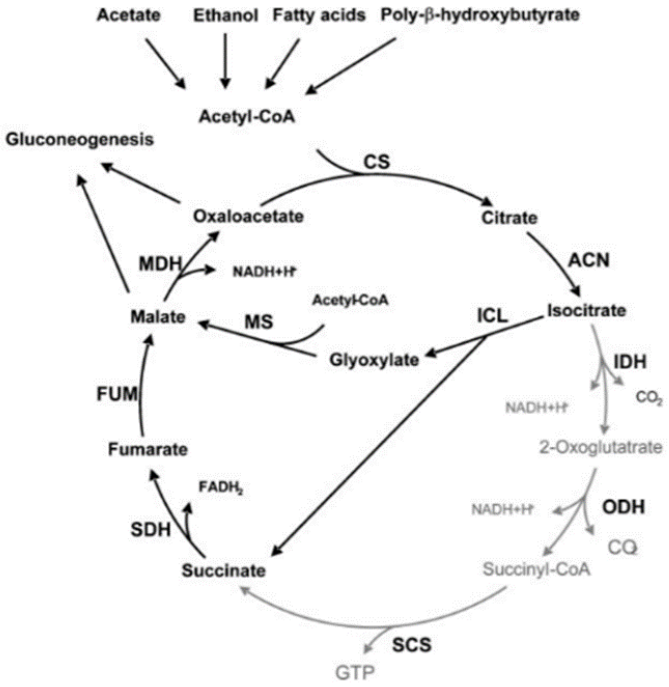


Figure 7 Enzymatic reactions of the glyoxylate (black arrows) and TCA (gray arrows) cycles. Abbreviations: CS, citrate synthase; ICL, isocitrate lyase; MS, malate synthase; MDH, malate dehydrogenase; IDH, isocitrate dehydrogenase; ACN, aconitase; ODH, 2-oxoglutarate dehydrogenase; SCS, succinyl-CoA synthetase; SDH, succinate dehydrogenase; FUM, fumarase; (Taken from (Dunn et al., 2009))

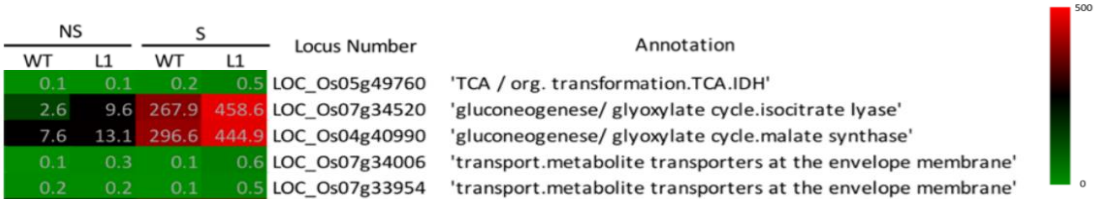


Figure 8 Expression levels of malate synthase in wild type (WT) and transgenic (L1) KDML105 rice overexpressing *OsCaM1-1* under normal (NS) and salt stress (S) conditions, presented as heat map (Taken from (Yuenyong et al., 2018))

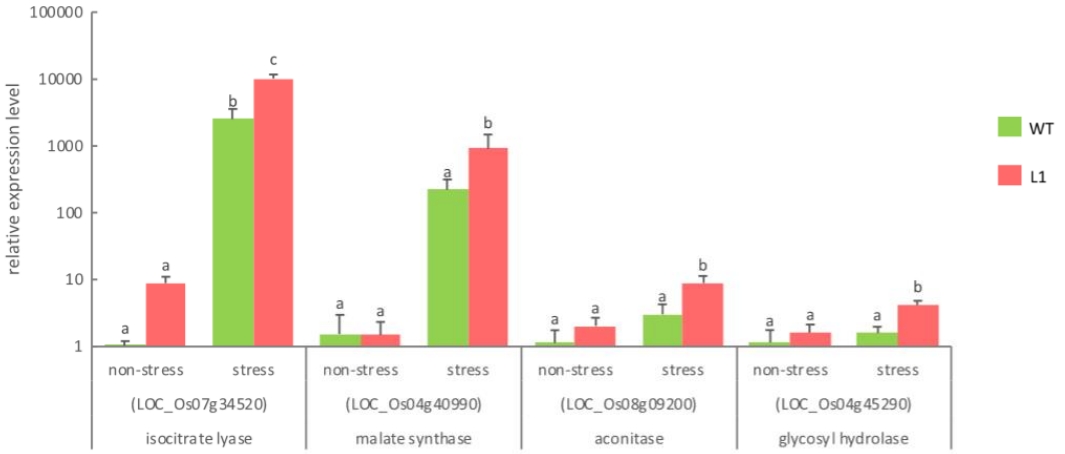


Figure 9 qRT-PCR confirming expression level of malate synthase in wild type (WT; green bars) and transgenic (L1; red bars) KDML105 rice overexpressing *OsCaM1-1* under non-stress and salt stress conditions (Taken from (Yuenyong et al., 2018))

In previous research, *Ricinus communis* malate synthase gene (*RcMLS)* was overexpressed in Arabidopsis. Transgenic Arabidopsis overexpressing *RcMLS* was phenotypically characterized by examining the seed germination under abiotic stress. The researcher found that overexpression of *RcMLS* enhanced seed germination under high temperature at 35 °C and salt stress at 75 mM NaCl comparing to the wild type Arabidopsis (Col-0) (Figure 10). Metabolomics analysis showed the high levels of accumulation of methionine, isoleucine, glucose, fructose, and sucrose in the transgenic Arabidopsis overexpressing *RcMLS* under abiotic stress. They suggested that *RcMLS* has modulated the glyoxylate cycle and gluconeogenesis pathway to maintain cellular homeostasis under abiotic stress (Brito et al., 2020).

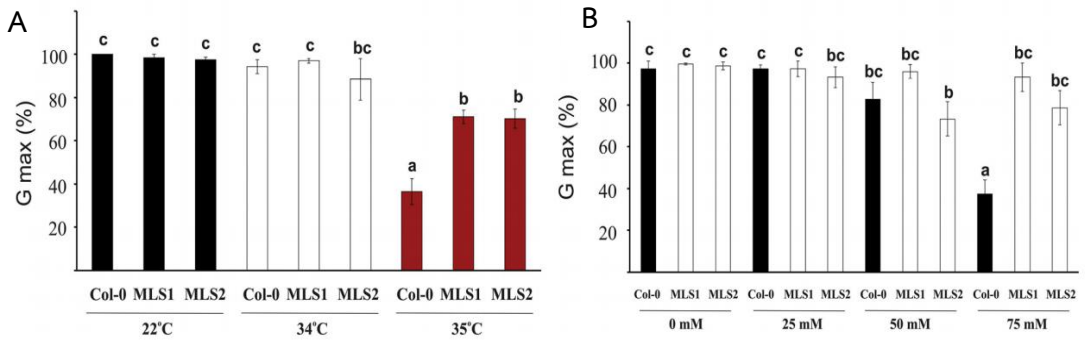


Figure 10 Maximum germination percentage of *Arabidopsis thaliana* seeds overexpressing *Ricinus communis* malate synthase under (A) high temperature, black bars represent temperature at 22 °C, white bars represent temperature at 34 °C, and red bars represent temperature at 35 °C, and (B) salt stress, black bars represent *Arabidopsis thaliana* Col-0 seeds, and white bars represent *Arabidopsis thaliana* seeds overexpressing *RcMLS* (Taken from (Brito et al., 2020))

In addition, Eastmond et al. (2000) studied the role of glyoxylate cycle in postgerminative growth of Arabidopsis and found that glyoxylate cycle is important during the post-germination in Arabidopsis. Arabidopsis plants were grown and kept in the dark treatment before transferred to the light. They found that seedling survival and recovery upon transfer to light are severely compromised in the *Aticl* Arabidopsis mutants, which lacked the isocitrate lyase, the key enzyme in glyoxylate cycle. They also found that the rate of lipid breakdown in *Aticl* Arabidopsis mutants were decreased in the absence of sugars whereas the rate of lipid breakdown was similar to that of wild type in the presence of sugars.

1.4 Sugar metabolism in plant under abiotic stress

Richter, Erban, Kopka, and Zörb (2015) analyzed the sugar concentration change in salt resistant maize (SR08) shoots compared to salt sensitive maize (Logo). They found that the concentrations of glucose, sucrose, fructose, and galactose in the salt resistant maize (SR08) were higher than the salt sensitive maize (Logo) and mono- and disaccharides increased at the salt stress level of 50 mM NaCl (Figure 11). They suggested that an increase in soluble sugars could have an osmolytic effect together with other compatible solutes, which are involved in stress signaling while regulating

genes concerned with salt-resistance mechanisms. This revealed that sugar metabolism was the main biochemical pathway in maize during salt stress.

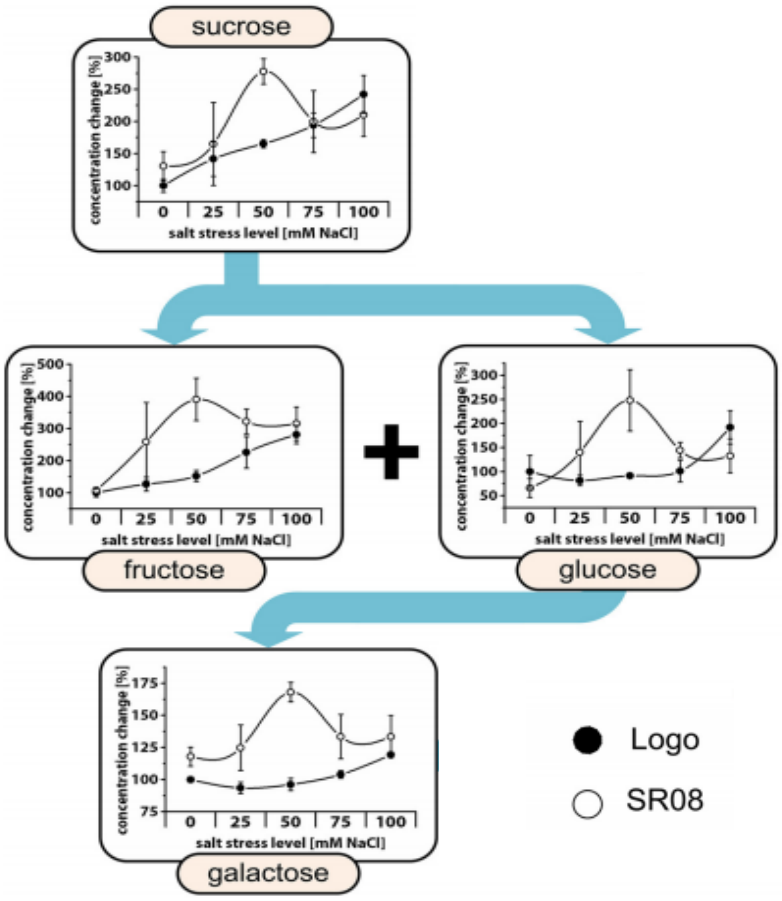


Figure 11 Concentration of sucrose, fructose, glucose and galactose in shoots of the two maize hybrids Logo (black dots) and SR08 (white dots) with increasing salt stress levels (Modified from (Richter et al., 2015))

1.5 Senescence in plants

Senescence is the important process in the life cycle of an organism. It occurs at many stages during the development of an organism and at many levels (Leopold, 1961). Leaf senescence is thought to have evolved to allow remobilization of nutrients and other molecules to younger tissues (Nam, 1997). Salt stress is one of the environmental factors that can modulate leaf senescence (Guo & Gan, 2005). Riyazuddin et al. (2020) have shown a schematic of gene regulation under salt stress.

After salt stress, MAPKs are activated and the phosphorylated ethylene biosynthesis genes enhances the ethylene production. Then, the downstream signaling activates the transcription of ethylene-responsive genes leading to an increase of senescence-associated genes (*SAG*s). Also, abscisic acid (ABA), which is induced by salt stress (Jia, Wang, Zhang, & Zhang, 2002), induces leaf senescence and ABA signaling transcription factors are reportedly involved in leaf senescence (Ueda et al., 2020).

Leaf senescence can also be induced by the darkness (Weaver & Amasino, 2001). Liebsch and Keech (2016) have shown the molecular regulation during dark-induced senescence as shown in Figure 12. PHYTOCHROME-INTERACTING FACTORS (PIFs) are major players regulating light deprivation-induced senescence. In darkness, PIFs activate ethylene biosynthesis genes (*ACS*s) and the transcription factors, *ETHYLENE INSENSITIVE 3* (*EIN3*), *ENHANCED EM LEVELS* (*EEL*) and *ABSCISIC ACID INSENSITIVE 5* (*ABI5*). Then, PIFs, *EIN3, ABI5*, and *EEL* activate the expression of the senescence regulator *ORESARA1* (*ORE1*). Finally, *ORE1*, together with *PIFs, ABI5* and *EIN3*, up-regulates the chlorophyll degradation genes. *ORE1* also activates senescence-associate genes (*SAG*s). In addition, PIFs activates *WRKY22*, *NAP*, and other senescence regulators.

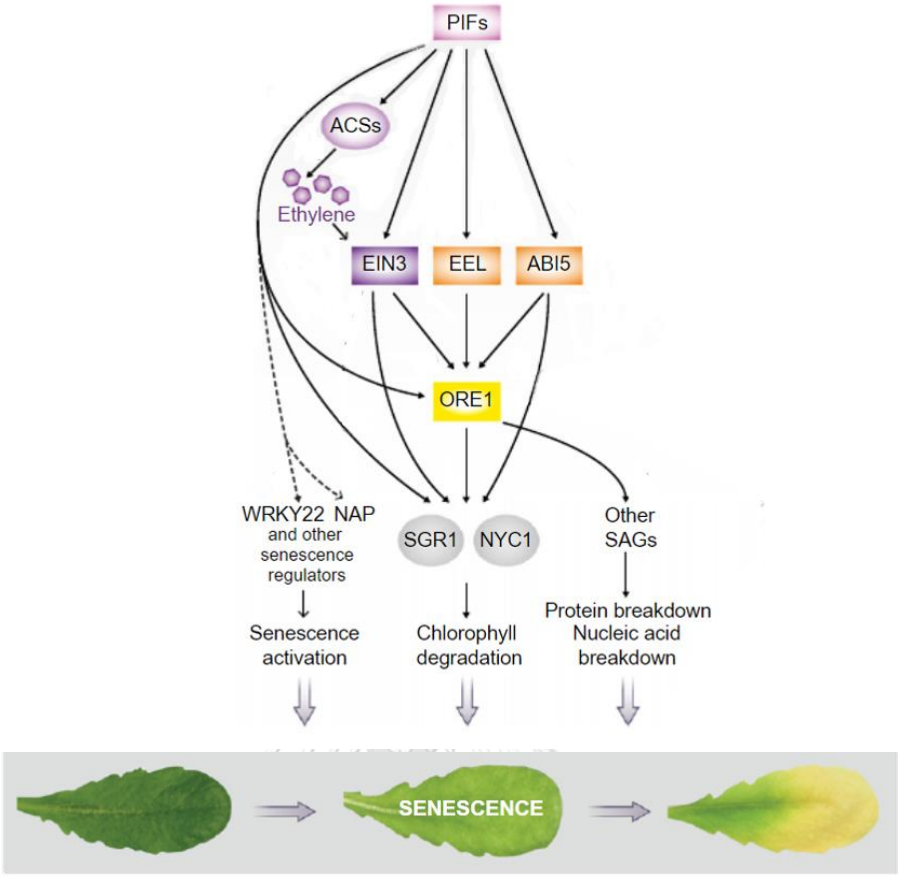


Figure 12 The molecular regulatory network during light deprivation-induced leaf senescence. Abbreviations: PIFs, PHYTOCHROME-INTERACTING FACTORS ; *ACS*s, 1-aminocyclopropane-1-carboxylate synthase; *EIN3*, *ETHYLENE INSENSITIVE 3*; *EEL*, *ENHANCED EM LEVELS*; *ABI5*, *ABSCISIC ACID INSENSITIVE 5*; *ORE1*, *ORESARA1*; *SGR*, *STAY-GREEN 1*; *NYC1*, *NON-YELLOW COLORING 1*; *SAG*s, Senescence-associated genes (Modified from (Liebsch & Keech, 2016))

1.6 Objectives of this study

To characterize the function of malate synthase from rice in revertant Arabidopsis and *OsMS*-overexpressing Arabidopsis responding to salt stress.

