การวิเคราะห์ระบบการต้านออกซิเดชันในข้าว *Oryza sativa* L. พันธุ์ขาวดอกมะลิ 105 ทรานสเจนิกที่มียีน *OsCaM1-1* แสดงออกเกินปกติภายใต้ความเครียดจากความเค็ม

นางสาวทวิพร แก้วเนรมิตร

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ANALYSIS OF ANTIOXIDANT SYSTEM IN TRANSGENIC 'KDML105' RICE *Oryza sativa* L. OVEREXPRESSING *OsCaM1-1* UNDER SALINITY STRESS

Miss Taviporn Kaewneramit

A Thesis Submitted in Partial Fulfillment of the Requirements
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ในการศึกษาพารามิเตอร์ต่างๆ (การเจริญเติบโต, การตอบสนองทางกายภาพและทางชีวเคมี) ที่เกี่ยวข้องกับการปรับปรุงการทนเค็มในข้าวทรานสเจนิกที่มีการแสดงออกเกินปกติของยีน *OsCaM1-1*, ที่เติบโตในภาวะเครียดจากความเค็มเทียบกับข้าวสายพันธุ์ควบคุม และข้าวขาวดอกมะลิ 105 สายพันธุ์ปกติ ภายหลังจากได้รับความเครียดจากความเค็ม (โซเดียมคลอไรด์ความเข้มข้น 150 มิลลิโมลาร์) เป็นเวลา 3 วัน พบว่าข้าวทรานสเจนิกที่มีการแสดงออกเกินปกติของยีน *OsCaM1-1* มีปริมาณรงควัตถุที่ใช้ในการสังเคราะห์แสง (คลอโรฟิลล์ *a*, *b* และแคโรทีนอยด์), ฤทธิ์การต้านอนุมูลอิสระ DPPH, อัตราการเจิญเติบโตสัมพัทธ์ (RGR) และแอกติวิตีของเอนไซม์ซูเปอร์ออกไซด์ดิสมิวเทส (SOD), คาทาเลส (CAT), แอสคอร์เบสเพอร์ออกซิเดส (APX) และกลูตาไธโอนรีดักเตส (GR) เพิ่มขึ้นสูงกว่าข้าวสายพันธุ์ควบคุมและสายพันธุ์ปกติ ในขณะที่การเกิดปฏิกิริยาออกซิเดชันของลิพิด และปริมาณน้ำสัมพัทธ์ (RWC) ลดลง ดังนั้นข้าวทรานสเจนิกที่มีการแสดงออกเกินปกติของยีน *OsCaM1-1* อาจลดหรือป้องกันความเสียหายจากภาวะออกซิเดชันจากความเค็มโดยการเพิ่มประสิทธิภาพระบบการต้านอนุมูลอิสระ

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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TAVIPORN KAEWNERAMIT: ANALYSIS OF ANTIOXIDANT SYSTEM IN TRANSGENIC ‘KDML105’ RICE *Oryza sativa* L. OVEREXPRESSING *OsCaM1-1* UNDER SALINITY STRESS. ADVISOR: NUCHANAT WUTIPRADITKUL, 111 pp.

To investigate various parameters (growth, physiological and biochemical responses) associated with improved tolerance under salt stress in the transgenic rice overexpressing *OsCaM1-1* gene, the control and the wild-type KDML105 differing in salt tolerance, comparatively. Three days after exposure to salt stress (150 mM NaCl), the results showed a greater increase in the photosynthetic pigment (chlorophyll *a*, *b* and carotenoid) contents, DPPH scavenging activity, relative growth rate (RGR), and the activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) in the transgenic rice plants than the control and wild-type, whereas lipid peroxidation and relative water content (RWC) showe decreased. Thus, the transgenic rice overexpressing *OsCaM1-1* gene might reduce or protect salt-induced oxidative damage by enhancing antioxidant system.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
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CONTENTS

Page

Page

List of Tables

Page

List of Figures

Page

CHAPTER I

INTRODUCTION

Salinity is a major abiotic stress in plant agriculture worldwide. Almost 71% of the earth's area is occupied by saline water. Due to the influence of salinization, alkalization and waterlogging about 95 million hectares of land worldwide is afflicted to high salinity. Salt-affected soils cause great economic losses by unacceptable yield reduction and in some cases, being far from any reasonable utilization.

Rice (*Oryza sativa* L.) is an important food crop for the entire world population. Especially, jasmine rice 'Khao Dok Mali 105' (KDML 105), a well known fragrant Thai rice, is an indica variety originally from Thailand. The grain is characterized by its unique appearance, cooking quality and aroma. Because of its characteristic shiny white color, resembling that of a jasmine flower, its soft texture and aromatic fragrance when cooked, it is increasingly in demand around the world. However, grain yield is limited because of its moderately salt-sensitive (Maas 1977).

Saline soil is enriched with salts which are readily water-soluble i.e. sodium chloride (NaCl), sodium sulfate (Na_2SO_4), calcium chloride ($CaCl_2$), and magnesium chloride ($MgCl_2$). It is a major barrier to rice cultivation, reducing productivity (Shannon et al. 1998). Sodium chloride salt is a small molecule when oxidized to sodium ions (Na^+) and chloride ions (Cl^-), which is easily absorbed by root cells and transferred to plant overall through its xylem vascular tissues (Maathuis and Amtmann 1999; Rodriguez-Navarro and Rubio 2006; Tester and Davenport 2003). Na^+ ions are well known as causing toxic damage to plant cells by both ionic and osmotic effects,

causing growth retardation, and low productivity (Chinnusamy et al. 2005; Mansour and Salama 2004). In addition, effects in plants experiencing salt stress are enhanced generation of reactive oxygen species (ROS) including superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$) and singlet oxygen ($^1O_2$) leading to oxidative stress. Excess of ROS triggers phytotoxic reactions such as lipid peroxidation, inactivating enzymes, protein degradation, denaturing DNA molecules and eventually, cell death (Bor et al. 2003; Jiang and Zhang 2001).

