สมบัติการจับคัลมอดุลินและบทบาทในการตอบสนองต่อความเครียดของ MYOSIN HEAVY CHAIN ในข้าว *Oryza sativa* L.

นางสาวจุฑามาศ อนันทยานนท์

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CAM-BINDING PROPERTY AND ROLES IN STRESS RESPONSE OF MYOSIN HEAVY CHAIN IN RICE *Oryza sativa* L.

Miss Jutamas Anantayanon

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
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Field of Study	Biochemistry and Molecular Biology
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จุฑามาศ อนันทยานนท์ : สมบัติการจับคัลมอดุลินและบทบาทในการตอบสนองต่อความเครียดของ MYOSIN HEAVY CHAIN ในข้าว *Oryza sativa* L. (CAM-BINDING PROPERTY AND ROLES IN STRESS RESPONSE OF MYOSIN HEAVY CHAIN IN RICE *Oryza sativa* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร.ธีรพงษ์ บัวบูชา, 145 หน้า.

เมื่อพืชได้รับความเครียดจากสิ่งแวดล้อมทั้งที่มีชีวิตและไม่มีชีวิตจะเกิดการตอบสนองโดยส่งสัญญาณด้วยการเพิ่มระดับความเข้มข้นของแคลเซียมอย่างรวดเร็วภายในไซโทซอล (cytosol) ของเซลล์พืช ซึ่งสัญญาณแคลเซียมนี้จะถูกถ่ายทอดโดยการทำงานของโปรตีนรับสัญญาณแคลเซียม (calcium sensor) คัลมอดุลิน (Calmodulin, CaM) ซึ่งเป็นหนึ่งในโปรตีนรับสัญญาณแคลเซียมจะทำงานโดยเข้าจับกับแคลเซียมไอออน แล้วส่งสัญญาณแคลเซียมต่อไปโดยเข้าจับและควบคุมการทำงานของโปรตีนเป้าหมายหลาย ๆ ชนิดภายในเซลล์ มีผลทำให้พืชตอบสนองต่อการเปลี่ยนแปลงของภาวะแวดล้อมต่าง ๆ อย่างเหมาะสม ในการศึกษาหน้าที่ของโปรตีนคัลมอดุลิน จำเป็นต้องค้นหาโปรตีนเป้าหมาย ซึ่งในการศึกษาก่อนหน้านี้ได้พบโปรตีนเป้าหมายของโปรตีน OsCaM1 จากข้าว (*Oryza sativa* L.) อยู่หลายชนิดด้วยวิธี cDNA expression library screening หนึ่งในโปรตีนเป้าหมายของโปรตีน OsCaM1 คือโปรตีน Myosin heavy chain ในงานวิจัยนี้ได้ใช้เทคนิค yeast two-hybrid system ในการตรวจสอบการจับกันของโปรตีน Myosin heavy chain กับโปรตีน CaM และ CML จากข้าว พบว่าโปรตีน Myosin heavy chain มีอันตรกิริยากับโปรตีน OsCML4, OsCML5 และ OsCML8 ทั้งในภาวะที่มีและไม่มีแคลเซียม แต่ไม่มีอันตรกิริยากับโปรตีน OsCaM1 นอกจากนี้ในงานวิจัยนี้ยังได้ใช้เทคนิค CaM Pull-down assay ในการยืนยันการจับกันของโปรตีนคัลมอดุลินกับโปรตีน Myosin heavy chain จากการทดลองพบว่า โปรตีน Myosin heavy chain มีปฏิสัมพันธ์กับโปรตีนคัลมอดุลินในภาวะที่มีแคลเซียม ในงานวิจัยนี้ยังได้ศึกษารูปแบบการแสดงออกของยีน *Myosin heavy chain* ในข้าวภายใต้ภาวะเครียดจากความเค็มและภาวะเครียดจากความแล้งด้วยวิธี real-time RT-PCR พบว่าในข้าวอายุ 2 สัปดาห์ ที่เนื้อเยื่อใบในภาวะเครียดจากความเค็มมีการแสดงออกที่ลดลงของยีน *Myosin heavy chain* ไม่ชัดเจน ส่วนในภาวะเครียดจากความแล้งมีการแสดงออกของยีนนี้ลดลงที่เวลา 6 ชั่วโมงอย่างมีนัยสำคัญทางสถิติที่ระดับ 0.05 นอกจากนี้ในงานวิจัยนี้ผู้วิจัยได้สร้าง อะราบิดอปสิสทรานส์จินิกส์ที่มีการแสดงออกเกินปกติของยีน *Myosin heavy chain* ได้สำเร็จจำนวน 4 ไลน์ได้แก่ C2, C6, E1 และ E3 ในการศึกษาร้อยละการงอกของเมล็ด พบว่าร้อยละการงอกของเมล็ด wild-type และเมล็ดทรานส์จินิกส์ภายใต้ภาวะเครียดจากความเค็มและภาวะเครียดจากความแล้งไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ผลการเปรียบเทียบน้ำหนักสดและน้ำหนักแห้งของต้น wild-type และทรานส์จินิกส์ภายใต้ภาวะเครียดจากความเค็มและภาวะเครียดจากความแล้ง พบว่าในภาวะเครียดทั้งน้ำหนักสดและน้ำหนักแห้งไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ เมื่อทำการเปรียบเทียบร้อยละการลดลงของน้ำหนักสดและน้ำหนักแห้ง พบร้อยละการลดลงของน้ำหนักสดภายใต้ภาวะเครียดจากความเค็มและภาวะเครียดจากความแล้งของไลน์ E1 และในภาวะเครียดจากความแล้งของไลน์ C6 ต่ำกว่าพืช wild type และพบว่าร้อยละการลดลงของน้ำหนักแห้งของไลน์ C6 และ E3 ต่ำกว่าพืช wild type ในภาวะเครียดจากความแล้งเท่านั้น

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2557

5472811223 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
KEYWORDS: CALMODULIN/ CALMODULIN TARGET PROTEIN/ RICE/ TRANSGENIC

JUTAMAS ANANTAYANON: CAM-BINDING PROPERTY AND ROLES IN STRESS RESPONSE OF MYOSIN HEAVY CHAIN IN RICE *Oryza sativa* L.. ADVISOR: ASSOC. PROF. TEERAPONG BUABOOCHA, Ph.D., 145 pp.

When plants encounter to environmental stimuli both biotic and abiotic stresses, plants will response using the signal transduction pathway by rapid increasing the concentration of calcium ion in the cytosol of plant cell. Calcium signals are then conveyed by the action of calcium sensors. Calmodulin (CaM), which is one of the calcium sensors, will bind to calcium ions and transmit the calcium signal by binding to and activating the target proteins within the cell. The activities of target proteins affect physiological responses to specific stimuli received by plant cells. To identify CaM function, it is necessary to find its target proteins. In previous study, the putative OsCaM1 target proteins were identified from rice (*Oryza sativa* L.) by cDNA expression library screening. One of the putative OsCaM1 target proteins is Myosin heavy chain. In this research, the yeast two-hybrid system was performed to examine the interaction of Myosin heavy chain with CaM and CML from rice. The result showed that Myosin heavy chain can interact only with OsCML4, OsCML5 and OsCML8 both in the presence and in the absence of calcium but not with OsCaM1. In the addition, in this research, the CaM Pull-down assay was performed to confirm the interaction of CaM with Myosin heavy chain. In the result, Myosin heavy chain was found to bind CaM in the presence of calcium. Moreover, the expression pattern of a *Myosin heavy chain* gene in rice under salt and drought stresses was investigated using real-time RT-PCR. In the two-week-old rice seedlings, this gene did not clearly exhibited lower expression level in the leaf tissues when rice plants exposed to salt stress. In the case of drought stress, the lower expression level in the leaf tissues of rice was detected ($p<0.05$). In addition, in this research, we have successfully generated four transgenic Arabidopsis lines overexpressing *Myosin heavy chain* gene including line C2, C6, E1 and E3. In order to characterize the role of Myosin heavy chain protein, the seed germination rate and percentage was observed. In the result, no significant change of the germination rate and percentage between wild-type and transgenic lines under both salt and drought stresses was detected. Next, fresh and dry weight of wild-type and transgenic lines were compared under both salt and drought stress conditions. We found that both fresh and dry weight measurement did not show significant difference in each stress treatment. When comparing the reduction percentage of fresh and dry weight, there was a decrease in the fresh weight reduction percentage in line E1 under both salt and drought stress conditions and only drought stress of line C6 was detected but, the dry weight reduction percentage was decrease only in drought stress condition of line C6 and E3.

Department: Biochemistry Student's Signature .....
Field of Study: Biochemistry and Molecular Biology Advisor's Signature .....
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LIST OF ABBREVIATIONS

A260	absorbance at 260 nm
bp	base pairs
^{o}C	degree Celsius
Ca^{2+}	Calcium ion
CaM	Calmodulin gene
CaM	Calmodulin protein
CML	Calmodulin-like gene
CML	Calmodulin-like protein
cDNA	complementary deoxyribonucleic acid
cyt	cytosol
Da	Dalton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
g	gram
h	hour
hr	hour
IPTG	isopropyl-thiogalactoside
kDa	kiloDalton
L	liter
LB	Luria-Bertani
µl	microliter
µM	micomolar
M	mole per liter (molar)
mg	milligram
ml	milliliter
mM	millimolar
min	minute

MS	Murashige and Skoog medium
ng	nanogram
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PEG	polyethylene glycol
pg	picogram
pH	power of hydrogen ion
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
V	voltage
v/v	volume by volume
w/v	weight by volume
WT	wild-type

CHAPTER I

INTRODUCTION

So as to survive and reproduce, many organisms respond to environmental stimuli in a timely and appropriate manner. Information perceived at the cellular level is translated to stimulus specific physiological responses via signal transduction pathways, often involving second messengers such as calcium ions (Ca^{2+}). Indeed, Ca^{2+} ions have been implicated in response to diverse external stimuli, including light, drought, salinity and pathogens, as well as internal developmental and hormonal cues (Neuhaus, Bowler, Hiratsuka, Yamagata, & Chua, 1997; Neuhaus, Bowler, Kern, & Chua, 1993; Oh, Kim, Wu, Clouse, Zielinski, & Huber, 2012; Whalley, Sargeant, Steele, Lacoere, Lamb, Saunders, et al., 2011; Wu, Hiratsuka, Neuhaus, & Chua, 1996).

1. Calcium signaling

Calcium ion (Ca^{2+}) plays a fundamental role in plant's growth and development. Many extracellular signals and environmental cues including light, abiotic and biotic stress factors, elicit change in the cellular calcium levels, termed as calcium signatures. Ca^{2+} has been well established as a second messenger. The concentration of Ca^{2+} is delicately balanced by the presence of 'Ca^{2+} stores' such as vacuoles, endoplasmic reticulum, mitochondria and cell wall (Tuteja & Mahajan, 2007).

In general, in the absence of a stimulus, the Ca^{2+} concentration in plant cytosol is maintained in the nanomolar range (100–200 nM) whereas it is in the millimolar range (1–10 mM) in the extracellular and intracellular Ca^{2+} stores as shown in Figure 1.1 (Vaka S. Reddy & Reddy, 2004). In response to a variety of stimuli, the cytosolic Ca^{2+} concentration is rapidly elevated via an increased Ca^{2+} influx and then quickly returns to the basal level by Ca^{2+} efflux (Evans, McAinsh, & Hetherington, 2001; A. S. N. Reddy, 2001a; Sanders, Pelloux, Brownlee, & Harper, 2002). This transportation of Ca^{2+} ions from the cytosol to the extracellular space or into

intracellular organelles is achieved by ATP-driven calcium-pumps and anti-porters (Vetter & Leclerc, 2003).

2. Calcium-binding proteins (Calcium sensors)

The transient Ca^{2+} elevations are sensed by several Ca^{2+} sensors or Ca^{2+}-binding proteins, which usually contain a conserved helix–loop–helix structure called EF-hand motif. A typical EF-hand comprises two short **α**-helices connected by a 12-amino-acid loop that is enriched in acidic residues being responsible for coordination of the Ca^{2+} ion (Gifford, Walsh, & Vogel, 2007). These motifs often exist in pairs that bind Ca^{2+} with positive cooperativity. Binding of calcium ion by Ca^{2+} sensors induces a conformational change, leading to exposure of hydrophobic regions, which allow the sensor to interact with downstream targets. In plants, three major groups of EF-hand Ca^{2+} sensors have been characterized, based on the number and organization of EF-hands and on the similarity of the amino acid sequences. These groups consist of calmodulin (CaM), calcium-dependent protein kinase (CDPK) and calcineurin B-like protein (CBL) (DeFalco, Bender, & Snedden, 2009; Gifford, Walsh, & Vogel, 2007).

CaM is one of the most conserved Ca^{2+}-binding proteins found in eukaryotic cells. Similar to the counterpart in animals, there are four EF-hands in a typical plant CaM (A. S. N. Reddy, 2001a; Wayne A. Snedden & Fromm, 2001). A new family of plant-specific Ca^{2+} sensors is CBL, which is similar to the regulatory B-subunit of calcineurin in animals, and usually contains three EF-hands (Luan, Kudla, Rodriguez-Concepcion, Yalovsky, & Gruissem, 2002). On the contrary to CaM and CBL, which serve only as calcium sensors, CDPK containing both a kinase domain and a CaM-like domain (four EF-hands) in a single protein acts as both Ca^{2+} sensor and effector (Cheng, Willmann, Chen, & Sheen, 2002; Harmon, Gribskov, & Harper, 2000). Apart from plants, CDPK is found in certain protozoans but not in other animals. Interestingly, plant genomes possess large families of each of the three classes of Ca^{2+} sensors. In *Arabidopsis*, a remarkable 11 CaM, 9 CBL and 34 CDPK genes are predicted to exist in its genome (Cheng, Willmann, Chen, & Sheen, 2002; Day, Reddy,

Ali, & Reddy, 2002; Harmon, Gribskov, & Harper, 2000; Luan, Kudla, Rodriguez-Concepcion, Yalovsky, & Gruissem, 2002).

3. Calmodulin (CaM) and Calmodulin-like (CML) proteins

Calmodulin (CaM) is a small heat-stable Ca^{2+} binding protein (approximately 150 amino acids) found in all eukaryotic cells and plays a key role in numerous cellular Ca^{2+} dependent signaling pathways (Cohen & Klee, 1988). CaM is evolutionarily highly conserved and comprises four EF-hands. The first two EF-hands combine to form a globular N-terminal domain tethered by a short flexible linker from a highly homologous C-terminal domain consisting of EF-hands 3 and 4 making CaM to be a dumbbell-shaped structure (Figure 1.2).

When CaM binds to calcium, the conformational change by CaM exposes its hydrophobic sites, which normally interact to its target proteins (Babu, Bugg, & Cook, 1988; Wriggers, Milligan, Schulten, & McCammon, 1998). Without binding of calcium, two EF-hands of the N-terminus stay at close proximity to each other, while the C-terminal domain of CaM exhibits a 'semi-open' conformation (Swindells & Ikura, 1996). In this structure, the target protein is pre-associated exclusively with the semi-open conformation of the C-terminal domain of apo-CaM. No interactions are detected with the N-terminal domain of CaM in the absence of calcium. This is different from the situation at elevated levels of calcium when both domains of CaM are engaged in interactions with the target protein (Schumacher, Rivard, Bachinger, & Adelman, 2001).

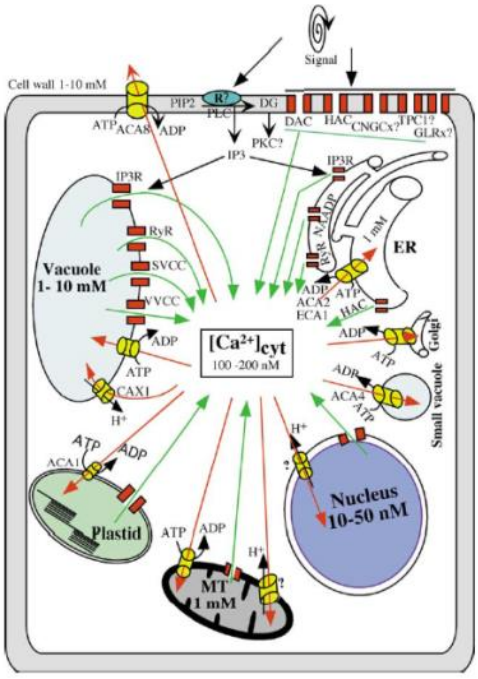


Figure 1.1 Schematic representation of Ca^{2+}-permeable channels, pumps and transporters on various membranes of a cell that are thought to be involved in generation of a signal-specific Ca^{2+} signature and restoring it to resting level. Different types of Ca^{2+}-permeable channels are shown with red-gates that allow Ca^{2+} entry from high-concentration sources (cell walls and organelles) into low-concentration cytosol. The channels are gated by voltage (HAC, DAC) and ligands such as Ca^{2+} (TPC1) Ca^{2+}-CaM, cNMP (CNGCs), and glutamate (GLRs), IP3 (IP3R), cADPR (RyR) and NAADP (NAADP receptor). Maintenance of low cytosolic Ca^{2+} is accomplished by Ca^{2+}-ATPases and transporters (shown with yellow-cylinders), which are driven by energy from the hydrolysis of ATP and proton-force, respectively. The precise location of some transporters, ATPases and antiporters marked with (?), the organellar Ca^{2+} sources and the molecular identity of IP3R, RyR and NAADP receptor remain to be identified. The estimated Ca^{2+} concentration in the cytosol, different organelles and the cell wall is indicated. Green and red arrows indicate influx and efflux sequestration of Ca^{2+} stream, respectively. PLC, phospholipase C; ER, endoplasmic reticulum; MT, mitochondrion (Vaka S. Reddy & Reddy, 2004)

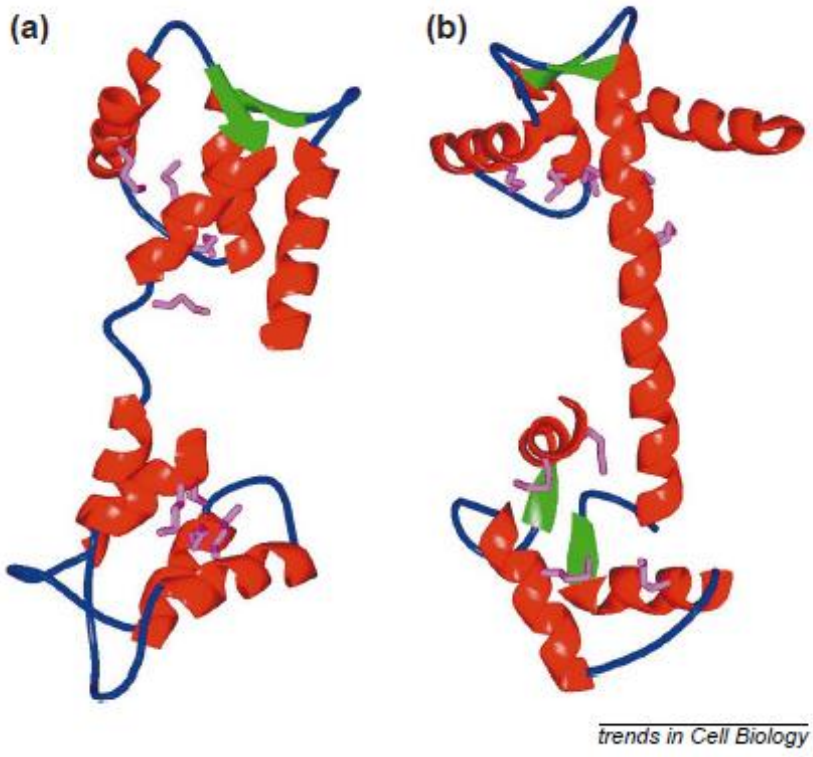


Figure 1.2 The Ca^{2+}-regulated conformational changes in calmodulin. The main chain structure of Ca^{2+}-free (apo) CaM (a) and Ca^{2+}_4–CaM (b) are shown in red with their respective N-terminal domains on top. Methionine side chains are shown in purple to denote the location of potential hydrophobic pockets in each of the two domains. Ca^{2+} binding produces large changes in the helices in both domains, resulting in the exposure of several hydrophobic residues (Chin & Means, 2000).

CaM has no an enzymatic activity but, in the presence of calcium ion, it is a multifunctional protein because it functions by transduction of the increased cytosolic calcium ion signal by binding to and altering the activities of various target proteins. The activities of these target proteins affect physiological responses to the vast array of specific stimuli received by plant cells (Yang & Poovaiah, 2003) as shown in Figure 1.3.

CML proteins belong to the sensor relay family of calcium sensor proteins. These proteins are composed of EF-hands with no identifiable functional domains and share at least 16% amino acid identity with CaM (McCormack & Braam, 2003). The differentiation of CaMs and CMLs includes differences in target specificity, subcellular localization and affinity for calcium (Luan, Kudla, Rodriguez-Concepcion, Yalovsky, & Gruissem, 2002; Zielinski, 2002). Different CML proteins have different role in stress perception and plant development (McCormack, Tsai, & Braam, 2005).

4. Calmodulin-binding proteins

Calmodulin (CaM) is an essential calcium-binding protein which binds to and activates a diverse population of downstream targets (calmodulin-binding proteins; CaMBPs) that carry out its critical signaling functions (O'Day, 2003).

Calmodulin-binding proteins (CaMBPs) comprise a diverse group related by the fact that they interact with calmodulin, an interaction that is usually regulated by the intracellular level of calcium ions. Some of the CaMBPs that have been identified, with their general sub-cellular locales and general functions, are shown in Figure 1.4. Based upon their calcium ion requirement for binding of CaM, CaMBPs are classified into three categories, Ca^{2+}-dependent, Ca^{2+}-independent and Ca^{2+}-inhibited (James, Vorherr, & Carafoli, 1995).

Therefore, the identification and characterization of novel CaMBPs are important to better understanding of Ca^{2+}-mediated signaling of CaM. In animals, CaMBPs have been identified including metabolic enzymes, protein kinases, receptors, ion channel proteins and G-proteins (W. A. Snedden & Fromm, 1998). In plants, a fast growing number of proteins have been characterized in recent years as

CaM targets, such as glutamate decarboxylase (Baum, Chen, Arazi, Takatsuji, & Fromm, 1993), kinesin (A. S. N. Reddy, Safadi, Narasimhulu, Golovkin, & Hu, 1996), chloroplast chaperonin (Yang & Poovaiah, 2000), chimeric calcium/ calmodulin-dependent protein kinase (Patil, Takezawa, & Poovaiah, 1995), and membrane transporter-like protein (Takezawa & Minami, 2004). More recently, 17 new CaMBPs were identified in Arabidopsis by Reddy *et al*. (2002) as shown in Table 1.1 however, none was from rice.

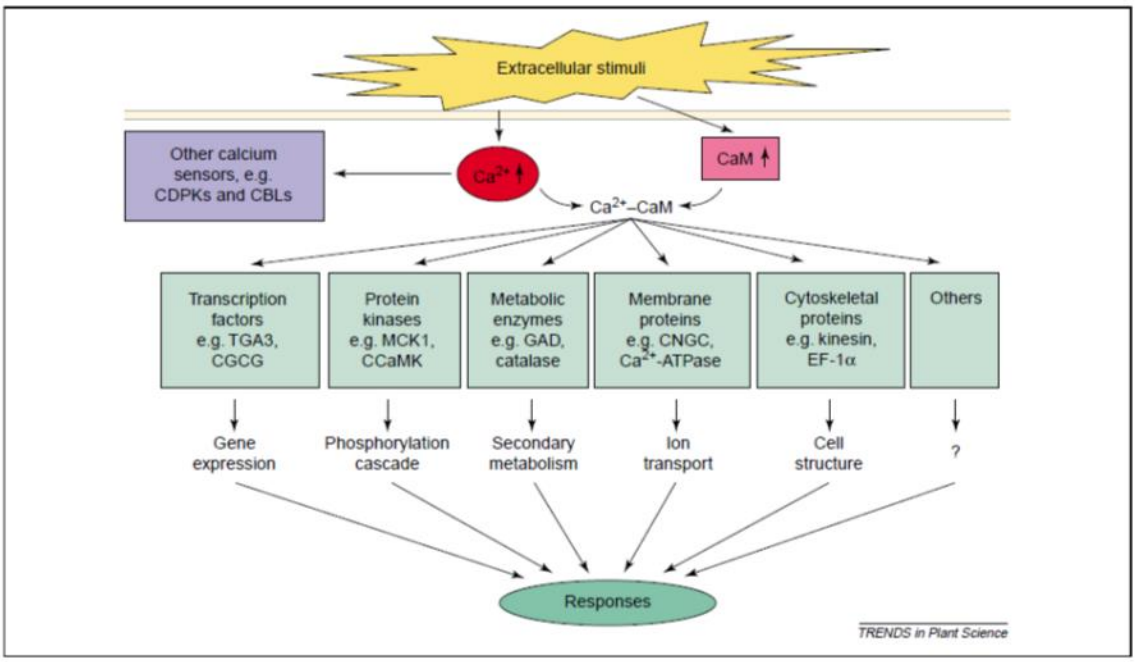


Figure 1.3 Model of Ca^{2+}/calmodulin-mediated network in plants. Ca^{2+} signal changes are triggered by environmental, hormonal or developmental signals. The calcium signatures are decoded by calcium sensors, such as calmodulin (CaM), calcium-dependent protein kinase (CDPK) and calcineurin-B like protein (CBL). Expressions of some CaM genes are also induced by these signals. The activated Ca^{2+} /CaM complex binds to numerous target proteins and modulates their activities. Those target proteins include transcription factors, protein kinases, metabolic enzymes, ion channels and transporters and cytoskeleton proteins. Finally, the Ca^{2+} /CaM-mediated signal network results in physiological responses, such as cell growth or differentiation, stress tolerance or growth arrest and cell death (Yang & Poovaiah, 2003).

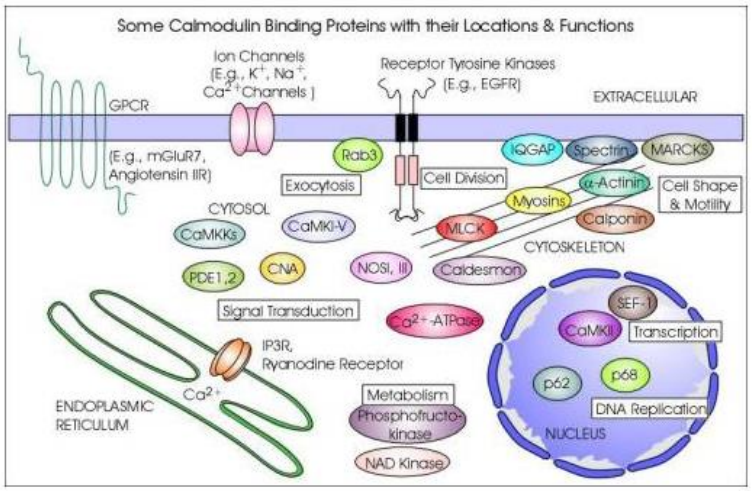


Figure 1.4 Some calmodulin binding proteins with their cellular localization and functions. As the targets of Ca^{2+}-CaM mediated signal transduction, CaMBPs have been shown to regulate various G-protein coupled receptors (GPCRs) such as the metabotropic glutamine receptor 7 (mGluR7) and angiotensin II receptor (Angiotensin IIR), as well as receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR). Intracellularly, ryanodine and inositol 1,4,5-trisphosphate receptors (IP3R) on the endoplasmic reticulum are CaMBPs. A multitude of cytosolic signaling elements are CaMBPs including the A subunit of calcineurin (CNA) a Ca^{2+}-CaM-dependent phosphatase, CaM-dependent kinases (CaMKI-V), CaMK kinases (CaMKKs), nitric oxide synthases I and III (NOSI, III) and phosphodiesterases 1 and 2 (PDE1,2), to name a few. A diversity of cell membrane ion channels is also regulated by CaM including various Na^{+}, K^{+} and Ca^{2+} channels. Other CaMBPs that associate, often transiently, with the cell membrane include Rab3, a monomeric GTPase involved in exocytosis, and several proteins that interact with the cytoskeleton including IQGAP, Spectrin and myrisoylated arginine-rich C kinase substrate (MARCKS). Cytoskeletal regulatory components such as a-Actinin, Caldesmon, Calponin, various myosin isoforms (Myosins), and myosin light chain kinase (MLCK) have been identified as CaMBPs. Ca^{2+}-ATPase is a CaMBP, as are various metabolic enzymes including Phosphofructokinase and NAD kinase. CaMBPs also localize to the nucleus where they regulate transcription (SEF-1, CaMKII), DNA replication (p68) and other nuclear events (p62) (O'Day, 2003).

Table 1.1 Identified CaM targets and their possible function/activity (a complete list of proteins are available at: www.arabidopsis.org/info/genefamily/CBP.html) (V. S. Reddy, Ali, & Reddy, 2002).