CHAPTER II
MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plants materials

Arabidopsis thaliana ecotype Columbia (Col-0)

Atms (malate synthase) *Arabidopsis thaliana* (SALK_002289) mutant

2.1.2 Microorganisms

E. coli TOP10 stain

Agrobacterium tumefaciens GV3101

2.1.3 DNA materials

OsMS cDNA clone (LOC_Os04g40990) (AK241214) (Genetic Resources Center, Naro Japan)

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

pGWB2 (Nakagawa Lab Plant Molecular Genetics, Japan)

DNA primers (synthesized by Bionics, South Korea)

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

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

2.1.4 Enzymes and Kits

2XTaq master mix (Vivantis, Malaysia)

5X iScript™ Reverse Transcription Supermix (Bio-Rad, USA)

DNase I (Thermo Fisher Scientific, USA)

LR Clonase II (Invitrogen, USA)

Luna® Universal qPCR Master Mix (New England Biolabs, UK)

Plant Genomic DNA Mini Kit (Geneaid, Taiwan)

RNeasy® Plant Mini Kit (QIAGEN, Germany)

Taq DNA polymerase (Thermo Fisher Scientific, USA)

TIANgel Midi Purification Kit (Tiangen, China)

TIANgel Midi Purification Kit (Tiangen, China)

2.1.5 Chemicals

5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich, USA)

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

Acetyl-CoA (Sigma-Aldrich, USA)

Agarose (Vivantis, Malaysia)

Agar (Himedia, India)

Bradford 5X (Bio-Rad, USA)

Bovine serum albumin (BSA) (Sigma-Aldrich, USA)

Coenzyme A hydrate (Sigma-Aldrich, USA)

Dipotassium hydrogen phosphate (K_2HPO4) (Carlo Erba Reagent, France)

DL-Dithiothreitol (DTT) (Vivantis, Malaysia)

Ethanol (QRëC, New Zealand and Fulltime, China)

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

Fructose (Carlo Erba Reagent, France)

Gentamycin (Bio Basic Canada Inc., Canada)

Glucose (Carlo Erba Reagent, France)

Hydroxyethyl piperazineethanesulfonic acid (HEPES) (Sigma-Aldrich, USA)

Hygromycin B (TOKU-E, USA)

Kanamycin (Bio Basic Canada Inc., Canada)

Liquid nitrogen (Linde, Ireland)

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

Methanol (Fulltime, China)

Murashige & Skoog basal medium with vitamins (MS) (PhytoTechnology Laboratories®, USA)

Potassium dihydrogen phosphate (KH_2PO4) (Carlo Erba Reagent, France)

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

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

Rifampicin (Bio Basic Canada Inc., Canada)

Silwet (PhytoTechnology Laboratories®, USA)

Sodium chloride (NaCl) (KEMAUS, Australia)

Sodium glyoxlate monohydrate ($HC(O)COONa·H_2O$) (Sigma-Aldrich, USA)

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

Sodium hypochlorite (NaOCl) (Haiter, Thailand)

Sucrose (Carlo Erba Reagent, France)

Tryptone (Himedia, India)

Tween20 (Fluka Chemika, Switzerland)

Tryptone (Himedia, India)

2.1.6 Instrument, glassware and plasticware

-80 °C utralow temperature freezer (New Brunswick Scientific, UK)

-20 °C freezer (Hitachi, Japan)

0.2 ml PCR tube (Axygen, USA)

1 ml syringe (Nipro, Japan)

1.5 ml microtube (Axygen, USA)

15 ml plastic centrifuge tube (Nest®, USA)

2 ml clear glass vial (Vertical Chromatography Co., Ltd., Thailand)

2 ml microtube (Axygen, USA)

250 µl glass insert vial (Agilent Technologies, USA)

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

50 ml plastic centrifuge tube (Nest®, USA)

60 °C oven (Memmert, Germany)

96 well plate (Corning, USA)

9 mm rib-sided open-top cap, PP (Vertical Chromatography Co., Ltd., Thailand)

Agilent Hi-Plex Ca (Duo) ligand exchange column (Agilent Technologies, USA)

Alcohol burner

Amersham™ Imager (GE Healthcare Life Sciences, Sweden)

Autoclave (Sanyo, Japan)

Beaker

Biological Safety Carbinet (Haier Biomedical, China)

C-MAG HS 7 magnetic stirrers (IKA®, USA)

CentriVap benchtop vacuum concentrators (Lanconco, USA)

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

Chlorophyll meter SPAD-502 Plus (Konica Minolta, Japan)

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

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

Disposable plastic pipette tip

Duran bottle (Duran, Germany)

FE20 FiveEasy™ benchtop pH meter (Mettler Toledo™, USA)

Flasks

Forceps

Fume Hood

Gel Documentation (Syngene, UK)

Glass spreader

Grinding balls stainless steel 2mm Ø (Retsch, Germany)

Innova 4000 Benchtop Incubator Shaker (Eppendorf, Germany)

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

Micro-Centrifuge (Hettich, Germany)

MicroPulser™ Electroporator (Bio-Rad, USA)

Mixer Mill MM 400 (Retsch, Germany)

MUPID-exU Horizontal Electrophoresis System (Mupid, Japan)

P100 µl pipette (Gilson, France)

P1000 pipette (Gilson, France)

P2 pipette (Rainin, USA)

P20 pipette (Gilson, France)

P200 pipette (Gilson, France)

Plastic petri dish

Plastic pipette tip (Axygen, USA)

Refrigerated centrifuge 5418 R (Eppendorf, Germany)

Refrigerated centrifuge 5804 R (Eppendorf, Germany)

Refrigerated centrifuge Sorvall Legend XTR (Thermo Fisher, USA)

Spectrophotometer (Eppendorf, Germany)

Synergy H1 microplate reader (BioTek®, USA)

Thermal camera (FLIR, USA)

Thermal Cycler T100™ (Bio-Rad, USA)

UV-gel pad (Thermo Fisher, USA)

Volumetric cylinder

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

Whatman® No.1 filter papers (Whatman International Ltd., UK)

2.1.7 DNA sequencing

Plasmid and PCR product DNA were sequenced by Bionics, South Korea

2.1.8 Planting materials

Peat moss

Perlite

2.2 METHODS

2.2.1 Arabidopsis cultivation and seeds harvesting

Arabidopsis were cultivated on half-strength Murashige and Skoog medium (MS) with 1% (w/v) (Appendix) sucrose under sterile condition. For salt stress condition, NaCl was added into the medium at the desired concentration. Arabidopsis seeds were decontaminated with 70% ethanol and soaking in 2% NaOCl diluted with sterilized water with a few drops of Tween 20 for 10 minutes. Then, the seeds were rinsed with sterilized water 4-5 times and sown on MS medium plate. The plates with seeds were incubated at 4 °C under dark condition for 3 days to break dormancy, then the plates were moved to the growth room at 25 °C under 16-hr/8-hr light/dark cycle for 1 week. The Arabidopsis plants were moved to pots containing 3:1 ratio of peat moss : perlite and watering every 2-3 days until the plants were matured. The seeds were harvested and stored at 4 °C.

2.2.2 The recombinant plasmid construction

OsMS was amplified from the OsMS cDNA clone using 2X *Taq* master mix with CACC_*OsMS*_F (5′ CACCATGGCCACCAACGCCGCAG 3′) and *OsMS*_R (5′ TCAGAGCTTGCACGGCGA 3′) primers. Then, *OsMS* fragments, containing “CACC” at the 5′ end, were directionally inserted to pENTR/D-TOPO vector using pENTR™/D-TOPO® cloning kit and TOP10 *E.coli* cells were transformed with the recombinant plasmids by heat shock method and the transformants were recovered by incubating in LB broth and shaking at 37 °C for 1 hr. The transformants were selected on LB agar with 50 µg/ml kanamycin and incubated at 37 °C overnight. After that, colony PCR was performed using *Taq* DNA polymerase with M13_F (5′ TGTAAAACGACGGCCAGT 3′) and *OsMS*_R (5′ TCAGAGCTTGCACGGCGA 3′) primers. The expected size of the PCR product was around 1,700 bp. Then, the clones were cultured in LB broth with the same antibiotic used in the previous step and the cells were collected for plasmid isolation.

Gateway cloning was performed to transfer the *OsMS* gene from the *OsMS*-pENTR/D-TOPO recombinant plasmid to pGWB2 vector, a destination vector driven by a 35SCaMV promoter, using LR clonase II. Then, TOP10 *E.coli* cells were transformed with the *OsMS*-pGWB2 recombinant plasmid and the transformants were selected on LB agar with 50 µg/ml kanamycin and 50 µg/ml hygromycin. Colony PCR was performed with 35S_F (5′ CTATCCTTCGCAAGACCCTTC 3′) and *OsMS*_R (5′ TCAGAGCTTGCACGGCGA 3′) primers. The PCR product size should be around 1,700 bp and the clones were cultured in LB broth containing 50 µg/ml kanamycin and 50 µg/ml hygromycin. Then, the cells were collected for plasmid isolation. The nucleotide sequence of the purified recombinant plasmid was determined and this recombinant plasmid would be used for transforming Arabidopsis.

2.2.3 *Agrobacterium*-mediated transformation (floral dipping)

Agrobacterium tumefaciens GV3101 was transformed with the recombinant plasmid from 2.2.2 by electroporation and the transformed cells were recovered in LB broth at 30 °C for 4 hr. Then, the transformants were selected on LB agar with 50 µg/ml kanamycin, 50 µg/ml hygromycin, 50 µg/ml rifampicin and 50 µg/ml gentamicin and incubated at 30 °C for 36-48 hr. After that, colony PCR was performed using *Taq* DNA polymerase and the clones were cultured in LB broth containing 50 µg/ml kanamycin, 50 µg/ml hygromycin, 50 µg/ml rifampicin and 50 µg/ml gentamicin. The cells were collected as the stock cultures by mixing with glycerol and stored at -80 °C

The wild type and *Atms* mutant Arabidopsis plants were grown until reaching the flowering stage. Any bloomed flowers or siliques must be eliminated before transforming with Agrobacterium to minimize the amount of non-transformant seeds.

The floral dipping protocol was modified from Clough and Bent (1998), the *Agrobacterium* stock culture carrying the interesting gene on the binary vector was cultured in LB broth with the same antibiotics and concentration used in the previous step and shaking at 30 °C overnight for using as the inoculum. The 1% (v/v) inoculum was add to the 400 ml LB broth with the same antibiotics and concentration and shaking at 30 °C for 18-24 hr. The cells were collected by centrifuged at 4,000xg for 15 min at 25 °C then resuspended with fresh 100 ml transformation buffer (Appendix). The Arabidopsis flowers (not bloomed) were soaked in *Agrobacterium* transformation mixture for 10-15 min and kept in the opaque box containing water under dark condition for 24 hr. Then, the transformed plants were moved to the normal growth

until the plants were matured and their seeds (T_1 generation) were separately harvested for selection.

2.2.4 Homozygous transgenic Arabidopsis selection

The transformed Arabidopsis seeds (T_1 generation) from 2.2.3 were grown on MS medium with 50 µg/ml hygromycin for selecting the transgenic Arabidopsis, which included;

- *Atms* Arabidopsis mutant lines carrying the *OsMS*-pGWB2 (OX/ms) cassette,
- wild type Arabidopsis lines carrying the *OsMS*-pGWB2 (OX/WT) cassette

Then, the resistant plants, which have the green expanded cotyledons and long hypocotyls, were picked and grown following step 1. A rosette leaf of 2-3 weeks old Arabidopsis was collected to confirm the existence of the inserted gene by PCR genotyping technique. The plants were grown until they were mature and produced T_2 generation seeds.

T_2 generation Arabidopsis seeds (100-200 seeds) were grown on MS medium with 50 µg/ml hygromycin for determining the survival rate of the segregated offspring. The expected survival rate from the hemizygous parent, which possessed only a single copy in a diploid cell or organism, was 75% with acceptance of χ^2 test at $p<0.05$. Then, the resistant plants were further grown for seeds (T_3 generation) and a rosette leaf from each plant was taken to confirm the existence of the inserted DNA by PCR.

Then, the harvested T_3 generation seeds were further grown on hygromycin selection plates for selecting the homozygous Arabidopsis among the mixture of hemizygous and homozygous T_2 plants. The expected survival rate of T_3 plants was 100% if their parent was homozygous. However, some seeds may not germinate because of the environmental effect, therefore the expected survival rate of ≥90% was used in χ^2 test. The plants were further grown and the leaf was taken to confirm the existence of the inserted DNA by PCR again. The Arabidopsis selection was shown in Figure 12. The plants were grown until they were mature and the homozygous T_4 seeds were harvested for the experiments.

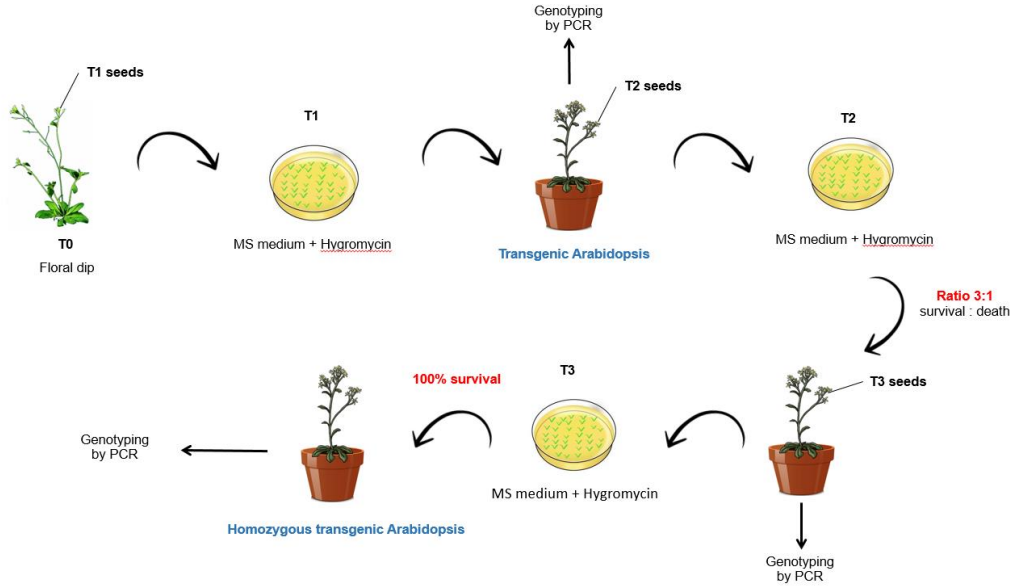


Figure 13 Homozygous transgenic Arabidopsis selection

2.2.5 Arabidopsis genotyping by PCR

The genomic DNA was used as template in PCR reaction. Fresh plant tissues around 100 mg were ground by Mixer Mill MM400. Then, the genomic DNA was isolated by Genomic DNA Mini Kit (Plant). The *OsMS* fragment insertion in the overexpression (OX/WT) Arabidopsis, which is the wild type Arabidopsis expressing *OsMS* under the control of 35SCaMV promoter, and revertant (OX/*ms*) Arabidopsis, which is the *Atms* mutant Arabidopsis expressing *OsMS* under the control of 35SCaMV promoter, was determined using *Taq* DNA polymerase with *OsMS*_M2_F (5′ CCGCGACGCTCTTCGTGAG 3′) and *OsMS*_R (5′ TCAGAGCTTGCACGGCGA 3′) primers. The expected size of PCR product was around 1,200 bp.