Reactive Oxygen Species (ROS)

ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. It has been estimated that about 1% of O_2 consumed by plants is diverted to produce ROS (Asada 2006) in various subcellular loci such as chloroplasts, mitochondria, peroxisomes. ROS are well recognized for playing a dual role as both deleterious and beneficial species depending on their concentration in plants. At high concentration ROS cause damage to biomolecules, whereas at low/moderate concentration it acts as secondary messenger in intracellular signalling cascades that mediate several responses in plant cells.

O_2 itself is a totally harmless molecule as in its ground state it has two unpaired electrons with parallel spin which makes it paramagnetic and, hence, unlikely to participate in reactions with organic molecules unless it is activated (Apel and Hirt 2004). Activation of O_2 may occur by two different mechanisms: (i) absorption of sufficient energy to reverse the spin on one of the unpaired electrons and (ii) stepwise monovalent reduction. In the former, $^1O_2$ is formed, whereas in latter, O_2 is sequentially reduced to $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$. Activation of O_2 occurs by two different mechanisms.

Stepwise monovalent reduction of O_2 leads to formation of $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$, whereas energy transfer to O_2 leads to formation of $^1O_2$. $O_2^{\bullet-}$ is easily dismutated to H_2O_2 either non-enzymatically or by superoxide dismutase (SOD) catalyzed reaction to H_2O_2. H_2O_2 is converted to H_2O by catalase (CAT), guaiacol peroxidase (GPX), and ascorbate peroxidase (APX) (Sharma et al. 2012) (Figure 1.1).

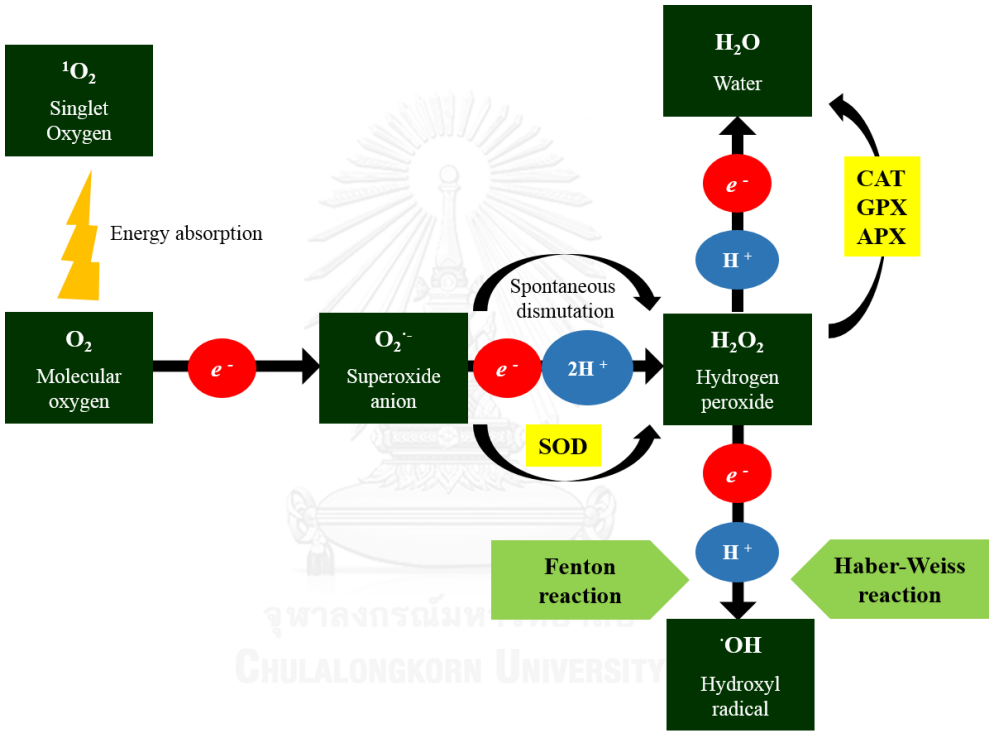

Figure 1.1 Schematic representation of generation of reactive oxygen species (ROS) in plants (Sharma et al. 2012).

The major site of superoxide radical ($O_2^{\bullet-}$) production is the reaction centers of photosystem I (PSI) and a photosystem II (PSII) in chloroplast thylakoids. In mitochondria, complex I, II and complex III in the electron transport chain (ETC) contribute to superoxide radical production. The terminal oxidases-cytochrome c oxidase and the alternative oxidase react with O_2, four electrons are transferred and H_2O

is released. There is situation when O_2 can react with other ETC components and there in only one electron transferred with the result of $O_2^{\bullet-}$ release. It has been shown that in plants 1-2% of O_2 consumption leads to $O_2^{\bullet-}$ production (Puntarulo et al. 1988).

Singlet oxygen is the first excited electronic state of O_2. Insufficient energy dissipation during photosynthesis can lead to formation of chlorophyll (Chl) triplet state. And the Chl triplet state can react with $3O_2$ to give up very reactive singlet oxygen. It has been proved that singlet oxygen formation during photosynthesis can have damaging effect on PSI and PSII and on whole machinery of photosynthesis.

Hydroxyl radicals ($^{\bullet}OH$) are the highest reactive ROS. It can be produced from $O_2^{\bullet-}$ and H_2O_2 at neutral pH and ambient temperature by iron-catalyzed.

H_2O_2 is produced by univalent reduction of $O_2^{\bullet-}$. H_2O_2 is moderately reactive (Table 1.1). It has been proved that excess of H_2O_2 leads to oxidative stress. This molecule may also inactivate enzymes by oxidizing their thiol groups. Moreover, H_2O_2 play dual role in plants. At low concentration it can act as a signal molecule involved in acclimatory signaling triggering tolerance to different biotic and abiotic stresses. At high concentration it leads to programmed cell death (Quan et al. 2008). It has been proved that H_2O_2 act as a key regulator of in a wide range of physiological processes like photorespiration and photosynthesis (Noctor and Foyer 1998b), stomatal movement (Bright et al. 2006), cell cycle (Mittler 2006) and growth and development (Foreman et al. 2003). H_2O_2 is taking as a second messenger for signals generated by means of ROS due to its relatively long life and high permeability across membranes. Many of the general stress genes are regulated by a signaling pathways using H_2O_2 as the messenger (Moller and Sweetlove 2010).