Target[a]	Domains	M[b]	Function/activity	Reference
CaM targets or CaM-binding proteins (CBPs)				
1. GADs	Decarboxylase	5	GABA signaling	Baum et al. (1993), Baum et al. (1996)
2. Catalase	Catalase	3	Removal of H_2O_2	Yang and Poovaiah (2002b)
3. MLO	TM	15	Defense against mildew in barley	Kim et al. (2002a), Kim et al. (2002b)
4. Hypothetical	–	2	Not known	Reddy et al. (2002)
5. Hypothetical	–	1	Not known	Reddy et al. (2002)
6. Hypothetical	NLS and unknown repeats	1	Not known	Reddy et al. (2002)
7. Hypothetical	RING	2	Not known	Reddy et al. (2002)
8. SAURs	–	5	Auxin signaling	Yang and Poovaiah (2000b)
9. ACBP60s	–	7	Stress tolerance?	Lu and Harrington (1994), Reddy et al. (1993)
10. TGA3	DNA binding domain	4	Transcription factor	Miao et al. (1994), Szymanski et al. (1996)
11. NPG1	TPRs	3	Pollen germination	Golovkin and Reddy (2003), Safadi et al. (2000)
12. CB-HSPs	–	3	Thermotolerance?	Lu et al. (1995)
13. C/CaMK[c]	Kinase	–	Phosphorylation	Levy et al. (2004), Liu et al. (1998), Mitra et al. (2004), Patil et al. (1995)
			Nodulation	
14. LRR-receptor kinases[d]	LRR and kinase	4	Developmental processes	Vanoosthuyse et al. (2003), Charpenteau et al. (2004)
15. KCBP[e]	Kinesin, MyTH4 and talin like	1	Cell division and trichome morphogenesis	Bowser and Reddy (1997), Oppenheimer et al. (1997), Reddy et al. (1996), Vos et al. (2000)
16. CNGCs	cNMP, TM	20	Ionic transport	Kohler et al. (1999)
17. EICBPs (SRs/CAMTAs)	CG1 and IPT/TIG DNA-binding domains, ankyrin motifs	6	Stress tolerance and gene regulation?	Bouche et al. (2002), Reddy et al. (2000), Taleb and Fromm (2004), Yang and Poovaiah (2002a)
			AtCAMTA1 and –5 enhance *AVP1* gene expression	Mitsuda et al. (2003)
18. ATPase		1	Cell division and vesicle fusion	Buaboocha et al. (2001)
19. Glyoxalase I and II		1	Salt tolerance	Singla-Pareek et al. (2003), Veena et al. (1999)
20. Apyrase		1	Light signaling and ATP transport	Hsieh et al. (1996)
21. Chaperonin		1	Salt tolerance	Yang and Poovaiah (2000a)
22. MDR-like	TM	1	Transport	Wang et al. (1996)
23. PP7	–	1	Phosphatase activity	Kutuzov et al. (2001)
24. HSP-70s	–	6	Thermotolerance	Sun et al. (2000)
25. ACAs[f]	TMs	13	Ca^{2+} efflux	Geisler et al. (2000), Harper et al. (1998)
26. PPI	–	2	Functions in pollen development	Vucich and Gasser (1996)
27. EF-1α	–	4	Translational regulation	Moore et al. (1998)
28. Ribosomal L19	–	3	Not known	Sonnemann et al. (1991)
29. NtMKP1 (tobacco CBP)	Kinase, Gelsolin, and Ser-rich	1	Wound and defense signaling	Yamakawa et al. (2003)
30. DsPTP1	Pohsphatase	1	Dephoshorylation of (phospho S, T, Y)	Yoo et al. (2003)
31. Protein phosphatase 2C (PCaMPP)	Phosphatase	1	Dephoshorylation	Takezawa (2003)
32. Nitricoxide synthase	Enzyme	1	Production of NO	Guo et al. (2003)
33. CaMK	Kinase	3	Phosphorylation	Zhang and Lu (2003)
34. Myosins	Myosin motor domain, IQs	17	Cytoskeletal functions	Reddy and Day (2001)

[a] The animal homologs to plant GAD and catalases do not bind CaM. CBPs 3-14 in this column are specific to plants. CBPs 16-34 are present in plants and animals. Myosins interact with CaM in a Ca^{2+}-independent manner.

[b] Number of family members (M).

[c] C/CaMK homolog in *Arabidopsis* is yet to be identified.

[d] Members include SRK, AtCaMRLK, AtRLK4 and CLV1 are shown to bind CaM in a Ca^{2+}-dependent manner; Vanoosthuyse et al. (2003), Charpenteau et al. (2004).

[e] Two sensors (CaM and KIC) interact with KCBP. A kinesin that binds to CaM has been reported in sea urchin but MyTH4 (myosin tail homology 4) and talin-like domains are absent in sea urchin kinesin-C.

[f] ACA2 is regulated differentially by CaM (positive) and CPK (negative).

5. CaM and CML proteins in rice

In the case of rice (*Oryza sativa* L.), OsCaM and OsCML proteins have been classified by Boonburapong and Buaboocha (2007). The summary of their characteristics of all OsCaM and OsCML proteins is shown in Table 1.2. They were named based on their percentage of amino acid identity with the amino acid sequence of OsCaM1 and the identity was calculated by dividing the number of identical amino acids by the total number of amino acids. These small proteins consist of 145 to 250 amino acid residues and share 30.2% to 84.6% amino acid identity with OsCaM1. The phylogenetic analysis of the OsCaM and OsCML proteins is shown in Figure 1.5. According to the phylogenetic analysis, these proteins were classified into 6 groups. Five proteins in group 1 having the highest degrees of amino acid sequence identity ($\geq$ 97%) to know typical CaMs from other plants were termed OsCaMs. The remaining proteins were termed OsCMLs, which were classified into 5 groups. More than 60% amino acid identity with OsCaM1 was observed in all the CML proteins in group 2 while the lower level of identity was found in other groups. The CML proteins in group 3, 4 and 5 share the average amino acid identity of 48.2%, 46.9% and 43.8% with OsCaM1, respectively. The CML proteins in group 6 were separated into 5 subgroups with no more than 40.7% identity with OsCaM1 (Boonburapong & Buaboocha, 2007).

6. The Yeast two-hybrid system

The yeast two-hybrid (Y2H) system, which is originally created by Fields and Song, is a technique that allows the detection of interacting proteins in living yeast cells or *in vivo*. The interaction between two proteins of interest, called bait and prey, is detected via the reconstitution of a transcription factor and the subsequent activation of reporter genes under the control of this transcription factor (Fields & Song, 1989).

As depicted in Figure 1.6 A, a protein of interest X is expressed as a fusion to a DNA binding domain (DBD). The DBD–X fusion is commonly called the “bait”. Because of the affinity of the DBD for its operator sequences, the bait is bound to

the upstream activator sequence (UAS) of the promoter but it does not activate the reporter gene because it lacks an activation domain. A second protein Y is expressed as a fusion to an activation domain (AD) and is commonly called the “prey”. The prey is capable of activating transcription but usually does not do so because it has no affinity for the promoter elements upstream of the reporter gene. If bait and prey are co-expressed and the two proteins X and Y have an interaction (Figure 1.6 B), then a functional transcription factor is reconstituted at the promoter site upstream of the reporter gene. Consequently, transcription of the reporter gene is activated.

Table 1.2 Characteristics of OsCam and OsCML genes and the encoded proteins (Boonburapong & Buaboocha, 2007).

Name	Locus[1]	Chr[2]	cDNA length[3]	Amino Acids[4]	EF hands[5]	% of Met[6]	Identity to OsCaM1 (%)[7]	Cys 27[8]	Lys 116[9]	Prenylation[10]	Myristoylation[11]	References
OsCam1-1	LOC_Os03g20370	3	450	149	4	6.0	100.0	+	+			[10]
OsCam1-2	LOC_Os07g48780	7	450	149	4	6.0	100.0	+	+			
OsCam1-3	LOC_Os01g16240	1	450	149	4	6.0	100.0	+	+			
OsCam2	LOC_Os05g41210	5	450	149	4	6.0	98.7	+	+			[10]
OsCam3	LOC_Os01g17190	1	450	149	4	6.0	98.7	+	+			
OsCML1	LOC_Os01g59530	1	564	187	4	4.3	84.6			+		[8,9,10]
OsCML2	LOC_Os11g03980	11	552	183	4	4.9	70.3			+		
OsCML3	LOC_Os12g03816	12	552	183	4	4.9	68.9			+		
OsCML4	LOC_Os03g53200	3	465	154	4	6.5	68.9	+	+			
OsCML5	LOC_Os12g41110	12	501	166	4	4.8	62.2	+	+			
OsCML6	LOC_Os11g37550	11	513	170	4	6.5	53.9	+				
OsCML7	LOC_Os08g02420	8	447	148	2	2.8	47.7		+			
OsCML8	LOC_Os10g25010	10	576	191	4	5.2	47.0					
OsCML9	LOC_Os05g41200	5	468	155	1	3.2	46.1					
OsCML10	LOC_Os01g72100	1	558	185	4	4.3	45.6		+			
OsCML11	LOC_Os01g32120	1	636	211	4	1.4	44.1					
OsCML12	LOC_Os01g41990	1	750	249	4	2.8	43.9					
OsCML13	LOC_Os07g42660	7	510	169	4	5.3	43.6					
OsCML14	LOC_Os05g50180	5	522	173	4	4.6	43.3					
OsCML15	LOC_Os05g31620	5	606	201	4	4.0	40.7					
OsCML16	LOC_Os01g04330	1	546	181	4	3.9	40.5					
OsCML17	LOC_Os02g39380	2	495	164	4	4.9	37.7		+			
OsCML18	LOC_Os05g13580	5	477	158	4	5.7	37.7		+			
OsCML19	LOC_Os01g72550	1	441	146	3	7.5	37.2					
OsCML20	LOC_Os02g50060	2	525	174	4	4.0	35.3				+	
OsCML21	LOC_Os05g24780	5	594	197	3	4.6	35.3					
OsCML22	LOC_Os04g41540	4	753	250	4	3.6	35.2					
OsCML23	LOC_Os01g72540	1	456	151	3	7.9	35.1					
OsCML24	LOC_Os07g48340	7	594	197	3	3.0	33.9					
OsCML25	LOC_Os11g01390	11	450	149	3	6.7	33.6					
OsCML26	LOC_Os12g01400	12	450	149	3	6.7	33.6					
OsCML27	LOC_Os03g21380	3	573	190	3	3.2	33.3					
OsCML28	LOC_Os12g12730	12	519	172	4	4.8	33.1		+			
OsCML29	LOC_Os06g47640	6	513	170	3	4.1	33.1					
OsCML30	LOC_Os06g07360	6	711	236	4	2.1	32.8					
OsCML31	LOC_Os01g72530	1	456	151	3	5.3	31.6					
OsCML32	LOC_Os08g04890	8	591	196	3	2.6	30.2					

[1] The Institute of Genomics Research (TIGR) gene identifier number.
[2] Chromosome number in which the gene resides.
[3] Length of the coding region in base pairs.
[4] Number of amino acids of the deduced amino acid sequence.
[5] Number of EF hands based on the prediction by InterProScan.
[6] Percentage of methionine (M) residues in the deduced amino acid sequence.
[7] Number of identical residues divided by the total number of amino acids that have been aligned expressed in percentage.
[8] Presence of a cysteine equivalent to Cys26 of typical plant CaMs at residue 7(-1) of the first EF-hand.
[9] Presence of a lysine equivalent to Lys115 of typical plant CaMs.
[10] Presence of a putative prenylation site.
[11] Presence of a putative myristoylation site.

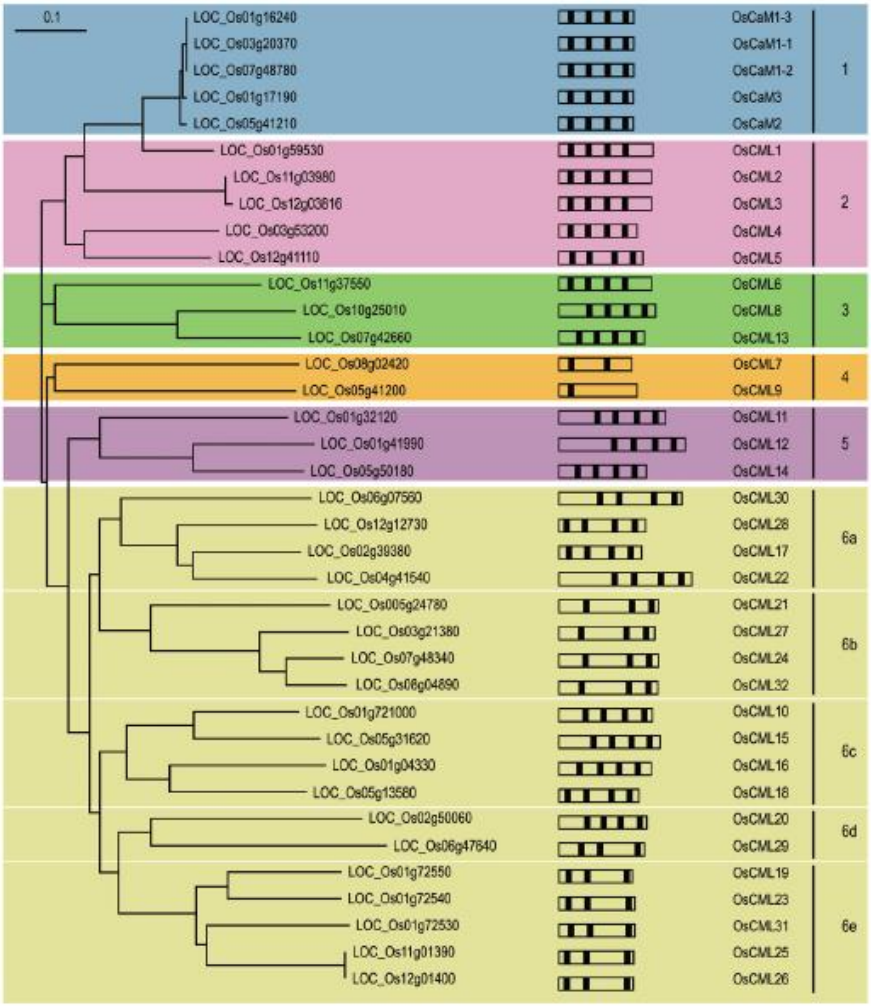


Figure 1.5 Neighbor-joining tree based on amino acid similarities among OsCaM and OsCML proteins. Tree construction using the neighbor-joining method and bootstrap analysis was performed with ClustalX. The TIGR gene identifier numbers are shown and the resulting groupings of CaM and CaM-like proteins designated as 1–6 are indicated on the right. Schematic diagrams of the OsCaM and OsCML open reading frames show their EF-hand motif distribution (Boonburapong & Buaboocha, 2007).

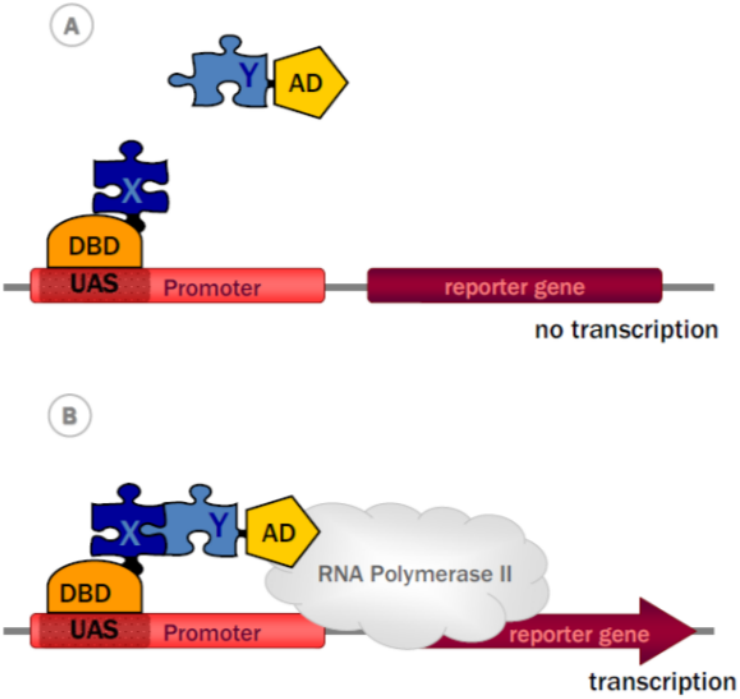


Figure 1.6 The classical yeast two-hybrid system. (A) The protein of interest X, is fused to the DNA binding domain (DBD), a construct called bait. The potential interacting protein Y is fused to the activation domain (AD) and is called prey. (B) The bait, i.e. the DBD-X fusion protein, binds the upstream activator sequence (UAS) of the promoter. The interaction of bait with prey, i.e. the AD-Y fusion protein, recruits the AD and thus reconstitutes a functional transcription factor, leading to further recruitment of RNA polymerase II and subsequent transcription of a reporter gene (Bruckner, Polge, Lentze, Auerbach, & Schlattner, 2009).

7. CaM Pull-down assay

Pull-down assays are the technique involving in isolation of a protein complex by adsorbing the complex onto beads. Immobilized ligands on the beads bind specifically to a component of the complex, either via an affinity tag (e.g., GST, histidine, maltose binding protein, etc.) or an antibody. Pull-down assays are important tools for mapping protein-protein interaction networks. They have been successfully used on a global scale to map protein-protein interactions in a number of organisms (e.g., yeast, *E. coli*, *C. elegans*). Genetic approaches involving the yeast two-hybrid (Y2H) system and similar technologies are useful complements to multiprotein complex isolation. False positives are a major concern associated with mapping protein-protein interactions on a global scale. Therefore, in addition to the relevant control experiments, it is useful to perform both complex isolation and Y2H experiments independently to validate data sets (S. Li, Armstrong, Bertin, Ge, Milstein, Boxem, et al., 2004).

Calmodulin-Sepharose^{TM} 4B is calmodulin immobilized by the CNBr method to the Sepharose 4B. This method is a form of affinity chromatography referred to as a CaM pull-down assay. It uses CaM-Sepharose beads to test proteins that bind to CaM and the influence of Ca^{2+} on this binding. It is considerably more time efficient and requires less protein relative to column chromatography and other assays. Altogether, this provides a valuable tool to explore Ca^{2+}/CaM signaling and proteins that interact with CaM (Kaleka, Petersen, Florence, & Gerges, 2012).

8. Real-time PCR

Reverse transcription (RT) followed by the polymerase chain reaction (PCR) represents a powerful tool for the detection and quantification of the mRNA level. Real-time RT-PCR is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range (Pfaffl & Hageleit, 2001). This technique is the most sensitive method for the detection and quantification of gene expression levels, in particular for low abundant transcripts in tissues with low RNA concentrations, from limited tissue sample and for the elucidation of small changes

in mRNA expression levels (Schmittgen, Zakrajsek, Mills, Gorn, Singer, & Reed, 2000; Wittwer & Garling, 1991).

In order to calculate the expression of a target gene in relation to an appropriate reference gene, various mathematical models are established. Calculations are based on the comparison of the distinct cycle determined by various methods. To determine the expression ratio between the target gene and a reference gene (ref), use the following equation:

$$\text{ratio} = \frac{(E_{target})^{\Delta CT\ target\ (MEAN\ control - MEAN\ sample)}}{(E_{ref})^{\Delta CT\ ref\ (MEAN\ control - MEAN\ sample)}}$$

where;

E_{target} = the amplification efficiency of the target gene.

E_{ref} = the amplification efficiency of the reference gene.

ΔCT_{target} (MEAN control – MEAN sample) = the mean CT of the target gene in the control minus the mean CT of the target gene in the test sample.

ΔCT_{ref} (MEAN control – MEAN sample) = the mean CT of the reference gene in the control minus the mean CT of the reference gene in the test sample.

9. Floral dip: method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*

Agrobacterium tumefaciens-mediated transformation is commonly used to create transgenic plants for research in plant molecular biology and for genetic improvement of crops since 1983 because it has several advantages compared with direct gene transfer methods. The advantages are stable gene expression because of the insertion of the foreign gene into the host plant chromosome; low copy number of the transgene; and large size of DNA segments can be transferred. (Ko & Korban, 2004; Lopez, Kumar, Pius, & Muraleedharan, 2004).

Plant transformation mediated by *Agrobacterium tumefaciens*, which is the soil plant pathogenic bacterium, has become the most popular method for

introduction of foreign genes into the plant genome and the subsequent regeneration of transgenic plants. The gene of interest can be introduced into the plant cell using the bacteria *Agrobacterium tumefaciens*. The *Agrobacterium* introduces a Ti plasmid containing T-DNA, into the plant cell, which is integrated randomly in the plant genome. The gene can be introduced stably in the plant cell by replacing part of the T-DNA for the gene of a desired protein. The simplified model of the events taking place during *Agrobacterium*-mediated transformation of plants is shown in Figure 1.7.

The *Arabidopsis thaliana* "floral dip" transformation is chosen as the best method for plant transformation. *Arabidopsis thaliana* flowering plants are simply dipped or sprayed with *Agrobacterium* culture carrying the gene of interest. After transformation process, seeds are collected from T0 plants and grown under the selection process in the presence of antibiotics for T1 individuals (Logemann, Birkenbihl, Ulker, & Somssich, 2006).

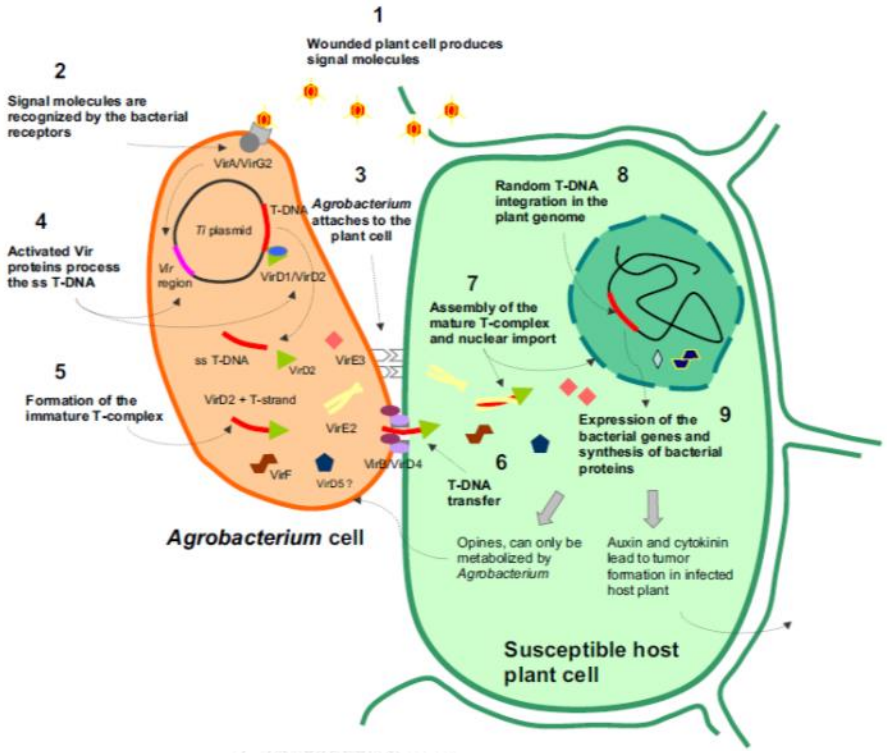


Figure 1.7 A simplified model of the Agrobacterium-mediated transformation process. The complex, and yet not fully deciphered, transformation process can, for didactical reason, be dissected in several distinct steps. Signal molecules released from wounded plant tissues (1) are recognized by the bacteria VirA/VirG2-component signal transduction system (2). Following a successful attachment to healthy susceptible plant cells (3), activation of the vir genes leads to the VirD1/VirD2 border-specific endonucleases targeting the T-DNA border sequences. They process the T-DNA from the Ti plasmid and release a single stranded T-DNA (ssT-strand) through a strand-replacement mechanism (4). Subsequently, the VirD2 protein covalently attaches in a polar manner to the 50 end of the T-strand (forming the so-called “immature T-complex”) (5). The VirD2/T-strand conjugate is then transferred to the plant cytoplasm via a type IV secretion system (T4SS) formed by 11 VirB proteins and VirD4 (6). Through this secretion apparatus, localized predominantly at the pole of bacteria, the T-DNA containing package is injected into the plant cell and passes through three membranes, the plant cell wall and cellular

spaces. Independently, and by using the same route, other bacterial virulence proteins (VirE2, VirE3, VirF and VirD5) that serve the transformation process in the plant, are exported to the host cell. Although its existence has not yet been demonstrated in plants, despite strong genetic and *in vitro* evidence, the "mature T-complex" is believed to be assembled inside the host cell by associating the VirD2-conjugated T-strand ("immature T-complex") with VirE2 (7). It has been suggested that VirD2 and VirE2 protect the ssT-strand (single stranded T-strand) from exonucleolytic attack inside the plant cytoplasm by attaching to its 50 end. Both the VirD2 and VirE2 proteins contain nuclear localization signals and serves as pilot proteins to guide the "mature T-complex" to the plant nucleus. Passing the nuclear membrane and targeting to the integration site inside the nucleus requires a cooperative action of bacterial and plant factors. Not much is known about the participating plant components, but there are indications that VirF also plays a role at this step, besides VirD2 and VirE2. Inside the nucleus, the escorting proteins are released via targeted proteolysis, and the uncoated single-stranded T-DNA is converted into a double-stranded molecule. Subsequently, it finds its place in the host chromatin and integrates into the host genome (8). Following successful integration, the expression of T-DNA-encoded genes leads to synthesis of bacterial proteins (9), promoting tumour formation (Păcurar, Thordal-Christensen, Păcurar, Pamfil, Botez, & Bellini, 2011).

As we know that CaM does not have an enzymatic activity but, in the presence of calcium, it functions by binding to and altering the activities of many target proteins, therefore identity of their specific interacting proteins is a prerequisite in understanding how CaM functions.

In the previous study, we have identified several putative OsCaM1 target proteins from rice (*Oryza sativa* L.) by cDNA expression library screening. The putative target proteins of OsCaM1 are showed in Table 1.3. So as to know what effectors or cellular processes OsCaM1 potentially regulates and how OsCaM1 acts in a molecular detail, its putative target proteins will be characterized. One of the putative OsCaM1 target proteins is Myosin heavy chain protein. In this study, we focus on the characterization of this protein.

Table 1.3 The putative OsCam1 target proteins

Identified OsCaM1-binding proteins	aa residues
Glutamate decarboxylase (GAD)	510
Kinesin motor domain-containing protein (KCBP)	1195
Cyclic nucleotide-gated ion channel (CNGC)	694
Hydroxyanthranilate hydroxyl cinnamoyltransferase (HHT)	442
CaM-binding transcription activator (CAMTA)	1003
Lipin	884
Response regulator receiver domain-containing protein (RR)	623
Myosin heavy chain	647
Unknown protein	232

10. Plant Myosins

Myosin is a molecular motor capable of producing motive force along actin filaments using the energy from ATP hydrolysis. A myosin molecule is generally composed of heavy and light chains. Most of the myosin heavy chains identified thus far have basically the N-terminal motor domain with ATP-hydrolysis and actin-binding sites, a neck domain with light chain binding sites, and a C-terminal tail region in which primary structures and sizes are diverse between myosin classes. The motor domain together with neck domain is often referred to as myosin head. The neck domain occupied by light chains works as a lever arm in the motor function. On the basis of sequence similarity of motor domain, myosins are divided into at least 24 classes (Foth, Goedecke, & Soldati, 2006). Among them, three classes of myosins, VIII, XI and XIII, are plant specific (A. S. N. Reddy, 2001b).

The plant myosins are believed to be involved in various cellular functions in such as cytoplasmic streaming or nuclear, organelle and vesicle transport, cytokinesis, membrane trafficking, signal transduction and intercellular communication through the plasmodesmata. Recently, plant myosins are also shown to be utilized for the intra and intercellular movement of some kinds of plant viruses in the host plant cells.