The wild type and *Atms* mutant Arabidopsis were confirmed by PCR using *Taq* DNA polymerase with LP (5′ TGACCGGAGAGTTGAGATCAC 3′), RP (5′ TAAGAGGGGACTAACCATGCC 3′) and LB (5′ ATTTTGCCGATTTCGGAAC 3′) primers. LP and RP were located on the gene while LB located on the left border of inserted T-DNA in the 2nd exon of Arabidopsis MS. The PCR product using the LP and RP primers should be around 1158 bp in the wild type Arabidopsis, which had no T-DNA insertion. While the PCR product using the LB and RP primers should be around 750 bp in the *Atms* mutant Arabidopsis, which contained the T-DNA-inserted *AtMS* (*Atms* mutant). In the

Atms mutant Arabidopsis, the inserted T-DNA is sufficiently long to obstruct PCR amplification using the LP and RP primers.

2.2.6 RNA isolation and cDNA synthesis

The plant tissues were ground with liquid nitrogen by Mixer Mill MM400 until they became powder then RNA was isolated by RNeasy® Plant Mini Kit. The quantity of RNA was checked by spectrophotometric method and the quality of RNA was checked by agarose gel electrophoresis. After that, RNA was treated with DNase I to remove the genomic DNA. Then, cDNA synthesis was performed using 5x iScript™ Reverse Transcription Supermix and cDNA would be used as the template for qRT-PCR.

2.2.7 Analysis of gene expression by qRT-PCR

The 4-week-old seedlings of the six Arabidopsis lines were used to analyze gene expression. The RNA was isolated using whole plants and cDNA synthesis was performed. The qRT-PCRs were performed using Luna Universal qPCR Master Mix.

The expression of *AtMS*, native malate synthase gene in Arabidopsis, was determined using *AtMS*_1_F (5′ CCAAGAGGTTGGCATCTCC 3′) and *AtMS*_1_R (5′ GAGATAGAAGAATGGACCGAAA 3′) primers.

The expression of *OsMS* was determined using *OsMS*_qRT_F (5′ CGTACAACCTCATCGTGGTG 3′) and *OsMS*_qRT_R (5′ CGGAGAAGTTACACGGAGAGA 3′) primers.

The expression of *SAG12* was determined using *SAG12*_F (5′ TCCAATTCTATTCGTCTGGTGTGT 3′) and *SAG12*_R (5′ CCACTTTCTCCCCATTTTGTTC 3′) primers.

The expression of *SAG13* was determined using *SAG13*_F (5′ CTTACGTGAATGGCAAGCAA 3′) and *SAG13*_R (5′ CCACATTGTTGACGAGGATG 3′) primers.

The expression of *WRKY22* was determined using *WRKY22*_F (5′ CGACAAAGTAATGCCGTCTCC 3′) and *WRKY22*_R (5′ CGTTTCTGGTTCTGTGGCTTT 3′) primers.

The expression of *AtEF1*α, reference gene, was determined using *AtEF1*α_qRT_F (5′ TTCGCTGTTAGGGACATGAGGC 3′) and *AtEF1*α_qRT_R (5′ CACCCTTCTTCACTGCAGCCTT 3′) to normalize gene expression by calculating $2^{-\Delta Ct}$ method (Livak & Schmittgen, 2001) (ΔCt is the difference between Ct values of reference gene and Ct values of interested gene).

2.2.8 Root length determination

Seeds of all Arabidopsis lines were grown on MS medium without 1% (w/v) sucrose. The plates with Arabidopsis seed were kept in the opaque box under dark condition for 7 days. Then, the Arabidopsis seedlings were photographed and the root length was measured by Image J program. We performed five biological replicates.

2.2.9 Arabidopsis germination percentage determination

Seeds of all Arabidopsis lines were sown on MS medium supplemented with 300 mM NaCl or without NaCl. We performed five biological replicates which each biological replicate contained 60 seeds of each line. Then, the Arabidopsis plates were kept in 4 ℃ for 3 days. The number of germinated seeds was daily counted for 7 days after the plates were moved to growing condition. Number of germinated seeds was reported as percentage.

2.2.10 Arabidopsis fresh weight and dry weight determination

Seeds of all Arabidopsis lines were sown on MS medium. The 5-day-old Arabidopsis were transferred to MS medium plates supplemented with 120 mM NaCl for 3 days then they were recovered by transferring to MS medium plates without NaCl for 5 days. The Arabidopsis plants of each biological replicated were weighed to determine the fresh weight. Then, the samples were oven-dried at 60 ℃ for 5-7 days and weighed to determine the dry weight. The fresh weight and dry weight reduced when compared values from plants grown under normal condition were calculated as reduction percentage. For this experiment, we performed 5 biological replicates.

2.2.11 Determination of growth and seedling establishment

Seeds of all Arabidopsis lines were grown on MS medium without 1% (w/v) sucrose supplemented with or without 120 mM NaCl for 20 days. The Arabidopsis plants from germinated seeds with true leaves that remained green in the absence of sucrose supplementation or under salt stress were counted as successful seedling establishment while the non-germinated or pale color plants with stunted growth were counted as unsuccessful. The numbers successfully established seedlings were used to calculate the percentage of seedling establishment. We performed four biological replicates in which each biological replicate, which contained 50 seeds of each line.

2.2.12 Leaf temperature measurement

The 4-week-old of all Arabidopsis lines at vegetative stage, growing in pots containing 3:1 of peat moss : perlite, were treated with 300 mM NaCl for 3 days. Rosette leaf temperature was measured using a FLIR C2 thermal camera (FLIR, USA). Six biological replicates were used.

2.2.13 Chlorophyll and total carotenoid content determination

The leaf samples were collected, frozen in liquid nitrogen. The samples were ground until became fine powder. Cholorophyll and carotenoid were extracted with 1 ml of 80% acetone. Then the extracts were centrifuged at 15,000xg for 5 min at 4 °C. The supernatants were collected and their absorbance was measured at wavelength 663 nm, 646 nm, and 470 nm with spectrophotometer. Chlorophyll a, chlorophyll b and total carotenoid concentrations were calculated using Lichtenthaler's equations shown in Figure 13 (Lichtenthaler & Wellburn, 1983). For leaf greenness index, the SPAD index of the treated 4-week-old plants of all Arabidopsis lines was determined by Chlorophyll Meter SPAD-502 Plus.

$$\text{Chlorophyll a } (\mu g/ml) = 12.21A_{663} - 2.81A_{646}$$

$$\text{Chlorophyll a } (\mu g/ml) = 20.31A_{646} - 5.03A_{663}$$

$$\text{Total carotenoid } (\mu g/ml) = \frac{1000A_{470} - 3.27(\text{Chlorophyll a}) - 104(\text{Chlorophyll b})}{229}$$

Figure 14 Lichtenthaler's equations (Lichtenthaler & Wellburn, 1983)

2.2.14 Statistical analysis

In all experiments including qRT-PCR; and determination of germination percentage, fresh weight, dry weight, root length, seedling establishment, SPAD value, leaf temperature, chlorophyll content, and total carotenoid content, were compared using analysis of variance (ANOVA) followed by comparing the means with Duncan's multiple range test, with significance accepted at $p<0.05$ using SPSS software version 22.0.

CHAPTER III
RESULTS

3.1 Recombinant plasmid construction for ectopic expression of *OsMS* in *malate synthase* Arabidopsis mutant and Arabidopsis wild type

OsMS coding sequence was successfully inserted into pGWB2 vector. *OsMS* should be overexpressed under the control of 35SCaMV promoter. PCR amplification was carried out to verify the insertion of *OsMS* coding sequence in the recombinant plasmid *OsMS*-pGWB2 (Figure 15). The size of PCR product was around 1,700 bp as expected. Nucleotide sequence of *OsMS* in pGWB2 vector was determined by DNA sequencing as shown in Appendix.

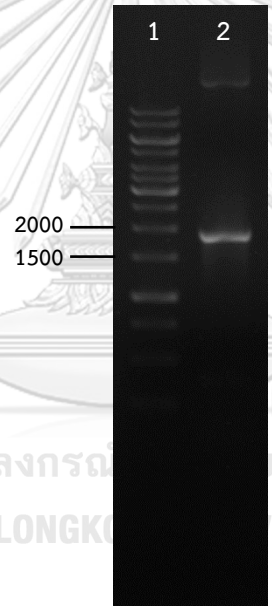


Figure 15 PCR amplification verifying the insertion of *OsMS* coding sequence in the recombinant plasmid *OsMS*-pGWB2. The PCR product was analyzed by agarose gel electrophoresis using 1% agarose gel.

Remark:

1 : DNA ladder

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

3.2 Homozygous transgenic Arabidopsis selection

According to the step 2.2.4 in the materials and methods chapter, the χ^2 test results at $p<0.05$ were shown in Appendix. The four independent lines of homozygous transgenic Arabidopsis were obtained including;

- Wild type Arabidopsis expressing *OsMS* line 13 driven by 35SCaMV promoter or OX/WT13
- *Atms* mutant Arabidopsis expressing *OsMS* line 4-2 driven by 35SCaMV promoter or OX/ms4-2
- *Atms* mutant Arabidopsis expressing *OsMS* line 5-2 driven by 35SCaMV promoter or OX/ms5-2
- *Atms* mutant Arabidopsis expressing *OsMS* line 13-3 driven by 35SCaMV promoter or OX/ms13-3

3.3 Transgenic Arabidopsis genotyping

Genotypes of the four independent lines of transgenic Arabidopsis expressing *OsMS* were confirmed by PCR using primers for the inserted *OsMS* and primers for the background genotype. The expected PCR product size of the inserted *OsMS* was around 1.2 kb. All transgenic Arabidopsis lines expressing *OsMS* gave ~1.2 kb PCR products, but wild type and *Atms* mutant Arabidopsis generated no PCR product as expected (Figure 16).

For confirming the background genotype using LP and RP primers, the 1% agarose gel electrophoresis results have shown that the PCR product size of wild type and OX/WT13 was around 1.1-1.2 kb, while *Atms* mutant Arabidopsis, OX/ms4-2, OX/ms5-2, and OX/ms13-3 had no PCR product (Figure 17A). For LB and RP primers, wild type and OX/WT13 had no PCR product, while PCR product size of *Atms* mutant Arabidopsis, OX/ms4-2, OX/ms5-2, and OX/ms13-3 was around 750 bp (Figure 17B).

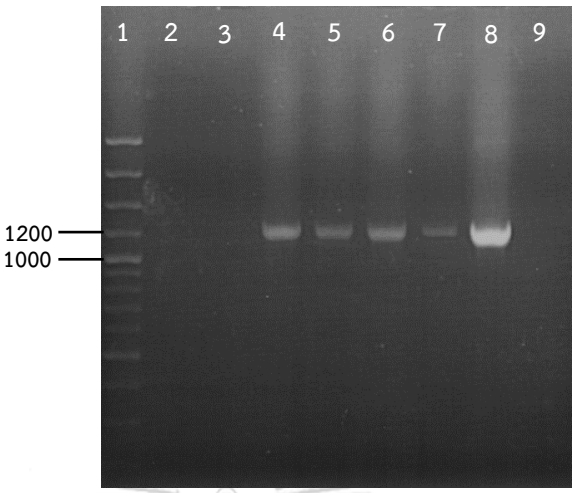


Figure 16 Inserted *OsMS* genotyping of transgenic Arabidopsis. The PCR product was analyzed by agarose gel electrophoresis using 1% agarose gel.

Remark

1 : DNA ladder

2 : Wilde type Arabidopsis (WT)

3 : *Atms* mutant Arabidopsis

4 : OX/WT13

5 : OX/ms4-2

6 : OX/ms5-2

7 : OX/ms13-3

8 : Positive control

9 : Negative control

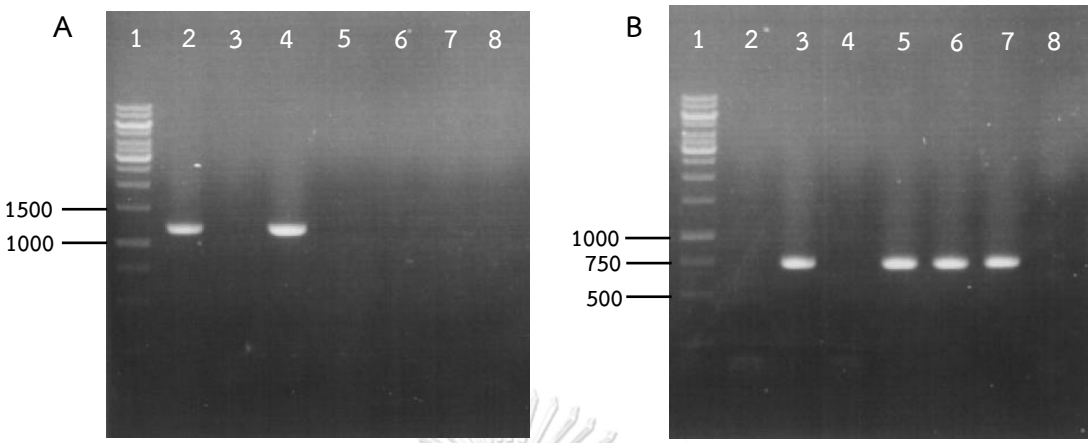


Figure 17 Background genotyping of transgenic Arabidopsis, (A) using LP and RP primers, (B) using LB and RP primers. The PCR product was analyzed by agarose gel electrophoresis using 1% agarose gel.

Remark

1 : DNA ladder

2 : Wilde type Arabidopsis (WT)

3 : *Atms* mutant Arabidopsis

4 : OX/WT13

5 : OX/ms4-2

6 : OX/ms5-2

7 : OX/ms13-3

8 : Negative control

3.4 Expression of *AtMS* and *OsMS* in Arabidopsis

The qRT-PCR was performed to verify the expression level of *AtMS* and *OsMS* genes. Firstly, the expression level of *AtMS* gene was determined and its absence was validated in the *Atms* mutant when compared with the wild type (Figure 18A). The expression level of *OsMS* gene was detected in all transgenic lines, OX/WT13, OX/ms4-2, OX/ms5-2, and OX/ms13-3, indicating that the *OsMS* gene was successfully expressed (Figure 18B).

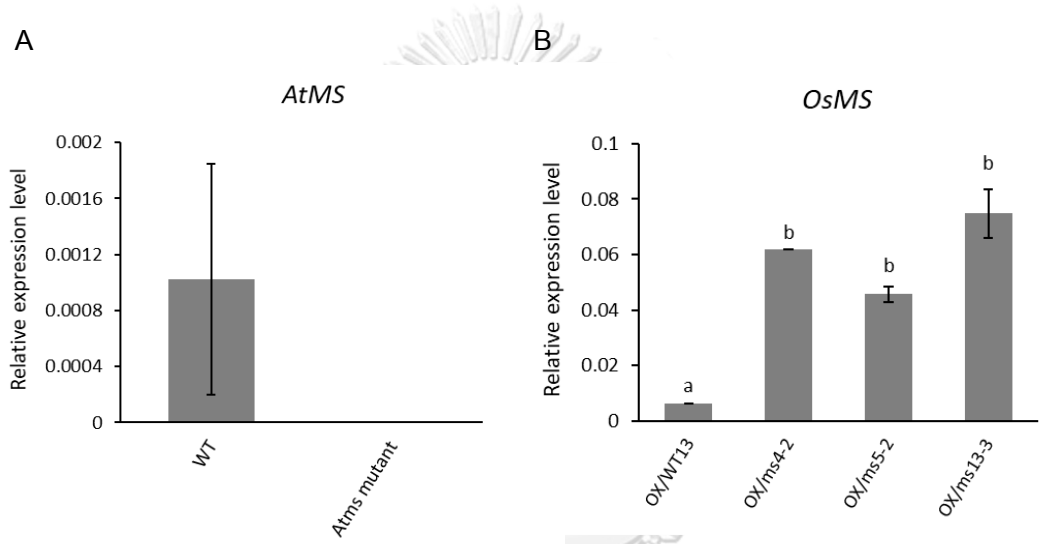


Figure 18 qRT-PCR analysis showing (A) *AtMS* gene expression level and (B) *OsMS* gene expression level in 4-week-old Arabidopsis leaves. *EF1α* gene was used as the internal control. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.5 Root length of transgenic Arabidopsis expressing *OsMS*

The six Arabidopsis lines were grown in MS medium without sucrose supplementation under dark condition for 7 days and root length was determined. The results showed that all six Arabidopsis lines exhibited no significant difference in root length (Figure 19).