Table 1.1 Key reactive oxygen species (ROS), their properties, and main scavenging systems in plant cells

ROS	Half-life and ability	Mode of action	Cellular sources	Main scavenging system
Superoxide radical ($O_2^{\bullet-}$)	1 μs, 30 nm	Reacts with double bond-containing compounds such as iron-sulphur (Fe-S) clusters of proteins; reacts with nitric oxide (NO) to form peroxynitrite ($ONOO^-$)	Formed in many photooxidation reactions (flavoprotein, redox cycling). Mehler reaction in chloroplasts, mitochondrial electron transport chains (ETCs) reactions, glyoximal photorespiration, peroxisomes, and plasma membrane. NADPH oxidase in membranes. Xanthine oxidase and membrane polypeptides in peroxisomes. Reactions of ozone (O_3) in apoplastic space	Superoxide dismutases (SODs)
Hydroxyl radical ($^{\bullet}OH$)	1 ns, 1 nm	Extremely reactive with protein, lipids, DNA, and other macromolecules	Reaction of H_2O_2 with $O_2^{\bullet-}$ (Haber-Weiss reaction), reactions of H_2O_2 with Fe^{2+} (Fenton reaction). Decomposition of O_3 in apoplastic space	Flavonoids, prevention of $^{\bullet}OH$ formation by sequencing Fe
Hydrogen peroxide (H_2O_2)	1 ms, 1 μm	Oxidized proteins; reacts with $O_2^{\bullet-}$ in a Fe-catalyzed reaction to form $^{\bullet}OH$	ETCs of mitochondria, chloroplasts, endoplasmic reticulum, and plasma membrane. Photorespiration,	Catalase, various peroxidases, peroxiredoxins, and flavonoids

			fatty acid β-oxidation, urate oxidase, and MnSOD in peroxisomes	
Singlet oxygen ($^1O_2$)	1 μs, 30 nm	Directly oxidized protein, polyunsaturated fatty acids, and DNA	Photoinhibition, photosystem II electron transfer reactions in chloroplasts	Carotenoids and α-tocopherols

Sites of production of ROS

ROS are produced in both unstressed and stressed cells at several locations in chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum, and cell walls (Blokhina and Fagerstedt 2010; Hossain et al. 2011; Mhamdi et al. 2010a) (Figure 1.2). ROS are always formed by the inevitable leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments.

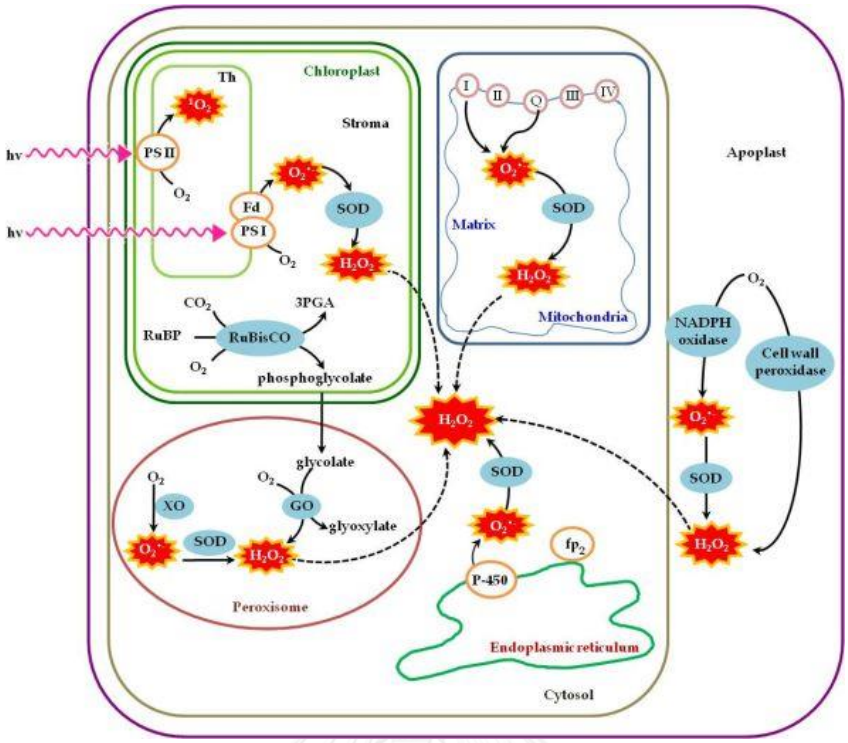

Figure 1.2 Sites of production of reactive oxygen species (ROS) at several location in plants (Blokhina & Fagerstedt, 2010; Hossain, Hasanuzzaman, & Fujita, 2011; Mhamdi et al., 2010).

ROS and Oxidative Damage to Biomolecules

Production and removal of ROS must be strictly controlled in order to avoid oxidative stress. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress". However, the equilibrium between production and scavenging of ROS is perturbed under a number of stressful conditions such as salinity, drought, high light, toxicity due to metals, pathogens, and so forth. Enhanced level of ROS can cause damage to biomolecules such as lipids, proteins and DNA (Figure 1.3). These reactions can alter intrinsic membrane properties like fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, DNA damage, and so forth ultimately resulting in cell death.