Thus, the objectives of this research are:

1. To examine the interaction of a Myosin heavy chain with CaM and CML proteins from rice (*Oryza sativa* L.).
2. To study the expression pattern of a *Myosin heavy chain* gene in rice (*Oryza sativa* L.) under salt and drought stresses.
3. To overexpress a *Myosin heavy chain* gene in *Arabidopsis thaliana* and characterize the transgenic plants.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Plant materials

Arabidopsis seeds (*Arabidopsis thaliana* Columbia): Wild-type

Rice 'KDML105' seeds (*Oryza sativa* L. spp. indica): Wild-type

2.1.2 Instruments

Autoclave: Labo Autoclave MLS-3020 (Sanyo Electric Co., Ltd., Japan)

Automatic micropipette: Pipetman P2, P20, P100, P1000 (Gilson Medical Electronics S.A., France)

Balance: PB303-L (Mettler Toledo, USA)

C-DiGit Chemiluminescence Western Blot Scanner (LI-COR, USA)

Electroporator 2510 (Eppendorf, Germany)

Gel document : Gel Doc™ (Syngene, England)

Gel electrophoresis apparatus: Mupid®–exU (Advance Co., LTD, Japan)

Growth Chamber

iCycler iQ real-time system (CFX96) (Bio-RAD Laboratories, USA)

Incubator: BM-600 (Memmert Gambh, Germany)

Incubator shaker: Innova™ 4000 (New Brunswick Scientific, UK)

Kodak x-ray film developer (USA)

Laminar flow: Bio Clean Bench (SANYO, Japan)

Magnetic stirrer: Fisherbrand (Fisher Scientific, USA)

Magnetic stirrer and heater: Cerastir (Clifton, USA)

Microcentrifuge: PMC-880 (Tomy Kogyo Co., Ltd., Japan)

Microwave oven (Panasonic, Japan)

Mixer mill model MM200 (Retsch, Germany)

NanoDrop® ND-100 Spectrophotometer (NanoDrop Technologies, Inc., USA)

PCR: T100TM Thermal Cycle (Bio-Rad, USA)

pH meter: FEP20 – FiveEasy Plus™ pH (Mettler Toledo, USA)

Power supply: Power PAC 1000 (Bio-RAD Laboratories, USA)

Refrigerator: Ultra low temperature freezer (New Brunswick Scientific, UK)

Refrigerated centrifuge: 5804R (Eppendorf, Germany)

Refrigerated centrifuge: 5417R (Eppendorf, Germany)

Spectrophotometer: DU®640 (Beckman Coulter, USA)

Vortex mixer: Model K 550-GE (Scientific Inc., USA)

XCell *SureLock*™ Mini-Cell (Invitrogen, USA)

2.1.3 Materials

Cellu·Sep® Regenerated Cellulose Tubular Membranes (Membrane filtration products, Inc., USA)

Microcentrifuge tube 0.6 and 1.5 ml (Axygen Hayward, USA)

0.22µm Millipore membrane filter (Millipore, USA)

Nipro disposable syringe (Nissho, Japan)

NuPAGE® Bis-Tris Mini Gels (Invitrogen, USA)

PVDF membrane (Thermo Fisher Scientific Inc., USA)

2.1.4 Chemicals and reagents

Absolute ethanol (Carlo Erba Reagenti, Italy)

Agarose: Seakem LE Agarose (FMC Bioproducts, USA)

Ammoniun sulfate (Sigma Chemical Co., USA)

Ammonium persulfate (Sigma Chemical Co., USA)

Bacto agar (Difco, USA)

Bacto tryptone (Difco, USA)

Bacto yeast extract (Difco, USA)

Basta (Bio World, USA)

Beta-mercaptoethanol (Fluka, Switzerland)

Boric acid (Merck, Germany)

Bovine Serum Albumin (Sigma Chemical Co., USA)

5-Bromo-4-chloro-3-indole-β-D-galactopyranoside; X-gal (Sigma Chemical co., USA)

Bromophenol blue (Merck, Germany)

Calcium chloride (Carlo Erba Reagenti, Italy)

Calcium sulfate (Carlo Erba Reagenti, Italy)

Calmodulin Sepharose 4B (GE Healthcare Bio-Sciences AB, Sweden)

Chloroform (Merck, Germany)

dATP, dCTP, dGTP, and dTTP (Fermentas Inc., USA)

Dithiothreitol: DTT (Sigma Chemical Co., USA)

Diethyl pyrocarbonate: DEPC (Sigma Chemical Co., USA)

Dimethylformamide: DMF (Sigma Chemical Co., USA)

Dimethyl sulfoxide (Sigma Chemical Co., USA)

Ethidium Bromide (Sigma Chemical Co., USA)

Ethylene diamine tetraacetic acid: EDTA (Carlo Erba Reagenti, Italy)

Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid: EGTA (Merck, Germany)

Formamide (Fluka, Switzerland)

GelCode® Blue Stain Reagent (Thermo Scientific, USA)

Gelatin from cold water fish skin (Sigma Chemical Co., USA)

Glycerol (Ajax Finechem Pty Ltd, New Zealand)

Glacial acetic acid (Carlo Erba Reagenti, Italy)

Hydrochloric acid (Merck, Germany)

Isopropanol (Merck, Germany)

Iso-1-thio-β-D-thiogalactopyranoside: IPTG (Serva, Germany)

Kelcogel® Gellan Gum (CP Kelco U.S., Inc., USA)

Lithium chloride (Sigma Chemical Co., USA)

Liquid nitrogen (Linde)

Magnesium sulfate (Sigma Chemical Co., USA)

Methanol (Merck, Germany)

Methylene blue (Carlo Erba Reagenti, Italy)

NuPAGE® LDS Sample Buffer (Invitrogen, USA)

NuPAGE® Transfer Buffer (Invitrogen, USA)

Ni-NTA Agarose (QIAGEN, Germany)

N, N′-methylene-bis-acrylamide (Sigma Chemical Co., USA)

Phenylmethylsulfonyl fluoride: PMSF (USB, USA)

Polyethylene glycol 3350, 6000 (Sigma Chemical co., USA)

Potassium acetate (Merck, Germany)

Potassium nitrate (BDH, England)

Ribonuclease inhibitor (Promega Co., USA)

Silwet L-77

Sodium chloride (Carlo Erba Reagenti, Italy)

Sodium dodecyl sulfate (Sigma Chemical Co., USA)

Sodium hydroxide (Carlo Erba Reagenti, Italy)

SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific, USA)

TRI Reagent® (Molecular research center, Inc., USA)

Tris-(hydroxyl methyl)-aminomethane (Fluka, Switzerland)

Triton X-100 (Merck, Germany)

Xylene Cyanol FF (Sigma Chemical co., USA)

2.1.5 Antibiotics

Ampicilin (Sigma Chemical Co., USA)

Gentamycin (Bio Basic Inc., USA)

Kanamycin (Bio Basic Inc., USA)

2.1.6 Antibodies

Anti-GFP Antibody from Rabbit (Rockland)

Anti-His Antibody (Amersham Biosciences Inc., USA)

Goat Anit-Rabbit IgG(H+L)-HRP (Bio-RAD Laboratories, USA)

Goat Anit-Mouse IgG(H+L)-HRP (Bio-RAD Laboratories, USA)

2.1.7 Enzymes

DNase I (Fermentas, USA)

Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Inc., USA)

LR Clonase™ II enzyme mix (Invitrogen, USA)

Restriction endonucleases: *Bam*HI, *Hin*dIII, *Msc*I, *Nco*I, *Nhe*I, *Not*I, *Kpn*I, *Sac*I (New England Biolabs, Inc., USA)

Taq DNA Polymerase (Fermentas, Inc., USA)

Quick ligase (New England Biolabs, Inc., USA)

2.1.8 Kits and Plasmids

Gel/PCR DNA Fragment Extraction Kit (Geneaid Biotech Ltd., Taiwan)

iScript™ Reverse Transcription Supermix kit (Bio-RAD Laboratories, USA)

pDEST™22 (Invitrogen, USA)

pDEST™32 (Invitrogen, USA)

pENTR™/D-TOPO (Invitrogen, USA)

pRZ850 (Modified by Professor Raymond E. Zielinski)

pRZ868a (Modified by Professor Raymond E. Zielinski)

pRZ869 (Modified by Professor Raymond E. Zielinski)

QIAprep Spin Miniprep Kit (QIAGEN, Germany)

SsoFast™ Evagreen® Supermix (Bio-RAD Laboratories, USA)

2.1.9 Oligonucleotide primers

The oligonucletide primers were synthesized by Integrated DNA Technologies, Singapore.

2.1.10 Microorganisms

Escherichia coli

Strain TOP10

(F– *mcr*A**Δ**(*mrr-hsd*RMS-*mcr*BC) **Φ**80*lacZ***Δ**M15 **Δ***lac*X74 *rec*A1 *ara*D139 **Δ**(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G)

Strain NiCo21(DE3)

*(can::CBD fhuA2 [lon] ompT gal (**λ** DE3) [dcm] arnA::CBD slyD::CBD glmS6Ala ΔhsdS **λ** DE3 = **λ** sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5*)

Strain DH5**α**

(F′/endA1 hsdR17 (r_k^- m_k^+) supE44 thi-1 recA1 gyrA96 (Nal^r) relA1**Δ**(lacZYA-argF)U169 deoR (**Φ**80dlacZ**Δ**(lacZ)M15)

Agrobacterium tumefaciens

Strain GV3101

GV3101 carrying pMP90

Saccharomyces cerevisiae

Strain MaV203

(MAT**α**, *leu*2-3,112, *trp*1-901, *his*3**Δ**200, *ade*2-101, *gal*4**Δ**, *gal*80**Δ**, *SPAL*10*::URA*3, *GAL*1::*lacZ*, *HIS*3$_{UAS\ GAL1}$*::HIS*3@*LYS*2, *can*1$_R$, *cyh*2$_R$) (Vidal, 1997)

2.2 GROWTH MEDIUM

2.2.1 Bacterial growth medium

Luria-Bertani broth (LB medium) (Maniatis & Sambrook, 1982)

LB medium containing 1% peptone, 0.5% NaCl and 0.5% yeast extract was prepared and pH was adjusted to 7.2 with NaOH. For agar plate, the medium was supplemented with 1.5% (w/v) agar. The medium was sterilized by autoclaving at 121°C for 20 minutes. Appropriate antibiotics were added to the culture medium.

YEP medium

YEP medium containing 10 g/L yeast extract, 10 g/L bacto-peptone and 5 g/L NaCl was made and pH was adjusted to 7.0. For agar plate, the medium was supplemented with 1.5% (w/v) agar. The medium was sterilized by autoclaving at 121°C for 20 minutes. Appropriate antibiotics were added to the culture medium.

YEBS liquid medium

YEBS liquid medium containing 1 g/L yeast extract, 5 g/L beef extract, 5 g/L sucrose, 5 g/L bacto-peptone and 0.5 g/L $MgCl_2$ was made and pH was adjusted to 7.0. For agar plate, the medium was supplemented with 1.5% (w/v) agar. The medium was sterilized by autoclaving at 121°C for 20 minutes. Appropriate antibiotics were added to the culture medium.

2.2.2 Yeast growth medium

YPAD medium

YPAD medium containing 10 g/L Bacto-yeast extract, 20 g/L Bacto-peptone, 20 g/L Dextrose and 100 mg/L Adenine sulfate was made and pH was adjusted to 6.0. For agar plate, the medium was supplemented with 2.0% (w/v) agar. The medium was sterilized by autoclaving at 121°C for 20 minutes.

SC medium

Synthetic Complete medium consists of a nitrogen base, a carbon source, and a “dropout” solution containing essential amino acids, nucleic acids, trace elements and vitamins. For selection purposes, certain amino acids are omitted or “dropped out” (e.g., leucine, tryptophan, histidine) from the dropout solution. For liquid medium, the agar is omitted.

SC medium containing 13.4 g/L Yeast nitrogen base without amino acids and 2.7 g/L amino acid powder mix was made and pH was adjusted to 5.9 with NaOH. The medium was sterilized by autoclaving at 121°C for 20 minutes.

2.2.3 Plant growth medium

Yoshida’s media solution (Yoshida, Forno, Cock, & Gomez, 1976)

Yoshida’s media stock solution was prepared in 5 separated glass bottles as following.

1.	$NaH_2PO_4.2H_2O$	40.3 g/L
2.	K_2SO_4	71.4 g/L
3.	$CaCl_2$	88.6 g/L
4.	$MgSO_4.7H_2O$	1.169 g/L
5.	$MnCl_2.4H_2O$	1.5 g/L
	$(NH_4)6Mo_7O_{24}.4H_2O$	0.07 g/L
	H_3BO_3	0.93 g/L
	$ZnSO_4.7H_2O$	0.035 g/L
	$CuSO_4.7H_2O$	0.031 g/L
	$FeCl_3.6H_2O$	7.7 g/L
	Citric acid (Monohydrate)	11.9 g/L

The stock solutions 5 were dissolved separately, and then the solutions were combined with 50 mL of concentrated H_2SO_4. Each stock solution was made up the volume to 1 L with deionized water. The stock solution was sterilized by autoclaving at 121°C for 20 minutes.

For every 4 L of culture solution to be prepared, five-milliliter of each of the stock solution was added. Citric acid should be added at the time of culture solution preparation to avoid the problem of fungal growth. The pH was adjusted to 5.0 with NaOH and the medium was sterilized by autoclave at 121°C for 20 minutes.

Murashige and Skoog (MS) media

MS medium contained 4.4 g/L Murashige and Skoog Basal Salt mixture, 30 g/L sucrose and 0.5 g/L Monohydrate 2-(N-morpholino)ethanesulfonic acid (MES) with pH adjusted to 5.7. For agar plate, the medium was supplemented with 0.2% (w/v) Kelcogel. The medium was sterilized by autoclaving at 121°C for 20 minutes. Appropriate antibiotics were added to the culture medium.

2.3 METHODS

2.3.1 Characterization of the OsCaM1 target protein

2.3.1.1 Yeast two-hybrid system

2.3.1.1.1 Construction of entry vector (pENTRTM/D-TOPO) containing *OsCaM*, *OsCML* and *Myosin heavy chain* genes

a) Primer design

The nucleotide sequences of *OsCaMs, OsCMLs* and *Myosin heavy chain* were obtained from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The forward and reverse primers of each gene were designed based on the cDNA sequences from the database. The sequence and the length of the oligonucleotide primers are shown in Table 2.1.

b) PCR amplification

The coding regions of *OsCaM, OsCML* and *Myosin heavy chain* genes were amplified from their respective cDNA clones, which were obtained from the National Institute of Agrobiological Sciences, Japan. The amplification reactions were performed in 50 µl reaction volume containing 1x Phusion buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 3% DMSO, 50-100 ng of DNA template and 1 unit of Phusion® High-Fidelity DNA polymerase (New England Biolabs). PCR amplification was performed as following: pre-denaturation at 98 ºC for 30 sec; and 30 cycles of denaturation at 98°C for 15 sec, annealing at a designated temperature (see Table 2.1) for 30 sec, and extension at 72°C for 45 sec. The final extension step was performed at 72 ºC for 5 min. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

c) Agarose gel electrophoresis

The DNA was run in 1% agarose gel electrophoresis by mixing the PCR product with 6x DNA loading dye. Tris-acetate-EDTA (TAE) buffer was used as a buffer system for this electrophoresis. After mixing the DNA sample with the loading dye,

the mixture was loaded into the gel. The electrophoresis was carried out at constant 100 volts. The duration of the running times depended on the size of DNA. Generally, the gel was run until the bromophenol blue moved to 3/4 of the gel. After electrophoresis, the gel was stained with ethidium bromide solution for 5 minutes and was destained with water for 5 minutes. The DNA fragments were visualized as fluorescent bands under the UV transilluminator and photographed.

d) Extraction of DNA fragment from agarose gel using Gel/PCR DNA Fragments Extraction Kit

To recover or concentrate DNA fragments (70 bp – 20 kb) from an agarose gel or a PCR reaction, respectively, Gel/PCR DNA Fragments Extraction Kit was performed following the manufacturer's instruction. For an agarose gel, after electrophoresis, DNA fragment was excised as gel slice from the agarose gel using a scalpel and transferred to a microfuge tube. Five-hundred-microliter of DF buffer was added to the sample and mixed by vortex. The sample was incubated at 55-60°C for 10-15 minutes to ensure the gel slice has been completely dissolved. During incubation, the tube was inverted every 2-3 minutes. The dissolved sample mixture was cooled to room temperature. Eight-hundred-microliter of sample mixture was transferred to the DF column. The column was centrifuged at 14-16,000xg for 30 seconds, then the flow through was discarded. Four-hundred-microliter of W1 buffer was added into the DF column. The column was centrifuged at 14-16,000xg for 30 seconds, then the flow through was discarded. Six-hundred-microliter of Wash buffer was added into the DF column, let stand at room temperature for 1 minute. The column was centrifuged at 14-16,000xg for 30 seconds, and then the flow through was discarded. After that, the column was centrifuged at 14-16,000xg for 3 minutes to dry the column matrix and the dried DF column was transferred to a new microfuge tube. The elution buffer was added into the center of column matrix. The column was allowed to stand for at least 2 minutes to ensure the elution buffer was completely absorbed and then the column was centrifuged at 14-16,000xg for 2 minutes to elute the purified DNA.

Table 2.1 The sequence and the length of oligonucleotide primers, and the annealing temperatures used in yeast two-hybrid system experiment.

Gene	**Primer**	**Sequence (5'---3')**	**Annealing temperature (^{o}C)**
OsCam1-1	Forward	5'-CACCATGGCGGACCAGCTCACC-3'	58
	Reverse	5'-TCACTTGGCCATCATGACCTTG-3'	
OsCam2	Forward	5'-CACCATGGCGGACCAGCTCACC-3'	55
	Reverse	5'-TCACTTGGCCATCATGACCTTA-3'	
OsCam3	Forward	5'-CACCATGGCGGACCAGCTCACC-3'	57
	Reverse	5'-TTACTTGGCCATCATGACTTTAACG-3'	
OsCML1	Forward	5'-CACCATGGCGGACCAGCTCTCC-3'	59
	Reverse	5'-TTACAGGATCACGCACTTCTGGC-3'	
OsCML4	Forward	5'-CACCATGGAAGGGCTGACGAGC-3'	57
	Reverse	5'-TCACCCAGATATCTTCCGTTCAG-3'	
OsCML5	Forward	5'-CACCATGGCGGAGGTGGAGGTG-3'	55
	Reverse	5'-TTATTGGTCGGAGAGCATCATC-3'	
OsCML8	Forward	5'-CACCATGGCGAGCAAATACAGAGGCT-3'	55
	Reverse	5'-CTAAAAAAACCCGGCCCCA-3'	
OsCML11	Forward	5'-CACCATGAGCGAGCCGGCCAC-3'	57
	Reverse	5'-TCAGGAGAAGATGTTGTCAAATGCG-3'	
OsCML13	Forward	5'-CACCATGTCTACTGTCAAGGGACAGA-3'	58
	Reverse	5'-CTAGTAACCATATCCAGTCCTCC-3'	
OsMyosin	Forward	5'-CACCATGGCTACAAAACTCCGT-3'	53
	Reverse	5'-TTATACAGATTTGTCCCCAGG-3'	

2.3.1.1.2 Ligation of PCR product to entry vector pENTR™/D-TOPO

To clone the PCR product of *OsCaM, OsCML* and *Myosin heavy chain* genes into the entry vector, reactions were set up following the manufacturer's instruction of Invitrogen. The following reaction components were prepared in a 0.2-mL PCR tube:

Fresh PCR product	0.5-4	µl
Salt solution	1	µl
Sterile water to a final volume of	5	µl
TOPO® vector	1	µl
Total reaction volume	6	µl

The reactions were mixed gently and incubated at room temperature for 1 hour. The reaction tubes were placed on ice and then transformed to *E. coli* strain TOP10.

To transform the reaction mixtures to competent *E. coli* cells, the procedure of One Short® Chemically Competent *E. coli* was performed following the manufacturer's instruction of Invitrogen. Two-microliter of the TOPO® cloning reaction was added into a vial of One Short® Chemically Competent *E. coli* cells and mixed gently. The reactions were incubated on ice for 5-30 minutes. Then, the cells were heat-shocked at 42°C for 30 seconds and immediately chilled on ice for 5 minutes. Two-hundred-microliter of S.O.C. medium was added into the cells. After that, the cells were incubated at 37°C for 1 hour with shaking. Finally, the cell suspension was spread on LB agar plate containing 50 µg/mL of Kanamycin and incubated at 37°C overnight. The recombinant clones containing inserted DNA were grown on the medium and were selected. Finally, the positive clones were confirmed by performing colony PCR and then DNA sequencing.

2.3.1.1.3 Construction of bait and prey plasmids by LR recombination

The LR recombination reaction between the entry clone and either pDEST™32 or pDEST™22 was performed to generate the bait and prey plasmid, respectively. Use the following procedure to perform the LR recombination reaction. The following components were added to a microfuge tube at room temperature and mixed.

Entry clone (100-300 ng)	1-10	µl
Destination vector (150 ng/µl)	2	µl
5x LR Clonase™ Reaction buffer	4	µl
TE buffer pH 8.0 to	16	µl

The LR Clonase™ II enzyme mix was removed from -80°C and thawed on ice for about 2 minutes. Then, the LR Clonase™ II enzyme mix was vortexed briefly twice (2 seconds each time). To each sample, four-microliter of the LR Clonase™ II enzyme mix was added to the reaction and mixed well by vortexing briefly twice. The reactions were incubated at 25°C for 1 hour.

The LR reaction was transformed to *E. coli* strain TOP10 as described previously (see 2.3.1.1.2) and the cell suspension was spread on an LB agar plate:

for pDEST™32, LB agar plate containing 50 µg/mL Gentamicin; and

for pDEST™22, LB agar plate containing 100 µg/mL Ampicillin.

And the plates were incubated at 37°C overnight. The recombinant clones containing inserted DNA were grown on the medium and were selected by colony PCR.

2.3.1.1.4 Transformation of the bait and prey plasmid to yeast strain MaV203

a) Making of the yeast competent cells

To make the yeast competent cells, the glycerol stock of yeast strain MaV203 was streaked onto YPAD agar plate and incubated at 30°C for 2 days. Single colony was picked and cultured in 15 mL of YPAD broth, then incubated at 30°C overnight with shaking at 250 rpm. In the next day, the OD600 of the overnight culture was determined and diluted to an OD600 of 0.4 in 50 mL of YPAD. The culture was grown an additional 2-4 hours at 30°C with shaking. The cells were centrifuged at 1,500xg, at 25°C for 10 minutes and resuspended with 40 mL of sterile deionized water. Then, the cells were centrifuged again at 1,500xg, 25°C for 10 minutes. The cells were resuspended with 1.5 mL of 1xLiAc/1xTE and mixed by pipetting.

*Note: Making the yeast competent cells fresh each time

b) Transformation of the bait and prey plasmid to yeast competent cells

For each transformation, one-microgram of each bait and prey plasmid was mixed together with 50 µl of yeast competent cells and then the cells were incubated at room temperature for 10 minutes. Three-hundred-microliter of 1xLiAc/40%PEG-3350/1xTE was added into the cells and incubated at 30°C for 30 minutes. After incubation, forty-microliter of DMSO was added and mixed well. Then, the cells were heat-shocked at 42°C for 10 minutes. The cells were centrifuged at 8,000 rpm for 2 minutes and resuspended with 1 mL of 1xTE. Then, the cells were centrifuged again at 8,000 rpm for 2 minutes. The supernatant was removed and the pellet was spread on SC-LT agar plate. The spread plates were incubated at 30°C for 2 days. The colonies growing on the selective plates had both the bait and prey plasmid.

2.3.1.1.5 Testing of the activation of the reporter genes

To test the activation of the reporter genes, the transformants growing on the selective plates were picked and streaked on new plates to make master plates.

a) *HIS3* reporter gene

Before testing of the activation of *HIS3* reporter gene, the bait auto-activation was performed by transforming the *gene*-pDEST^{TM}32 and the empty vector pDEST^{TM}22 into yeast strain MaV203. The transformed yeast cells were diluted by autoclaved ultrapure water. Serial dilutions of the transformed yeast cells were dropped on the SC-LTH +3AT plates containing either $CaCl_2$ or EGTA. For testing the bait auto-activation, yeast cells that expressed the fusion protein chosen as a bait protein could not grow on the SC-LTH +3AT medium.

Testing of the activation of *HIS3* reporter gene was performed in the same as testing of the bait auto-activation by dropping the serial dilution of yeast cells on the SC-LTH +3AT medium plates. If the bait and prey fusion proteins have interaction, the yeast cells would grow on the SC-LTH +3AT medium.

After the serial dilutions of the yeast cells were dropped on the SC-LTH +3AT medium plates, they were incubated at 30°C for 2 days.

b) *LacZ* reporter gene

To test the activation of the *LacZ* reporter gene, the transformed yeast cells were streaked on the membrane that was placed on the YPAD agar plate and incubated at 30°C for 2 days. After 2 days, for each membrane, the following components were prepared in 15 mL corning tube:

10 mg X-gal in 100 µl DMF
60 µl 2-mercaptoethanol
10 mL Z-buffer

Stack two round 125 mm Whatman 541 filter papers in a 15 cm petri dish. Saturate with 8 mL of the X-gal solution and remove any air bubbles. The streaked

membrane was carefully removed from the surface of the YPAD plate using forceps and completely immersed in liquid nitrogen for 20-30 seconds. The frozen membrane was placed on the top of soaked Whatman filters colony side up and any air bubbles were removed. The membrane was incubated at 37°C. The appearance of blue color was monitored over 24 hour period. Final results were scored at hour 24.

Strong interactors show blue color within 1 hour. Weak interactors show blue color within 24 hours, but can exhibit very faint blue color.

2.3.1.2 CaM Pull-down assay

2.3.1.2.1 Searching for the CaM binding site

Nucleotide and protein sequences of *Myosin heavy chain* were obtained from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). The protein sequence of *Myosin heavy chain* was used as a query sequence to search the CaM binding site on the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html). Oligonucleotide primers were designed to generate the gene encoding the CaM binding site.

2.3.1.2.2 Construction of Calmodulin binding protein (Myosin heavy chain) into expression vector pRZ850

To generate expression plasmid, *Myosin heavy chain* was amplified by PCR using specific primers which restriction sites *Bam*HI and *Not*I engineered at the 5' and 3' ends, respectively. The sequence and the length of the oligonucleotide primers are shown in Table 2.2.

To amplify the *Myosin heavy chain* gene, the PCR amplification reactions were performed in 50 µl reaction volume containing 1x Phusion buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 3% DMSO, 50-100 ng of DNA template and 1 unit of Phusion® High-Fidelity DNA polymerase (New England Biolabs). PCR amplification was performed as following: pre-denaturation at 98 ºC for 30 sec; and 30 cycles of

denaturation at 98°C for 15 sec, annealing at 68°C for 30 sec, and extension at 72°C for 45 sec. The final extension step was performed at 72 °C for 5 min. Then, PCR product was purified using Gel/PCR DNA fragments extraction kit (Geneaid) (see 2.3.1.1.1 d).

Table 2.2 The sequence and the length of oligonucleotide primers, and the annealing temperatures used for recombinant protein expression. The underlined sequences represent the restriction enzyme recognition sequences.

Gene	**Primer**	**Sequence (5'---3')**	**Annealing temperature (^{o}C)**
OsMyosin	Forward: *Bam*HI-Myo	5'-ATGCGGATCCGTCAAAGCTGCAGTAAGCTTA-3'	68
	Reverse: *Not*I-Myo	5'-ATGCGCGGCCGCTAACTCTTTCTGGGCCAAGTG-3'	

2.3.1.2.3 Ligation of PCR product to pRZ850 expression vector

To clone the *Myosin heavy chain* gene into expression vector, the purified PCR product and pRZ850 vector were double digested with *Bam*HI and *Not*I restriction endonucleases. The following reaction components were prepared in a 1.5 mL microfuge tube:

10x CutSmart buffer	2	µl
*Bam*HI-HF	0.5	µl
*Not*I-HF	0.5	µl
10x BSA	2	µl
Sterile water to	19	µl
DNA	1	µg
Total reaction volume	20	µl

The reactions were incubated at 37°C for 3 hours, and then separated by the agarose gel electrophoresis. The DNA fragment at specific size was cut and purified using Gel/PCR DNA fragments extraction kit (Geneaid). The purified DNA was ligated using Quick ligase following the manufacturer's instruction:

pRZ850 vector	300	ng
Myosin heavy chain insert	1	µg
2x Quick ligation buffer	10	µl
H_2O to	19	µl
Quick T4 DNA ligase	1	µl
Total reaction volume	20	µl

The reaction was incubated at 25°C for 30 minutes and then chilled on ice. The reaction was followed by transformation to *E. coli* or storage at -20°C.

2.3.1.2.4 Transformation of the ligation reaction to *E. coli* strain DH5α

a) Preparation of *E. coli* competent cells

To make *E. coli* competent cells, the glycerol stock of *E. coli* strain DH5α was streaked onto LB agar plate and incubated at 37°C overnight. Single colony was picked and cultured in 5 mL of LB broth, then incubated at 37°C overnight with shaking at 250 rpm. Two-hundred milliliter of LB broth was inoculated with 2% of *E. coli* culture grown overnight and incubated at 37°C for 3-4 hours until the optical density at 600 nm (OD_{600}) of cells reached 0.4-0.6. All following steps were on ice. The cells were harvested by centrifugation at 4°C, 4500 rpm for 10 minutes, and then the pellet was resuspended with 100 mM $CaCl_2$. The previous step was repeated, and then the cells were incubated on ice for 20 minutes. The cells were centrifuged at 4°C, 4500 rpm for 10 minutes. The small amount of supernatant was removed and glycerol was added to resuspend the cells. The competent cells were divided into 50 µl aliquots and stored in the -80°C freezer for later use.

b) Transformation of plasmid to *E. coli* competent cells

Competent cells were gently thawed on ice. Fifty-microliter of competent cells were mixed well with ligation reaction, and then chilled on ice for 30 minutes. The cells were heat shock at 42°C for 45 seconds and immediately chilled on ice for 5 minutes. After that, Five-hundred microliter of S.O.C. medium was added to the tube and the cells were resuspended with a pipette. The cell suspension was incubated at 37°C with shaking at 250 rpm for 60 minutes. Finally, the cell suspension was spread on LB agar plate containing 50 µg/mL of Kanamycin and incubated at 37°C overnight. The recombinant clones containing inserted DNA were grown on the medium and were selected.

c) Screening of the transformants

To make sure that the transformants grown on the medium had the DNA insert, double digestion by restriction endonucleases was performed. A few colonies on the plate were chosen and cultured in 5 mL of LB broth containing 50 µg/mL of

Kanamycin at 37°C overnight with shaking at 250 rpm. Then, plasmid DNA was extracted using QIAprep Spin Miniprep Kit (QIAGEN) and cut with *Bam*HI and *Not*I for 3 hours. The reaction was then analyzed by agarose gel electrophoresis with suitable DNA marker. The recombinant plasmid that contained DNA insert would show the band at specific size. Finally, the selected clones were confirmed by DNA sequencing.

2.3.1.2.5 Protein production and extraction

Myosin heavy chain recombinant protein was expressed in *E. coli* strain NiCo21(DE3). Bacterial cultures were grown under antibiotic selection (50 µg/mL of Kanamycin) with shaking at 37°C overnight as the starter. Bacterial cells were inoculated into 200 mL of LB broth containing 50 µg/mL of Kanamycin at 37°C to an OD600 of 0.4-0.6 (Approximately 3 hours). Protein expression was induced over the course of 3 hours by adding 1 mM IPTG. The cells were then harvested by centrifugation at 3,000xg at 4°C for 10 minutes. The pellet was resuspended in extraction buffer (50 mM Tris-HCl, 2 mM EDTA, 1 mM DTT, 0.1 mg/mL PMSF, 1x Bugbuster® Protein extraction Reagent, pH 7.5) and incubated on a shaking platform or rotating mixer at room temperature for 10-20 minutes. The extract was then centrifuged at 16,000xg at 4°C for 20 minutes. The supernatant was transferred to a fresh tube and kept at -80°C.

2.3.1.2.6 Protein purification

The Ni-NTA Agarose was used for purification of His-tagged proteins by gravity-flow chromatography. First of all, the resin was resuspended in the stock bottle and then 1 mL of slurry was transferred into a 15 mL corning tube. The resin was batch-equilibrated 2 times with 1 mL of equilibration/wash buffer each time. Then, five-hundred-microliter of equilibration/wash buffer was added into the resin and 1 mL of slurry was transferred to the soluble fraction* from NiCo21(DE3). The sample was incubated end-over-end at 4°C for 30 minutes. The resin was centrifuged at 5,000xg for 5 minutes and the unbound fraction was saved. The resin was batch equilibrated

3 times with 10 column volumes (5 mL) of equilibration/wash buffer each time. After that, the slurry was transferred to the column for elution 3 times with 500 µl of elution buffer each time. All of the samples were frozen at -80°C.

*150 mM NaCl and 60 mM Imidazole were added into the soluble fraction before incubation with resin.