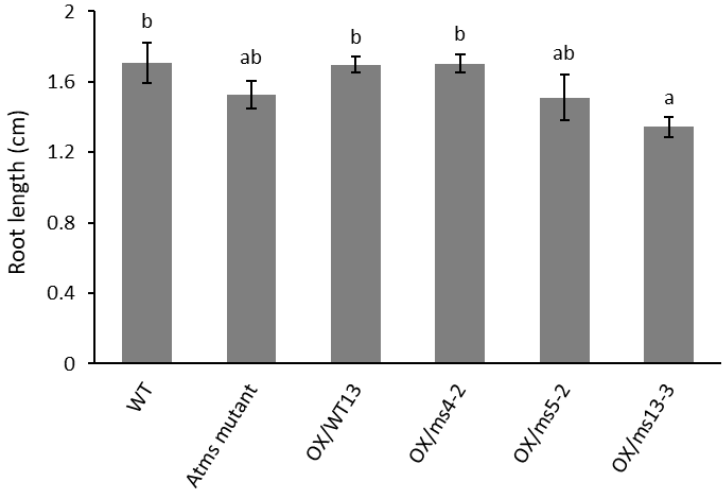


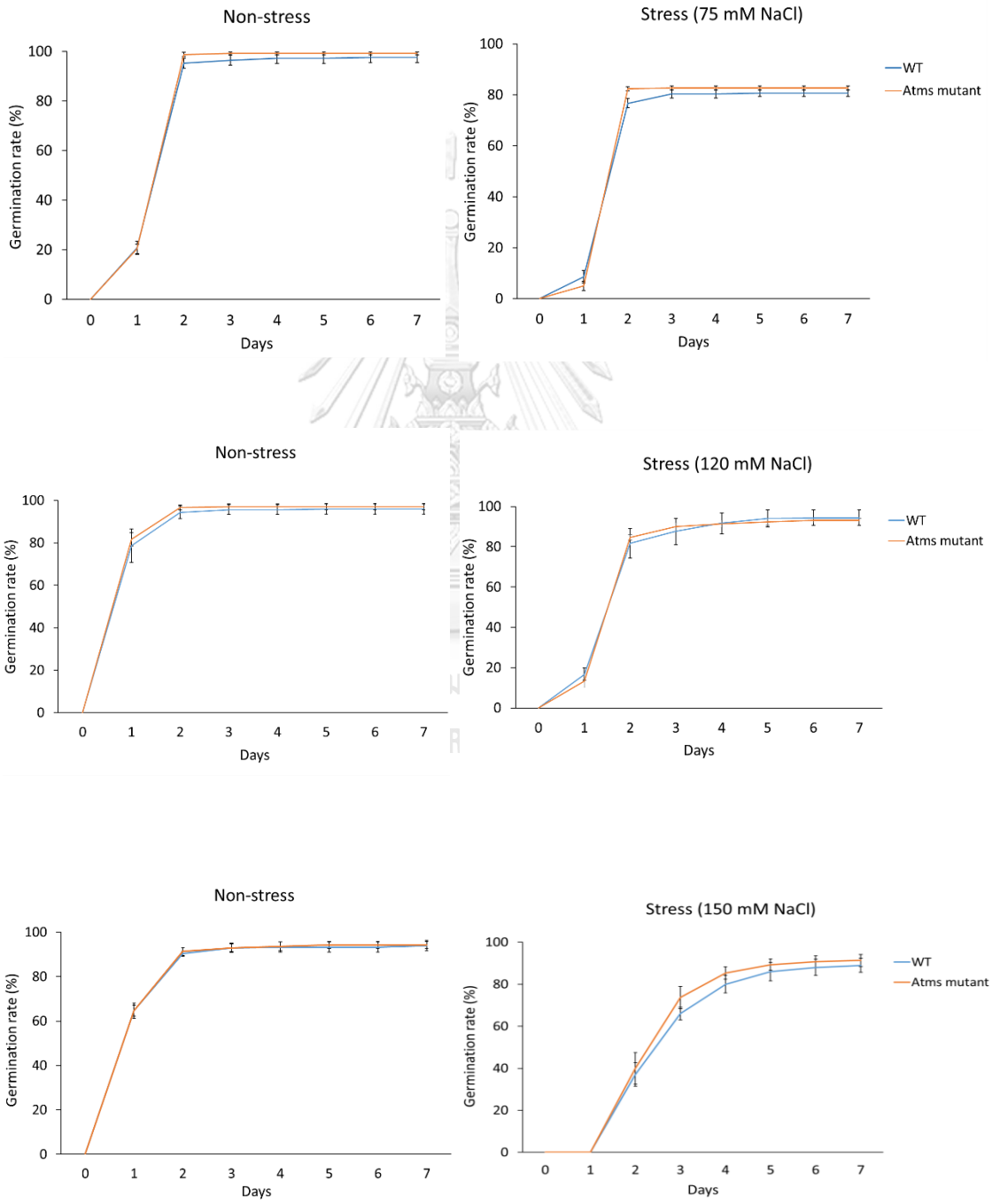
Figure 19 Root length of six Arabidopsis lines (*N* = 5) growing in medium without sucrose under dark condition for 7 days (*N* = 5). One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.6 Germination percentage of transgenic Arabidopsis expressing *OsMS*

Seeds of wild type and *Atms* mutant Arabidopsis lines were sown on MS medium supplemented with at 75, 120, 150, 200, 250, and 300 mM NaCl or without NaCl in five biological replicates and germination percentage was determined. Under control condition, wild type and *Atms* mutant almost completely germinated after 2 days. Under salt stress condition at 75, 120, 150, and 200 mM NaCl, there were no difference in germination percentages in all Arabidopsis lines. Meanwhile, under salt stress condition at 250 and 300 mM NaCl, *Atms* mutant had the lower germination percentage than wild type after 4 days and 5 days of sowing, respectively (Figure 20).

Then, wild type and *Atms* mutant Arabidopsis lines were compared with transgenic Arabidopsis expressing *OsMS* gene when grown under 250 and 300 mM NaCl conditions. Under normal condition, all six Arabidopsis lines almost completely germinated after 2 days. Under salt stress condition at 250 mM NaCl, althought *Atms* mutant had the lower germination percentage than wild type, three lines of revertant did not exhibit higher percentage when compared with *Atms* mutant (Figure 21). Under salt stress condition at 300 mM NaCl, *Atms* mutant similarly exhibited the lower

germination rate than wild type. Moreover, two of the three revertant lines, OX/ms4-2 and OX/ms5-2, exhibited higher germination rate than *Atms* mutant indicating that *OsMS* gene somewhat reverted the phenotype of the *Atms* mutant under high salt condition. However, OX/WT13 and OX/ms13-3 showed similar germination rate with the *Atms* mutant (Figure 22).



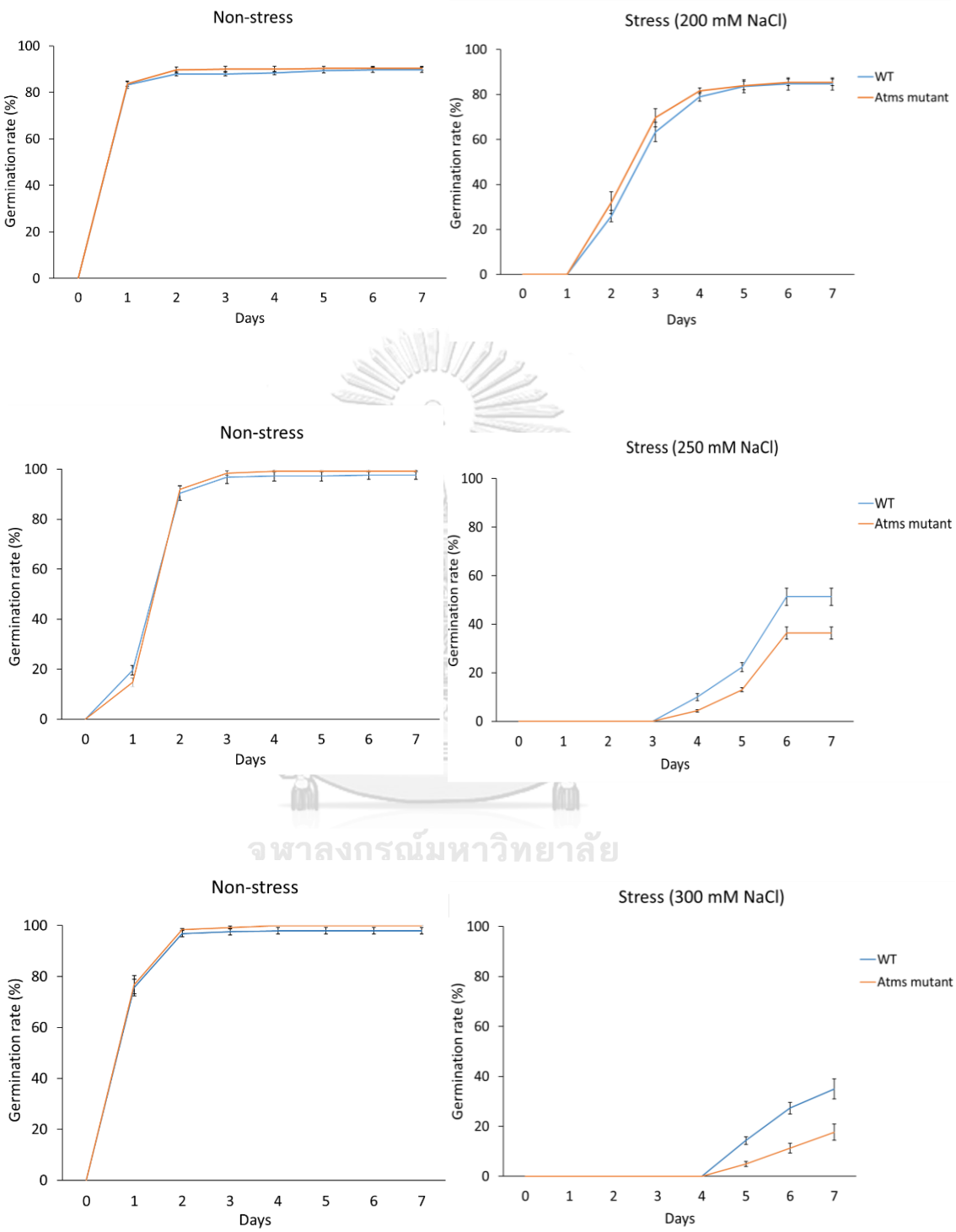


Figure 20 Germination rate of wild type (WT) and *Atms* mutant under normal (Non-stress) and varies salt treatment (Stress) at 75, 120, 150, 200, 250, and 300 mM NaCl (N=5). The error bars represent SD.

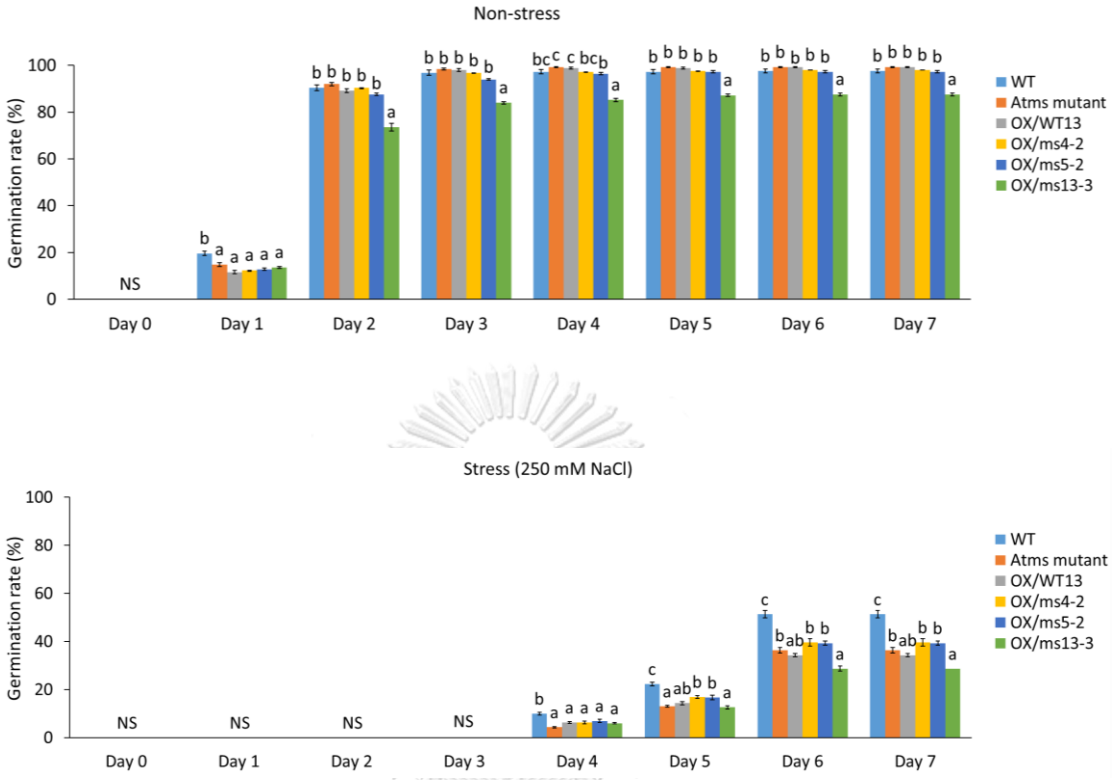


Figure 21 Germination rate of six Arabidopsis lines under normal (Non-stress) and 250 mM NaCl (Stress) conditions (N = 5). One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

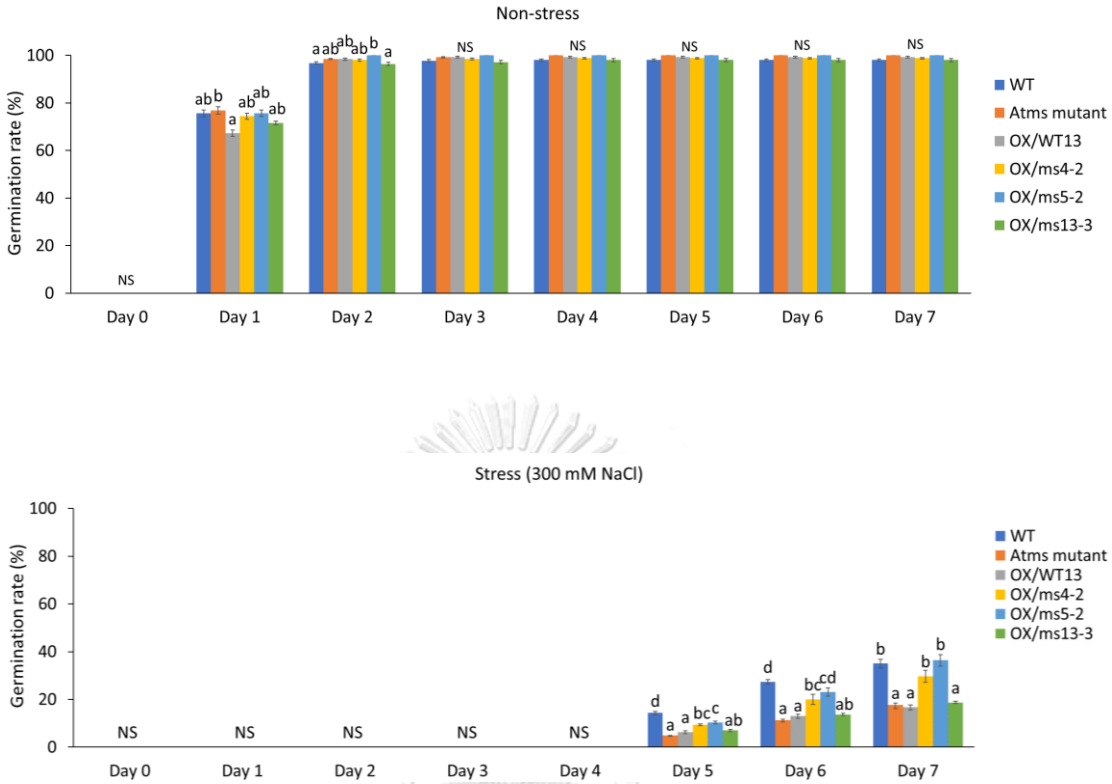


Figure 22 Germination rate of six Arabidopsis lines under normal (Non-stress) and 300 mM NaCl (Stress) conditions (N = 5). One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.7 Fresh weight and dry weight of transgenic Arabidopsis expressing *OsMS*

The 5-day-old Arabidopsis were transferred to MS medium with 120 mM NaCl for 3 days, and recovered for another 5 days. Then, fresh weight and dry weight were determined and shown as reduction percentage to investigate the role of malate synthase in water maintenance. For fresh weight, compared to the other Arabidopsis lines, *Atms* mutant line had higher reduction percentage, around 39%, which was statistically higher than those of the wild type and all *OsMS*-expressing Arabidopsis lines, indicating that *OsMS* gene reversed the salt sensitivity phenotype of the mutant. However, dry weight did not have the same pattern as fresh weight. For dry weight,

there were no difference in reduction percentage in most Arabidopsis lines when compared with wild type (Figure 23).