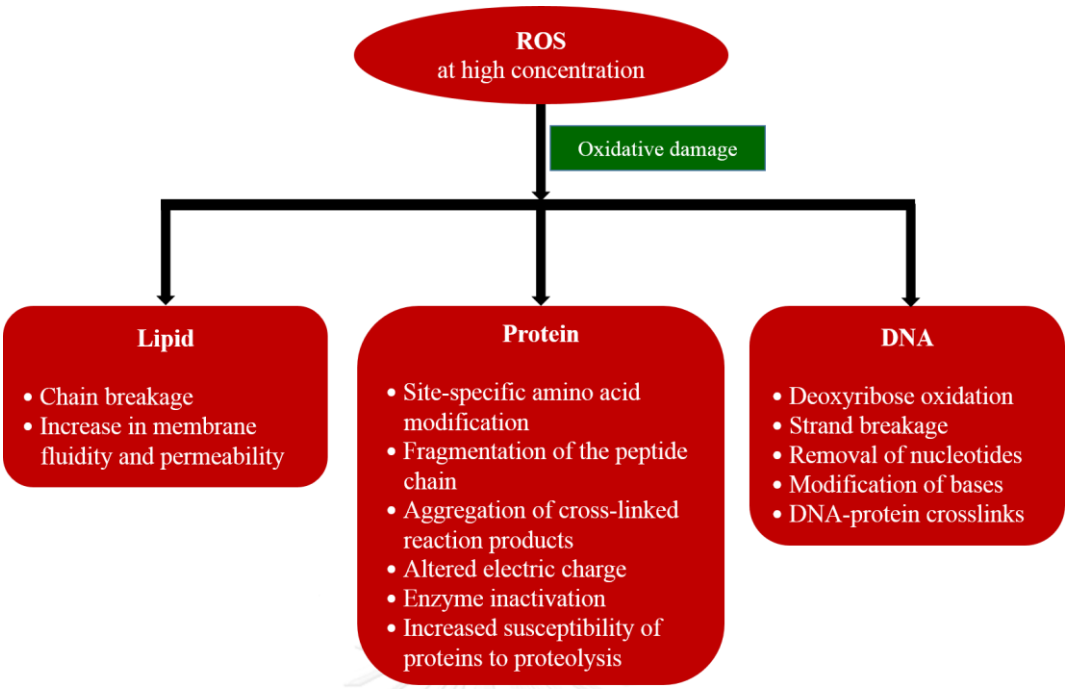

Figure 1.3 Reactive oxygen species (ROS) induced oxidative damage to lipids, proteins, and DNA (Sharma et al. 2012).

Lipids

When ROS level reaches above threshold, enhanced lipid peroxidation takes place in both cellular and organellar membranes, which, in turn, affect normal cellular functioning. Lipid peroxidation aggravates the oxidative stress through production of lipid-derived radicals that themselves can react with and damage proteins and DNA. The level of lipid peroxidation has been widely used as an indicator of ROS mediated damage to cell membranes under stressful conditions. Increased peroxidation (degradation) of lipids has been reported in plants growing under environmental stresses (Sharma and Shanker Dubey 2005; Tanou et al. 2009). Increase in lipid peroxidation under these stresses parallels with increased production of ROS. Malondialdehyde (MDA) is one of the final products of peroxidation of unsaturated

fatty acids in phospholipids and is responsible for cell membrane damage (Halliwell 1989). Two common sites of ROS attack on the phospholipid molecules are the unsaturated (double) bond between two carbon atoms and the ester linkage between glycerol and the fatty acid. The polyunsaturated fatty acids (PUFAs) present in membrane phospholipids are particularly sensitive to attack by ROS. A single $^{\bullet}OH$ can result in peroxidation of many polyunsaturated fatty acids because the reactions involved in this process are part of a cyclic chain reaction. The overall process of lipid peroxidation involves three distinct stages: initiation, progression, and termination steps. The initial phase of lipid peroxidation includes activation of O_2 which is rate limiting. $O_2^{\bullet-}$ and $^{\bullet}OH$ can react with methylene groups of PUFA forming conjugated dienes, lipid peroxy radicals and hydroperoxides (Smirnoff 1995).

Proteins

Proteins are the most abundant cellular component oxidized by ROS constituting up to 68% of the oxidized molecules in the cell (Rinalducci et al. 2008). Protein oxidation is a covalent modification induced by ROS or by products of oxidative stress. Protein oxidation mostly is irreversible, however, a few involving sulfur-containing amino acid are reversible (Ghezzi and Bonetto 2003). The most susceptible residues to oxidation are the sulphur containing cysteine and methionine. The thiol of cysteine may be oxidized by hydroxyl radicals, superoxide and hydrogen peroxide to a disulfide that can be readily reversible. Oxidation of methionine in many proteins has little effect on protein structure and function.

DNA

Due to biotic and abiotic stresses DNA is exposed to damage. Endogenously generated damage to DNA is known as "spontaneous DNA damage", which is produced by reactive metabolites ($^{\bullet}OH$, $O_2^{\bullet-}$ and $NO^{\bullet}$). High level of ROS can influence on damage to cell structures, nucleic acids, lipids and proteins. It has been considered that one of the most reactive is $^{\bullet}OH$ causing damage to all components of DNA molecules. This molecule damages purine, pyrimidine and deoxyribose backbone. $^1O_2$ damages guanine, and H_2O_2 and $O_2^{\bullet-}$ do not react at all. Result of DNA damage can be various physiological effects like reduced protein synthesis, cell membrane destruction, damage to photosynthetic proteins what consequently leads to growth and development disorders (Britt 1999).

A wide range of unfavorable environmental conditions like mentioned drought, extreme temperatures, salt stress etc. can induce stresses that alter seriously plant metabolism and may increase production of ROS (H_2O_2, $O_2^{\bullet-}$,$^1O_2$, $^{\bullet}OH$) inducing an oxidative stress in organelles. Plants are unable to escape exposure to these environmental constraints and evolved mechanisms in order to survive. To prevent appearance of these toxic compounds and their consequences plants have a variety of constitutively expressed antioxidant defense mechanisms to scavenge the ROS generated.

Physiological and Biochemical Mechanisms of Salt Tolerance

Plants develop various physiological and biochemical mechanisms in order to survive in soils with high salt concentration. Principle mechanisms include, but are not limited to, activation of antioxidant enzyme and synthesis of antioxidant compounds

and ion homeostasis and compartmentalization. Research advances elucidating these mechanisms are discussed below.