2.3.1.2.7 SDS-Polyacrylamide gel electrophoresis

Before running the electrophoresis, the protein samples were prepared as following:

Protein sample	x	µl
NuPAGE® LDS Sample Buffer (4x)	5	µl
1M DTT	2	µl
Deionized water to	20	µl
Total reaction volume	20	µl

The protein samples were incubated at 70°C for 10 minutes. Then, the samples were loaded into the NuPAGE® Bis-Tris Mini Gels and run at constant 200 volts for 35 minutes with MES buffer. So as to estimate size of polypeptides, SeeBlue® Pre-Stained (Invitrogen) and prestained SDS-PAGE standards (Bio-RAD) were used as protein marker. When the electrophoresis was run completely, the gel was incubated in the protein fixing solution (50% methanol and 7% acetic acid) for 1 hour to overnight. After incubation, the gel was washed 3 times with water for 5-10 minutes each time. To see the protein band after electrophoresis, the gel was stained with GelCode® Blue Stain Reagent for about 20 minutes.

2.3.1.2.8 Dialysis and Protein determination

After purification, dialysis of the purified protein was performed using Cellu·Sep® Cellulose Membranes. The dialysis tubing was soaked in deionized water for at least 1 hour. The dialysis clamps were used by folding one end of the tubing

and pinching the tubing in the clamp. Then, the tubing was filled with the protein solution using pipette. The air bubbles were removed and then the tubing was clamped or tied off at the open end. The tubing was placed in a large beaker, which was filled with dialysis buffer (20 mM MOPS-NaOH, pH 7.5 and 1mM DTT) and incubated with stirring at 4°C. The buffer was changed at least once.

After dialysis, the protein concentration was estimated by Bradford assay using bovine serum albumin (BSA) as standard. The absorbance at 595 nm was measured and the protein concentration was calculated using the standard curve, plotted between OD595 on the Y-axis and BSA concentration (µg/mL) on the X-axis. Five-microliter of the standard and the sample were each pipetted into separate clean tubes. The 250 µl of Bradford 1x dye reagent was added to each tube and then mixed by vortexing. The reaction was incubated for at least 5 minutes at room temperature. The absorbance at 595 nm was measured using the UV-VIS spectrophotometer.

2.3.1.2.9 CaM-Sepharose binding assay

Calmodulin Sepharose 4B, calmodulin immobilized by the CNBr method to Sepharose 4B, was used for testing of the interaction of Myosin heavy chain with the calmodulin. The protein sample was prepared in a 1.5 mL microfuge tube as follows:

20 mM MOPS	x	µl
1M DTT	1	µl
1M $CaCl_2$	1	µl
Protein	20	µg
---	---	---
Total reaction volume	1	mL

The protein sample was placed on ice before adding to the CaM-Sepharose resin. The resin was resuspended in the stock bottle and then 25 µl of slurry was transferred into a 1.5 mL microfuge tube. The resin was batch-equilibrated 2 times with 250 µl (10 bed volumes) of wash buffer each time. Then, the protein sample

was added into the resin and incubated end-over-end at room temperature for 30 minutes. The resin was centrifuged at 5,000xg for 5 minutes and the unbound fraction was saved. The resin was batch-equilibrated 2 times with 50 µl of wash buffer each time and the wash fraction was saved. Finally, elution of protein was conducted by batch-equilibrating 3 times with 50 µl of elution buffer each time and the elution fractions were saved. All of the samples were frozen at -80°C.

2.3.1.2.10 Western Blotting

When the SDS-PAGE was run completely (see 2.3.1.2.7), the gel was incubated in NuPAGE® transfer buffer for a while and then the protein samples were transferred to the PVDF membrane. The membrane was incubated in 25 mL of blocking solution (5% (w/v) cold water fish skin gelatin in PBST or TBST) for 1 hour to overnight with gentle agitation on an orbital shaker or rocking platform. Then, the blocking solution was discarded and replaced with 1° antibody solution made up in blocking solution. The membrane was incubated for 1-2 hours with agitation. The 1° antibody was decanted and replaced with PBST or TBST. The membrane was incubated for 5-10 minutes with agitation. The wash solution was then decanted and discarded. The wash step was repeated 4 more times. Then, the membrane was incubated in blocking solution containing 2° antibody for 1-2 hours with agitation. The 2° antibody was decanted and replaced with PBS or TBS. The membrane was then incubated for 5-10 minutes with agitation. The wash solution was decanted and discarded. The wash step was repeated 4 more times. The membrane was incubated in freshly mixed SuperSignal® reagents, no agitation needed, for 5 minutes in a container covered with the lid of a freezer box. The membrane was exposed to x-ray film or photographed by C-digit blot scanner.

2.3.2 Expression pattern of Myosin heavy chain gene in rice using real-time RT-PCR under salt and drought stresses.

2.3.2.1 Rice growing

Seeds of Rice 'KDML105' (*Oryza sativa* L.) were sterilized by rinsing with 70% ethanol for 5 min and then soaking in 35% Clorox® (2% w/v sodium hypochlorite, Clorox Co, USA) with shaking for 20 min. After that, seeds were washed with sterile distilled water several times and immersed in sterile water in the dark container. Sterile water was changed every day until germination of cotyledon (Figure 2.1). One inch long leaf of rice was transferred to a mesh immersing with Yoshida's media solution. Rice seedlings were cultured under 25°C air-temperature, 60 ± 5% relative humidity (RH) with 16-h light/ 8-h dark photoperiod provided by fluorescent lamps in the growth chamber. After one week, seeds were removed and then rice seedlings were transferred to 50 ml Yoshida's media solution in clear glass bottle under the same condition (Figure 2.1).

2.3.2.2 Rice stress treatment

a) Salt stress

Two-week-old rice seedlings were transferred to Yoshida's media solution containing 150 mM NaCl and cultured under 25°C air-temperature, 60 ± 5% relative humidity (RH) with 16-h light/ 8-h dark photoperiod provided by fluorescent lamps in the growth chamber. Control treatment of rice was grown in Yoshida's media solution under the same condition. After treatment, rice seedlings were collected at various times (0, 1, 3, 6, 12, 24 and 48 hours) and kept in -80°C freezer before using as starting materials.

b) Drought stress

Two-week-old rice seedlings were transferred to Yoshida's media solution containing 20% PEG 6000 and cultured under 25°C air-temperature, 60 ± 5% relative humidity (RH) with 16-h light/ 8-h dark photoperiod provided by fluorescent lamps in

the growth chamber. Control treatment of rice was grown in Yoshida's media solution under the same condition. After treatment, rice seedlings were collected at various times (0, 1, 3, 6, 12, 24 and 48 hours) and kept in -80°C freezer before using as starting materials.

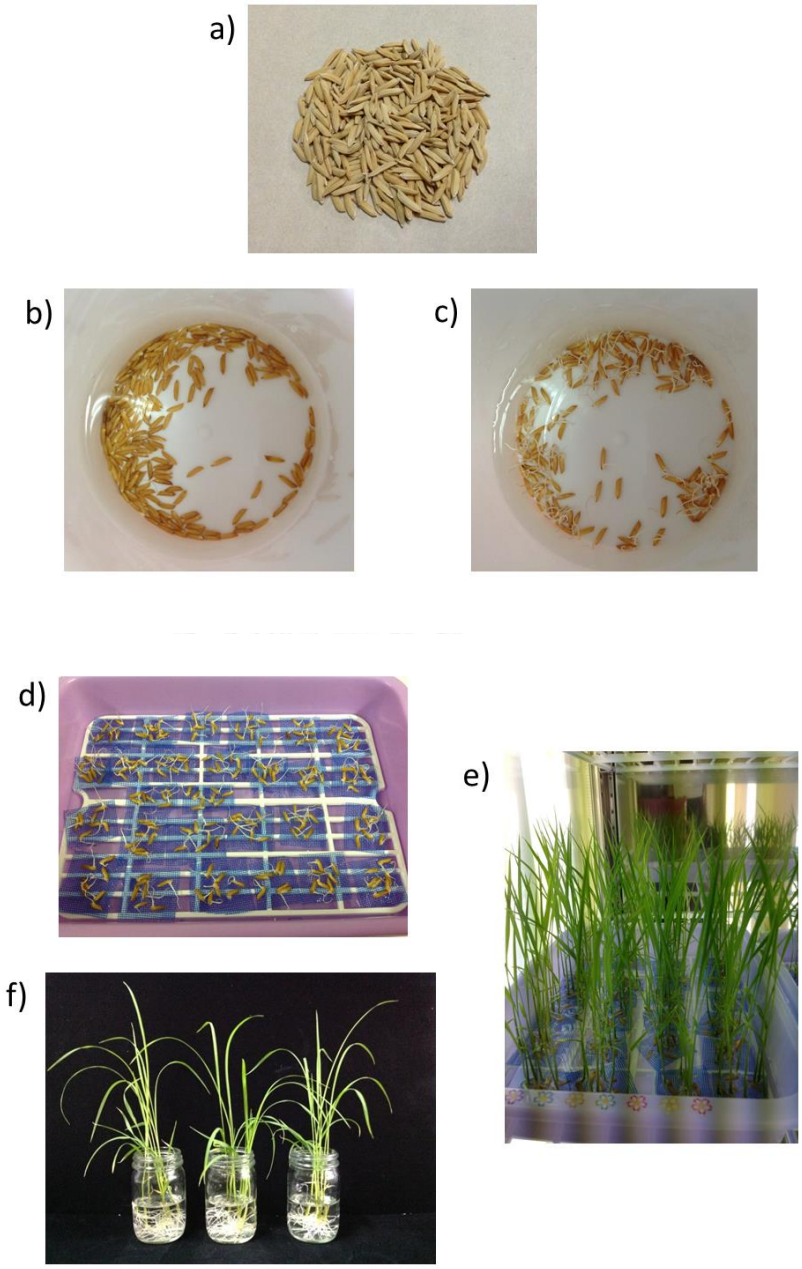


Figure 2.1 Rice 'KDML105' (*Oryza sativa* L.)

a) Mature rice seeds
b) Sterilized seeds immersed in sterile water
c) Seeds starting to germinate
d) Germinated seeds transferred the mesh
e) One-week old seedlings
f) Two-week-old rice seedlings

2.3.2.3 Rice RNA extraction

Total Rice RNA was isolated by TRI Reagent® (Molecular Research Center, USA). Leaf of rice samples were ground using the mixer mill. One hundred milligrams of tissue samples were homogenized in 1 mL of TRI Reagent®. The homogenate was incubated at room temperature for 5 minutes. After that, the homogenate was supplemented with 0.2 mL of chloroform per 1 mL of TRI Reagent® and followed by vortexing vigorously for 15 seconds. The mixture was stored at room temperature for 2-15 minutes and followed by centrifugation at 12,000xg at 4°C for 15 minutes. The aqueous phase was transferred to a new tube. RNA in the aqueous phase was precipitated by mixing with 0.5 mL of isopropanol and stored at room temperature for 5-10 minutes. Then, samples were centrifuged at 12,000xg at 4-25°C for 8 minutes. The supernatant was removed, and the RNA pellet was resuspended in 1 mL of 75% ethanol. The samples were centrifuged at 7,500xg at 4-25°C for 5 minutes. The supernatant was removed and the RNA pellet was air-dried for 5-10 minutes. Sample of the extracted RNA was dissolved in 50 µl RNase-free water (DEPC-treated water) and incubated at 55-65°C for 10-15 minutes. The concentration of the extracted RNA was determined by the UV spectrophotometer.

2.3.2.4 RNA quantitation

RNA was quantified by measuring the absorbance of a diluted RNA solution at 260 nm and calculating the concentration in ng/µl using the equation:

RNA concentration (ng/µl/OD) = A260 x Dilution factor x 40

2.3.2.5 Agarose gel electrophoresis for RNA

Quality of the extracted RNA was examined by TAE/formaldehyde electrophoresis. A 2% agarose gel in 1xTAE buffer was prepared and 1xTAE was used as a running buffer. The RNA samples were mixed with 6x loading dye and formamide at the final concentration of at least 60% (v/v) formamide. The samples were denatured by heating at 65°C for 5 minutes, and then immediately chilled on

ice for 5 minutes. Samples were loaded into the gel and run at 100V for 30 minutes. The gel was stained by ethidium bromide solution and then destained in water. The stained gel was viewed under the UV transilluminator and photographed.

2.3.2.6 DNase Treatment

In order to remove the contaminated DNA, total RNA was treated with DNase I. The following components were prepared in a 0.2 mL PCR tube:

RNA	10	µg
10x DNase buffer	3	µl
RNasein (40 U/µl)	0.5	µl
DEPC-treated water to	29	µl
DNase I (1U/µl)	1	µl
Total reaction volume	30	µl

The reaction was incubated at 37°C for 1 hour in the thermocycler, then heat-inactivated at 65°C for 10 minutes and cooled down to 12°C. The sample was then used for reverse transcription or kept in the -80°C freezer.

2.3.2.7 Reverse transcription

In order to synthesize cDNA, the reverse transcription reaction was performed by iScript™ Reverse Transcription Supermix kit following the manufacturer's instruction. The following components were prepared in a 0.2 mL PCR tube:

5x iScript reverse transcription supermix	4	µl
Nuclease-free water	x	µl
DNase treated-RNA (1 pg to µg concentration RNA)	y	µl
Total reaction volume	20	µl

The reaction mixture was incubated at 25°C for 5 minutes, and the temperature was increased to 42°C for 30 minutes. The enzyme was heat-inactivated at 85°C for 5 minutes and cooled down to 12°C. The cDNA was used as template for PCR amplification or kept at -20°C freezer.

2.3.2.8 Real-time PCR amplification

The Real-time PCR reactions were performed using the SsoFast™ EvaGreen® Supermix. The following components were prepared in a 0.2 mL PCR tube:

SsoFast™ EvaGreen® Supermix	10	µl		
Forward Primer 8 µM	1	µl	Final conc.	400 nM
Reverse Primer 8 µM	1	µl	Final conc.	400 nM
Sterile water	6	µl		
DNA template	2	µl		
Total reaction volume	20	µl		

The Real-time PCR was conducted using the iCycler iQ real-time system (CFX96, Bio-RAD). The reaction was heated at 95°C for 5 minutes as an initial denaturation step, then following by 40 cycles of denaturing at 95°C for 30 seconds, annealing at a designated temperature (see Table 2.3) for 30 seconds, and extension at 72°C for 30 seconds. The signal was read after extension of each cycle. The melting curve was constructed by increasing the temperature of the PCR product after amplification from 60 to 95°C with the increment of 0.5°C and held for 10 seconds. The melting curve results were read at every increment.

Table 2.3 The sequence and the length of oligonucleotide primers, and the annealing temperatures used for real-time RT-PCR amplification.

Gene	**Primer**	**Sequence (5'---3')**	**Annealing temperature (°C)**
OsMyosin	Forward	5'-TGTACAGAAAGCTTCTGCTGG-3'	59
	Reverse	5'-TTATACAGATTTGTCCCCAGG-3'	
OsEF1-alpha	Forward	5'-ATGGTTGTGGAGACCTTC-3'	57.9
	Reverse	5'-TCACCTTGGCACCGGTTG-3'	

2.3.3 Characterization of transgenic Arabidopsis over-expressing a target protein

2.3.3.1 Arabidopsis growing

All experiments with *Arabidopsis thaliana* were performed using the Columbia ecotype (Col-0, wild-type). Seeds were surface-sterilized by rinsing with 70% ethanol and then soaking in bleaching solution with shaking. After that, seeds were washed with sterile distilled water three times and imbibed in the dark at 4°C for 3 days and then transferred to the plant growth room under 16-h light/ 8-h dark photoperiod at 22°C.

2.3.3.2 Construction of Myosin heavy chain gene into binary vector

a) Primer design

The nucleotide sequence of the *Myosin heavy chain* gene was obtained from Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and then oligonucleotide primers were designed based on the cDNA sequence from the database to place the coding sequence of *Myosin heavy chain* gene under the control of 35SCaMV promoter and nos terminator of the binary vector.

For constructing c-terminal fusions to GFP, a pair of primers for amplifying the coding region of the *Myosin heavy chain* gene (LOC_Os12g17310) was designed with *Bam*HI and *Not*I restriction sites engineered at the 5′ and 3′ ends, respectively. The sequence and the length of the oligonucleotide primers are shown in Table 2.4.

For constructing n-terminal fusions to GFP, a pair of primers for amplifying the coding region of the *Myosin heavy chain* gene (LOC_Os12g17310) was designed with *Nco*I and *Bam*HI restriction sites engineered at the 5′ and 3′ ends, respectively. The sequence and the length of the oligonucleotide primers are shown in Table 2.4.

b) Cloning of *Myosin heavy chain* into binary vector

The protocols for cloning the *Myosin heavy chain* gene into pRZ868a binary vector are the same as cloning *Myosin heavy chain* gene into the pRZ850 expression vector (see 2.3.1.2.2-2.3.1.2.4). After sequencing, the recombinant binary vector was transformed to *Agrobacterium tumefaciens* strain GV3101.

Table 2.4 The sequence and the length of oligonucleotide primers, and the annealing temperatures used for preparing the constructs in the transgenic Arabidopsis experiment. The underlined sequences represent the restriction enzyme recognition sequences.

Gene	**Primer**	**Sequence (5'---3')**	**Annealing temperature (^{o}C)**
OsMyosin	Forward: *Bam*HI-Myo	5'-ATGCGGATCCGGAGCTACAAAACTCCGTCAAC-3'	68
	Reverse: *Not*I-Myo	5'-ATGCGCGGCCGCTTATACAGATTTGTCCCCAGGAAG-3'	
OsMyosin	Forward: *Nco*I-Myo	5'-ATGCCCATGGGCTACAAAACTCCGTCAACCGTC-3'	68
	Reverse: *Bam*HI-Myo	5'-ATGCGGATCCTACAGATTTGTCCCCAGGAAGAT-3'	

2.3.3.3 Transformation of the binary vector to Agrobacterium tumefaciens strain GV3101 by electroporation

A. tumefaciens GV3101 were transformed with the pRZ868a: 35SCaMV-*gfp-Myo-nos* and pRZ869-868a: 35SCaMV-*Myo-gfp-nos* recombinant vector by electroporation.

All steps were performed on ice as much as possible. The competent cells were gently thawed on ice and the cuvettes were chilled on ice as well. DNA was added (1 µl containing 10 to 500 ng) to cells on ice and mixed briefly by stirring with a micropipette tip. All liquid was added to the electroporation cuvette, then transform by electroporation at 2000 mV (1500 to 2500 mV is the usual working range – the best condition depend on the strain). After that, one milliliter of YEP broth was added immediately to the cuvette. The cell suspension was transferred to a 1.5 mL microfuge tube and incubated at 26-30°C (no shaking) for 1 hour. Finally, the cell suspension was spread on YEP agar containing 35 µg/mL of Kanamycin and incubated at 28°C for 3 days.

2.3.3.4 Transformation of a Myosin heavy chain gene in Binary vector to Arabidopsis thaliana by floral dip method

A. tumefaciens strain GV3101 harboring the pRZ868a: 35SCaMV-*gfp-Myo-nos* and pRZ869-868a: 35SCaMV-*Myo-gfp-nos* was streaked on YEP agar containing 35 µg/mL of Kanamycin and incubated at 28°C for 3 days. A single colony was picked and cultured in 50 mL of YEBS containing 35 µg/mL of Kanamycin. The cultures were grown at 28-30°C until cell density was saturated (typically 2-3 days). A few days later, 50 mL culture was poured into 450 mL YEBS. Further antibiotics for bacterial selection are not required, but addition will not interfere with subsequent steps. The cells were grown for 8 hours. One-hundred-microliter of Silwet L-77 was added into the cultures and mixed. *A. thaliana* lines having bolted where visible flowers were present were dipped into the *Agrobacterium* culture. Plants were sealed in closed plastic bag overnight to increase humidity. The next day, plants were removed from

their sealed environment before 24 hours have passed. It was critical that the enclosure was not left over 24 hours, as extended humidity in the presence of *A. tumefaciens* leads to plant death. When the plants had completed their life cycles and were well dried, the seeds of each plant were collected.

2.3.3.5 Selecting *A. thaliana* transgenics on agar

A. thaliana seeds were sterilized (see 2.3.3.1). The sterile seeds were carefully applied onto 0.5xMS agar plate containing 1% sucrose and 30 μM Basta. The plates were stratified at 4°C for a few days. Then, the plates were moved to the plant growth room. The selected transgenic seedlings were removed with forceps and transferred to soil for further growth.

2.3.3.6 Expression analysis of the Myosin heavy chain mRNA in transgenic plants

To confirm the expression of *Myosin heavy chain* gene in the transgenic Arabidopsis, total Arabidopsis RNA was isolated by TRI Reagent® (Molecular Research Center, USA). Then, the total RNA was treated with DNase I to remove the contaminated DNA and cDNA was synthesized by iScript™ Reverse Transcription Supermix kit (see 2.3.2.3-2.3.2.7). After that, the real-time RT-PCR was performed using the gene specific primers as shown in Table 2.5.

Table 2.5 The sequence and the length of oligonucleotide primers, and the annealing temperatures used for real-time RT-PCR analysis of the transgenic Arabidopsis.

Gene	**Primer**	**Sequence (5'---3')**	**Annealing temperature (^{o}C)**
OsMyosin	Forward	5'-ACCTCACAAGAACAGCAGAA-3'	56.6
	Reverse	5'-TTATACAGATTTGTCCCCAGG-3'	
AtEF1-alpha	Forward	5'-GAGACTTTCTCCGAGTACCCACC-3'	56.6
	Reverse	5'-AGTCTCATCATTTGGCACCC-3'	

2.3.3.7 Characterization of Transgenic Arabidopsis

Phenotypes including salt and drought stress tolerance in the transgenic Arabidopsis lines compared with the wild-type were examined.

a) Seed germination assay

The germination assay was carried out on the transgenic seeds subjected to salt or drought stresses. Arabidopsis seeds (four transgenic lines and wild-type) were sterilized and carefully plated on the 0.5xMS agar containing either NaCl or PEG6000 for salt and drought stress, respectively. The experiment was performed in 4 biological replicates (each 25 seeds) for each treatment condition (control, salt and drought). The plates were then stratified at 4°C for a few days and they were moved to the plant growth room. The germination rate was scored on different days; germination was considered to have occurred when the radicles have penetrated the seed coats. Data were compared using two-way ANOVA statistical analysis to analyze the effect of treatment conditions on the germination percentage and germination rate of Arabidopsis seeds.

b) Measurement of transgenic seedling fresh weight

Arabidopsis seeds (four transgenic lines and wild-type) were sterilized and sown on the 0.5xMS agar plate. The plates were then stratified at 4°C for a few days and they were then moved to the plant growth room. Seven days later, Arabidopsis plants were transferred to the 0.5xMS agar plate containing either NaCl or PEG6000 for salt and drought stress, respectively. The experiment was performed in 4 biological replicates (each 15 plants) for each treatment condition (control, salt and drought). To monitor salt and drought tolerance, whole two-week-old-plants grown in either NaCl or PEG6000 were weighed. Fresh weight measurements were estimated using a precision balance. Data were compared using ANOVA statistical analysis to analyze the effect of treatment conditions on the Arabidopsis growth.

c) Measurement of transgenic seedling dry weight

From 2.3.3.7 b, after fresh weight was measured, Arabidopsis samples were baked at 60°C for 3 days and were then weighed using the precision balance. Data were also compared using ANOVA statistical analysis to analyze the effect of treatment conditions on the Arabidopsis growth.

CHAPTER III

RESULTS

3.1 Characterization of the OsCaM1 target protein

3.1.1 Yeast two-hybrid system

Identification and characterization of target proteins of CaM is particularly important to understand how these proteins have a role in translating calcium signals into cellular responses. Previous study has identified Myosin heavy chain protein as one of the putative target proteins of OsCaM1 from rice (*Oryza sativa* L.) by cDNA expression library screening. In the present study, the interaction of OsCaM and OsCML proteins with Myosin heavy chain protein and their calcium dependence were tested by the yeast two-hybrid system.

3.1.1.1 Construction of entry vector (pENTR™/D-TOPO) containing OsCaM, OsCML and Myosin heavy chain genes

OsCaM, *OsCML* and *Myosin heavy chain* cDNA clones, obtained from the National Institute of Agrobiological Sciences, Japan, were used as templates for PCR amplification of the coding regions of *OsCaM*, *OsCML* and *Myosin heavy chain* genes. A pair of primers for amplifying the coding region of each gene was designed with CACC engineered at their 5' ends. The sequence and the length of the oligonucleotide primers are shown in Table 2.1.

The amplified PCR products were obtained as shown by agarose gel electrophoresis in Figure 3.1. Each DNA fragment was purified using the Gel/PCR DNA Fragments Extraction Kit and the purified PCR products were ligated into pENTR™/D-TOPO entry vector (Appendix A). The competent TOP10 cells were transformed with the ligation reactions and the transformants were selected by screening on LB agar plate containing 50 μg/mL of Kanamycin. The colonies were randomly selected and used as templates for colony PCR amplification so as to verify the insertion of the PCR products into the entry vector. To confirm the nucleotide sequence of genes

inserted into pENTR™/D-TOPO entry vector, the recombinant plasmids were subjected to DNA sequencing using M13F (-20) and M13R as universal primers. The result showed that the sequence of the cloned genes in the recombinant plasmids shared 100% nucleotide sequence identity with their respective cDNA sequences. The nucleotide sequences of the ORF these genes were shown in Figure 3.2.

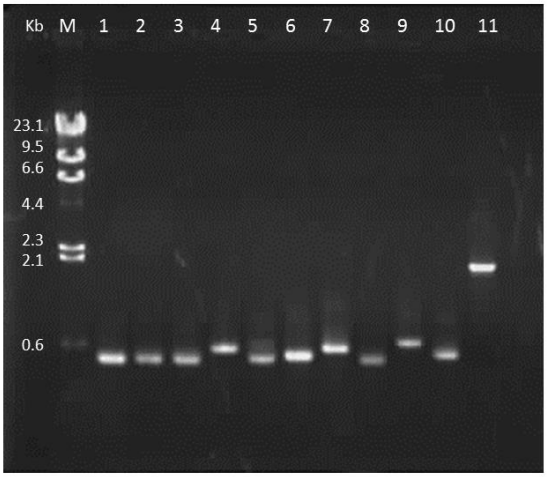


Figure 3.1 Agarose gel electrophoresis of the amplified fragments of *OsCaM*, *OsCML* and *Myosin heavy chain* genes. The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Lane M λ/*Hin*dIII standard marker
Lane 1 *OsCaM1* fragment
Lane 2 *OsCaM2* fragment
Lane 3 *OsCaM3* fragment
Lane 4 *OsCML1* fragment
Lane 5 *OsCML4* fragment
Lane 6 *OsCML5* fragment
Lane 7 *OsCML8* fragment
Lane 8 *OsCML9* fragment
Lane 9 *OsCML11* fragment
Lane 10 *OsCML13* fragment
Lane 11 *Myosin heavy chain* fragment

OsCam1-1 (LOC_Os03g20370.1)

ATGGCGGACCAGCTCACCGACGACCAGATCGCCGAGTTCAAGGAGGCCTTCAGCCTCTTC
GACAAGGACGGCGATGGTTGCATCACAACCAAGGAGCTGGGAACCGTGATGCGTTCGCTG
GGGCAGAACCCAACGGAGGCCGAGCTCCAGGACATGATCAACGAGGTCGACGCGGACGGC
AACGGCACCATCGACTTCCCGGAGTTCCTCAACCTGATGGCACGCAAGATGAAGGACACC
GACTCGGAGGAGGAGCTCAAGGAGGCGTTCAGGGTGTTCGACAAAGACCAGAACGGCTTC
ATCTCCGCCGCCGAGCTCCGCCACGTCATGACCAACCTCGGCGAGAAGCTGACCGACGAG
GAGGTCGACGAGATGATCCGCGAAGCCGACGTCGACGGTGACGGCCAGATCAACTACGAG
GAGTTCGTCAAGGTCATGATGGCCAAGTGA

OsCam2 (LOC_Os05g41210.1)

ATGGCGGACCAGCTCACCGACGAGCAGATCGCCGAGTTCAAGGAGGCGTTCAGCCTCTTC
GACAAGGACGGCGACGGTTGCATCACTACTAAGGAGCTTGGAACCGTGATGCGGTCCCTT
GGTCAGAACCCAACTGAGGCGGAGCTGCAGGACATGATCAACGAGGTTGATGCTGATGGC
AATGGGACCATTGACTTCCCAGAGTTCCTGAACCTGATGGCGAAGAAGATGAAGGATACC
GACTCTGAGGAGGAGCTCAAGGAGGCCTTCCGTGTGTTTGACAAGGACCAGAACGGTTTC
ATCTCGGCTGCTGAGCTCCGCCACGTCATGACCAACCTTGGTGAGAAGCTGACCGACGAG
GAAGTCGACGAGATGATCCGTGAGGCTGACGTCGATGGCGATGGCCAGATCAACTACGAG
GAGTTCGTTAAGGTCATGATGGCCAAGTGA

OsCam3 (LOC_Os01g17190.1)

ATGGCGGACCAGCTCACCGACGACCAGATCGCCGAGTTCAAGGAGGCCTTCAGCCTCTTC
GACAAGGACGGCGACGGTTGTATTACTACTAAGGAGTTGGGAACTGTCATGCGTTCGCTG
GGGCAGAACCCAACTGAGGCAGAACTACAAGACATGATCAATGAGGTGGATGCTGACGGC
AATGGAACAATCGATTTCCCTGAATTTCTCAACCTGATGGCCCGCAAGATGAAAGATACT
GATTCTGAGGAAGAGCTCAAGGAGGCGTTCCGTGTGTTCGACAAGGACCAAAATGGTTTT
ATCTCTGCTGCTGAATTGCGTCACGTCATGACTAACCTTGGTGAGAAGCTGACAGATGAG
GAGGTTGAAGAGATGATCCGTGAGGCCGATGTTGATGGTGATGGGCAGATCAACTACGAT
GAATTCGTTAAAGTCATGATGGCCAAGTAA