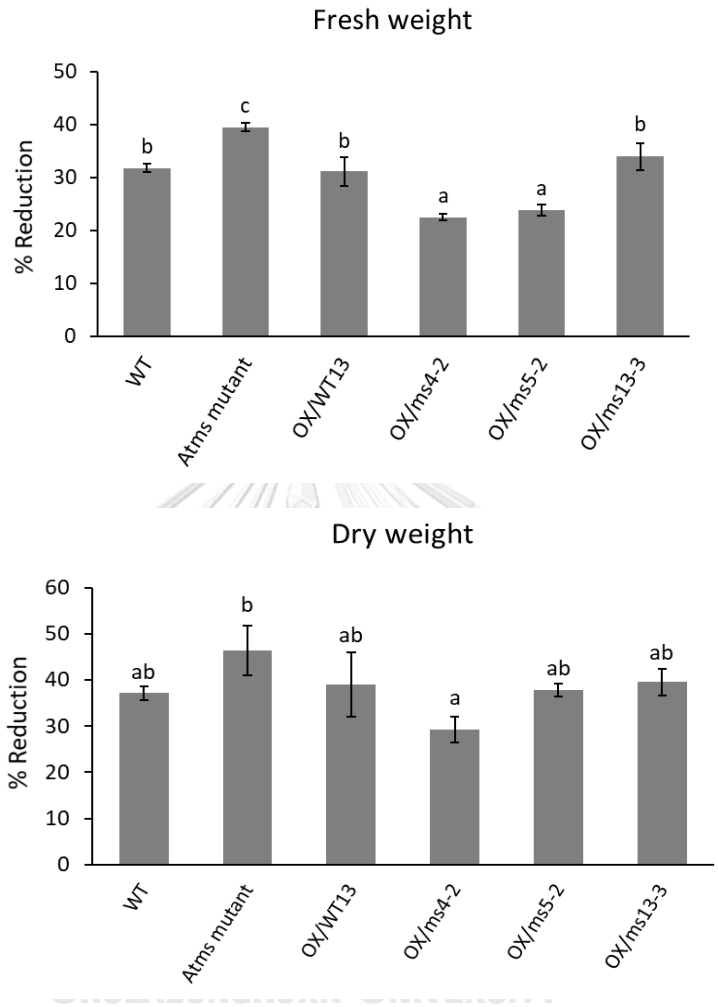


Figure 23 Fresh weight and dry weight of six Arabidopsis lines in medium without sucrose under salt stress shown as reduction percentage (N=5). The 5-day-old Arabidopsis were transferred to MS medium with 120 mM NaCl for 3 days, and recovered for another 5 days. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.8 Seedling establishment of transgenic Arabidopsis expressing *OsMS*

Seedling establishment of the six Arabidopsis lines, which were grown in medium without sucrose for 20 days, were determined. The *Atms* mutant line exhibited the lower percentage of seedling establishment than wild type. However, the three revertant lines exhibited the similar percentage of seedling establishment compared with the *Atms* mutant (Figure 24). After 20 days in medium without sucrose and supplemented with salt treatment at 120 mM NaCl, the results showed that *Atms* mutant line had the lower percentage of seedling establishment than wild type and OX/WT13, though not statistically. The three lines of revertant also did not show the difference in percentage of seedling establishment compared with *Atms* mutant indicating that *OSMS* gene did not revert this phenotype (Figure 25).

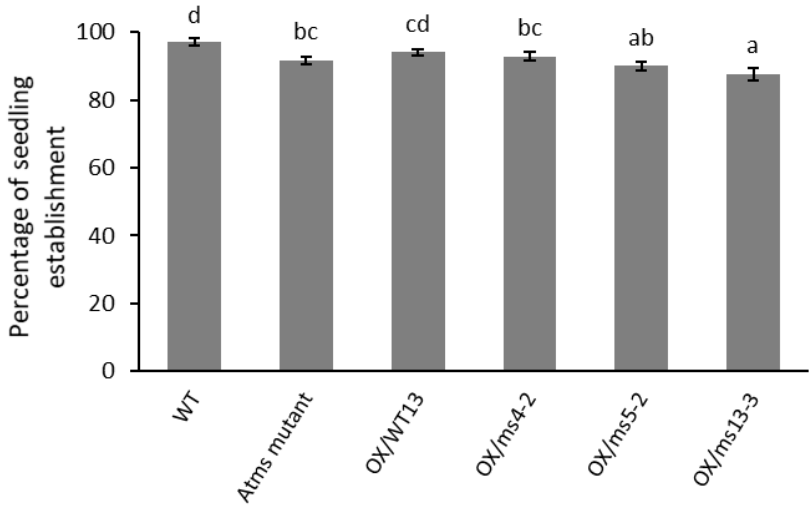


Figure 24 Percentage of seedling establishment of six Arabidopsis lines after 20 days in medium without sucrose (*N* = 5). One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

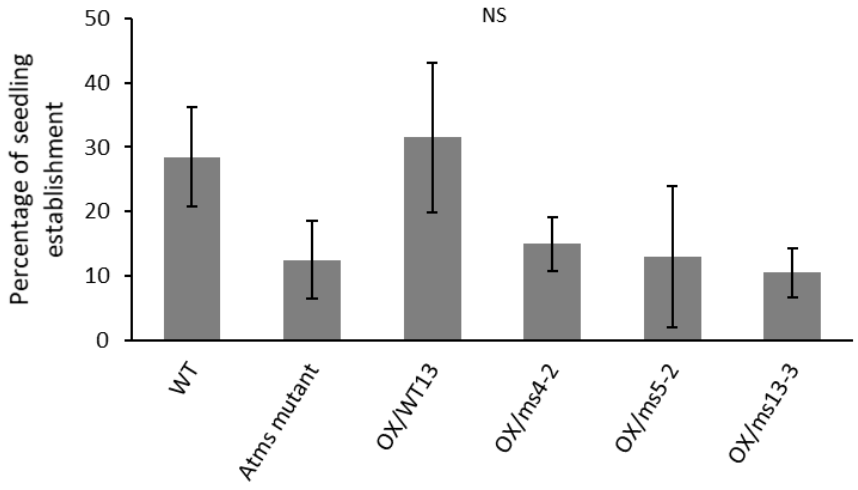


Figure 25 Percentage of seedling establishment of six Arabidopsis lines after 20 days in medium without sucrose under salt stress (*N* = 5). One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.9 Leaf temperature of transgenic Arabidopsis expressing *OsMS*

To examine the stomatal response under salt stress, rosette leaf temperature of the six Arabidopsis lines were determined. The results showed that all Arabidopsis lines did not exhibit difference in leaf temperature under normal condition. Under salt treatment for 5 days, leaf temperature of all Arabidopsis lines increased when compared to the normal condition. However, leaf temperature among different Arabidopsis lines was not different either after 3 days or 5 days of salt treatment (Figure 26).

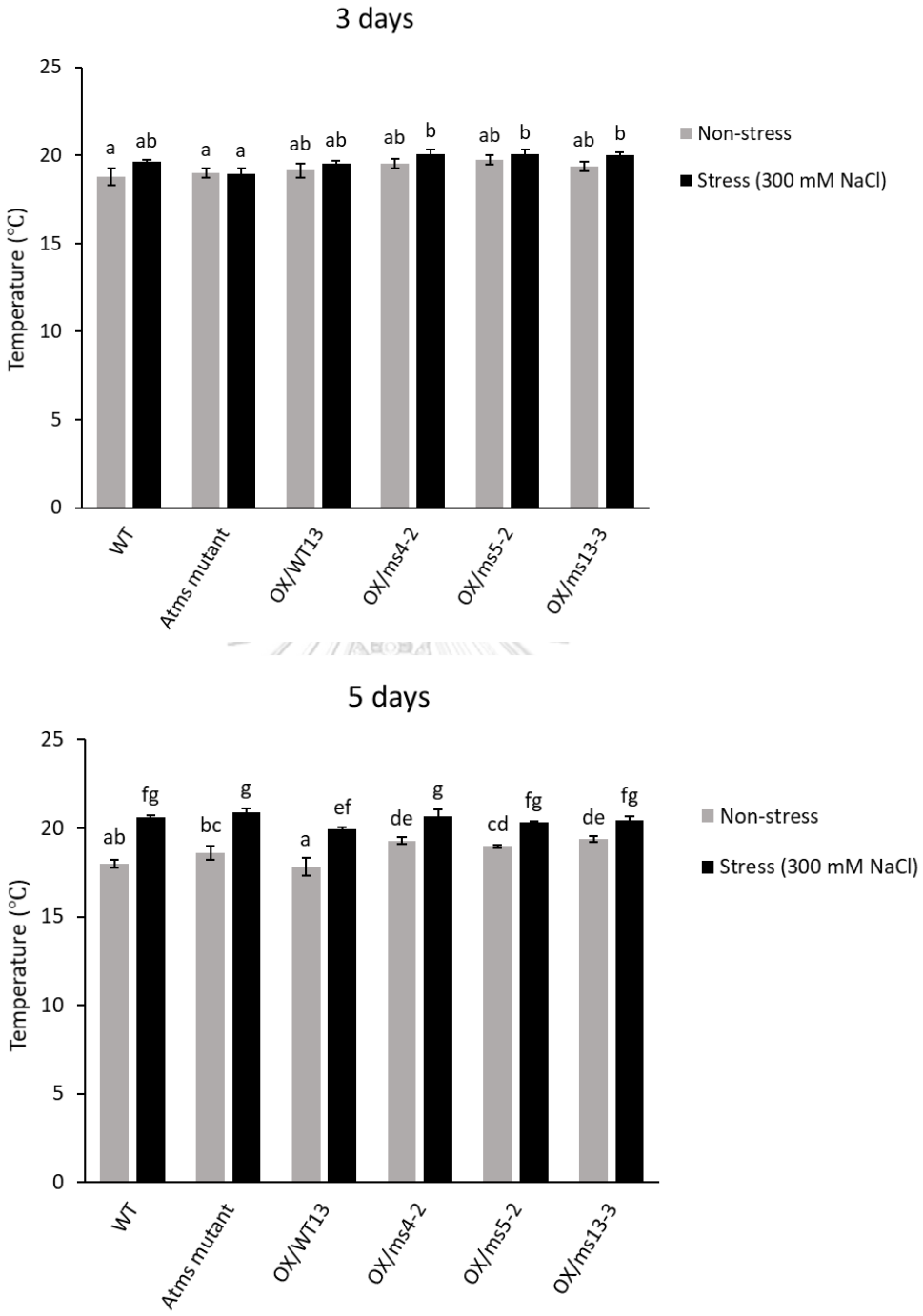


Figure 26 Arabidopsis rosette leaf temperature of six Arabidopsis lines under non-stress or salt stress at 300 mM NaCl for 3 and 5 days. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.10 Chlorophyll and total carotenoid contents of transgenic Arabidopsis expressing *OsMS*

To investigate the role of malate synthase in leaf senescence regulation, the SPAD value, which is proportional to the amount of chlorophyll, of six Arabidopsis lines was measured to determine the leaf greenness index of plants. Under normal condition, all Arabidopsis lines did not exhibit the difference in SPAD values (Figure 27A and 28A). Under salt stress condition at 300 mM NaCl, there were no difference in SPAD value of all Arabidopsis lines after 3 days of salt treatment (Figure 27B). Meanwhile, *Atms* mutant line had the highest SPAD values when compared with WT and OX/WT13 and two of the three revertant lines exhibited lower SPAD values than the *Atms* mutant after 5 days of salt treatment (Figure 28B), suggesting that *OsMS* has reverted the phenotype under 5-day salt stress of the *Atms* mutant. Chlorophyll and carotenoid contents of 4-week-old Arabidopsis treating with 300 mM NaCl solution for 5 days were also determined. The results showed that *Atms* mutant tended to have higher chl *a* and total carotenoid contents than wild type and OX/WT13 while the three revertant lines exhibited values more similar to those of *Atms* mutants (Figure 29).

To further examine senescence, six Arabidopsis lines were kept in dark condition for 4 days to induce senescence. The results showed the difference in chlorophyll and total carotenoid contents in all Arabidopsis lines, especially in *Atms* mutant line, which had the significantly higher chlorophyll and total carotenoid contents than wild type and OX/WT13. Also, all revertant lines exhibited somewhat lower contents of chlorophyll and total carotenoid contents than *Atms* mutant line and closer to those of wild type suggesting that *OsMS* gene could revert the phenotype (Figure 30).