Antioxidative Defense System in Plants

Plants possess complex antioxidative defense system comprising of nonenzymatic and enzymatic components to scavenge ROS. In plant cells, specific ROS producing and scavenging systems are found in different organelles such as chloroplasts, mitochondria, and peroxisomes. ROS-scavenging pathways from different cellular compartments are coordinated (Pang and Wang 2008). Under normal conditions, potentially toxic oxygen metabolites are generated at a low level and there is an appropriate balance between production and quenching of ROS. The balance between production and quenching of ROS may be perturbed by a number of adverse environmental factors, giving rise to rapid increases in intracellular ROS levels (Noctor et al. 2002) , which can induce oxidative damage to lipids, proteins, and nucleic acids. In order to avoid the oxidative damage, higher plants raise the level of endogenous antioxidant defense (SHARMA 2010). Given the challenge imposed by plant oxygen-evolving capability, each organelle or compartment has evolved mechanisms for the elimination of excess ROS accumulation (Figure 1.4).

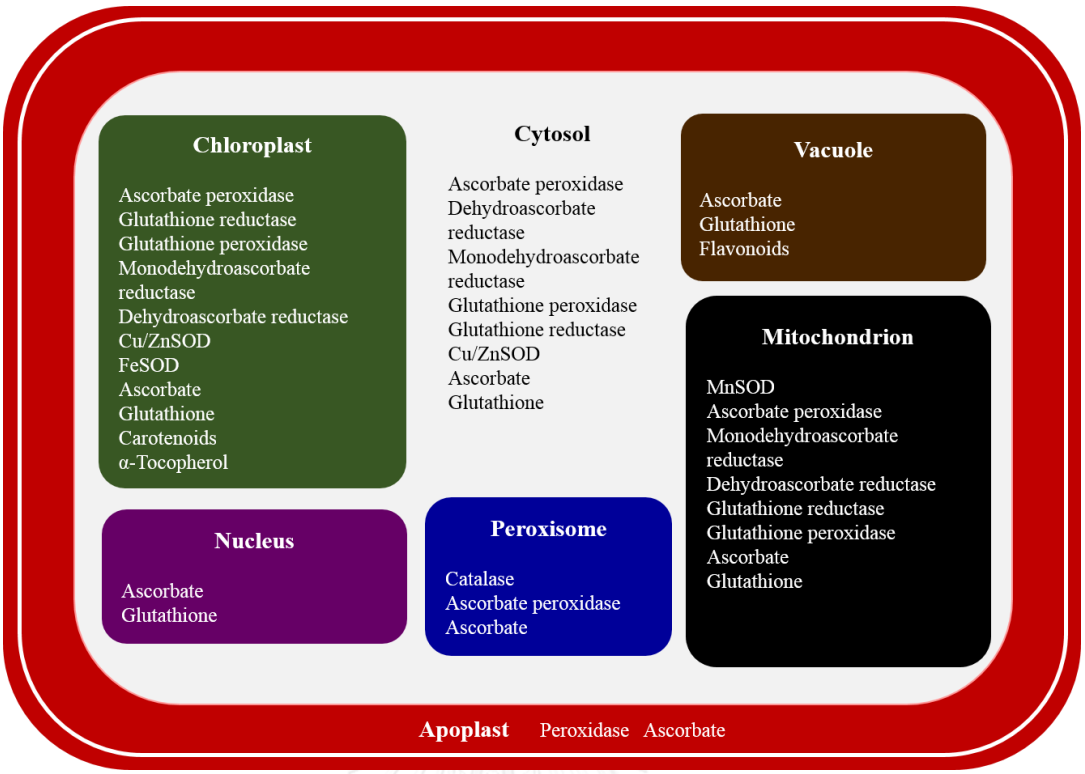

Figure 1.4 Distribution of the main antioxidant resources in plant cell (Racchi 2013).

Nonenzymatic Components of Antioxidative Defense System

The non-enzymatic antioxidants refer to the biological activity of numerous vitamins, secondary metabolites and other phytochemicals aimed to protect plants against ROS activity. Among the most important non-enzymatic antioxidants are ascorbic acid (AsA), glutathione (GSH), carotenoids, flavonoids, etc.

Ascorbic acid (vitamin C)

Ascorbic acid is the most abundant, powerful and water soluble antioxidant which minimizes or prevents damage caused by ROS in plants. Ascorbic acid is one of the most studied one and has been detected in majority of plant cell types, organelles

and apoplast (Smirnoff 2000). Ascorbic acid reacts not only with H_2O_2, but also with $O_2^{\bullet-}$, $^{\bullet}OH$ and lipid hydroperoxidases. In turf grass, ascorbic acid concentration significantly increases during water deficiency (Hong-Bo et al. 2006). Ascorbic acid can also directly scavenge $^1O_2$, $O_2^{\bullet-}$ and $^{\bullet}OH$ and regenerate tocopherol from tocopheroxyl radicals providing membrane protection. Moreover, antioxidants like ascorbic acid and glutathione are involved in neutralization of secondary products of ROS reaction. Fundamental role of ascorbic acid in the plant defense system is to protect metabolic processes against H_2O_2. And also ascorbic acid reacts non-enzymatically with superoxide, hydrogen peroxide and singlet oxygen (Smirnoff 2000).

Glutathione (GSH)

Glutathione (GSH) is a tripeptide (α-glutamyl-cysteinyl-glycine), which is considered as the most important intracellular defense against ROS-induced oxidative damage. Glutathione has been detected in all cell compartments such as cytosol, chloroplasts and endoplasmatic reticulum (Foyer and Noctor 2003). Glutathione is the major source of non-protein thiol groups. The nucleophilic nature of the thiol group is important in the formation of mercaptide bonds with metals for reacting with selected electrophiles. Glutathione is involved in control of H_2O_2 levels. The change in the ratio of its reduced (GSH) to oxidized (GSSG) form during the degradation of H_2O_2 is very important in certain signaling pathway. It has been considered that GSH/GSSG ratio, indicative of the cellular redox balance, may be involved in ROS perception (Li and Jin 2007). Glutathione is important in plant chloroplasts because it helps to protect the photosynthetic apparatus from oxidative damage.