OsCML1 (LOC_Os01g59530.1)

ATGGCGGACCAGCTCTCCGAAGAGCAGATTGGAGAGTTCAGGGAGGCCTTCAGCCTCTTC
GACAAGGACGGCGACGGTTCTATCACCACCAAGGAGCTAGGAACCGTGATGCGAAGTCTA
GGGCAGAACCCAACGGAGGCGGAGCTGCAGGACATGATCAGCGAGGTGGACACGGACAGC
AACGGCAACATCGAATTCAAGGAGTTCCTGGGCCTGATGGCGCGCAAGCTGAGGGACAAG
GACTCCGAGGAGGAGCTGAAGGAGGCATTCCGCGTCTTCGACAAGGACCAGAACGGTTTC
ATCTCTGCCACCGAGCTCCGCCACGTGATGGCCAACATCGGGGAGCGGCTCACCGACGAG
GAGGTCGGCGAGATGATCAGCGAGGCCGACGTCGACGGCGACGGGCAGATCAACTACGAG
GAGTTCGTCAAGTGCATGATGGCCAAGAAGAGGAGGAAGAGGATAGAGGAGAAGAGGGAC
CACGACGGCGGCAGCAGGACGAAGAGTGCAGGGCCCTCCGCCGCGCCGGCGAGCAAGCGT
GGCCAGAAGTGCGTGATCCTGTAA

OsCML4 (LOC_Os03g53200.1)

ATGGAAGGGCTGACGAGCGAGCAGATGGTGGCGTTCCAGGAGGCGTTCTTGCTCTTCGAC
AAGAACGGCGATGGATGCATCACCTTGGAAGAGCTGGCTGCGGTGACTCGTTCTCTTGGC
CTTGAGCCGACCGATCAGGAGCTCAACGACATGATGAGGGAAGTTGATACAGATGGCAAT
GGGATCATAGATTTTCAGGAGTTCCTGAGCCTCATTGCCAGGAAGATGAAGGATGGGGAT
GGCGACGAAGAGCTGAAAGAAGCTTTCGAGGTCCTAGACAAGGATCAGAATGGTTTTATC
TCCCCAACTGAGCTGAGGACAGTGATGACCAATCTTGGGGAGAAGATGACAGACGAGGAG
GTTGAGCAGATGATAAGGGAGGCGGATACCGATGGCGACGGGCAGGTGAACTACGATGAA
TTTGTGATAATGATGAAAAACGCTGAACGGAAGATATCTGGGTGA

OsCML5 (LOC_Os12g41110.1)

ATGGCGGAGGTGGAGGTGAGGGTGAGGCAGGAGCAGGTGGCGGAGTTCAGGGAGACGTTC
GCGTTCTTCGACAAGGACGGGGACGGGTGCATCACGCTGGAGGAGCTGGACACGGTGGTG
CGGTCGCTGGGGCAGACGCCGACGAGGGAGGAGCTCGCCGAGATGATCCGCGACGTCGAC
GTCGACGGCAACGGCACCATCGAGTTCGCCGAGTTCCTCGCCCTCATGGCCCGCAAGGCC
TCGCGCGGCGGCGAGAACGGCGGAGGAGGAGACGACTCCGGCGACGCCGCCGACGAGGAG
CTCCGGGAGGCGTTCAAGGTGTTCGACAAGGACCAGGACGGGCTCATCTCCGCCGCCGAG
CTCCGCCACGTGATGATCAGCCTCGGCGAGAAGCTCACCGACGAGGAGGTGGAGCAGATG
ATCCGGGAGGCCGACCTCGACGGCGACGGCCAGGTCAACTTCGACGAGTTCGTCAGGATG
ATGATGCTCTCCGACCAATAA

OsCML8 (LOC_Os10g25010.1)

ATGGCGAGCAAATACAGAGGCTACTACCACGACGAGGCATCGTCGGCCGCCGGCGGCGGC
GGCGGCGGCGGCGGCGGCGACGGGTACAGGAGGGAGAAGCAGGTGAGGAAGAAGCGGCTG
ACGGCGCAGAAGAGGAAGGAGATCAAGGAGGCGTTCGACCTCTTCGACACCGATGGATCA
GGTACCATTGATCCAAAGGAGCTCAACGTCGCCATGAGAGCACTGGGGTTTGAGATGACA
CCAGAGCAAATACACCAGATGATCGCGGAGGTGGACAAGGACGGGAGCGGGACGATCGAC
TTCGACGAGTTCGTGCACATGATGACCGACAAGATGGGCGAGCGCGACGCCAGGGAGGAG
CTCAACAAGGCCTTCAAGATCATCGACAAGGACAACAACGGGAAGATCTCGGACGTGGAC
ATCCAGCGGCTGGCCATCGAGACCGGCGAGCCCTTCACGCTGGACGAGGTCAGGGAGATG
ATCGAAGCCGCCGACGAGAACGGTGATGGCGAGGTCGACCACGAGGAGTTTCTCAAGATG
ATGAAGAGGATAGGATTTGGGGCCGGGTTTTTTTAG

OsCML11 (LOC_Os01g32120.1)

ATGAGCGAGCCGGCCACCACCACCCCCACCCCCACCCCCGCCGGAGACCACGACGCAGCC
GCCACCGCATGCAAGCCTGCGGAGACGACCACGGCCCTGATCACCTGCAGGAGCAGCAGC
TGCAGCGCCCAGCAGCAGCAGCAGCAGCAGCAGCAGGAGGAGCCGCTCGGCGACGAC
CAGCTGGGTGAGCTGCGGGAGATCTTCCGCTCCTTCGACCGCAACGGCGACGGCAGCCTG
ACGCAGCTGGAGCTCGGGTCCCTCCTCCGCTCCCTCGGCCTCAAGCCCAGCACCGACGAG
CTGGACTCCCTCATCCAGCGCGCCGACACCAACTCCAACGGCCTCATCGAGTTCTCCGAG
TTCGTCGCCCTCGTCGCGCCCGAGCTCCTCTACGACCGCGCCCCCTACTCCGAGGACCAG
ATCCGCCGCCTCTTCAACATCTTCGACCGCGACGGCAACGGCTTCATCACCGCCGCCGAG
CTCGCCCACTCCATGGCCAAGCTCGGCCACGCGCTCACCGTCAAGGAGCTCACCGGCATG
ATCAAGGAAGCCGACACCGACGGCGACGGCCGCATCAGCTTCCAGGAATTCTCCCGCGCC
ATCACCGCCGCCGCATTTGACAACATCTTCTCCTGA

OsCML13 (LOC_Os07g42660)

ATGTCTACTGTCAAGGGACAGACCAGGAGGGAGAGGCCTAGAGGAGCTCGCCCTCATGGA
TTGACGAAGCAGAAGAGGCAGGAAATAAAGGAGGCGTTTGATCTGTTCGACACAGATAAC
TCTGGAACCATTGATGCCAAAGAGTTGAATGTTGCCATGAGAGCCTTGGGATTTGAGATG
ACAGAAGAACAAATTAACCAGATGATTGCTGATGTGGACAAAGATGGTAGTGGATCCATA
GATTATGAGGAGTTTGAGCATATGATGACTGCTAAGATTGGAGAGAGAGACAGTAAAGAA
GAACTTACGAAAGCATTCAGTATTATCGACCAAGATAAAAATGGGAAGATATCAGATGTT
GATATTCAGCGAATTGCCAAGGAATTAGGTGAAAACTTCACTTATCAAGAGATTCAAGAA
ATGGTGCAAGAGGCAGATCGAAATGGTGATGGTGAGATAGATTTTGATGAGTTCATTAGG
ATGATGAGGAGGACTGGATATGGTTACTAG

Myosin heavy chain (LOC_Os12g17310.1)

ATGGCTACAAAACTCCGTCAACCGTCAACTGATGCAGAAAAAACAGAGGTTGGAGAAATA
GACACAAGGGCTCCTTTTGAATCCGTCAAAGCTGCAGTAAGCTTATTTGGGGAAGTTCGA
TTTTCATCTGACAAATCAGCTGCAAGGAAGCCAAAGCCTCCTCAGGCAGAGAGGGTGTTA
GCTAAGGAGACAGAACTGCACTTGGCCCAGAAAGAGTTAAATAAATACAAGGATCAGCTC
AACAATGCTGAGACAACCAGAGTGCAAGCGTTATCTGAGCTGGAGAAAGCTAAGAAAACT
GTTGAGGACCTGACCAATAAGCTGGATGCTATCAACAAGTCCAAAGAGCTGGCTATTCAA
GCAACAGAGGATGCAAAAACTCGAACAAAGCAGCTTGAAGGTGGAGACTCGCTTGAGGCT
GTTGGAAAAGATGGCCCTTTAAAGCAGGAATTGGATGTTGCAAGGGAACAGTATGTTGTT
GCTTTGGCAGATCTTGATGCAGCAAAACAGGAGCTTAGAAAGCTCAAGAAGGATTTTGAA
GCTTCATTGGATATGAGGTTGGCTGCAGCACAGCAGGAAGAGGAATCATTGCACTTAGCT
GAAACAAACAAGCAAAAGGCTGATCAGCTTCGCAAGGAGATTGCTACAATTCAAGAGTCT
CTTACGCATGTGAAGGCAGCCACTGAACAAGCACATGAAGAAGAGGCTCAAATCCTTGCT
GAGAAAGATGTTACTAGGAAAACATACAAACAAGCTTTGGAAGAAGCCGAGAAGAAATTA
TCCTCTTTGAAAAAGGATTTTGATCCTGCTGTTTATAAAAGCCTCAAAGAAAAGCTAGAT
GAGACCAATTTGGAGATTTCATCTATGCAGAAAAAGATTGAAGATGCTCGAGCTCAAGAT
TTGGAGTCTATTGCTACTGTCAGCACAGAGTTGGATGATGCTAAGGAAATGTTACAGAAA
GTGGCAGAGGAGGAAAGTTCTCTTCGGAGTTTAGTAGAATCACTTAAACAAGAGTTAGAA
GCTGTTAAGGAGGAGCATGATCAATTGAAACAGAAGGATACAGAAACTGAATCCATAGTT
GGAGACCTACATGTGAAGCTTCAGAAATGCAAATCTGAGCTTGAGGCAGCCGTAGCTGCT
GAATCAAAAGCAACGTCAGCTTCTGATGACTTGATGTTGGCCCTCCAACAGTTGTCTTCC
GAGTCAAAAAATGCCCTGCAGGAAGCTGAAGTAATGCAAAAGAGTGCTGCAGATTTAAGG
GATGAAGCTGAAGCTGCACGAGTAGCATTAGCAGAAGCTGAACAAAAGTTGCAATCTGCT
TTAAAAGAAGCAGAAGAGGCAAAATCAGCTGAAGCAAAGGCCCTTGATCAGATCAAGCAA
CTATCAGAAAGAGCAAGCGCTGCTCGGGCCTCAACATCTGAATCAGGTGCGAAGATAACA
ATATCAAAAGAAGAGTTTGAATCTCTTAGCCGAAAGGTGGAGGAGTCAGAGAAATTGAGT
GAGATGAAAGTTGCTGCCGCTATGGCTCAAGTGGAGGCTGTCAGAGCCAGTGAGAATGAG
GCGATTAAGAAATTGGAGGCGGCTCGGAAAGAGATGGAAGACATGGAATTGGCAACAGAG
GAAGCACTAAAGAGGGCAGAGATGGCTGAAGCAGCAAAGAGAGCTGTAGAAGGTGAGCTC
AGGAGGTGGCGCGAGAAGGAGCAGAAGAAAGCTGCTGAAGCTCAGCCTGCTCCAGAAGCG
CAAGCACATGGAACTGCATCTTCCCCTGTACAGAAAGCTTCTGCTGGAAAAGCCAATGAG
AAGAATGATGGACCTCACAAGAACAGCAGAACACTATTGAAAAAGAGCTTTATGCTACCA
AATATTACAAGCATGTTCCATAAGAAGAAGAATCATGCCGACGGCAGTTCTCCTTCACAT
CTTCCTGGGGACAAATCTGTATAA

Figure 3.2 Nucleotide sequences of the genes used in the yeast two-hybrid system.

3.1.1.2 Construction of the bait and prey plasmids using LR Recombination and transformation to yeast MaV203

The LR recombination reaction between an entry clone and either pDEST™32 or pDEST™22 (Appendix A) was performed to generate bait or prey plasmid, respectively. The pDEST™32, which is used to clone the gene in frame with the sequence encoding the GAL4 DBD (forming the bait), is the GAL4 DNA binding domain (GAL4 DBD)-containing Gateway® Destination vector. The pDEST™22, which is used to clone the gene in frame with the sequence encoding GAL4 AD (generating the prey), is a GAL4 activation domain (GAL4 AD)-containing Gateway® Destination vector.

In this study, *Myosin heavy chain* was used as a bait, so *OsCaM* and *OsCML* genes were used as preys. Competent TOP10 cells were transformed with the LR recombination reactions and the transformants were selected by screening on LB agar plate containing 50 µg/mL of Gentamicin and 100 µg/mL of Ampicillin for bait and prey, respectively. The resulting recombinant bait plasmid was called pDEST™32/MYO and the recombinant prey plasmids were called pDEST™22 or pDEST™22/CMLs.

Competent yeast cells strain MaV203 were co-transformed with pDEST™32/MYO and each of the pDEST™22/CaMs or pDEST™22/CMLs and the transformants were selected by screening on SC-LT agar plate. The result for transformation of bait and prey into MaV203 cells is shown in Figure 3.3.

3.1.1.3 Testing of the bait auto-activation

Testing of the bait (GAL4 DBD fusion) for nonspecific activation was performed by transforming the pDEST™32/MYO and the empty-pDEST™22 into yeast strain MaV203. The extent of self-activation was accessed on the reporter gene *HIS3* by determining the concentration of *HIS3* inhibitor 3AT necessary to repress growth. This concentration would later be used in two-hybrid screening to suppress growth of yeast cells not containing interacting bait and prey.

The transformed yeast cells were serially diluted with ultrapure water and dropped on the SC-LT (non-selective condition) and SC-LTH+3AT containing either 50

mM $CaCl_2$ or 25 mM EGTA (selective conditions). The result (Figure 3.4) showed that Myosin heavy chain protein can be used as a bait protein without auto-activation of the reporter gene *HIS3*. The concentration of 3AT used in two-hybrid screening was 100 mM as determined in Figure 3.4.

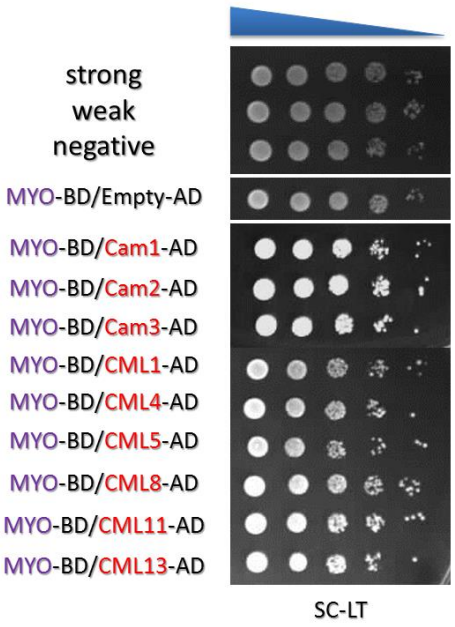


Figure 3.3 Transformation of bait and prey plasmids into yeast cells. Serial dilutions of the transformed cells are shown by narrowing triangles.

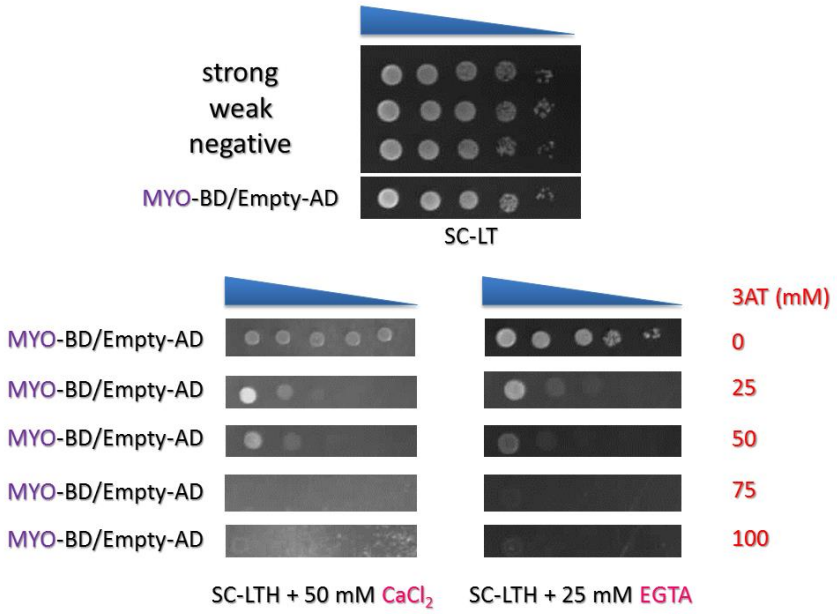


Figure 3.4 Testing of the bait auto-activation. Yeast strain MaV203 co-transformed with the test constructs was grown at 30 °C for several days under non-selective (SC-LT) and selective (SC-LTH + 3AT) conditions. Serial dilutions of transformed cells are shown by narrowing triangles.

3.1.1.4 Testing of the activation of the reporter genes

MaV203 cells that contain bait and prey proteins that strongly interact will induce reporter genes (*HIS3* and *LacZ*) in this system.

a) *HIS3* reporter gene

To confirm that the Myosin heavy chain is a CaM binding protein, pDEST^{TM}32/MYO and each pDEST^{TM}22/CaM or pDEST^{TM}22/CML were transformed into MaV203 cells. The colonies, which contain bait and prey were streaked on SC-LT plates to generate master plates. Four isolated colonies of each transformant were picked and serially diluted with ultrapure water. The diluted cells were dropped on the SC-LTH+100 mM 3AT containing either 50 mM $CaCl_2$ or 25 mM EGTA. The result of the activation of the reporter gene *HIS3* in these transformants showed that the Myosin heavy chain can interact with OsCML4, OsCML5 and OsCML8 both in the presence and in the absence of calcium as shown in Figure 3.5.

b) *Lac*Z reporter gene

An X-gal assay was performed for examining of the *Lac*Z reporter gene. Same as the *HIS3* reporter gene, four isolated colonies of the transformants were streaked on YPAD plate with a membrane placed on top of the agar. After incubation, the streaked cells on membrane were lysed by immersing the membrane in liquid nitrogen and the membrane was then incubated in the X-gal solution (see 2.3.1.1.5 b). The result of the activation of the reporter gene *Lac*Z in these transformants showed that the Myosin heavy chain also interact with OsCML4, OsCML5 and OsCML8, similarly to the result on the *HIS3* reporter gene as shown in Figure 3.6.

3.1.2 CaM Pull-down assay

This method is a form of affinity chromatography referred to as a CaM pull–down assay. It uses CaM-Sepharose beads to test proteins that bind to CaM and the influence of calcium on this binding. It is considerably more time efficient and requires less protein relative to column chromatography and other assays. Altogether, this provides a valuable tool to explore Ca^{2+}/CaM signaling and proteins that interact with CaM.

3.1.2.1 Searching for the CaM binding site

The CaM binding site of the Myosin heavy chain protein was searched on the Calmodulin Target Database (http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html) as shown in Figure 3.7. The oligonucleotide primers were designed at the flanking region of the CaM binding site and engineered with *Bam*HI and *Not*I restriction sites at the 5' and 3' ends, respectively. The sequence and the length of the oligonucleotide primers were shown in Table 2.2.

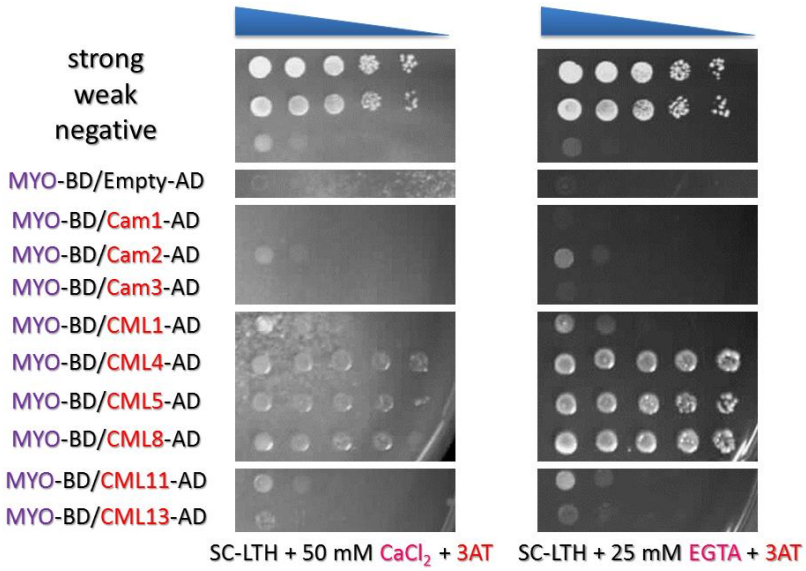


Figure 3.5 Interaction of Myosin heavy chain protein with OsCaMs and OsCMLs examined by the yeast two-hybrid system. Yeast strain MaV203 co-transformed with Myosin heavy chain and CaM/CML constructs were grown at 30 °C for several days under selective (SC-LTH +100 mM 3AT containing either 50 mM $CaCl_2$ or 25 mM EGTA) condition. Serial dilutions of the transformed cells are shown by narrowing triangles.

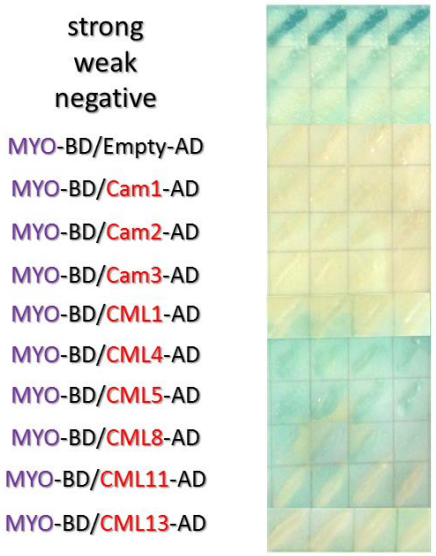


Figure 3.6 Testing of the activation of the *Lac*Z reporter gene.

3.1.2.2 Construction of Myosin heavy chain gene into pRZ850 expression vector

According to searching of the CaM binding site in Figure 3.7, the oligonucleotide primers were designed at the flanking region of the first putative CaM binding site (CBS) of *Myosin heavy chain* and engineered with *Bam*HI and *Not*I restriction sites at the 5' and 3' ends, respectively. The amplified PCR product was obtained as shown by agarose gel electrophoresis in Figure 3.8. The DNA fragment of approximately 0.15 kb, which contained a putative CaM binding site of *Myosin heavy chain* (CBS), was purified using the Gel/PCR DNA Fragments Extraction Kit. The purified PCR product and pRZ850 vector (Appendix A) were double digested with *Bam*HI and *Not*I. The digested CBS fragment was ligated into the same restriction sites in the pRZ850 vector. The ligation reaction was used to transform *E. coli* DH5α. The transformants were selected by kanamycin resistance and colonies were randomly picked for plasmid extraction. The plasmid was then analyzed by *Bam*HI and *Not*I digestion. To confirm the nucleotide sequence of the CBS inserted into the pRZ850 vector, the recombinant plasmid was subjected to DNA sequencing. The resulting nucleotide sequence of the putative CaM binding site of *Myosin heavy chain* fused to GFP was shown in Figure 3.9.

3.1.2.3 Expression and purification of recombinant fusion protein

The recombinant vector harboring the putative CaM binding site of *Myosin heavy chain* (CBS) was transformed into *E. coli* NiCo21(DE3) to produce the fusion protein. The predicted molecular mass for the CBS fusion protein is around 45 kDa. To efficiently produce the recombinant protein, the system for protein expression was optimized by culturing at 16 °C, 30 °C, 37 °C or room temperature. The result showed that the recombinant CBS could be expressed well at all temperatures, but the temperature at 37 °C was selected. The CBS fusion protein was purified using Ni-NTA Agarose beads. The result of the protein purification is shown in Figure 3.10.

3.1.2.4 CaM-Sepharose binding assay

To further characterize the CaM binding domain of Myosin heavy chain (CBS), the expressed CBS fusion protein was purified and its binding ability to CaM-Sepharose was analyzed. As shown in Figure 3.11, the purified CBS elution 4 (Lane 8) was able to bind to CaM. This binding occurred in the presence of Ca^{2+}. To confirm that the CBS fusion protein bound CaM not resin in the column, the Western blot analysis was performed. The proteins from SDS-PAGE were transferred to the PVDF membrane (see 2.3.1.2.10) and blotted with Biotinylated AtCaM2. The result showed that the CBS fusion protein bound AtCaM2 as shown in Figure 3.12.

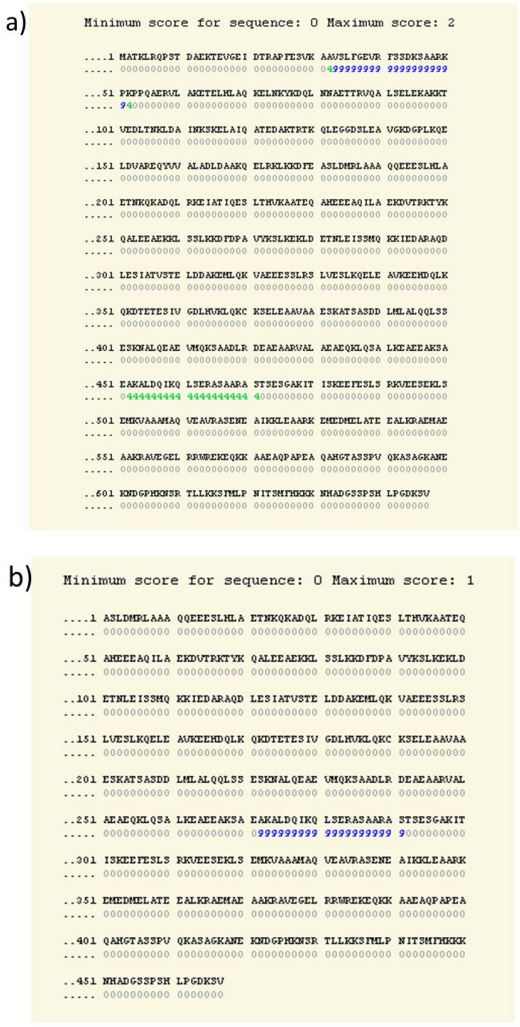


Figure 3.7 Searching for the CaM binding site. From the Calmodulin Target Database, there are two CaM binding sites predicted in the full length of Myosin heavy chain (a). When the first CaM binding site of Myosin heavy chain was deleted, the maximum score of the sequence was reduced (b). So, the first putative CaM binding site was selected for study in this experiment.

a) Prediction of CaM binding site on the full length Myosin heavy chain protein

b) Prediction of CaM binding site on the Myosin heavy chain protein with the first predicted CaM binding site deleted.

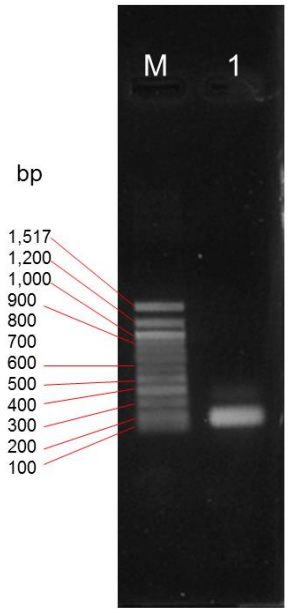


Figure 3.8 Agarose gel electrophoresis of the amplified fragment of *Myosin heavy chain* encoding the putative CaM binding site. The PCR product was separated on 1.2% agarose gel and visualized by ethidium bromide staining.

Lane M100 bp DNA ladder

Lane 1 DNA fragment encoding the putative CaM binding site of Myosin heavy chain protein (CBS)

```
CATCACCACCACCATCACCATCATTCGGCAGGTGTTAGCAAAGGCGAAGAACTGTTTACT        60
H  H  H  H  H  H  H  H  S  A  G  V  S  K  G  E  E  L  F  T         20

GGTGTTGTACCTATACTTGTGGAACTTGACGGGGACGTCAATGGACACAAGTTCAGTGTG       120
G  V  V  P  I  L  V  E  L  D  G  D  V  N  G  H  K  F  S  V         40

CGAGGTGAGGGCGAGGGAGATGCTACAAACGGGAAGTTAACCTTAAAATTTATTTGTACG       180
R  G  E  G  E  G  D  A  T  N  G  K  L  T  L  K  F  I  C  T         60

ACTGGGAAACTCCCGGTCCCTTGGCCAACGCTAGTGACTACTTTGACTTATGGTGTTCAG       240
T  G  K  L  P  V  P  W  P  T  L  V  T  T  L  T  Y  G  V  Q         80

TGCTTCTCTAGGTATCCAGATCACATGAAGCAACATGATTTTTTCAAATCTGCTATGCCA       300
C  F  S  R  Y  P  D  H  M  K  Q  H  D  F  F  K  S  A  M  P        100

GAAGGTTATGTGCAAGAAAGAACAATCTCATTCAAAGATGATGGAACTTATAAGACCCGT       360
E  G  Y  V  Q  E  R  T  I  S  F  K  D  D  G  T  Y  K  T  R        120

GCGGAAGTTAAATTCGAGGGAGACACACTGGTTAATAGAATAGAGCTTAAAGGTATTGAT       420
A  E  V  K  F  E  G  D  T  L  V  N  R  I  E  L  K  G  I  D        140

TTCAAGGAAGATGGAAATATTTTAGGTCATAAGCTCGAATACAATTTTAACTCTCATAAC       480
F  K  E  D  G  N  I  L  G  H  K  L  E  Y  N  F  N  S  H  N        160

GTGTATATTACTGCCGATAAACAGAAAAACGGGATCAAGGCAAACTTTAAAATCAGACAT       540
V  Y  I  T  A  D  K  Q  K  N  G  I  K  A  N  F  K  I  R  H        180

AACGTAGAGGATGGTTCCGTGCAATTGGCTGACCATTACCAGCAAAACACACCTATCGGA       600
N  V  E  D  G  S  V  Q  L  A  D  H  Y  Q  Q  N  T  P  I  G        200

GATGGACCCGTTCTCTTGCCAGACAATCATTACCTAAGTACACAATCAGTTTTGAGCAAG       660
D  G  P  V  L  L  P  D  N  H  Y  L  S  T  Q  S  V  L  S  K        220

GACCCGAATGAGAAGCGGGATCATATGGTACTTCTTGAGTTTGTCACGGCAGCTGGAATA       720
D  P  N  E  K  R  D  H  M  V  L  L  E  F  V  T  A  A  G  I        240

ACTCATGGAATGGATGAACTGTACAAGGGCGGAGGATCCGTCAAAGCTGCAGTAAGCTTA       780
T  H  G  M  D  E  L  Y  K  G  G  G  S  V  K  A  A  V  S  L        260

TTTGGGGAAGTTCGATTTTCATCTGACAAATCAGCTGCAAGGAAGCCAAAGCCTCCTCAG       840
F  G  E  V  R  F  S  S  D  K  S  A  A  R  K  P  K  P  P  Q        280

GCAGAGAGGGTGTTAGCTAAGGAGACAGAACTGCACTTGGCCCAGAAAGAGTTATAA         897
A  E  R  V  L  A  K  E  T  E  L  H  L  A  Q  K  E  L  *           298
```

Figure 3.9 Nucleotide and deduced amino acid sequences of the putative CaM binding site of Myosin heavy chain protein (CBS) fused at the C-terminal end of GFP. The underlined sequence is the CBS and the double underlined is the 8x His tag. The stop codon is marked with an asterisk (*).