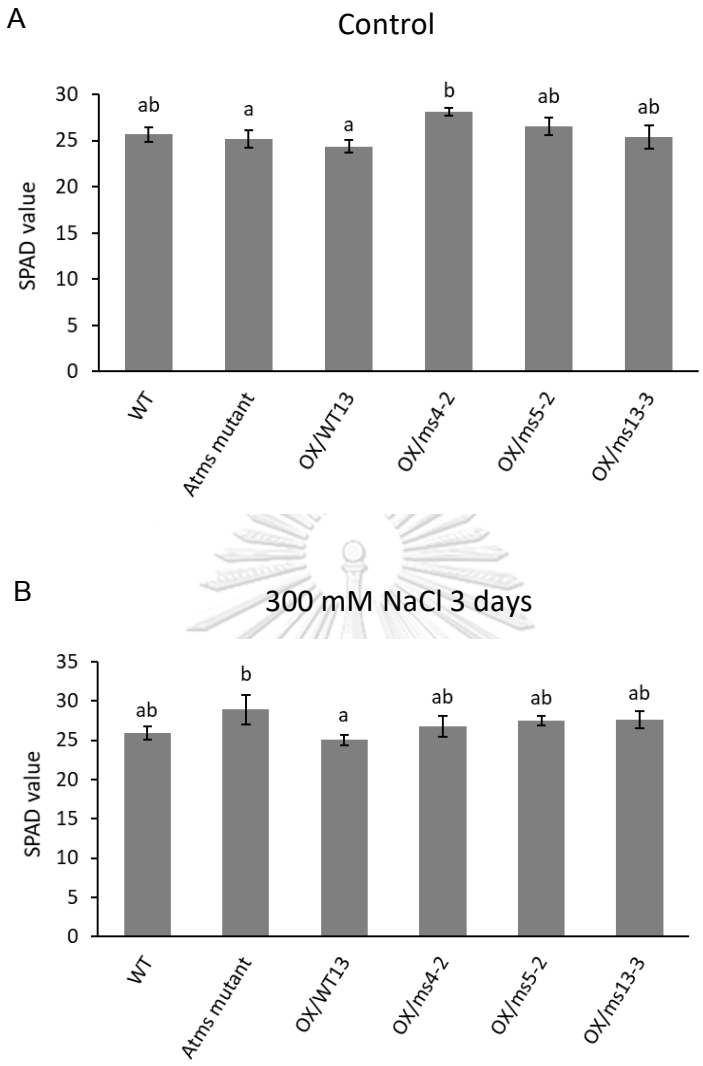


Figure 27 SPAD value of six Arabidopsis lines (A) under normal condition and (B) under salt stress at 300 mM NaCl for 3 days. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

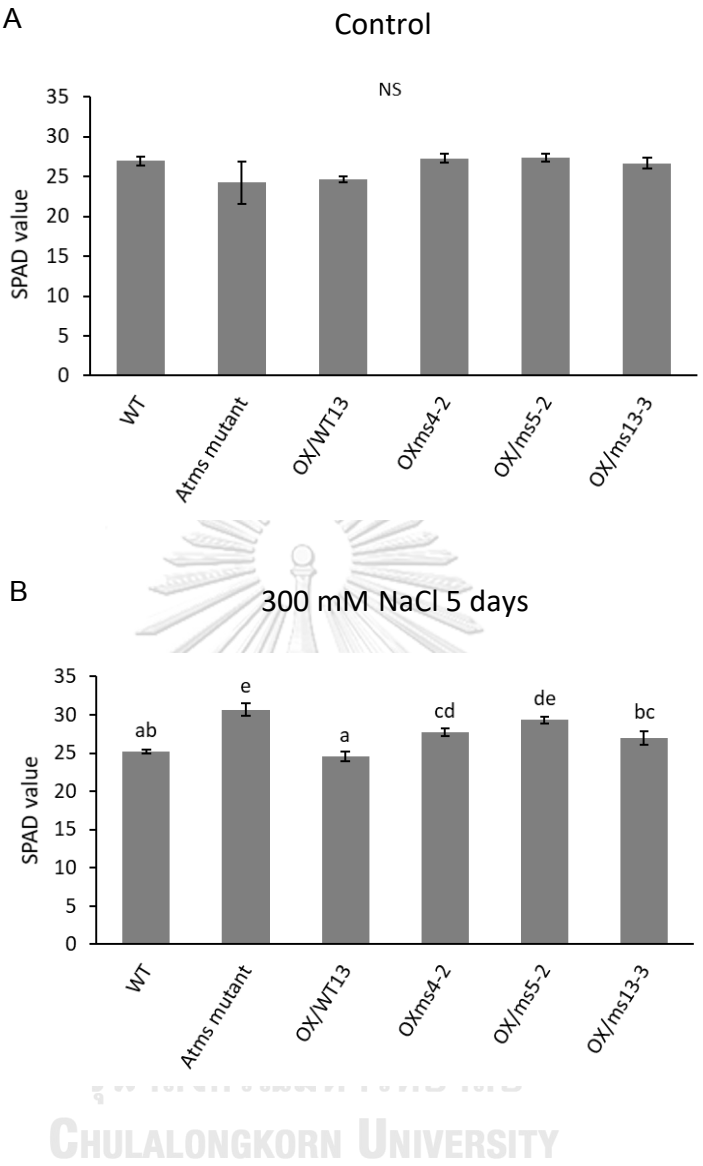


Figure 28 SPAD value of six Arabidopsis lines (A) under normal condition and (B) under salt stress at 300 mM NaCl for 5 days. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

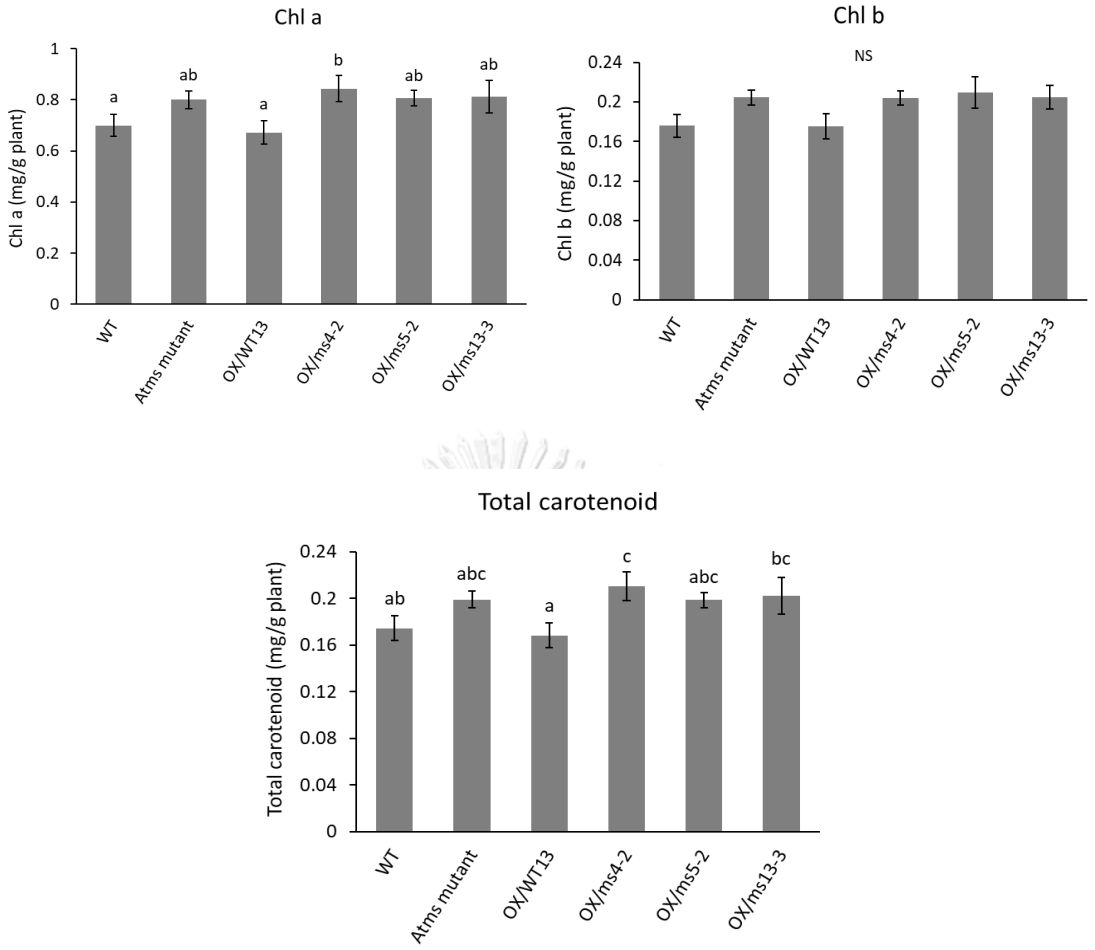


Figure 29 Chlorophyll and total carotenoid contents of six Arabidopsis lines growing under salt stress. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in survival rate data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

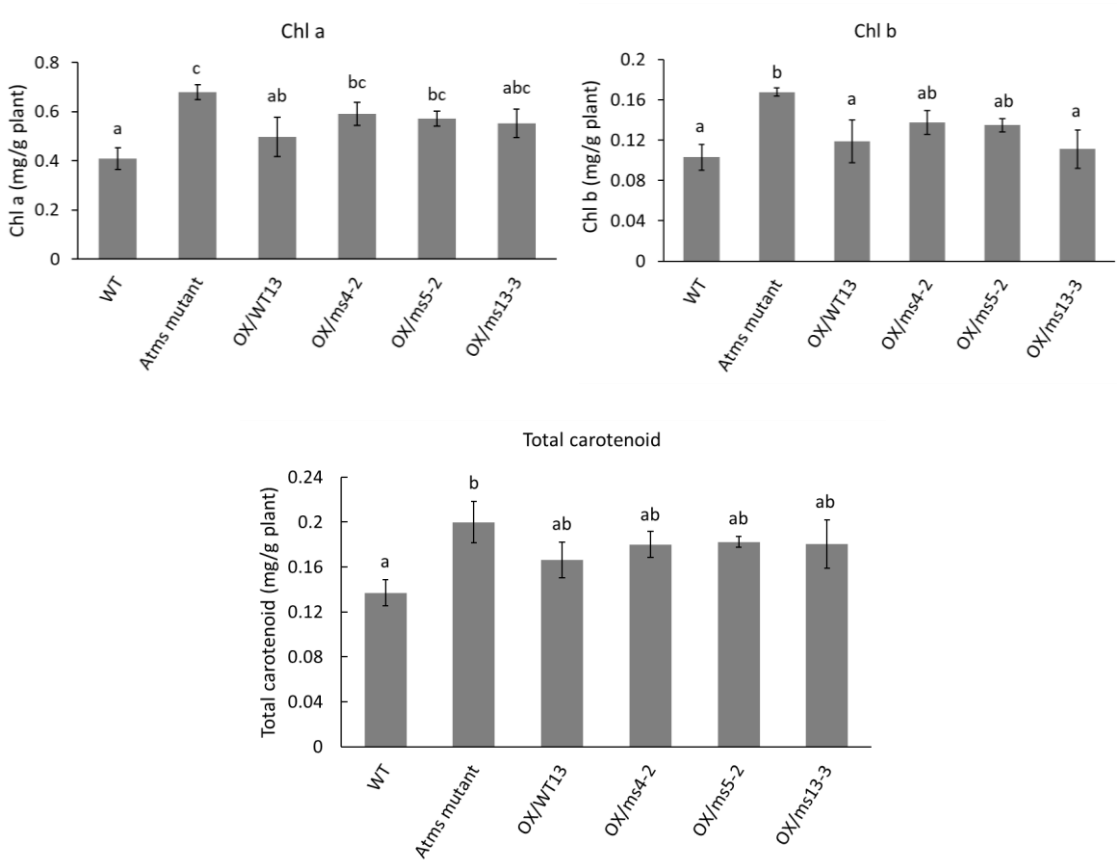


Figure 30 Chlorophyll and total carotenoid content of six Arabidopsis lines growing under dark condition for 4 days. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in survival rate data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

3.11 Expression of senescence-associated genes in Arabidopsis

To investigate the role of malate synthase in leaf senescence regulation, expression level of senescence-associated gene, *SAG12*, *SAG13*, and *WRKY22* gene, were determined after dark condition for 4 days to support the chlorophyll and carotenoid contents determination. The results showed that the expression levels of *SAG12* were higher in wild type and OX/WT13 whereas *Atms* mutant line had the lowest expression level of *SAG12*. In addition, all *OsMS*-overexpressing *Atms* mutant lines tended to exhibit *SAG12* expression levels that were higher than the *Atms* mutant and closer to those of wild type. The expression levels of *SAG12* were in agreement with the chlorophyll and total carotenoid contents that were possibly affected by the

induced senescence. For expression levels of *SAG13* and *WRKY22* gene, there were no difference among the six Arabidopsis lines (Figure 31).

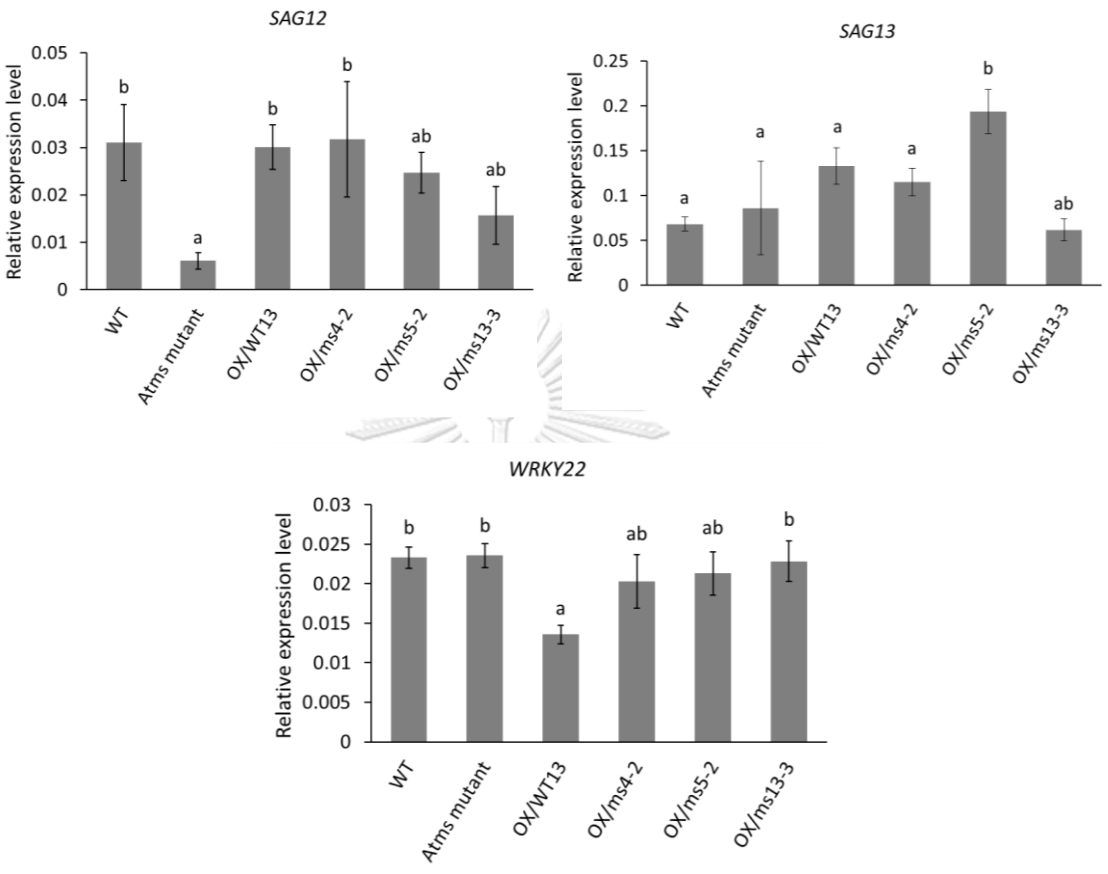


Figure 31 Relative expression level of senescence-associated genes, *SAG12*, *SAG13*, and *WRKY22*, of the six Arabidopsis lines in 4-week-old leaves under dark condition for 4 days. *EF1α* gene was used as the internal control. One-way ANOVA with Duncan multiple range test ($p < 0.05$) was used in survival rate data analysis. Data are shown as the mean ± 1 SE, and means with the same letter are not significantly different.

CHAPTER IV
DISCUSSIONS

In previous report, transcriptome data of KDML105 rice showed that expression of *malate synthase* (*MS*) gene (LOC_Os04g40990) was upregulated under salt stress. Here, the revertant Arabidopsis lines (OX/ms4-2, OX/ms5-2, and OX/ms13-3), which were the *Atms* mutant expressing *OsMS* gene, and *OsMS*-overexpressing Arabidopsis (OX/WT13), which was the wild type Arabidopsis expressing *OsMS* gene, were constructed to investigate the role of *OsMS* in response to salt stress. The transgenic Arabidopsis lines were successfully constructed and the insertion and expression of *OsMS* were confirmed by genotyping and qRT-PCR, respectively. According to the qRT-PCR results, the expression level of *AtMS* gene was determined and its absence was validated in the *Atms* mutant when compared with the wild type. The expression level of *OsMS* gene was detected in all transgenic lines, OX/WT13, OX/ms4-2, OX/ms5-2, and OX/ms13-3, indicating that the inserted *OsMS* gene in all transgenic lines was successfully expressed. These results are consistent with the genotyping results.

For root length determination, the result showed that there were no difference in root length in the six Arabidopsis lines under dark and sucrose deficiency condition (Figure 19). Seedling establishment in medium without sucrose and seedling establishment in medium without sucrose under salt stress treatment were similar among the six Arabidopsis lines. *Atms* mutant and revertants exhibited the lower percentage of seedling establishment than wild type and OX/WT13 expressing *OsMS* gene (Figure 24 and 25) suggesting that *OsMS* gene did not revert the phenotype of the Arabidopsis mutant. This observation was supported by the findings by Cornah, Germain, Ward, Beale, and Smith (2004) when *mls-1* and *mls-2* mutants, which lacked malate synthase, and *icl-2* mutant, which lacked isocitrate lyase, were grown on MS medium without sucrose in dark treatment for 7 days. The results showed that *mls-1* and *mls-2* mutants had only a slightly stunted phenotype while *icl-2* mutant had a more noticeable stunted phenotype suggesting that AtMS was only partially dispensable for lipid utilization and gluconeogenesis in Arabidopsis seedlings (Cornah et al., 2004). Moreover, photosynthesis may be established and became the main energy source rather than the carbon source stored in seed during germination. Identity of MS amino acid sequences between rice and Arabidopsis were 71.5%, therefore OsMS may not function effectively in *Atms* mutant. Different species often have a preference for a particular codon for encoding an amino acid (Comeron & Aguadé, 1998).