Carotenoids

Carotenoids are pigments that are found in plants and microorganisms. There are over 600 carotenoids in nature. Carotenoids are lipid soluble antioxidants that plays multitude of function in plant metabolism including oxidative stress tolerance. Carotenoids take part in three different functions in plants. First one, they absorb the light at wavelength between 400 and 550 nm and transfer it to the Chl. Secondly, they protect photosynthetic apparatus by quenching a triplet sensitizer (Chl3), $^1O_2$ and other harmful free radicals which are naturally formed during photosynthesis (an antioxidant function). Thirdly, they are important for the PSI assembly and the stability of light of light harvesting complex protein as well as thylakoid membrane stabilization (structural function) (Siefermann-Harms 1987).

Enzymatic Components

The enzymatic components of the antioxidative defense system comprise of several antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POX), enzymes of ascorbate-glutathione (AsA-GSH) cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (Noctor and Foyer 1998a). These enzymes operate in different subcellular compartments and respond in concert when cells are exposed to oxidative stress.

Table 1.2 Major ROS scavenging antioxidant enzymes.

Enzymatic antioxidants	**Enzyme code**	**Reaction catalyzed**
Superoxide dismutase (SOD)	EC 1.15.1.1	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2$
Catalase (CAT)	EC 1.11.1.6	$H_2O_2 \rightarrow H_2O + ½O_2$
Ascorbate peroxidase (APX)	EC 1.11.1.11	H_2O_2 + AsA $\rightarrow 2H_2O$ + DHA

Guaiacol peroxidase (POX)	EC 1.11.1.7	guaiacol + $4H_2O_2$ → tetraguaiacol + $8H_2O$
Monodehydroascorbate reductase (MDHAR)	EC 1.6.5.4	MDHA + NAD(P)H → AsA + $NAD(P)^+$
Dehydroascorbate reductase (DHAR)	EC 1.8.5.1	DHA + 2GSH → AsA +GSSG
Glutathione reductase (GR)	EC 1.6.4.2	GSSG + NAD(P)H → 2GSH + $NAD(P)^+$

Superoxide Dismutase

Superoxide dismutase (SOD, EC 1.15.1.1) is the primary scavenger in the detoxification of active oxygen species in plants discovered by Irwin Fridovich and Joe McCord (McCord and Fridovich 1969). SOD constitutes the first line of defense against ROS. Specialization of function among SODs may be due to combination of the influence of subcellular localization of the enzyme and upstream sequences in genomic sequence. SOD remove $O_2^{\bullet-}$ by catalyzing its dismutation, one $O_2^{\bullet-}$ is reduced to H_2O_2 and another to O_2 (Table 1.2). SODs are metalloproteins and based on their metal cofactor they are classified into three known types: the copper/zinc (Cu/ZnSOD), the manganese (MnSOD) and the iron (FeSOD) that are localized in different cellular compartment (Mittler 2002). The activity of SOD isoenzymes can be detected by negative staining and identified on the base of their sensitivity to KCN and H_2O_2. Cu/ZnSOD is sensitive to both inhibitors; the MnSOD is resistant on both inhibitors, whereas FeSOD is resistant to KCN and sensitive to H_2O_2. The distribution of SOD isoenzymes is also distinctive. The Cu/ZnSOD is found in the cytosolic fraction and also in chloroplasts in higher plants. MnSOD is found in the mitochondria of eukaryotic cells and in peroxisomes. And the FeSOD is usually present in chloroplasts, but they are not often found in plants. The up regulation of SODs has been observed in plants

subjected to both abiotic (Boguszewska et al. 2010) and biotic stresses (Torres 2010). Overexpression of SODs in transgenic plants resulted in higher salt or drought tolerance (Badawi et al. 2004). Thus, SOD have a critical role in the survival of plants under environmental stresses.

Catalase

Among antioxidant enzymes, catalase (CAT, EC 1.11.1.6) was the first enzyme to be discovered and characterized. It is a ubiquitous tetrameric heme-containing enzyme that catalyzes the dismutation of two molecules of H_2O_2 into water and oxygen. It has high specificity for H_2O_2, but weak activity against organic peroxides. Plants contain several types of H_2O_2-degrading enzymes, however, CATs are unique as they do not require cellular reducing equivalent. CATs have a very fast turnover rate, but a much lower affinity for H_2O_2 than APX. The peroxisomes are major sites of H_2O_2 production. CAT scavenges H_2O_2 generated in this organelle during photorespiratory oxidation, β-oxidation of fatty acids, and other enzyme systems such as XOD coupled to SOD (Corpas et al. 2008; Scandalios et al. 1997). Though there are frequent reports of CAT being present in cytosol, chloroplast, and mitochondria, the presence of significant CAT activity in these is less well established (Mhamdi et al. 2010b). To date, all angiosperm species studied, contain three CAT genes. Willekens et al. (Willekens et al. 1995) proposed a classification of CAT based on the expression profile of the tobacco genes. Class I CATs are expressed in photosynthetic tissues and are regulated by light. Class II CATs are expressed at high levels in vascular tissues, whereas Class III CATs are highly abundant in seeds and young seedlings.

H_2O_2 has been implicated in many stress conditions. When cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, H_2O_2 is degraded by CAT in an energy efficient manner (Mallick and Mohn 2000). Environmental stresses cause either enhancement or depletion of CAT activity, depending on the intensity, duration, and type of the stress (Han et al. 2009; Moussa and Abdel-Aziz 2008; Sharma and Shanker Dubey 2005). In general, stresses that reduce the rate of protein turnover also reduce CAT activity. Stress analysis revealed increased susceptibility of CAT-deficient plants to paraquat, salt and ozone, but not to chilling (Willekens et al. 1997). Overexpression of a CAT gene from *Brassica juncea* introduced into tobacco, enhanced its tolerance to Cd induced oxidative stress (Guan et al. 2009).

Ascorbate Peroxidase

Ascorbate peroxidase (APX, EC 1.11.1.11) exists as isoenzymes and plays an important role in the metabolism of H_2O_2 in higher plants. It is clear that a high level of endogenous ascorbate is essential to maintain effectively the antioxidant system that protects plants from oxidative damage due to biotic and abiotic stresses. APX is involved in scavenging of H_2O_2 into water-water and ascorbate-glutathione cycles and utilizes ascorbate as an electron donor. There are five different isoforms of APX base on the localization: thylakoid tAPX, glyoxysome membrane APX (gmAPX), chloroplast stromal soluble form (sAPX) and cytosolic form of APX (cAPX). It has been shown enhanced expression of APX in plants growing under unfavorable environmental conditions.