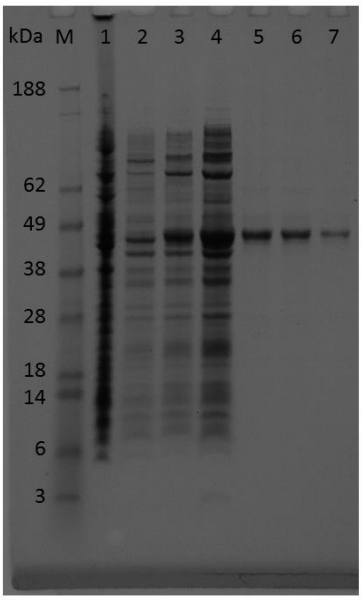


Figure 3.10 SDS-PAGE analysis of the purified recombinant CBS protein.

Lane M	SeeBlue® Pre-Stained standard
Lane 1	Pellet
Lane 2	Uninduced condition
Lane 3	Induced condition
Lane 4	Unbound protein
Lane 5	Purified CBS elution 1
Lane 6	Purified CBS elution 2
Lane 7	Purified CBS elution 3

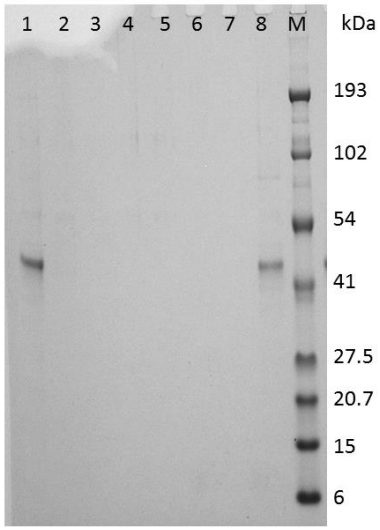


Figure 3.11 Analysis of the putative CaM binding domain of Myosin heavy chain protein (CBS) by CaM-Sepharose pull-down assay. The purified CBS fusion protein was incubated with Calmodulin Sepharose™ 4B resin in the presence of Ca^{2+} for 30 minutes and then eluted with elution buffer containing EGTA. The gel was stained with GelCode® Blue Stain Reagent.

Lane M Prestained SDS-PAGE standards (Bio-RAD)
Lane 1 Input protein
Lane 2 Unbound protein
Lane 3 Wash 1
Lane 4 Wash 2
Lane 5 Purified CBS elution 1
Lane 6 Purified CBS elution 2
Lane 7 Purified CBS elution 3
Lane 8 Purified CBS elution 4

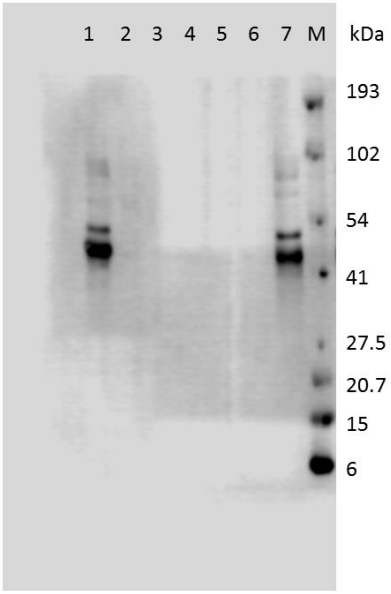


Figure 3.12 Western blot analysis of the CaM-Sepharose binding assay. The PVDF membrane was incubated with Biotinylated AtCaM2 as the primary antibody and Streptavidin-HRP was used as the secondary antibody. The membrane was incubated in freshly mixed SuperSignal® reagents and photographed by the C-digit blot scanner.

Lane M	Prestained SDS-PAGE standards (Bio-RAD)
Lane 1	Input protein
Lane 2	Unbound protein
Lane 3	Wash 2
Lane 4	Purified CBS elution 1
Lane 5	Purified CBS elution 2
Lane 6	Purified CBS elution 3
Lane 7	Purified CBS elution 4

3.2 Expression pattern of *Myosin heavy chain* gene in rice using real-time RT-PCR under salt and drought stresses

3.2.1 Expression pattern of *Myosin heavy chain* gene under salt stress

The seeds of rice 'KDML105' were sterilized and grown in Yoshida's media solution. Two-week-old 'KDML105' rice seedlings were challenged by salt stress (150 mM NaCl) as shown in Figure 3.13. The rice plants also had survived for at least 48 hours within the experimental period (Figure 3.13). The expression level of *Myosin heavy chain* gene compared to *EF1-alpha* as an internal control in leaf tissues after induction of salt stress was examined by real-time RT-PCR. Data were compared with t-test at $p<0.05$ (Appendix D-1). The result showed that no significant change in the expression level of *Myosin heavy chain* gene was detected (Figure 3.14).

3.2.2 Expression pattern of *Myosin heavy chain* gene under drought stress

The seeds of Rice 'KDML105' were sterilized and grown in Yoshida's media solution. Before starting the experiment, the concentration of PEG6000 was varied from 10% to 20%. Rice plants began to wilt after 1 hour of treatment with 20% PEG6000, which indicated loss of water content, while the others had not changed the phenotype. The rice plants also had survived for at least 48 hours within the experimental period (Figure 3.15). Therefore, two-week-old 'KDML105' rice seedlings were challenged by drought stress (20% PEG6000). The expression level of *Myosin heavy chain* gene compared to *EF1-alpha* as an internal control in leaf tissues after induction of drought stress was examined by real-time RT-PCR. Data were compared with t-test at $p<0.05$ (Appendix D-2). The result showed that significant difference in the expression level of *Myosin heavy chain* gene was found at 6 hours after treatment, but at the other time point, no significant change in the expression level of *Myosin heavy chain* gene was detected (Figure 3.16).

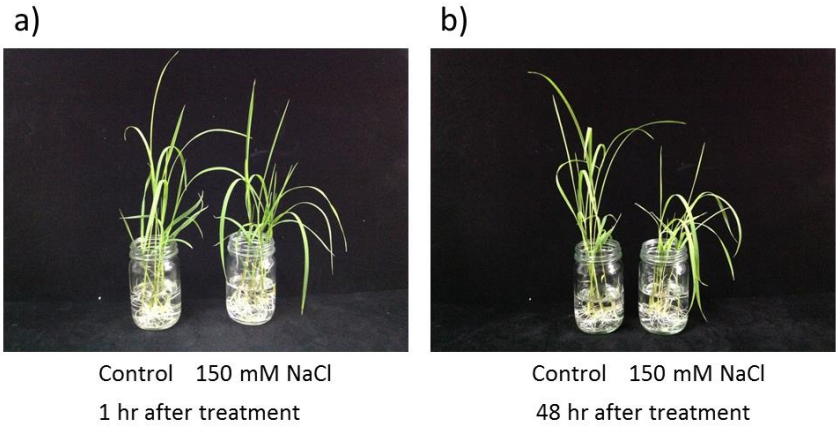


Figure 3.13 Appearances of the 'KDML105' rice plants under salt stress (150 mM NaCl) and normal (Control) conditions.

a) Rice at 1 hr after treatment

b) Rice at 48 hr after treatment

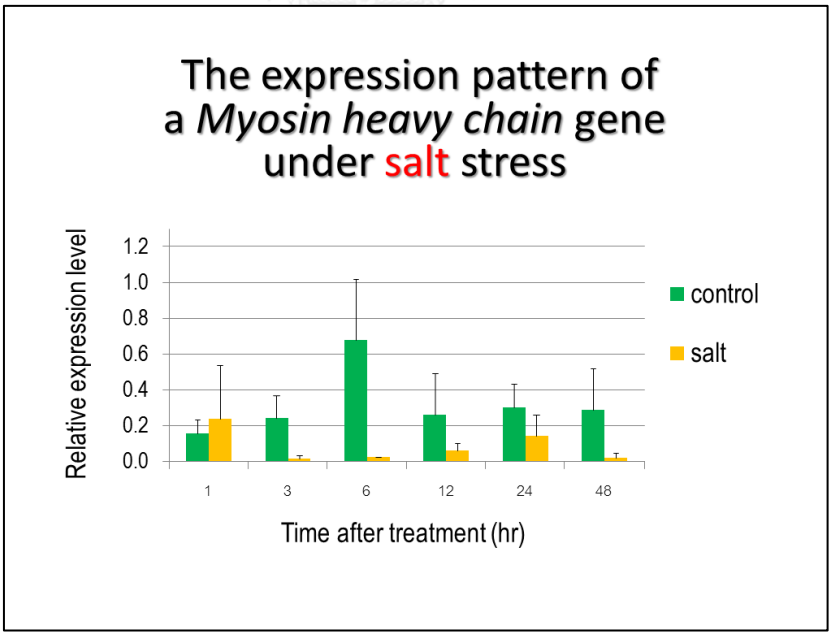


Figure 3.14 *Myosin heavy chain* gene expression in the leaf of the 'KDML105' rice seedlings under normal (control) and salt stress (salt) conditions. Bar is the standard deviation (S.D.)

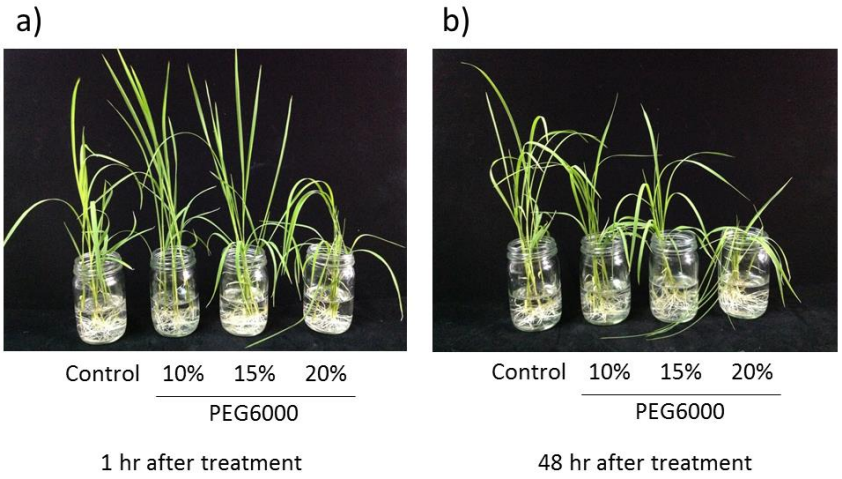


Figure 3.15 Appearances of the 'KDML105' rice plants under drought stress (10-20% PEG6000) and normal (Control) conditions.

a) Rice at 1 hr after treatment

b) Rice at 48 hr after treatment

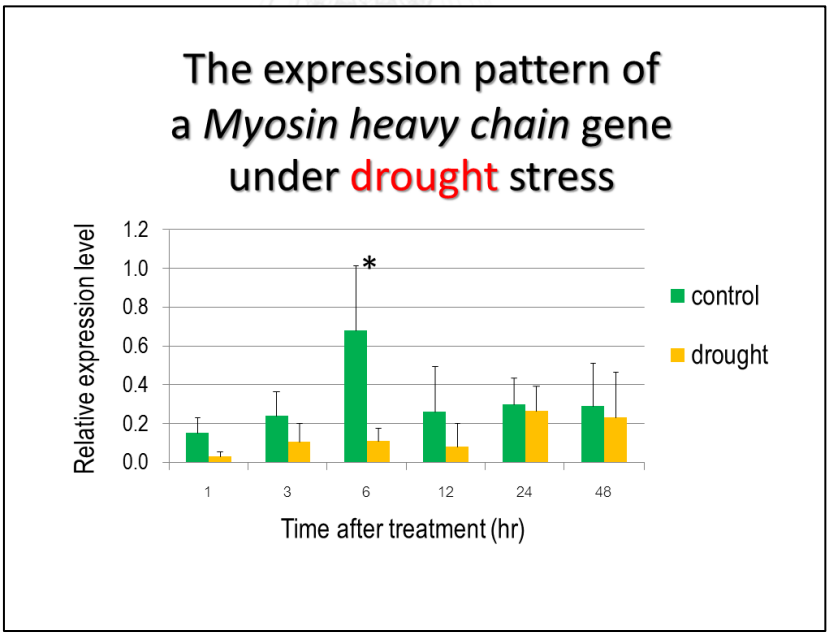


Figure 3.16 *Myosin heavy chain* gene expression in the leaf of the 'KDML105' rice seedlings under normal (control) and drought stress (drought) conditions. Bar is the standard deviation (S.D.)

(* represent $p<0.05$).

3.3 Characterization of transgenic Arabidopsis over-expressing the *Myosin heavy chain* gene

3.3.1 Construction of *Myosin heavy chain* gene into Binary vector

For plant transformation, pRZ868a and pRZ869-868a (Appendix A) were employed to generate the C- and N-terminal fusions to GFP, respectively. *E. coli* expression plasmid pRZ869 has the *Nco*I sequence at the N-terminus of GFP and as the *Sac*I sequence at the C-terminus of GFP. To generate the vector that could be used to fuse GFP to the C-terminus of the gene of interest, the pRZ869 and pRZ868a plasmids were cut at the *Nco*I and *Sac*I restriction sites and the GFP fragment from pRZ869 was then ligated at the same restriction sites into the pRZ868a to replace the GFP of pRZ868a. The resulting plasmid was called pRZ869-868a.

Base on the *Myosin heavy chain* cDNA sequence in Figure 3.2, primers for amplifying the coding region of the *Myosin heavy chain* were designed with restriction endonuclease sites as shown in Table 2.4. The amplified PCR products of approximately 2.0 kb, which were the expected size of *Myosin heavy chain*, were obtained as shown by agarose gel electrophoresis in Figure 3.17. Each DNA fragment was purified using the Gel/PCR DNA Fragments Extraction Kit.

For constructing the C-terminal fusion to GFP, the purified PCR product and pRZ868a vector were double digested with *Bam*HI and *Not*I. For constructing the N-terminal fusion to GFP, the purified PCR product and pRZ869-868a vector were double digested with *Nco*I and *Bam*HI. The digested fragments were ligated into the binary vector at the same restriction sites. The ligation reaction was transformed to *E. coli* DH5α. The plasmids were then extracted and subjected to DNA sequencing.

The pRZ868a binary vector contains a kanamycin-resistant gene (*npt* II) as a bacterial selectable marker and a *Bar* gene as a plant selectable marker within the T-DNA. The resulting recombinant plasmids were called pRZ868a: 35SCaMV-*gfp-Myo-nos* and pRZ869-868a: 35SCaMV-*Myo-gfp-nos*, in which the coding regions of the *Myosin heavy chain* were fused under the control of the 35SCaMV promoter and the *nos* terminator.

3.3.2 *Arabidopsis thaliana* floral dip transformation

To generate transgenic plant that harbors the *Myosin heavy chain* gene driven by the 35SCaMV promoter, each of the pRZ868a: 35SCaMV-*gfp-Myo-nos* and pRZ869-868a: 35SCaMV-*Myo-gfp-nos* were transformed to *Agrobacterium tumefaciens* by electroporation and the transformants were confirmed by colony PCR.

For Arabidopsis transformation, *Agrobacterium tumefaciens* strain GV3101 harboring each of pRZ868a: 35SCaMV-*gfp-Myo-nos* and pRZ869-868a: 35SCaMV-*Myo-gfp-nos* were used. Arabidopsis seeds were sterilized (see 2.3.3.1) and grown in soil in the plant growth room. *A. thaliana* lines that have bolted, where visible flowers were present (Figure 3.18 a), were dipped into the *Agrobacterium* culture (see 2.3.3.4). When the plants had completed their life cycles and were well dried, the seeds of each plant were collected (Figure 3.18 b).

3.3.3 Selecting *A. thaliana* transgenic plants

To screen the transgenic plants, the T1 seeds were sterilized and carefully applied onto 0.5xMS agar plate containing Basta as an herbicide. The plants growing on the plates were moved to soil one plant per pot and the genomic DNA was then extracted from their leaves. The PCR amplification was performed using the genomic DNA as template to confirm the gene insertion. The result showed that the specific bands were detected in the four putative Arabidopsis transgenic lines including line C2, C6, E1 and E3 (Figure 3.19). C represents Myosin heavy chain in pRZ868a vector; E represents Myosin heavy chain in pRZ869-868a vector.

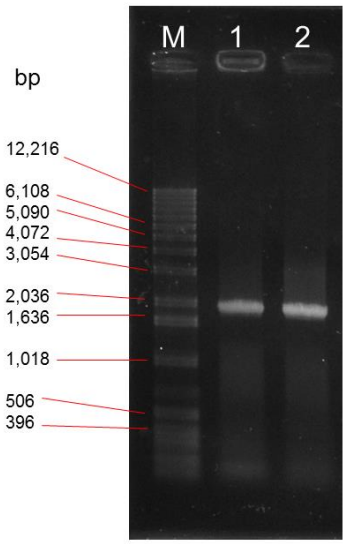


Figure 3.17 Agarose gel electrophoresis of the amplified fragments of the *Myosin heavy chain* for insertion into the binary vector. The PCR products were separated on 1% agarose gel and visualized by ethidium bromide staining.

Lane M1 kb DNA ladder

Lane 1 *Myosin heavy chain* fragment for constructing the C-terminal fusion to GFP

Lane 2 *Myosin heavy chain* fragment for constructing the N-terminal fusion to GFP

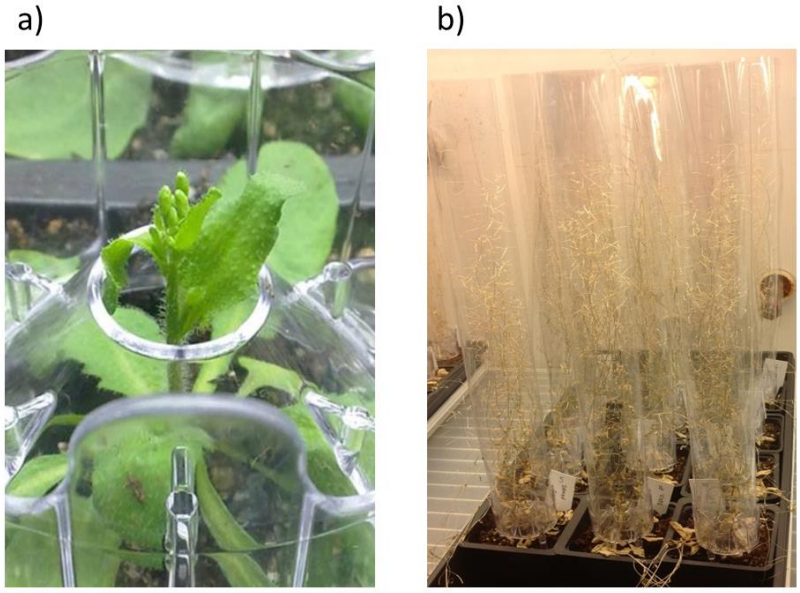


Figure 3.18 *Arabidopsis thaliana*

a) *A. thaliana* line having bolted is ready to be dipped into *Agrobacterium* culture

b) Dry seeds are ready for harvesting

3.3.4 Detection of the *Myosin heavy chain* mRNA in transgenic plants by real-time RT-PCR

To determine whether the *Myosin heavy chain* gene inserted into genomic DNA of the transgenic Arabidopsis was expressed at the mRNA level, total RNA was isolated from leaves of the plants using TRI Reagent® and used to perform real-time RT-PCR. Total RNA extracted from leaves was reverse transcribed to cDNA by iScript™ Reverse Transcription Supermix kit. The first strand cDNA was used as a template for amplification. The result in Figure 3.20 showed all transgenic Arabidopsis expressed the transgene with higher expression levels of the *Myosin heavy chain* in the transgenic Arabidopsis plants lines C2 and E3, and relatively lower expression levels in lines C6 and E1.

3.3.5 Characterization of transgenic Arabidopsis lines

Seed germination and seedling establishment are the most critical stages for survival during the life cycle of an individual plant. Water and salt stresses are two important environmental factors that limit the germination of seeds in most ecological environments. As the soil dries, or the salt level builds up, water and osmotic potentials in the soil decline, limiting the germination of seeds, which become conditioned by negative water potentials. Seed responses to abiotic stresses probably involve and integrate an interconnected signaling network to cope with drought and salinity (Vallejo, Yanovsky, & Botto, 2010).

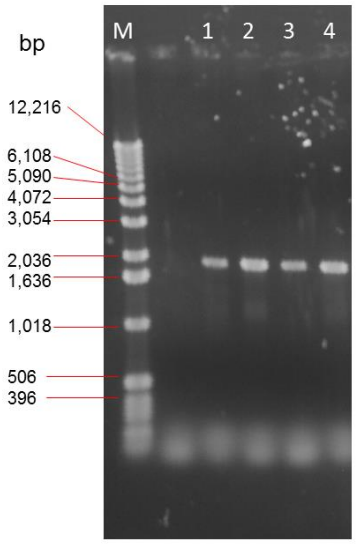
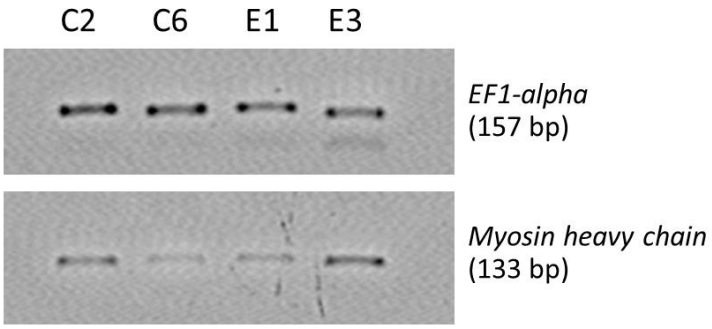


Figure 3.19 PCR analysis of *Myosin heavy chain* gene insertion in the genome of the transgenic Arabidopsis lines transformed with pRZ868a: 35SCaMV-*gfp-Myo-nos* or pRZ869-868a: 35SCaMV-*Myo-gfp-nos*. PCR products were analyzed on 1% agarose gel and visualized by ethidium bromide staining.

Lane M 1 kb DNA ladder
Lane 1 Line C2
Lane 2 Line C6
Lane 3 Line E1
Lane 4 Line E3

a)



b)

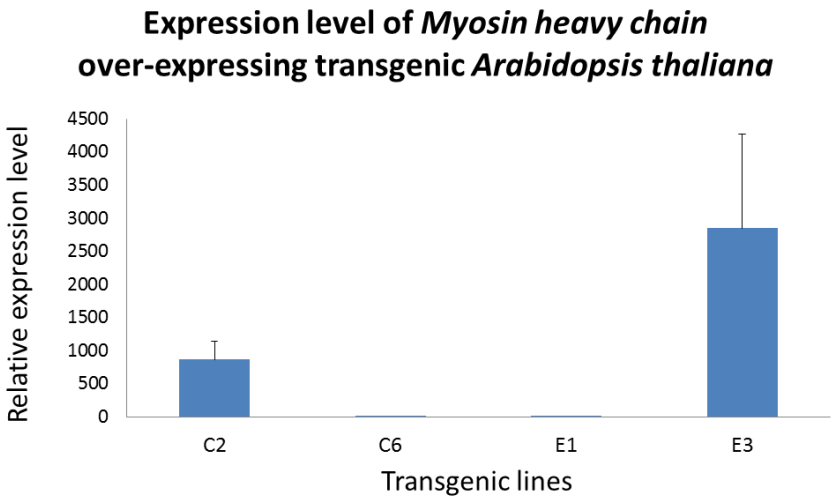


Figure 3.20 Expression of the *Myosin heavy chain* gene in the transgenic Arabidopsis lines.

a) Expression examined by RT-PCR

b) Relative expression levels determined by real-time RT-PCR using the level in E1 as reference.

3.3.5.1 Arabidopsis seed germination assay

T3 seeds were sterilized and carefully applied on Petri dishes containing the growth media: half-strength Murashige and Skoog (0.5x MS) salts, 0.2% kelcogel with addition of 100 mM NaCl or 20%PEG6000. To synchronize germination, seeds were stratified for 3 days at 4 °C and subsequently transferred to a plant growth room (23 °C, photoperiod 16-h light/ 8-h dark). Germination rates were scored daily for 7 days following stratification. Seeds were considered to have germinated when the radicles have penetrated the seed coats. The result of germination assay was shown in Figure 3.21 and 3.22. According to Figure 3.22, there may be some differences of germination rate at day 2 in this condition, so we performed statistical analysis (analysis of variance, ANOVA) to analyze whether the effect of individual treatment led to different germination rates, as shown in Appendix D-3. As a result, no significant change in the seed germination rate in each treatment was detected.

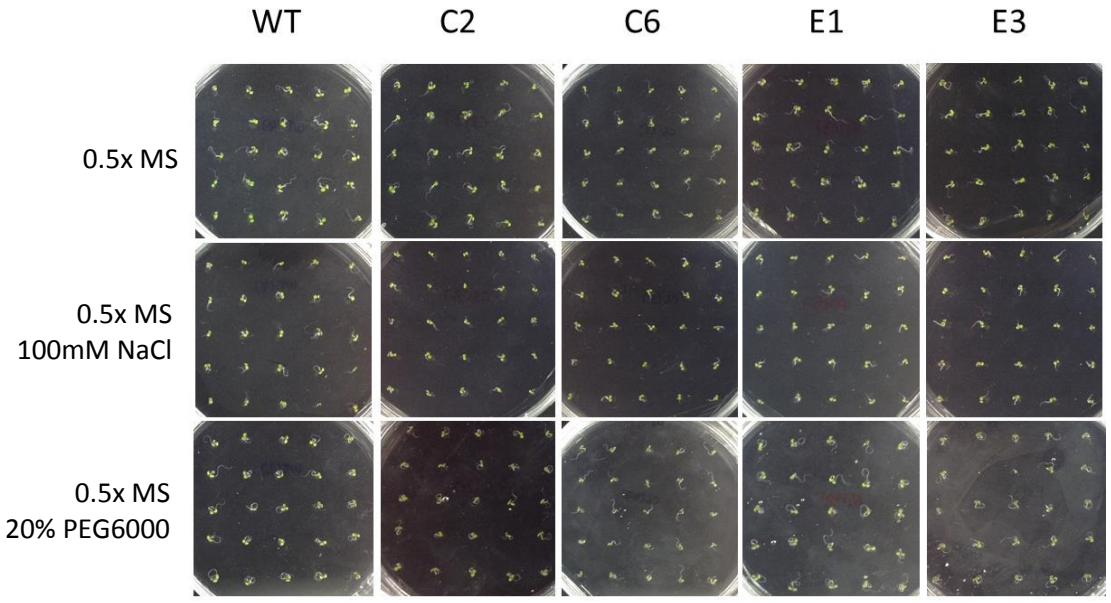
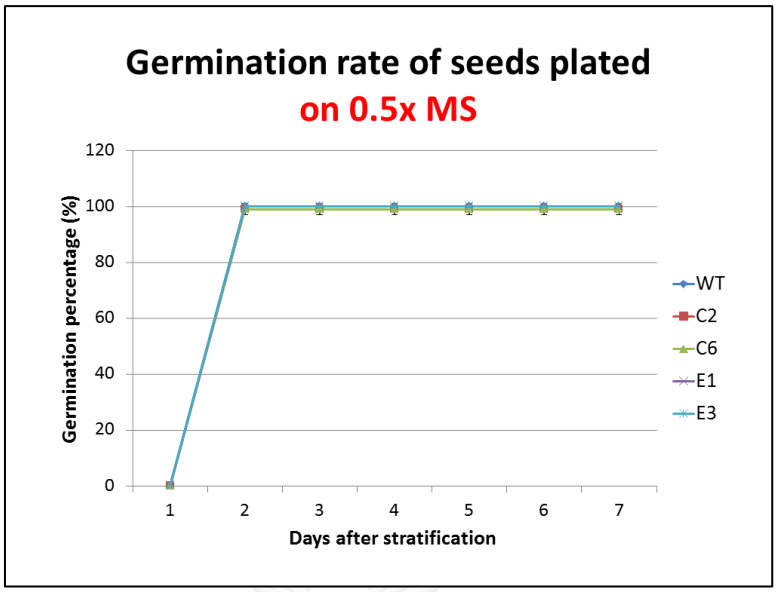
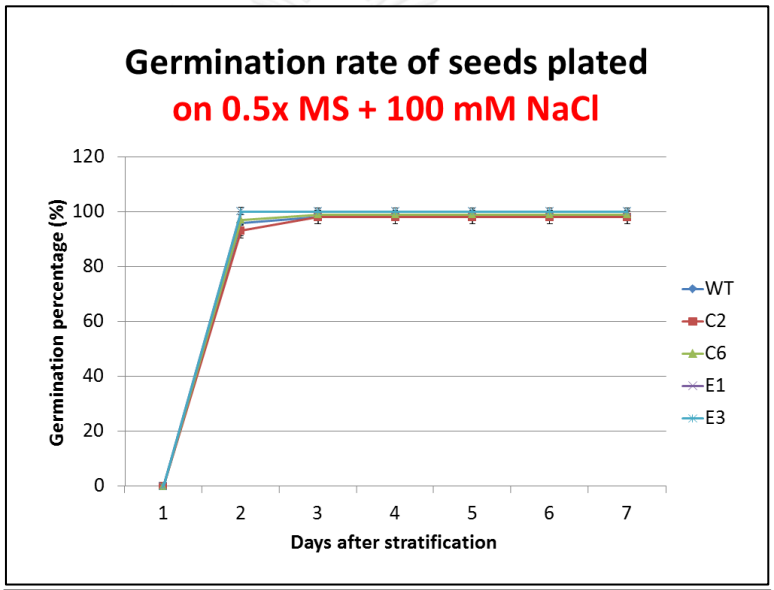


Figure 3.21 Representative seed germination images of Col-0 (wild-type), and the transgenic lines C2, C6, E1 and E3. Images were taken four days after stratification from plates containing 0.5x MS media, 0.5x MS + 100 mM NaCl or 0.5x MS + 20% PEG6000.