That codon usage bias can make it less efficient for gene expression in different species. Thereby, codon usage bias may be the reason of the similar phenotypes in all Arabidopsis lines.

Atms mutant was more sensitive to salt stress. According to the germination rate results under 300 mM NaCl, *Atms* mutant had the lower germination percentages than the wild type (Figure 22). In previous study, Yuenyong, Sirikantaramas, Qu, and Buaboocha (2019) found that *Aticl* mutant Arabidopsis, which lacked isocitrate lyase, had the lower germination rate than wild type under salt stress. They suggested that isocitrate lyase, a key enzyme in glyoxylate cycle, affected salt stress tolerance in the germination stage. Oil is the most common storage compound in seeds (Eastmond, 2006) and Arabidopsis is an oil seed plant (Li, Beisson, Pollard, & Ohlrogge, 2006). Therefore, stored lipid in the seed is converted to gluconeogenic substrates as a carbon source for generating energy during seed germination (Beevers, 1961). This suggests that malate synthase may play a role under salt stress during germination by using the acetyl unit from acetyl-CoA, the product of lipid breakdown, to generate sugars via the glyoxylate cycle and gluconeogenesis. In addition, OX/ms13-3, one of the three revertant lines, exhibited the lower germination rate while the others revertant lines exhibited the higher germination rate. This observation was demonstrated by *Agrobacterium*-mediated transformation (Step 2.2.3 in the chapter of materials and methods) that *Agrobacterium tumefaciens* T-DNA normally integrates into random sites in the plant genome (Chilton & Que, 2003). *OsMS* overexpression also appeared to have a negative effect in OX/WT13. Previous study reported that high levels of glucose cause ABA accumulation resulting in a delay of germination. Therefore, the higher level of malate synthase may cause the negative effect in germination of OX/WT13. Glucose and ABA content should be further determined to prove this hypothesize.

Following the fresh weight and dry weight results, there were no difference in reduction percentage of dry weight in all Arabidopsis lines under salt stress. However, *Atms* mutant exhibited the higher reduction percentage of fresh weight than the other Arabidopsis lines under salt stress indicating that *Atms* mutant is slightly more sensitive to salt stress and *OsMS* gene somewhat reverted the phenotype of the Arabidopsis mutant. Sugar is reported to be involved in the defense mechanisms of plants against salt stress and water deficiency by adjusting osmotic potential for better water uptake. Watanabe, Kojima, Ide, and Sasaki (2000) reported that sugars promote osmotic and salt tolerance and the effects of accumulated total soluble sugars are discussed in

relation to osmotic adjustment. In another report, Nemati, Moradi, Gholizadeh, Esmaeili, and Bihamta (2011) demonstrated that soluble sugars accumulation associated with salt tolerance in rice and high total soluble sugars concentration in the salt tolerant rice was found . Moreover, Cha-um, Charoenpanich, Roytrakul, and Kirdmanee (2009) demonstrated that soluble sugar contents were related to the fresh weight. They reported that the fresh weight and total soluble sugars in the salt tolerant rice (Homjan; HJ) were higher than the salt sensitive rice (Pathumthani 1; PT1) under salt stress. Therefore, malate synthase may play a role in salt tolerance mechanism by synthesizing sugars via gluconeogenesis following glyoxylate cycle, leading to osmotic adjustment and water maintenance.

For rosette leaf temperature, under salt stress for 5 days, leaf temperature of flowering stage Arabidopsis was increased compared to normal growing condition (Figure 26). These results correspond to the Orzechowska et al. (2021) report, which showed that the exposure of Arabidopsis to different levels of salt concentrations resulted in an increased rosette temperature. They suggest that the decrease in stomatal conductance leads to an increase in leaf temperature. However, there were no difference in rosette leaf temperature among the Arabidopsis lines under salt stress.

The SPAD value was used to estimate the leaf greenness index of plant leaves. Under salt stress for 5 days, the results showed that *Atms* mutant had the highest SPAD values (Figure 28B). The results correspond to the chlorophyll and total carotenoid contents in the six lines Arabidopsis lines either after treating with salt solution (Figure 29) or after dark-induced condition (Figure 30). *Atms* mutant had the higher chlorophyll and total carotenoid contents than WT and OX/WT13. Also, revertant lines exhibited more similar contents to those of wild type. These results showed that senescence in *Atms* mutant was delayed supported by the expression level of senescence-associated gene, *SAG12*. *SAG12*, which encodes a cysteine protease involving in nitrogen remobilization and RuBisCo degradation (James et al., 2019; James et al., 2018), is reported to be highly associated with senescence and is a commonly used marker for leaf senescence (Huo, Wang, Teng, & Liu, 2015; J. Zhou et al., 2015). The expression level of *SAG12* in *Atms* mutant was lower than the other Arabiadopsis lines (Figure 31). *SAG13* is stress-induced senescence marker (J. Zhou et al., 2015) and *WRKY22* is transcription factor involved in regulation of dark-induced leaf senescence (X. Zhou, Jiang, & Yu, 2011). However, there were no difference in *SAG13* and *WRKY22* gene in all Arabidopsis lines. Taken together, delayed stress-induced senescence may result in negative effect on *Atms* mutant growth during salt stress.

Some previous reports revealed that sugars were involved in the regulation of leaf senescence (Kim, 2019). In another study, Pourtau, Jennings, Pelzer, Pallas, and Wingler (2006) demonstrated that sugar induced genes that are characteristic for developmental senescence including *SAG12* gene. In addition, previous reports revealed that leaf senescence during drought stress contributes to plant survival. It allows nutrients mobilization from senescing leaves to young leaves. Also, it prevents the water loss through transpiration and contributes to the maintenance of water balance (Munné-Bosch & Alegre, 2004; Wingler & Roitsch, 2008). Expression of plant malate synthase gene was found highly induced during senescence (De Bellis & Nishimura, 1991; Graham, Leaver, & Smith, 1992) and was found highly upregulated in the rice overexpressing calmodulin gene (Yuenyong et al., 2019), which its homologous gene in Arabidopsis has been shown to regulate leaf senescence (Dai, Lee, Lee, Nam, & Kwak, 2018). Therefore, malate synthase may contribute to the resistance to salt stress by generating sugars via glyoxylate cycle and gluconeogenesis resulting in the induction of the senescence-associated gene that regulates the leaf senescence, which may contribute to the plant survival under salt stress.

Breeding for salt stress tolerance in crop plants is an important approach that should be given priority. Therefore, functional characterization of rice malate synthase is one of the necessary knowledge of molecular mechanisms for abiotic stress tolerance and these data can be used for applying in the breeding program in the future.

CHAPTER V
CONCLUSIONS

1. *OsMS* gene can revert some phenotypes of the *Atms* mutant under salt stress including the germination rate, the reduction of fresh weight and the leaf greenness.

2. Rice malate synthase and the glyoxylate cycle play important roles in seed germination, water maintenance and leaf senescence during salt stress.

3. Salt tolerance ability conferred by malate synthase may be achieved through the upregulation of glycoxylate cycle to induce leaf senescence, which may lead to nutrient remobilization and result in better adjustment and survival under salt stress.

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APPENDIX

APPENDIX A

SOLUTION PREPARATION

1. Half-strength Murashige and Skoog (MS) medium

MS medium powder		2.215	g
Sucrose		10	g
Distilled water	up to	1	l

Adjust pH to 5.8 by KOH

Sterile by autoclave at 121 °C for 15 min before use

2. LB medium

Tryptone		10	g
NaCl		10	g
Yeast extract		5	g
Distilled water	up to	1	l

Sterile by autoclave at 121 °C for 15 min before use

3. 10X tris boric EDTA buffer (TBE)

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

pH is should be around 8.3

4. Transformation buffer for Arabidopsis floral dipping

Murashige and Skoog (MS) medium		0.22	g
Sucrose		5	g
Silwet		20	µl

Distilled water		100	ml

5. Plant lysis buffer for crude plant genomic extraction

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
Distilled Water	up to	1	l

Sterile by autoclave at 121 ℃ for 15 min before use

6. DNase I reaction mixture for removal of genomic DNA from RNA

RNA		1	µg
10x reaction buffer with $MgCl_2$		1	µl
DNase I, RNase-free		1	µl
DEPC-treated water	up to	10	µl

7. Reverse transcription reaction mixture for cDNA synthesis

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

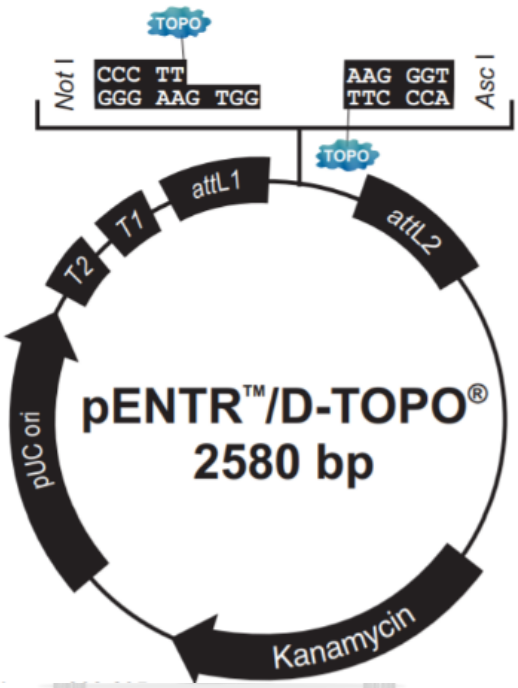
8. qRT-PCR reaction mixture

2x Luna® universal qPCR master mix		5	µl
1,000 nM forward primer		0.5	µl
1,000 nM reverse primer		0.5	µl
cDNA template		1	µl
Nuclease-free water		3	µl

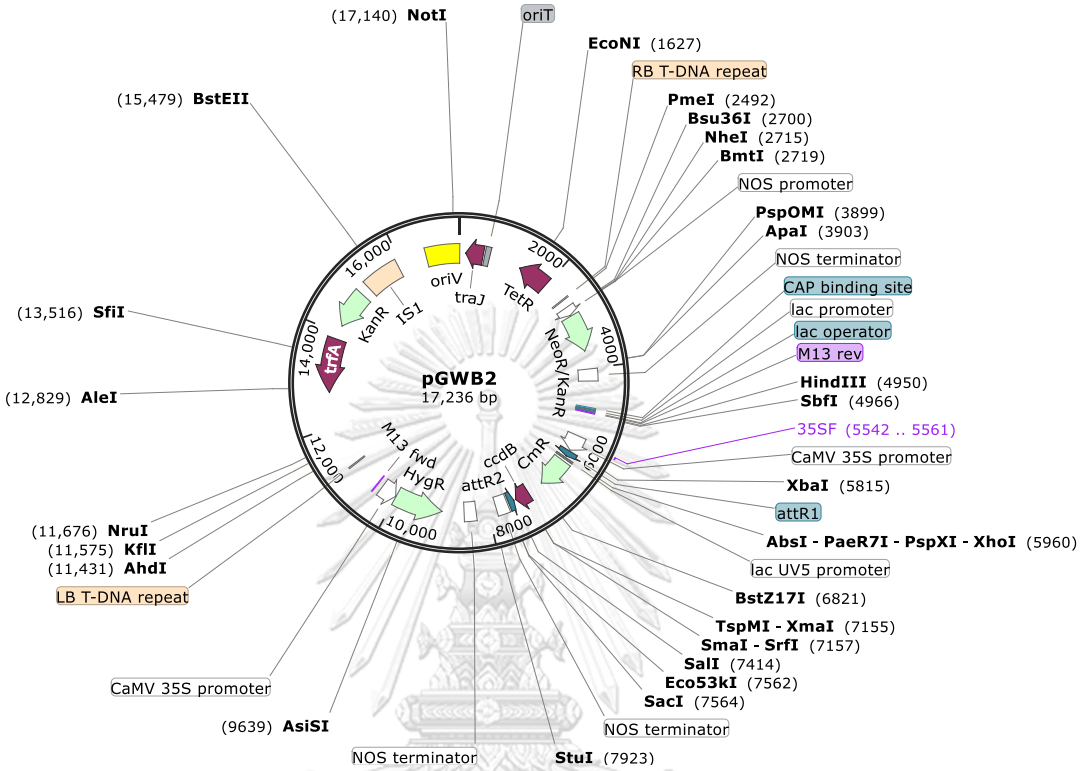
APPENDIX B

VECTORS

1. pENTR/D-TOPO



2. pGWB2



APPENDIX C

HOMOZYGOUS TRANSGENIC ARABIDOPSIS SELECTION

1. Hemizygous selection of T2 generation transgenic Arabidopsis over-expressing *OsMS* under the control of 35SCaMV promoter

Line	Total seed	Observed germinated	Expected germinated	Chi-germinated	Observe non-germinated	Expected non-germinated	Chi-non-germinated	SUM-Chi*	Status
OX/WT1	148	96	111	2.027	52	37	6.081	8.081	NO
OX/WT4	151	101	113.25	1.325	50	37.75	3.975	5.300	NO
OX/WT6	136	84	102	3.177	52	34	9.529	12.706	NO
OX/WT13	137	108	102.75	0.268	29	34.25	0.805	1.073	YES
OX/WT15	80	9	60	43.35	71	20	130.05	173.4	NO

*Remark : SUM-Chi < 3.84 indicating that Chi-Square test follow the criteria at significant level p < 0.05

YES = Hemizygous, NO = Non-Hemizygous

2. Hemizygous selection of T2 generation *AtMS* Arabidopsis mutant expressing *OsMS* or revertants under the control of CaMV 35SCaMV promoter

Line	Total seed	Observed germinated	Expected germinated	Chi-germinated	Observe non-germinated	Expected non-germinated	Chi-non-germinated	SUM-Chi*	Status
OX/ms4-1	146	131	109.5	4.222	15	36.5	12.664	16.886	NO
OX/ms4-2	152	106	114	0.561	46	38	1.684	2.246	YES
OX/ms5-2	150	106	112.5	0.376	44	37.5	1.127	1.502	YES
OX/ms13-3	148	114	111	0.081	34	37	0.243	0.324	YES
OX/ms14-1	132	87	99	1.455	45	33	4.364	5.818	NO
OX/ms14-2	133	88	99.75	1.384	45	33.25	4.152	5.536	NO

*Remark : SUM-Chi < 3.84 indicating that Chi-Square test follow the criteria at significant level $p < 0.05$

YES = Hemizygous, NO = Non-Hemizygous

3. Homozygous selection of T3 generation transgenic Arabidopsis over-expressing *OsMS* under the control of 35SCaMV promoter

Line	Total seed	Germinated seed	Expected 90% of germinated seed	Status
OX/WT13/1	60	20	54	NO
OX/WT13/2	56	6	50.4	NO
OX/WT13/4	56	13	50.4	NO
OX/WT13/7	60	36	54	NO
OX/WT13/8	66	30	59.4	NO
OX/WT13/9	42	21	37.8	NO
OX/WT13/10	60	43	54	NO
OX/WT13/11	50	47	45	YES
OX/WT13/13	66	30	59.4	NO
OX/WT13/15	50	37	45	NO
OX/WT13/16	50	36	45	NO
OX/WT13/20	40	30	36	NO
OX/WT13/23	44	44	39.6	YES
OX/WT13/24	50	38	45	NO
OX/WT13/25	48	39	43.2	NO
OX/WT13/27	38	31	34.2	NO
OX/WT13/28	40	36	36	YES
OX/WT13/29	50	35	45	NO
OX/WT13/30	53	40	47.7	NO

YES = Hemizygous, NO = Non-Hemizygous

4. Homozygous selection of T3 generation *AtMS* Arabidopsis mutant expressing *OsMS* or revertants under the control of CaMV 35SCaMV promoter