Glutathione Reductase

Glutathione reductase (GR, EC 1.6.4.2) is a flavoprotein oxidoreductase. It is an enzyme that is thought to play an essential role in defence system against ROS (Gill and Tuteja 2010b). Reducing glutathione disulfide (GSSG) to the sulfhydryl form (GSH), which is an important cellular antioxidant in defense against ROS, it sustains the reduced status of GSH. Glutathione disulfide contains of two GSH linked by a disulphide bridge which can be converted back to GSH by GR (Reddy 1986). GR is localized mainly in chloroplasts and small amount of this enzyme has been found in mitochondria and cytosol. By catalyzing the reduction of GSH, GR is an enzyme involved in regulation of cell energy metabolism. GR catalyzes the NADPH-dependent reduction of disulfide bond of GSSG what is important in the maintaining of GSH pool. Increased level of GR has been observed in plants subjected to metal, drought and salt stresses.

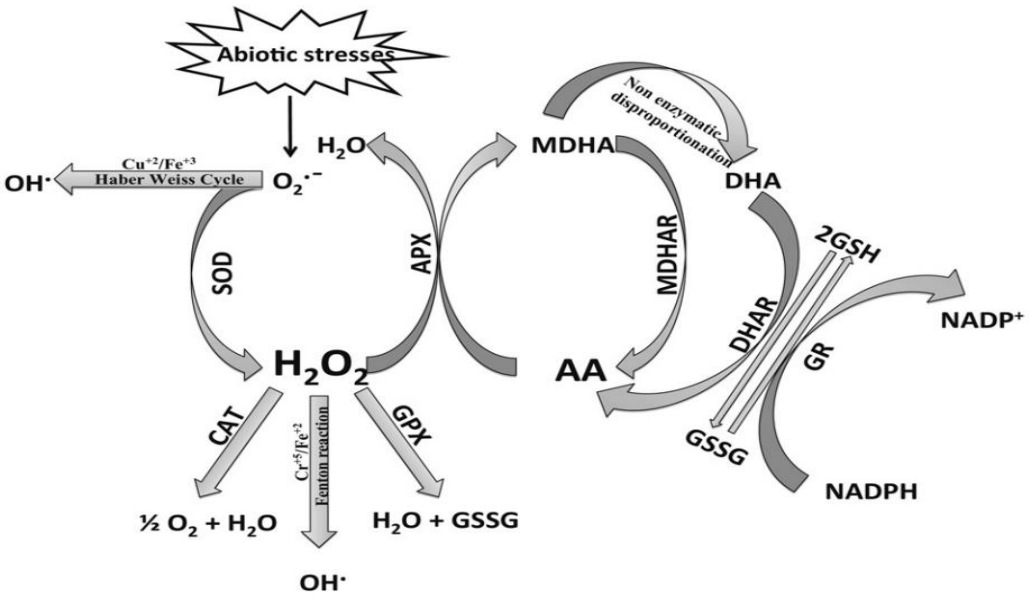

Figure 1.5 ROS and antioxidants defense mechanism (Gill and Tuteja 2010a).

Ion Homeostasis and Salt Tolerance

Maintaining ion homeostasis by ion uptake and compartmentalization is not only crucial for normal plant growth but is also an essential process for growth during salt stress. (Hasegawa 2013; Niu et al. 1995; Serrano et al. 1999). Irrespective of their nature, both glycophytes and halophytes cannot tolerate high salt concentration in their cytoplasm. Hence, the excess salt is either transported to the vacuole or sequestered in older tissues which eventually are sacrificed, thereby protecting the plant from salinity stress (Reddy et al. 1993; Zhu 2003).

Major form of salt present in the soil is NaCl, so the main focus of research is the study about the transport mechanism of Na^+ ion and its compartmentalization. The Na^+ ion that enters the cytoplasm is then transported to the vacuole via Na^+/H^+ antiporter. Two types of H^+ pumps are present in the vacuolar membrane: vacuolar type H^+-ATPase (V-ATPase) and the vacuolar pyrophosphatase (V-PPase) (Dietz et al. 2001; Otoch et al. 2001; Wang et al. 2001). Of these, V-ATPase is the most dominant H^+ pump present within the plant cell. During nonstress conditions it plays an important role in maintaining solute homeostasis, energizing secondary transport and facilitating vesicle fusion. Under stressed condition the survivability of the plant depends upon the activity of V-ATPase (Dietz et al. 2001). In a study performed by De Lourdes Oliveira Otoch and workers (2001) in hypocotyls of *Vigna unguiculata* seedlings, it was observed that the activity of V-ATPase pump increased when exposed to salinity stress, activity of V-PPase was inhibited, whereas in the case of halophyte *Suaeda salsa*, V-ATPase activity was upregulated and V-PPase played a minor role (Wang et al. 2001).

Salt Overly Sensitive pathway (SOS)

The SOS signalling pathway (Figure 1.6) consists of three major proteins, SOS1, SOS2, and SOS3-SOS2, which encodes a plasma membrane Na^+/H^+ antiporter, is essential in regulating Na^+ efflux at cellular level. Overexpression of this protein confers salt tolerance in plants (Shi et al. 2000). The interaction between SOS2 and SOS3 protein results in the activation of the kinase (Guo et al. 2004). The activated kinase then phosphorylates SOS1 protein thereby increasing its transport activity (Quintero et al. 2002). Besides conferring salt tolerance it also regulates pH homeostasis, membrane vesicle trafficking, and vacuole functions (Oh et al. 2010; Quintero et al. 2011). Thus with the increase in the concentration of Na^+ there is a sharp increase in the intracellular Ca^{2+} level which in turn facilitates its binding with SOS3 protein. The SOS3 protein then interacts and activates SOS2 protein by releasing its self-inhibition. The SOS3-SOS2 complex is then loaded onto plasma membrane where it phosphorylates SOS1 (Figure 1.6). The phosphorylated SOS1 results in the increased Na^+ efflux, reducing Na^+ toxicity (Martínez-Atienza et al. 2007).