Germination rate of seeds plated
on 0.5x MS
Germination percentage (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Days after stratification
WT
C2
C6
E1
E3


Germination rate of seeds plated
on 0.5x MS + 100 mM NaCl
Germination percentage (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Days after stratification
WT
C2
C6
E1
E3

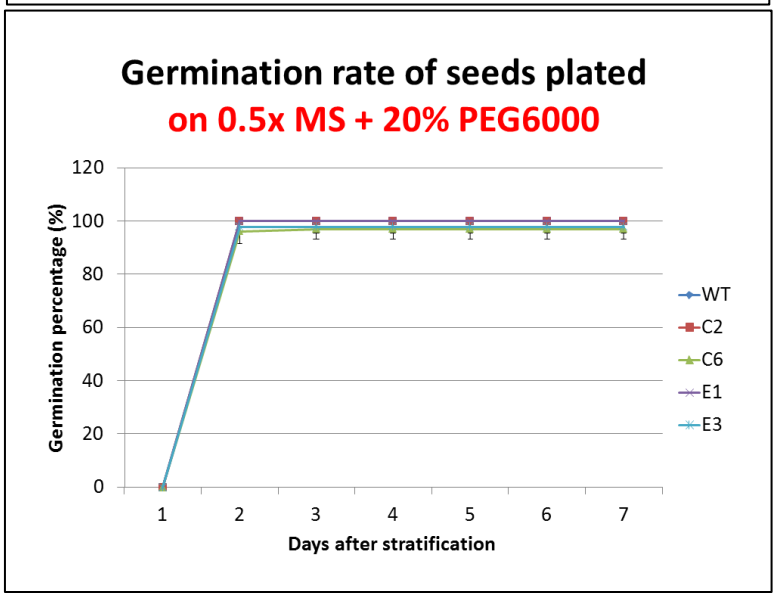

Germination rate of seeds plated
on 0.5x MS + 20% PEG6000
Germination percentage (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Days after stratification
WT
C2
C6
E1
E3

Figure 3.22 Germination percentage of Col-0 (wild-type, WT), and the T3 transgenic lines C2, C6, E1 and E3 plated on 0.5x MS (upper panel), 0.5x MS supplemented with 100 mM NaCl (middle panel) or with 20% PEG6000 (lower panel). Data represent average germination percentages ± SD of four biological replicates, 25 seeds each, observed daily for 7 days after stratification. Statistical analysis was shown in Appendix D-3.

3.3.5.2 Arabidopsis fresh weight measurement

Arabidopsis seeds (Col-0 (wild-type, WT), T3 transgenic lines C2, C6, E1 and E3) were sterilized and carefully applied on 0.5x MS agar plates and were then stratified for 3 days at 4°C before transferred to the plant growth room (23°C, photoperiod 16-h light/ 8-h dark). Seven-day-old seedlings were then transferred to the treatment conditions (control, salt and drought). Seven days after treatment, Arabidopsis whole seedlings were weighed. The result of fresh weight measurement in Figure 3.23 was compared using statistical analysis (analysis of variance, ANOVA) as shown in Appendix D-4. As a result, no significant change in the fresh weight in each treatment of Arabidopsis lines was detected.

To easily compare the effect of salt and drought stresses on the inhibition of plant growth, the reduction percentage of each treatment was calculated. The result of reduction percentage of fresh weight showed that E1 showed lower reduction percentage of fresh weight under both salt and drought stresses as shown in Figure 3.24. Data were compared using statistical analysis (ANOVA) as shown in Appendix D-5.

3.3.5.3 Arabidopsis dry weight measurement

After 3.3.5.2, Arabidopsis samples (Col-0 (wild-type, WT), T3 transgenic line C2, C6, E1 and E3) were dried at 60°C for 3 days and then weighed. The result of dry weight measurement was shown in Figure 2.25. Data were compared using statistical analysis (analysis of variance, ANOVA) as shown in Appendix D-6. The result showed that no significant change in the dry weight in each treatment of Arabidopsis lines was detected.

Similarly to the fresh weight, the reduction percentage of dry weight was calculated. The result of reduction percentage of dry weight showed that C6 and E3 showed lower reduction percentage of dry weight under drought stress as shown in Figure 3.26. Data were compared using statistical analysis (ANOVA) as shown in Appendix D-7.

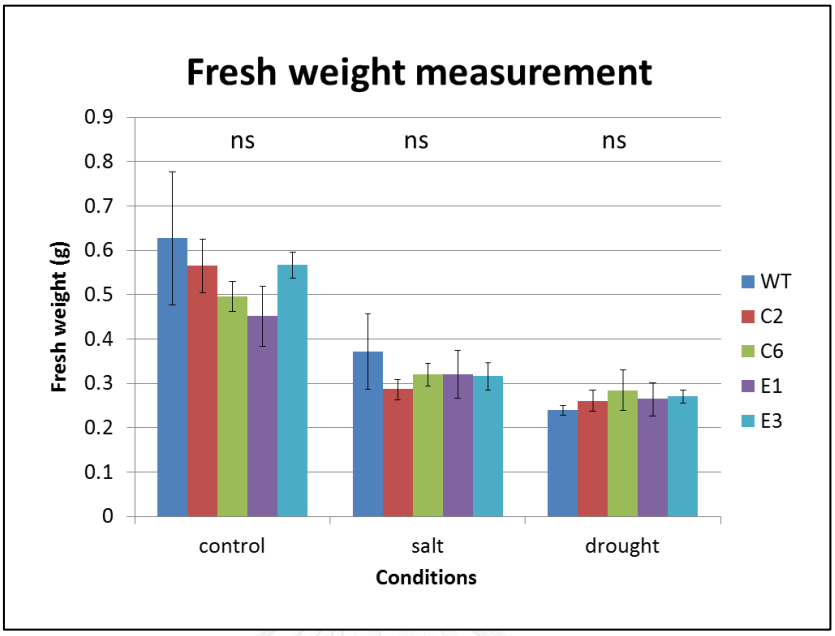


Figure 3.23 Fresh weight measurement of Col-0 (wild-type, WT), and the transgenic lines C2, C6, E1 and E3 grown on 0.5x MS (control), 0.5x MS supplemented with 100 mM NaCl (salt) or with 20% PEG6000 (drought). Data represent average fresh weight (g) ± SD of four biological replicates, 15 plants each, observed at day 7 after treatment. Statistical analysis was shown in Appendix D-4.

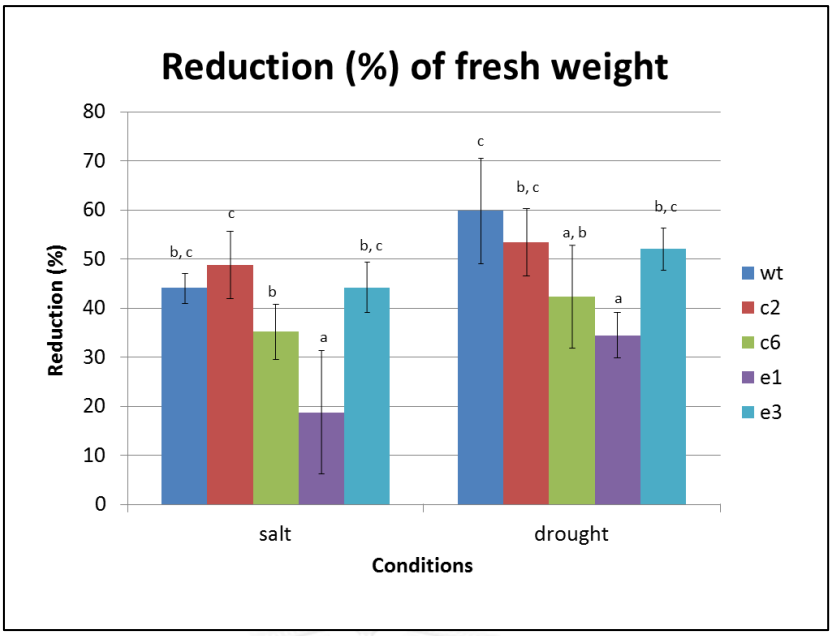


Figure 3.24 Reduction (%) of fresh weight measurement of Col-0 (wild-type, WT), and the transgenic lines C2, C6, E1 and E3 grown on 0.5x MS supplemented with 100 mM NaCl (salt) or with 20% PEG6000 (drought) compared with those grown on 0.5x MS (control). Data represent average fresh weight (g) ± SD of four biological replicates, 15 plants each, observed at day 7 after treatment. Statistical analysis was shown in Appendix D-5.

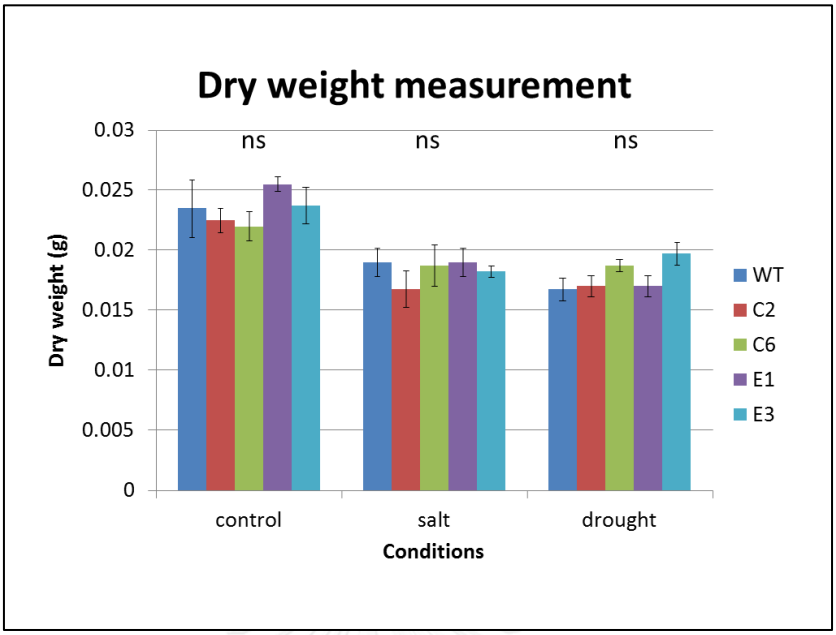


Figure 3.25 Dry weight measurement of Col-0 (wild-type, WT), and the transgenic lines C2, C6, E1 and E3 grown on 0.5x MS (control), 0.5x MS supplemented with 100 mM NaCl (salt) or with 20% PEG6000 (drought). Data represent average fresh weight (g) ± SD of four biological replicates, 15 plants each, observed at day 7 after treatment. Statistical analysis was shown in Appendix D-6.

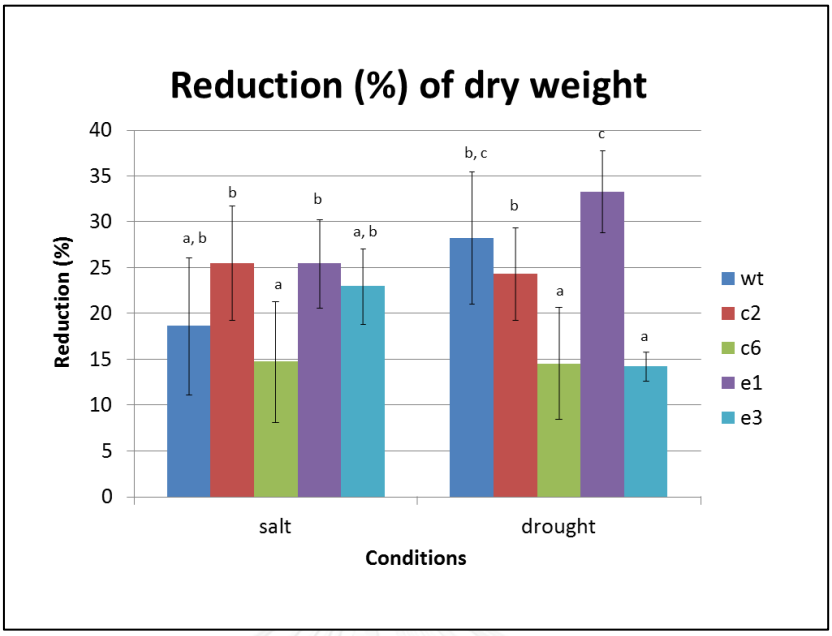


Figure 3.26 Reduction (%) of dry weight measurement of Col-0 (wild-type, WT), and the transgenic lines C2, C6, E1 and E3 grown on 0.5x MS supplemented with 100 mM NaCl (salt) or with 20% PEG6000 (drought) compared with those grown on 0.5x MS (control). Data represent average fresh weight (g) ± SD of four biological replicates, 15 plants each, observed at day 7 after treatment. Statistical analysis was shown in Appendix D-7.

CHAPTER IV
DISCUSSION

4.1 Interaction of Myosin heavy chain with OsCaMs and OsCMLs from rice using the yeast two-hybrid system

According to the testing of the bait auto-activation of the *HIS3* reporter gene (Figure 3.4), we found that the more 3AT concentration, the less bait auto-activation of the reporter gene *HIS3*. It was demonstrated that Myosin heavy chain was able to be used as a bait protein without auto-activating the transcription of the reporter gene *HIS3* at the high concentration of 3AT.

In a previous study, Myosin heavy chain has been identified as one of the putative target proteins of OsCaM1 from rice (*Oryza sativa* L.) by cDNA expression library screening. In order to examine the specificity of the interaction between Myosin heavy chain and OsCaM1, we tested the ability of Myosin heavy chain to bind other OsCMLs and other typical CaMs in yeast cells. By testing of the activation of *HIS3* reporter gene, the growth of yeast cells on the selective medium (SC-LTH+3AT containing either 50 mM $CaCl_2$ or 25 mM EGTA) indicates that the bait and prey fusion proteins are co-expressed and the two proteins, Myosin heavy chain and either OsCaMs or OsCMLs, have an interaction. In the results, Myosin heavy chain can interact with OsCML4, OsCML5 and OsCML8 both in the presence and in the absence of calcium (Figure 3.5). However, Myosin heavy chain did not exhibit an interaction with OsCaM1 and the other OsCaMs/OsCMLs tested.

By testing of the activation of *Lac*Z reporter gene, the blue color of yeast cells also indicates that bait and prey fusion proteins are co-expressed and the two proteins, Myosin heavy chain and either OsCaM or OsCML, have an interaction. The result of the testing of the activation of *Lac*Z reporter gene (Figure 3.6) was the same as the testing of the activation of *HIS3* reporter gene. OsCML4, OsCML5 and OsCML8 have also shown a strong interaction with Myosin heavy chain, but OsCaM1 and the other OsCaMs/OsCMLs tested did not exhibit an interaction with Myosin heavy chain.

In the previous study, Chinpongpanich *et al* (2012) reported that *CaM* and *CML* genes in rice exhibited different expression patterns in tissues/organs. The expression of OsCML4, OsCML5 and OsCML8 was induced under salt and drought stresses. The mRNA level of OsCML4 and OsCML8 significantly increased by 3 hours after treatment and remained elevated for at least 24 hours while the expression of OsCML5 was up-regulated as early as 1-3 hours before rapidly returning to the normal level (Chinpongpanich, Limruengroj, Limpaseni, & Buaboocha, 2012). According to the results, Myosin heavy chain is one of the target protein of OsCML4, OsCML5 and OsCML8, suggesting that the Myosin heavy chain protein from rice possibly involve in the calcium signal transduction of rice under salt and drought stresses.

As we know that CaM molecules have a high flexibility (Yamniuk & Vogel, 2004), which could be one possible explanation why we could not see an interaction of Myosin heavy chain with OsCaM1 although it was obtained from the cDNA expression library screening using OsCaM1 as probe. Chinpongpanich *et al* (2011) reported the conformational changes upon Ca^{2+} binding of OsCaMs and OsCMLs by circular dichroism spectroscopy and fluorescence spectroscopy using ANS. The OsCMLs were found exhibiting a spectrum of both structural and functional characteristics that ranged from typical CaMs. OsCML4 and OsCML5 were found to behave very similar to OsCaMs except on the Ca^{2+} binding; their ANS emission maxima did not increase as much. OsCML8 exhibited only a small increase in its molar ellipticity of the CD spectrum as well as a smaller blue shift and intensity increment of the ANS fluorescence emission maxima (Chinpongpanich, Wutipraditkul, Thairat, & Buaboocha, 2011). As it would be expected for a protein of such a paramount importance, CaM is susceptible to undergo post-translational modifications (Benaim & Villalobo, 2002). Sequence conservation related to functionality of plant CaMs also includes lysine (K) at position 116 which is assumed to be trimethylated. All OsCaM proteins possess a lysine residue at this position. Trimethylation of lysine 116 is believed to be a post-translational modification that helps regulate CaM activity. Another possible reason is that the calcium ion from the medium did not enter into the yeast cells and produce proper cytosolic calcium

signals because the yeast cells can protect themselves using the calcium homeostasis system (Cui, Kaandorp, Sloot, Lloyd, & Filatov, 2009). So, we attempted to confirm the interaction of Myosin heavy chain and CaM by *in vitro* CaM pull-down assay.

4.2 Interaction of Myosin heavy chain with Calmodulin using the CaM pull-down assay

To test whether the Myosin heavy chain, which is a putative OsCaM1-binding protein, can interact with CaM, the CaM binding site of Myosin heavy chain was searched on the Calmodulin Target Database. The result from the CaM target database (Figure 3.7) showed that Myosin heavy chain had two putative CaM binding sites, but the first one gave a higher score. So, in this study, we focused on the first CaM binding site of the Myosin heavy chain protein (CBS).

We verified the interaction between the putative CaM binding site of Myosin heavy chain (CBS) and CaM by *in vitro* pull-down assay. The CaM pull-down assay was performed using CaM-Sepharose beads. As shown in Figure 3.11, we found that CBS was detected as a CaM-Sepharose-bound protein in the presence of calcium. This result was further demonstrated the binding affinity of the CaM binding site of Myosin heavy chain (CBS) using the biotinylated AtCaM2 in the binding assay. Figure 3.12 showed the binding of the putative CaM-binding site of Myosin heavy chain (CBS) with AtCaM2. The result demonstrated that Myosin heavy chain is one of the CaM-binding proteins and might be involved in the calcium signaling pathway.

4.3 Expression pattern of *Myosin heavy chain* gene under salt and drought stresses.

a) Salt stress

Expression level of the *Myosin heavy chain* gene in the leaf tissues of the 'KDML105' rice under salt stress induced by 150 mM NaCl was determined. According to the GSE6901 data from the DNA microarray database (Appendix E), lower expression level of the *Myosin heavy chain* gene under salt stress was found.

In the present study, the result obtained from real-time RT-PCR showed that *Myosin heavy chain* gene did not clearly exhibit lower expression level in the leaf tissues of the 'KDML105' rice under salt stress. As the Figure 3.14 showed that expression of the *Myosin heavy chain* gene looked slightly lower than that of the control at 3 and 6 hours after salt stress, but the statistical analysis (t-test at $p<0.05$) showed no significant change in the expression level of *Myosin heavy chain* gene.

b) Drought stress

The effect of drought stress (20% w/v PEG6000) on expression of the *Myosin heavy chain* gene in leaf tissues of the 'KDML105' rice was investigated. The DNA microarray data (GSE6901, GSE24048 and E-MEXP-2401 in Appendix E), which were obtained from whole plants, revealed that the expression level of *Myosin heavy chain* gene was decreased under drought stress. Same as the DNA microarray database, lower expression level in leaf tissues of the 'KDML105' rice of *Myosin heavy chain* gene at 6 hours after drought stress was detected by real-time RT-PCR.

Although myosin heavy chain is one of the CaM binding proteins as described above, in this study using the real-time RT-PCR, the *myosin heavy chain* gene did not exhibit higher expression level in the leaf tissues of 'KDML105' rice under both salt and drought stresses. It might be possible that Myosin heavy chain is not involved in the response to both salt and drought stresses, but it may involve in other stresses. In the previous study, Myosin heavy chain has been identified in plants in response to heavy metal (Roth, von Roepenack-Lahaye, & Clemens, 2006) and cold stress (Yan, Zhang, Tang, Su, & Sun, 2006). However, the role of this protein is unclear. As this protein was expressed in fully expanded leaves, it might be related to actin organization, organelle movement or signal transduction (Imogen Sparkes, 2011). As the *myosin heavy chain* gene was down-regulated in drought stress and likely down-regulated in salt stress treatment in the present study, we were unable to relate the function of this protein to both salt and drought stresses.

4.4 Characterization of transgenic Arabidopsis over-expressing Myosin heavy chain protein

a) Effect of salt and drought stresses on seed germination

Seed germination and early seedling growth are critical stages for plant establishment (F.-L. Li, Bao, & Wu, 2011), and plants are more sensitive to both salt and drought stresses during these stages. Research on physiological and biochemical responses under both salt and drought stresses in the phases of seed germination and early seedling growth is important to fully understand the traits in the early life stage and, to a certain extent, to understand the interior reasons for low seedling recruitment under natural conditions.

Abiotic stresses such as salt and drought stresses greatly affect seed germination, but the response intensity and harmful effects of stress depend on species. In this study, we have successfully generated transgenic Arabidopsis over-expressing the *Myosin heavy chain* gene from rice (*Oryza sativa* L.) in order to characterize the role of Myosin heavy chain protein, which is one of the CaM binding proteins. According to the result of seed germination, although there was some difference of the germination rate at day 2 after stratification, the analyzed data from the statistical analysis showed that no significant change in the seed germination percentage in each treatment at day 2 was found. It might be possible that the experimental designs using moderate salt and drought stresses (Vallejo, Yanovsky, & Botto, 2010) in this study did not have enough effect on the seed germination. Another possible reason is that the *Myosin heavy chain* gene might not involve in the response to both salt and drought stresses, but *Myosin heavy chain* gene may involve in other stresses as described above.

b) Effect of salt and drought stresses on plant growth

In order to examine the stress tolerance of the different transgenic Arabidopsis lines over-expressing the *Myosin heavy chain* gene from rice (*Oryza sativa* L.), seedlings of the transgenic plants were subjected to salt and drought stresses. As controls, we used seedlings of wild-type plant. According to the result of

fresh and dry weight measurement, both salt and drought stresses had significant effect on plant growth. In the stress conditions, both fresh and dry weights of the wild-type and the transgenic Arabidopsis lines were significantly different from the control condition. However, the transgenic Arabidopsis over-expressing Myosin heavy chain did not exhibit stress tolerance when compared to the wild-type.

As known, soil is an essential component of wetland ecosystem, which can support, hold, and regulate water and nutrients (Huang, Bai, Shao, Gao, Xiao, Huang, et al., 2012). However, the effects of drought stress and salt stress on the growth and development of plants are coupled together, by reducing the soil solution water potential (Zhuang, Li, & Caom, 2010).

Although, the fresh and dry weight measurement did not show significant difference in each treatment between the wild-type and the transgenic Arabidopsis lines, there are some differences in the weight reduction percentage of both fresh and dry weight measurement.

Even though, statistically significant differences in the reduction percentage of fresh weight were observed in salt and drought stress conditions, the standard deviation of some lines was relatively high especially in salt stress of line E1. Line E1 had the lowest reduction percentage of fresh weight in both salt and drought stresses, although the result from the expression of *Myosin heavy chain* gene in the E1 transgenic line was lower than other lines. Also, line C6 under drought stress had lower reduction percentage in fresh weight, even though the result from the expression of *Myosin heavy chain* gene showed that line C6 exhibited lower expression level when compared with those of lines C2 and E3.

For dry weight, statistically significant differences in the reduction percentage were observed only in drought stress. The reduction percentage of line C6 and E3 of drought stress condition was significantly lower when compared with wild-type, although the result from the expression of *Myosin heavy chain* gene showed that line C6 exhibited lower expression level compared with those from the other lines. In the case of salt stress, no significant change in the reduction percentage of dry weight was detected.

From the results, we were also unable to conclusively relate the function of Myosin heavy chain to both salt and drought stresses, although we have generated the transgenic Arabidopsis over-expressing this protein to characterize its function. It could be possible that *Myosin heavy chain* gene did not involve in response to both salt and drought stresses. It might involve in response to others as described above. Another possible reason why we could not observe a changed phenotype is that stress might have effects on suppressing of the transcription and translation of some genes. Holzinger and Karsten (2013) reported that the actin filament system was substantially destroyed by consequence of desiccation in green algae (Holzinger & Karsten, 2013). As we know that myosin is a molecular motor capable of producing motive force along actin filaments using the energy from ATP hydrolysis. The movement does not occur when the actin filaments are destroyed.

Myosins, a large family of actin-based motors, have one or two heavy chains with one or more light chains associated with each heavy chain. The heavy chains of myosin have a N-terminal head domain with an ATPase and actin-binding site (Korn, 2000). Myosin heavy chains alone retain their ATPase activities in the absence of light chains. The light chains apparently function to modify and regulate myosin heavy chain activity (Kiehart, 1990).

Recent years have seen major progress in understanding of myosin function in plant cells. It is now established that myosin motors are responsible for the rapid organelle movements that lead to cytoplasmic streaming (Shimmen, 2007). Thus, myosins have been attributed to the movement of the ER (Liebe & Menzel, 1995; Quader, Hofmann, & Schnepf, 1987; I. Sparkes, Runions, Hawes, & Griffing, 2009), Golgi (Boevink, Oparka, Cruz, Martin, Betteridge, & Hawes, 1998; Nebenführ, Gallagher, Dunahay, Frohlick, Mazurkiewicz, Meehl, et al., 1999), mitochondria (Van Gestel, Köhler, & Verbelen, 2002; Zheng, Beck, Müller, Chen, Wang, Wang, et al., 2009), peroxisomes (Jedd & Chua, 2002; Mano, Nakamori, Hayashi, Kato, Kondo, & Nishimura, 2002), plastids (Paves & Truve, 2007; Wada, Kagawa, & Sato, 2003), and the vacuole (Higaki, Kutsuna, Okubo, Sano, & Hasezawa, 2006).

Houdusse *et al* (1996) constructed the model of CaM bound to IQ motif in the neck region of myosin. The model accounts for aspects of the regulation of

many myosins where CaM is bound at low levels of Ca^{2+} and released or changed in conformation at high levels of Ca^{2+}. The conformational changes as a function of Ca^{2+} depend not only on the precise sequence of the IQ motifs but also on the interactions between CaM molecules bound to adjacent sites on the myosin heavy chain. Calcium regulates the activity, and thus, it is important to understand how calcium affects CaM binding to the IQ motifs (Trybus, Gushchin, Lui, Hazelwood, Krementsova, Volkmann, et al., 2007). According to the model, the full versatility of CaM binding to target peptides is displayed in the regulation of myosins. At higher Ca^{2+} concentrations, CaM changes conformation and acts as a switch to regulate the activity of the myosin molecules (Houdusse, Silver, & Cohen, 1996).

Myosin motor proteins have recently been shown to control the movement of the ER and other organelles such as Golgi bodies, peroxisomes and mitochondria. Griffing *et al* (2014) explore myosins with a focus on their role in regulating ER network remodeling by affecting ER tubulation, sheet formation and the persistence of these structures (Griffing, Gao, & Sparkes, 2014). In addition to the importance of these ER network dynamics for organelle communication, the ER may further directly provide the source membrane for vacuole biogenesis (Brandizzi, Frigerio, Howell, & Schäfer, 2014)

Although Myosin heavy chain was identified as the CaM-binding protein from rice (*Oryza sativa* L.) in this study, the role of this protein on calcium signaling remained unclear. Nonetheless, OsCaM1 and its related Ca^{2+} sensor proteins: OsCML4, OsCML5, and OsCML8 may have some regulatory role. Therefore, more studies should be conducted in order to understand the role of OsCaM and these OsCMLs in conjunction with the Myosin heavy chain in translating calcium signals into cellular responses.

CHAPTER V

CONCLUSIONS

1. By using the yeast two-hybrid system and CaM pull-down assay in order to examine the interaction of a Myosin heavy chain with either OsCaMs or OsCMLs, we have identified Myosin heavy chain as a new CaM-binding protein from rice (*Oryza sativa* L.). However, Myosin heavy chain also interacts with OsCML4, OsCML5 and OsCML8 in the calcium-independent manner.

2. According to the gene expression analysis, *Myosin heavy chain* did not exhibit higher expression level in the leaf tissues of 'KDML105' rice during the period of 2 days under both salt and drought stresses. Based on PCR of their genomic DNA, RT-PCR, and real-time RT-PCR, four Arabidopsis transgenic lines over-expressing the *Myosin heavy chain* gene were successfully made by Agrobacterium-mediated transformation using floral dip method.