Line	Total seed	Germinated seed	Expected 90% of germinated seed	Status
OX/ms4-2/1	60	25	57.6	NO
OX/ms4-2/2	64	20	54	NO
OX/ms4-2/3	64	14	54	NO
OX/ms4-2/4	56	21	50.4	NO
OX/ms4-2/5	60	58	54	YES
OX/ms4-2/6	60	40	54	NO
OX/ms4-2/11	50	24	45	NO
OX/ms5-2/4	60	15	54	NO
OX/ms5-2/5	52	48	46.8	YES
OX/ms5-2/6	64	45	57.6	NO
OX/ms5-2/7	60	43	54	NO
OX/ms5-2/8	56	34	50.4	NO
OX/ms5-2/9	60	38	54	NO
OX/ms5-2/10	58	26	52.2	NO
OX/ms5-2/28	50	30	45	NO
OX/ms5-2/30	60	34	54	NO
OX/ms13-3/1	65	28	58.5	NO
OX/ms13-3/21	45	35	40.5	NO
OX/ms13-3/24	60	21	54	NO
OX/ms13-3/25	35	32	31.5	YES
OX/ms13-3/26	50	34	45	NO
OX/ms13-3/27	40	32	36	NO
OX/ms13-3/29	50	39	45	NO
OX/ms13-3/33	60	49	54	NO

YES = Hemizygous, NO = Non-Hemizygous

APPENDIX D

DNA SEQUENCES

```
Ref      ----ATGGCCACCAACGCCGCAGCGCCGCCGTGCCCGTGCTACGACACGC      46
             ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CACCATGGCCACCAACGCCGCAGCGCCGCCGTGCCCGTGCTACGACACGC      50

Ref      CGGAGGGCGTGGACATCCTCGGCCGGTACGACCCGGAGTTCGCGGCCATC      96
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CGGAGGGCGTGGACATCCTCGGCCGGTACGACCCGGAGTTCGCGGCCATC     100

Ref      CTCACCCGCGACTCGCTGGCCTTCGTGGCCGGCCTGCAGCGCGAGTTCCG     146
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CTCACCCGCGACTCGCTGGCCTTCGTGGCCGGCCTGCAGCGCGAGTTCCG     150

Ref      CGGCGCCGTCCGGTACGCCATGGAGCGCAGGCGGGAGGCGCAGCGGCGGT     196
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CGGCGCCGTCCGGTACGCCATGGAGCGCAGGCGGGAGGCGCAGCGGCGGT     200

Ref      ACGACGCCGGCGAGCTCCCCCGGTTCGACCCGGCCACGAGGCCCGTCCGC     246
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     ACGACGCCGGCGAGCTCCCCCGGTTCGACCCGGCCACGAGGCCCGTCCGC     250

Ref      GAGGCAGGCGGCTGGGCGTGCGCCCCCGTGCCGCCGGCCATCGCCGACCG     296
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GAGGCAGGCGGCTGGGCGTGCGCCCCCGTGCCGCCGGCCATCGCCGACCG     300

Ref      CACCGTCGAGATCACCGGCCCCGCCGAGCCGCGCAAGATGGTCATCAACG     346
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CACCGTCGAGATCACCGGCCCCGCCGAGCCGCGCAAGATGGTCATCAACG     350

Ref      CCCTCAACTCCGGCGCCAAGGTCTTCATGGCTGACTTCGAGGACGCGCTG     396
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CCCTCAACTCCGGCGCCAAGGTCTTCATGGCTGACTTCGAGGACGCGCTG     400

Ref      TCGCCGACGTGGGAGAACCTGATGCGCGGGCAGGTGAACCTGCGCGACGC     446
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     TCGCCGACGTGGGAGAACCTGATGCGCGGGCAGGTGAACCTGCGCGACGC     450

Ref      GGTTGCCGGCACGATCACCTACCGCGACGCGGCGCGAGGGCGGGAGTACA     496
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GGTTGCCGGCACGATCACCTACCGCGACGCGGCGCGAGGGCGGGAGTACA     500

Ref      GGCTCGGCGACCGCCCCGCGACGCTCTTCGTGAGGCCGCGCGGCTGGCAC     546
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GGCTCGGCGACCGCCCCGCGACGCTCTTCGTGAGGCCGCGCGGCTGGCAC     550

Ref      CTCCCCGAGGCGCACGTCCTCGTCGACGGCGAGCCGGCCATCGGCTGCCT     596
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CTCCCCGAGGCGCACGTCCTCGTCGACGGCGAGCCGGCCATCGGCTGCCT     600

Ref      CGTCGACTTCGGCCTCTACTTCTTCCACAGCCACGCCGCCTTCCGCTCCG     646
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CGTCGACTTCGGCCTCTACTTCTTCCACAGCCACGCCGCCTTCCGCTCCG     650
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Ref      GCCAGGGCGCCGACTTCGGCCCCTTCTTCTACCTCCCCAAGATGGAGCAC     696
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GCCAGGGCGCCGACTTCGGCCCCTTCTTCTACCTCCCCAAGATGGAGCAC     700

Ref      TCTAGGGAGGCGAGGATATGGAAGGGGGTGTTCGAGAGGGCGGAGAAGGA     746
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     TCTAGGGAGGCGAGGATATGGAAGGGGGTGTTCGAGAGGGCGGAGAAGGA     750

Ref      GGCGGGGATAGGGAGGGGGAGCATCAGGGCGACGGTGCTGGTGGAGACGC     796
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GGCGGGGATAGGGAGGGGGAGCATCAGGGCGACGGTGCTGGTGGAGACGC     800

Ref      TGCCGGCGGTGTTCCAGATGGAGGAGATCCTGCACGAGCTGCGCGACCAC     846
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     TGCCGGCGGTGTTCCAGATGGAGGAGATCCTGCACGAGCTGCGCGACCAC     850

Ref      TCGGCGGGGCTCAACTGCGGCCGCTGGGACTACATCTTCAGCTACGTCAA     896
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     TCGGCGGGGCTCAACTGCGGCCGCTGGGACTACATCTTCAGCTACGTCAA     900

Ref      GACGTTCCGCGCCCGCCCCGACCGCCTCCTCCCCGACCGCGCCCTCGTCG     946
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GACGTTCCGCGCCCGCCCCGACCGCCTCCTCCCCGACCGCGCCCTCGTCG     950

Ref      GCATGGCCCAGCACTTCATGCGCTCCTACTCCCACCTCCTCATCCAGACC     996
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GCATGGCCCAGCACTTCATGCGCTCCTACTCCCACCTCCTCATCCAGACC    1000

Ref      TGCCACCGCCGCGGCGTCCACGCCATGGGCGGCATGGCGGCGCAGATCCC    1046
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     TGCCACCGCCGCGGCGTCCACGCCATGGGCGGCATGGCGGCGCAGATCCC    1050

Ref      GATCAAGGACGACGCGGCGGCGAACGAGGCGGCGCTGGAGCTGGTGCGCA    1096
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GATCAAGGACGACGCGGCGGCGAACGAGGCGGCGCTGGAGCTGGTGCGCA    1100

Ref      AGGACAAGCTGCGGGAGGTGCGCGCCGGGCACGACGGGACGTGGGCGGCG    1146
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     AGGACAAGCTGCGGGAGGTGCGCGCCGGGCACGACGGGACGTGGGCGGCG    1150

Ref      CACCCGGGGCTCATCCCGGCGATCCGGGAGGTGTTCGAGGGACACCTCGG    1196
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     CACCCGGGGCTCATCCCGGCGATCCGGGAGGTGTTCGAGGGACACCTCGG    1200

Ref      AGGGAGGCCGAACCAGATCGACGCGGCGGCTGGCGACGCCGCCCGTGCCG    1246
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     AGGGAGGCCGAACCAGATCGACGCGGCGGCTGGCGACGCCGCCCGTGCCG    1250

Ref      GCGTCGCCGTCACGGAGGAGGACCTGCTCCAGCCGCCGCGCGGGGCGCGC    1296
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     GCGTCGCCGTCACGGAGGAGGACCTGCTCCAGCCGCCGCGCGGGGCGCGC    1300

Ref      ACGGTGGAGGGCCTGCGCCACAACACGCGCGTCGGCGTGCAGTACGTCGC    1346
         ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS     ACGGTGGAGGGCCTGCGCCACAACACGCGCGTCGGCGTGCAGTACGTCGC    1350
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Ref       GGCGTGGCTATCCGGGTCGGGCTCCGTGCCGCTGTACAACCTGATGGAGG     1396
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      GGCGTGGCTATCCGGGTCGGGCTCCGTGCCGCTGTACAACCTGATGGAGG     1400

Ref       ACGCCGCCACCGCGGAGATCAGCCGGGTGCAGAACTGGCAGTGGCTCCGG     1446
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      ACGCCGCCACCGCGGAGATCAGCCGGGTGCAGAACTGGCAGTGGCTCCGG     1450

Ref       CACGGCGCGGTGCTGGACGCCGGCGGCGTGGAGGTCCGGGCCACGCCCGA     1496
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      CACGGCGCGGTGCTGGACGCCGGCGGCGTGGAGGTCCGGGCCACGCCCGA     1500

Ref       GCTGCTGGCGCGCGTCGTGGAGGTGGAGATGGCGAGGGTGGAGGCCGAGG     1546
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      GCTGCTGGCGCGCGTCGTGGAGGTGGAGATGGCGAGGGTGGAGGCCGAGG     1550

Ref       TGGGCGCCGAGAGGTTCCGGCGCGGCCGGTACGCGGAGGCCGGCAGGATC     1596
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      TGGGCGCCGAGAGGTTCCGGCGCGGCCGGTACGCGGAGGCCGGCAGGATC     1600

Ref       TTCAGCCGGCAGTGCACCGCGCCGGAGCTGGACGACTTCCTCACGCTCGA     1646
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      TTCAGCCGGCAGTGCACCGCGCCGGAGCTGGACGACTTCCTCACGCTCGA     1650

Ref       CGCGTACAACCTCATCGTGGTGCACCACCCCGGAGCATCGTCGCCGTGCA     1696
          ||||||||||||||||||||||||||||||||||||||||||||||||||
OsMS      CGCGTACAACCTCATCGTGGTGCACCACCCCGGAGCATCGTCGCCGTGCA     1700

Ref       AGCTCTGA   1704
          ||||||||
OsMS      AGCTCTGA   1708
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= TOPO cloning site

APPENDIX E

MALATE SYNTHASE GENOMIC SEQUENCES

>A.thaliana Araport11|AT5G03860|Chr5:1032098..1034973 reverse

ACTTTAACAAAAAAAAAAAAAAAAAAAGAGTTTAAGACCAAATTTCAAAACATTTGGATTGAACCCAAA
ACAGTGCCTCGATTTTATCTGAATCAATCCGTTAACAAGGATGTTTTGGTCTCATCATCCAATAGAAGC
AATCAGTAAACTAATTTGCATGACAACTAAAACTGTTTTAGGTTTTATGCAGAGCCAGAGGTATTGGAT
GATGAAAACGATATGGTGACGAAAAGGATATTATTATAAATAGAAAATTTGTGATAATCCTATCCTAAC
CGATATAGTGACGAAAATCATACAGAACTTGGCACACTTTCCCAAGGAGGCACACATGTCAATGCGTTT
CACCGATTATACCTATCCATTTCCACCTTTATAATTCATATTACACGATCACATTTATATATCACTATA
TCACGTTACAAAATTTAGACAAAAAAATAAATATGGAGCTCGAGACCTCAGTTTATCGACCAAACGTCG
CCGTTTATGATTCTCCGGATGGTGTGGAGGTTCGAGGACGGTACGACCAAATCTTCGCCAAGATTTTGA
CGAGAGAAGCTCTTAGCTTTGTGGCTGAGTTACAGAGAGAGTTTCGTGGCCATGTGAAGTATGCCATGG
AGTGCCGGAGAGAGGCAAGACGGCGTTATAACTCCGGTGCCGTTCCTGGGTTTGATCCTTCCACTAAGT
TCATCAGAGACGGTGATTGGAGCTGTGCCTCCGTCCCTCCTGCCGTTGCTGACCGGAGAGTTGAGATCA
CCGGTCCGGTCGAGAGGAAGATGATCATCAACGCTCTTAACTCCGGAGCTAAAGTCTTTATGGTCCATT
TTCTTTCACTCTCTCTTTATACAACACATTAGTTGGTTTGGATTGGATTTGACCGACATTGGTGAAACT
TTTGAATAATACTGTAACAAACTTTTAATTGGACCGCGTTCAATTTGGTATCAGTTTAAATTTGGTTTT
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GTGTTGGGGTTTGAACAGGCTGATTTCGAGGACGCTTTGTCTCCAAGCTGGGAGAATCTGATGAGAGGG
CATGTGAACCTGAAAGATGCAGTGGATGGATCCATTACGTTCCACGACAAGTCAAGGAACAGAGTTTAC
AAGCTCAACGACCAAACCGCTAAGCTTTTTGTCCGGCCAAGAGGTTGGCATCTCCCGGAGGCTCACATC
CTCATCGACGGTGAACCCGCCACTGGATGTCTTGTCGATTTTGGCCTTTACTTCTTCCACAACTACGCC
AAATTTAGACAGACTCAAGGTTCCGGTTTCGGTCCATTCTTCTATCTCCCCAAAATGGAACATTCTAGG
TACCTTCCTGGTTCGGTCATCAAATTGATGTTTTTCGGTCTAGGGTTAATTAGGTTACTTGGTTTCTTG
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GCATTAGAGCAACTGTGCTTATCGAGACCCTCCCGGCTGTCTTCCAGATGAATGAGATACTCTATGAAC
TACGTGATCACTCAGTCGGTTTAAACTGTGGAAGATGGGATTATATCTTCAGCTATGTCAAAACCTTCC
AAGCTCACCCTGACCGGCTCTTACCAGACCGTGTCCTAGTTGGCATGGGCCAGCATTTCATGAGAAGTT
ACTCTGATCTTCTCATCCGTACTTGTCATAAGCGCGGTGTCCACGCCATGGGTGGCATGGTTAGTCCCC
TCTTAACACTAGTTCACTATATGTCTCTTTCAAACTACATGAATGCTCTGTGAAAACATTATAAATAAA
GTTGAAAATGTTTTGATTTGTTCGGTGGTATGATGATCAGGCGGCTCAGATTCCGATAAGAGATGACCC
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AAGTCCAAACCAAATCAAATCAGTGAAGCGCGAGGACGCAGCCGCAATAACAGAGGAAGATCTGCTTCA

AATACCGCGAGGAGTGCGTACACTCGAGGGTTTAAGGCTGAACACACGTGTAGGAATTCAATACTTAGC
CGCATGGTTAACAGGATCAGGATCTGTCCCACTCTATAACCTAATGGAAGATGCTGCTACGGCTGAGAT
AAGCCGTGTCCAGAATTGGCAATGGATTAGGTATGGAGTGGAACTTGATGGAGACGGGCTTGGAGTTCG
AGTGAGTAAAGAGTTGTTTGGGAGAGTTGTCGAAGAAGAGATGGAAAGAATTGAGAAAGAAGTTGGGAA
AGACAAGTTTAAGAATGGAATGTATAAGGAAGCTTGCAAGATGTTTACAAAGCAGTGTACTGCACCTGA
GCTTGATGATTTTCTTACACTTGCTGTTTATAATCACATTGTAGCTCACTACCCTATCAATGTCTCAAG
GCTCTGAAAGAAAGAAGATGAAGAAGGAAAGGGGAGAGAGGGGTTGAGATATGAGTTTGTGCTTTTGTT
TCTTTGTGTCTGTTTCTCAAAATGTTATTGTTCTTTCTGTATTTTGGTATATTGTAATAAGCAAAACTT
GTTCTTTTATTTCCAGTAAGAAATAAATGTTCTATATCATCCTTAA

= 5'UTR = Coding sequences = 3'UTR

= T-DNA insertion site for *Atms* mutant Arabidopsis

VITA

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