3. The germination percentage of transgenic Arabidopsis seeds over-expressing *Myosin heavy chain* gene from rice (*Oryza sativa* L.) was not different from wild-type seeds when exposed to both salt and drought stress conditions. When comparing both fresh and dry weight of the wild-type and the transgenic Arabidopsis lines under salt and drought stress conditions, both fresh and dry weight measurement did not show significant difference between the wild-type and all of the transgenic lines.

4. Based on the fresh weight reduction, line E1 had lower percentage under both salt and drought stress conditions while line C6 had lower percentage only under drought stress. When the dry weight reduction is considered, lines C6 and E3 exhibited lower percentages under drought stress condition.

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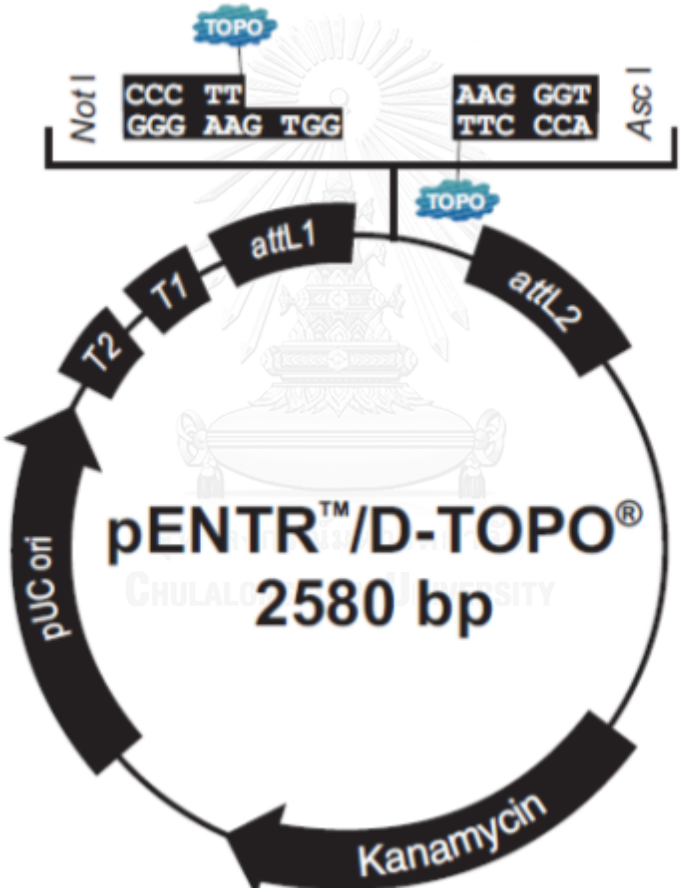
APPENDICES

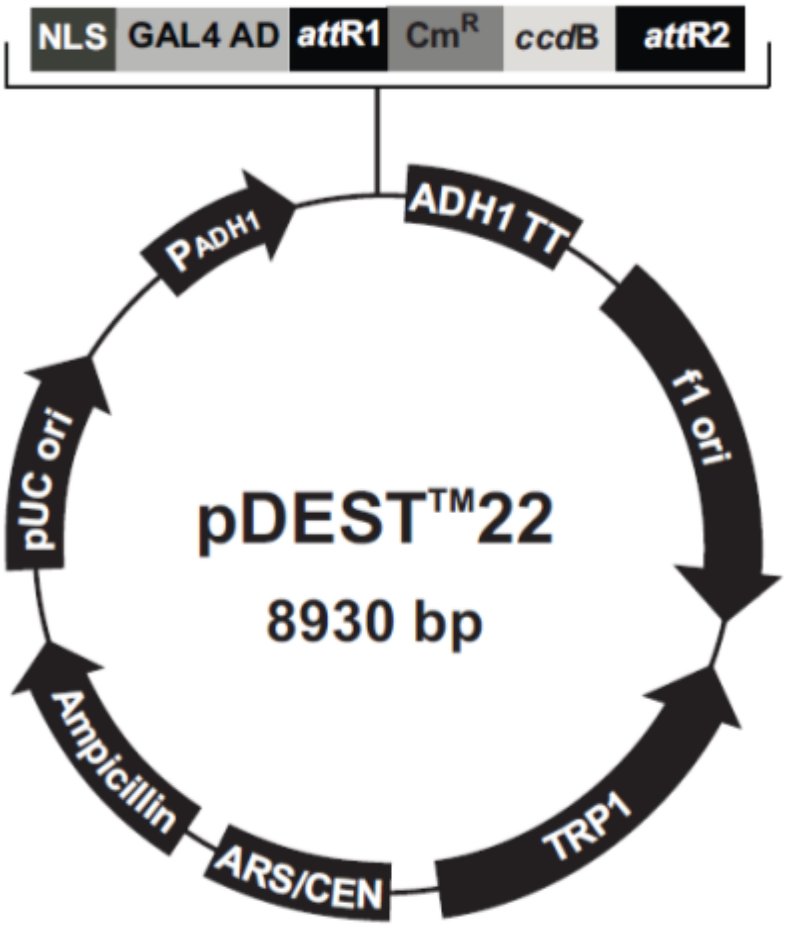
APPENDIX A

Vector maps

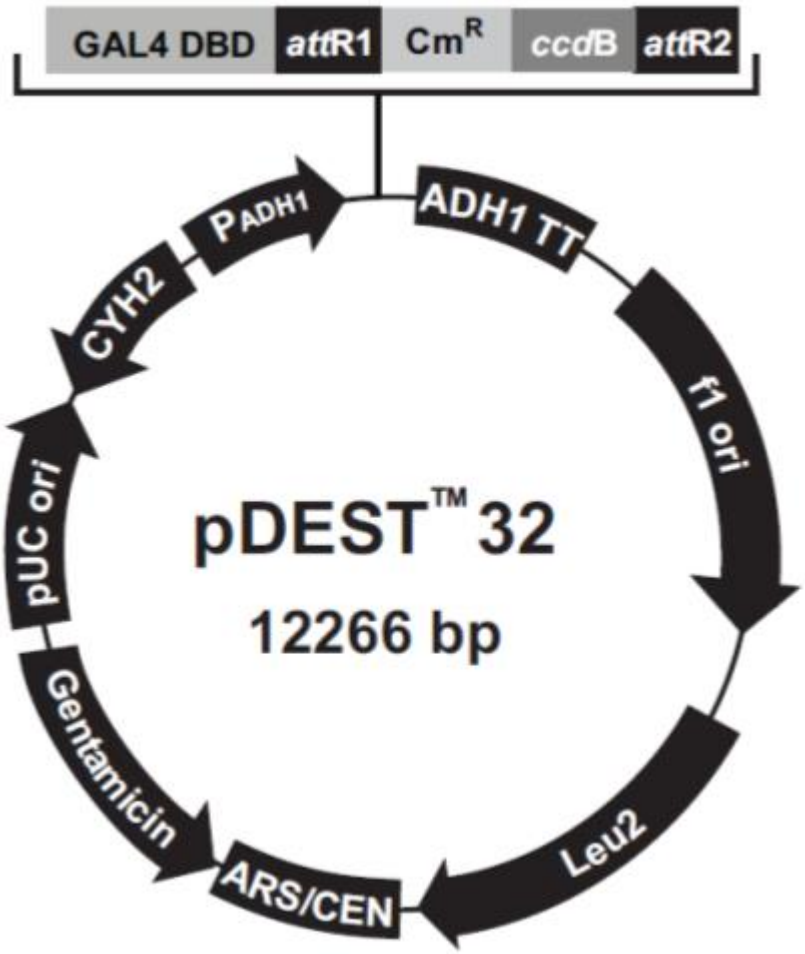
1. pENTR™/D-TOPO® map



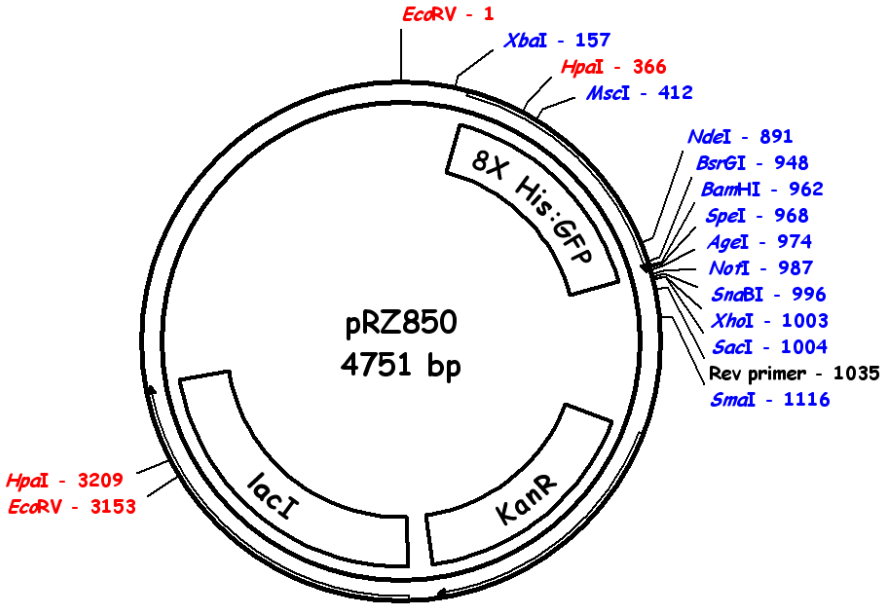
2. pDEST™22 map



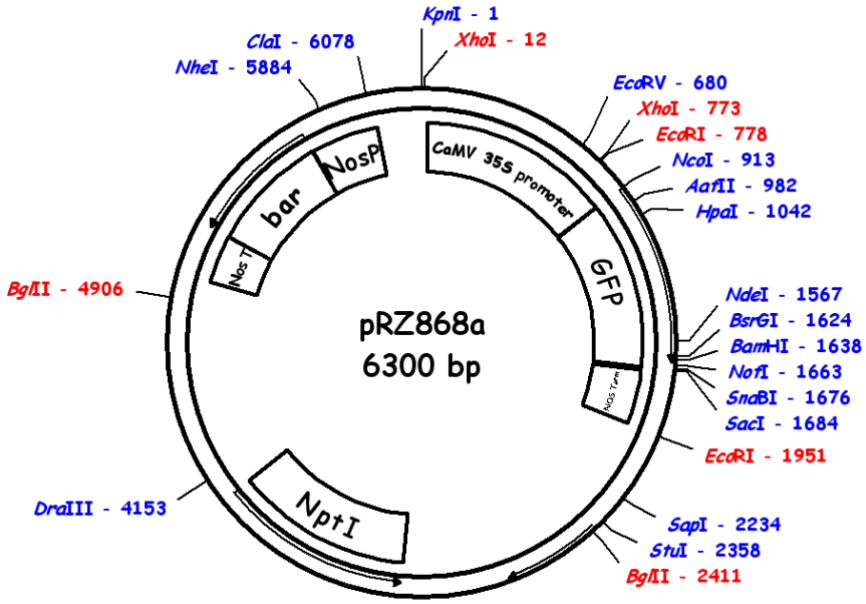
3. pDEST^{TM}32 map



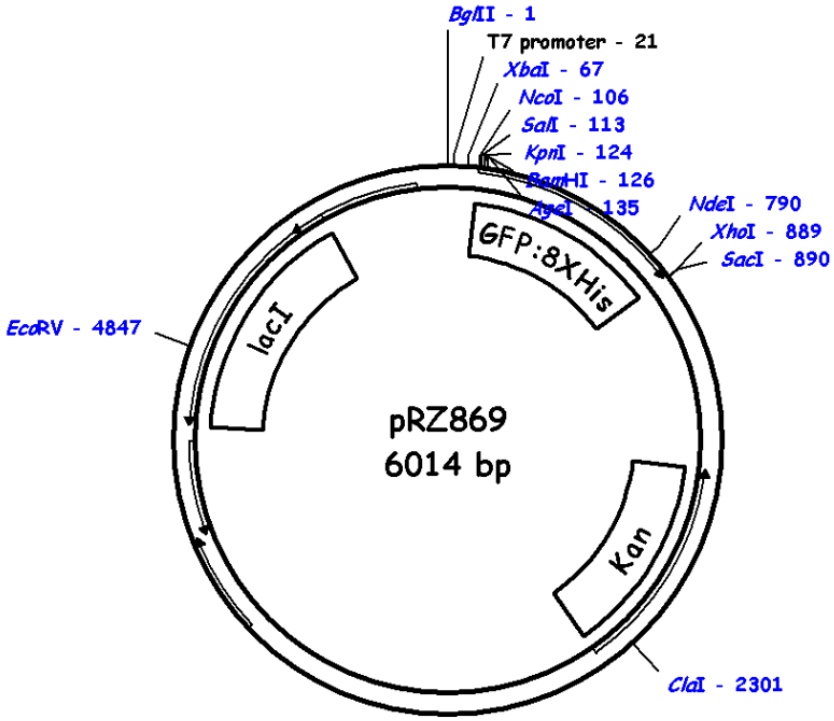
4. pRZ850 map



5. pRZ868a map



6. pRZ869 map



APPENDIX B

B-1. TAE buffer 40X

Tris base	193.6 g
Sodium acetate·$3H_2O$	108.9 g
$Na_2EDTA·2H_2O$	15.2 g
H_2O	700 ml

Dissolve, and adjust pH to 7.2 with acetic acid.

Add water to a final volume of 1 L.

Filter through a 0.22-μm nitrocellulose filter.

Autoclave and store at room temperature.

B-2. DNA gel loading buffer (10X sample buffer)

0.5 M EDTA/NaOH pH 7.5	2 ml	(0.1 M)
Glycerol	5 ml	(50% v/v)
H_2O	2.5 ml	
20% (w/v) SDS	0.5 ml	(1% w/v)
Xylene cyanol	5-10 mg	
Bromophenol Blue	5-10 mg	

Divide into 1-ml aliquots.

Store at room temperature or at 4°C.

Warm at 65°C before using to insure that SDS is dissolved.

B-3. 1 mg/ml Ethidium bromide

Caution: EtBr is a potent mutagen! Always wear gloves when handling EtBr-containing solutions.

Ethidium bromide	0.1 g
H_2O	to 100 ml

Store at 4°C in a darken bottle.

B-4. DEPC-treated deionized water

Add 50 µl diethyl pyrocarbonate to 500 ml deionized water in glass bottle, and shake vigorously.

Let stand in a fume hood overnight.

Autoclave.

B-5. 1M Tris-HCl, pH 7.5 (1 L)

Tris base	141.12 g

Add 800 ml of deionized water.

Adjust pH to 7.5 with concentrated HCl.

Adjust volume to 1 L with deionized water.

Autoclave and store at room temperature.

B-6. 10x TBS (Tris-buffered saline)

Tris base	30 g	(250 mM)
NaCl	80 g	(1.37 M)
KCl	2 g	(0.027 M)

Dissolve the Tris and NaCl in 800 ml of deionized water, and adjust the pH to 8.0 with HCl.

Bring the volume to 1 L with double deionized water.

B-7. 10x PBS (Phosphate-buffered saline)

NaCl	80 g	(1.37 M)
KCl	2 g	(0.027 M)
Na_2HPO_4	14.4 g	
KH_2PO_4	2.4 g	

Dissolve all components in 900 ml of double deionized water.

Adjust the pH to 7.2 with H_3PO_4.

Bring the volume to 1 L with double deionized water.

B-8. TBST or PBST

10x PBS or TBS	100 ml	(1X)
Deionized water	895 ml	
10% Tween-20	5 ml	0.05% (v/v)

APPENDIX C

Protocol

C-1. RNA extraction

TRI-REAGENT – RNA/DNA/Protein isolation reagent protocol (Molecular Research Center, USA)

1. Homogenize tissue samples in TRI Reagent (1 ml per 50-100 mg of tissues) in a Polytron homogenizer. Store the homogenate at room temperature for 5 minutes.
2. Add 0.2 ml of chloroform per ml of TRI Reagent, cover the samples tightly and shake vigorously for 15 seconds. Store the resulting mixture at room temperature for 2-15 minutes and centrifuge at 12,000xg at 4°C for 15 minutes.
3. Transfer the aqueous phase to a fresh tube and precipitate RNA by mixing with 0.5 ml of isopropanol per ml of TRI Reagent. Store at room temperature for 5-10 minutes and centrifuge at 12,000xg at 4-25°C for 8 minutes.
4. Remove the supernatant and wash the RNA pellet with 75% ethanol and subsequent centrifuge at 7,500xg at 4-25°C for 5 minutes.
5. Remove the ethanol wash and briefly air-dry the RNA pellet for 5-10 minutes. Dissolve RNA in 50 µl RNase-free water by diethyl pyrocarbonate (DEPC) treatment and incubate at 55-60 °C for 10-15 minutes.

C-2. Protein gel blots

Antigen detection protocol: all steps are carried out at room temperature in a square petri plate**.**

1. Incubate the filter in 20 - 25 ml of Starting Block or Sea Block: TBST or PBST (1:4 to 1:9, v/v) for 1 hr to overnight with gentle agitation on an orbital shaker or rocking platform. Alternatively, 5% (w/v) cold water fish skin gelatin is a good blocking agent.
2. Discard the blocking solution and replace it with 1° antibody solution made up in Starting Block or Sea Block: TBST or PBST (1:9, v/v). Incubate the filter for 1-2 hr with agitation.
3. Decant the antibody solution and replace it with Starting Block or Sea Block: TBST or PBST (1:9, v/v). Incubate the filter for 5-10 min with agitation. Decant and discard this wash solution.
4. Repeat step 3 FOUR more times.
5. Incubate the filter in Starting Block or Sea Block: TBST or PBST (1:19, v/v) containing 2° antibody. Incubate the filter for 1-2 hr with agitation. Secondary antibodies can be diluted 1: 10,000 to 25,000. Determine the optimum level empirically. See the SuperSignal instructions for dilution guidelines.
6. Decant the secondary antibody solution and replace it with TBS or PBS.
   Note: it is better not to use buffers with Tween at this point!
   Incubate the filter for 5-10 min with agitation. Decant and discard this wash solution.
7. Repeat step 6 FOUR more times.
8. Incubate the filter in freshly mixed SuperSignal reagents, no agitation needed, for 5 min in a container covered with the lid of a freezer box.
9. Decant and discard the SuperSignal reagent and blot the filter gently to dryness with filter paper.
10. Place the filter in a clear plastic sheet protector or wrap it in plastic wrap.

11. Expose the filter to x-ray film in the darkroom in total darkness. Begin with an exposure of 30 to 60 sec. A range of exposures from 10 sec to 5 min may be needed to observe the correct exposure.
12. Place the exposed films in a cassette, take them to the darkroom and develop them in the automatic processor.

C-3. Plant Genomic DNA extraction

QuickExtract™ Plant DNA Extraction Solution

1. Cut a 3-5 mm leaf disc, using a leaf punch or the cap of a 500-µl microfuge tube (simply snap the tube closed over the portion of the leaf to be sampled).
2. Place the leaf disc into a 500-µl tube or a well of a 96-well plate, add 100 µl of QuickExtract Plant DNA Extraction Solution and immerse the leaf tissue. DO NOT GRIND THE LEAF TISSUE!
3. Heat the samples at 65°C for 6 minutes then at 98°C for 2 minutes.
4. Place the samples on ice. Use 1 µl of sample as template for PCR (25-50-µl reaction volumes).

APPENDIX D

Table D-1. T-test for expression pattern of *Myosin heavy chain* in response to normal and salt stress conditions

a) Salt 1 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.153667	.0771060	.0445172
	salt	3	.238667	.2973051	.1716492

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	5.525	.078	-.479	4	.657	-.0850000	.1773280	-.5773415	.4073415
	Equal variances not assumed			-.479	2.268	.674	-.0850000	.1773280	-.7678137	.5978137

b) Salt 3 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.240000	.1268109	.0732143
	salt	3	.014667	.0172143	.0099387

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	8.994	.040	3.050	4	.038	.2253333	.0738858	.0201935	.4304732
	Equal variances not assumed			3.050	2.074	.089	.2253333	.0738858	-.0819881	.5326548

c) Salt 6 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.680000	.3315117	.1913984
	salt	3	.024333	.0076376	.0044096

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	9.354	.038	3.425	4	.027	.6556667	.1914492	.1241186	1.1872147
	Equal variances not assumed			3.425	2.002	.076	.6556667	.1914492	-.1672360	1.4785694

d) Salt 12 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.260000	.2307553	.1332266
	salt	3	.061333	.0349476	.0201770

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	6.438	.064	1.474	4	.214	.1986667	.1347459	-.1754478	.5727811
	Equal variances not assumed			1.474	2.092	.273	.1986667	.1347459	-.3574099	.7547432

e) Salt 24 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.300000	.1350111	.0779487
	salt	3	.100333	.1079367	.0623173

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	.356	.583	2.001	4	.116	.1996667	.0997970	-.0774143	.4767476
	Equal variances not assumed			2.001	3.815	.119	.1996667	.0997970	-.0827915	.4821249

f) Salt 48 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.289333	.2234487	.1290082
	salt	3	.019667	.0280416	.0161898

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	4.391	.104	2.074	4	.107	.2696667	.1300201	-.0913270	.6306603
	Equal variances not assumed			2.074	2.063	.170	.2696667	.1300201	-.2737128	.8130461

Table D-2. T-test for expression pattern of *Myosin heavy chain* in response to normal and drought stress conditions

a) Drought 1 hr

[DataSet1] D:\Thesis_book\SPSS\salt.sav

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.153667	.0771060	.0445172
	drought	3	.032000	.0222711	.0128582

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	4.170	.111	2.626	4	.058	.1216667	.0463369	-.0069853	.2503186
	Equal variances not assumed			2.626	2.331	.102	.1216667	.0463369	-.0528645	.2961978

b) Drought 3 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.240000	.1268109	.0732143
	drought	3	.105333	.0978281	.0564811

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	.459	.535	1.456	4	.219	.1346667	.0924686	-.1220674	.3914007
	Equal variances not assumed			1.456	3.758	.223	.1346667	.0924686	-.1287235	.3980568

c) Drought 6 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.680000	.3315117	.1913984
	drought	3	.112000	.0645833	.0372872

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	6.099	.069	2.913	4	.044	.5680000	.1949966	.0266027	1.1093973
	Equal variances not assumed			2.913	2.152	.092	.5680000	.1949966	-.2168252	1.3528252

d) Drought 12 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.260000	.2307553	.1332266
	drought	3	.083333	.1183821	.0683480

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	1.708	.261	1.180	4	.303	.1766667	.1497357	-.2390663	.5923996
	Equal variances not assumed			1.180	2.985	.323	.1766667	.1497357	-.3012568	.6545901

e) Drought 24 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.300000	.1350111	.0779487
	drought	3	.264000	.1291666	.0745743

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	.053	.829	.334	4	.755	.0360000	.1078765	-.2635131	.3355131
	Equal variances not assumed			.334	3.992	.755	.0360000	.1078765	-.2637443	.3357443

f) Drought 48 hr

Group Statistics

	condition	N	Mean	Std. Deviation	Std. Error Mean
expression	control	3	.289333	.2234487	.1290082
	drought	3	.230333	.2330243	.1345367

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
expression	Equal variances assumed	.052	.831	.317	4	.767	.0590000	.1863953	-.4585164	.5765164
	Equal variances not assumed			.317	3.993	.767	.0590000	.1863953	-.4588756	.5768756

Table D-3. Two-way ANOVA for seed germination rate at day 2 in all treatment conditions

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	31.122[a]	6	5.187	1.358	.335
Intercept	145685.480	1	145685.480	3.814E4	.000
treatment	14.696	2	7.348	1.924	.208
line	16.426	4	4.107	1.075	.429
Error	30.555	8	3.819		
Total	145747.157	15			
Corrected Total	61.677	14			

a. R Squared = .505 (Adjusted R Squared = .133)

Table D-4. Two-way ANOVA for fresh weigh measurement

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.221[a]	6	.037	18.084	.000
Intercept	2.126	1	2.126	1.046E3	.000
Treatment	.213	2	.107	52.416	.000
Line	.007	4	.002	.918	.498
Error	.016	8	.002		
Total	2.363	15			
Corrected Total	.237	14			

a. R Squared = .931 (Adjusted R Squared = .880)

Table D-5. One-way ANOVA for reduction (%) of fresh weight

a) Salt stress

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1859.255[a]	4	464.814	9.427	.001
Intercept	25791.880	1	25791.880	523.094	.000
line	1859.255	4	464.814	9.427	.001
Error	640.983	13	49.306		
Total	29858.135	18			
Corrected Total	2500.238	17			

a. R Squared = .744 (Adjusted R Squared = .665)

value

	line	N	Subset 1	Subset 2	Subset 3
Duncan[a]	e1	3	1.8744E1		
	c6	4		3.5260E1	
	wt	3		4.4114E1	4.4114E1
	e3	4		4.4217E1	4.4217E1
	c2	4			4.8812E1
	Sig.		1.000	.131	.414

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 49.306.

a. Uses Harmonic Mean Sample Size = 3.529.

b) Drought stress

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1395.500[a]	4	348.875	5.412	.008
Intercept	43960.048	1	43960.048	681.950	.000
line	1395.500	4	348.875	5.412	.008
Error	902.472	14	64.462		
Total	48205.688	19			
Corrected Total	2297.972	18			

a. R Squared = .607 (Adjusted R Squared = .495)

value

Duncan

line	N	Subset 1	Subset 2	Subset 3
e1	3	3.4466E1		
c6	4	4.2349E1	4.2349E1	
e3	4		5.2049E1	5.2049E1
c2	4		5.3379E1	5.3379E1
wt	4			5.9856E1
Sig.		.200	.095	.227

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 64.462.

Table D-6. Two-way ANOVA for dry weigh measurement

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.000[a]	6	1.709E-5	11.969	.001
Intercept	.006	1	.006	4.152E3	.000
treatment	9.603E-5	2	4.802E-5	33.622	.000
line	6.525E-6	4	1.631E-6	1.142	.403
Error	1.142E-5	8	1.428E-6		
Total	.006	15			
Corrected Total	.000	14			

a. R Squared = .900 (Adjusted R Squared = .825)

Table D-7. One-way ANOVA for reduction (%) of dry weight

a) Salt stress

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	350.369[a]	4	87.592	2.444	.092
Intercept	9223.958	1	9223.958	257.398	.000
line	350.369	4	87.592	2.444	.092
Error	537.531	15	35.835		
Total	10111.858	20			
Corrected Total	887.900	19			

a. R Squared = .395 (Adjusted R Squared = .233)

value

Duncan

line	N	Subset 1	Subset 2
c6	4	1.4751E1	
wt	4	1.8666E1	1.8666E1
e3	4	2.2980E1	2.2980E1
e1	4		2.5461E1
c2	4		2.5517E1
Sig.		.084	.156

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 35.835.

b) Drought stress

Tests of Between-Subjects Effects

Dependent Variable:value

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1053.260[a]	4	263.315	9.192	.001
Intercept	9852.875	1	9852.875	343.953	.000
line	1053.260	4	263.315	9.192	.001
Error	401.044	14	28.646		
Total	11841.258	19			
Corrected Total	1454.304	18			

a. R Squared = .724 (Adjusted R Squared = .645)

value

Duncan

line	N	Subset 1	Subset 2	Subset 3
e3	3	1.4226E1		
c6	4	1.4544E1		
c2	4		2.4327E1	
wt	4		2.8250E1	2.8250E1
e1	4			3.3269E1
Sig.		.936	.333	.220

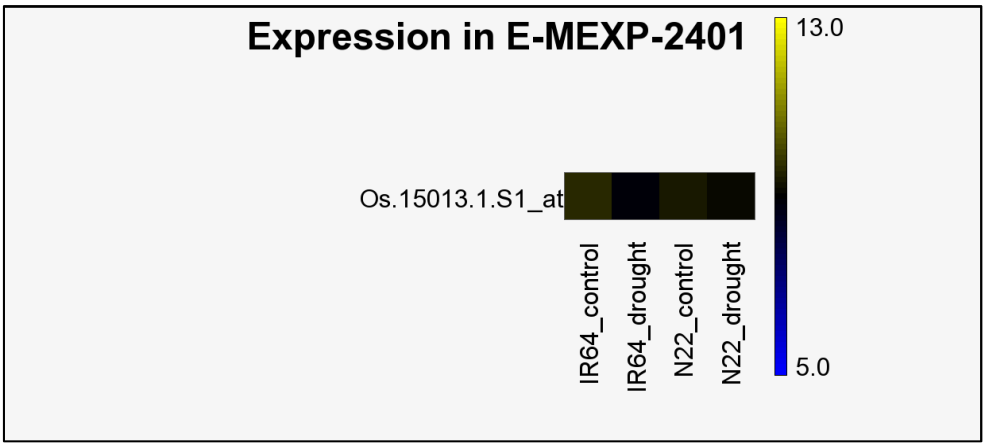
Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = 28.646.

APPENDIX E

Expression profiles of *Myosin heavy chain* gene in rice under various conditions

From Rice Oligonucleotide Array Database

(http://www.ricearray.org/expression/expression_search.php)



VITA

Miss Jutamas Anantayanon was born in 1989 in Bangkok, Thailand. She is a student under the Development and Promotion of Science and Technology Talents (DPST) Project jointly administered by the Ministry of Science and Technology, the Ministry of Education, and the Institute for the Promotion of Teaching Science and Technology (IPST). She graduated with the second class honors degree of Bachelor of Science in Chemistry from the Department of Chemistry, Faculty of Science, Silpakorn University in 2010. Now, she has studied for the degree of Master of Science at the Department of Biochemistry, Faculty of Science, Chulalongkorn University since 2011. She was a participant in The 18th National Genetics Conference 2013 (NGC2013) under the theme “Genetics towards ASEAN” in Bangkok, 2013. In this conference, her research was published in the Thai Journal of Genetics, Vol.6 (2013) entitled “Interaction studies of a Prefoldin with Calmodulin and CML proteins from rice (Oryza sativa L.)”.