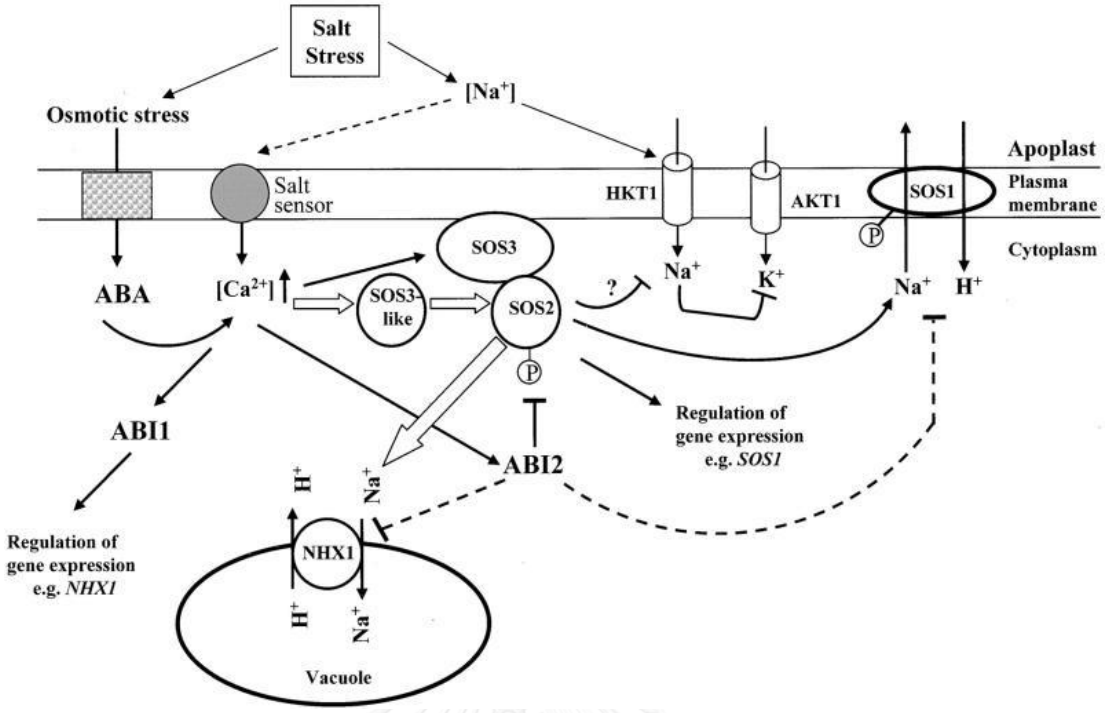

Figure 1.6 Model of SOS pathway for salinity stress responses (Chinnusamy et al. 2004).

Roles of Ca^{2+} in plants

Calcium (Ca^{2+}) is an element that is crucial for numerous biological functions. In addition to its key roles in the structural integrity of the cell wall and the membrane system, it has been shown to act as an intracellular regulator in many aspects of plant growth and development including stress responses (White and Broadley 2003). An increased cytosolic Ca^{2+} has dual roles in regulating H_2O_2 homeostasis (Figure 1.7), initiating the stress signal transduction pathways for stress tolerance. Ca^{2+} release can be primarily from an extracellular source (apoplastic space) as the addition of EGTA or BAPTA blocks calcineurin mediated activity (Figure 1.8). Furthermore, calcium-binding proteins (calcium sensors) can provide an additional level of regulation in

calcium signaling. These sensor proteins recognize and decode the information provided in the calcium signatures and relay the information downstream to initiate a phosphorylation cascade, leading to regulation of gene expression. However, as high Ca^{2+} concentrations can be toxic to cellular energy metabolism (Wu et al. 1996).

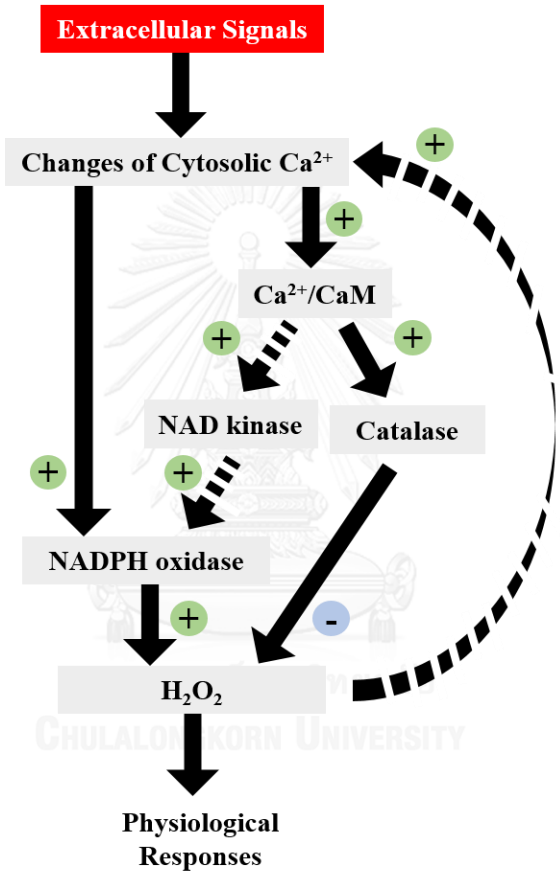

Figure 1.7 Model showing Ca^{2+}-triggered changes leading to the positive and negative regulation of H_2O_2 level in plants. For positive regulation, extracellular signals trigger an influx of Ca^{2+}, which increases the generation of H_2O_2. This may occur by activating NADPH oxidase, which has affinity to Ca^{2+}, and increasing the production of NADPH by means of CaM-regulated NAD kinase. For negative regulation, Ca^{2+} binds to CaM, and the Ca^{2+}/CaM complex

stimulates the catalytic activity of catalase, leading to the rapid degradation of H_2O_2. The increase in H_2O_2 can boost the Ca^{2+} influx by activating the calcium channel (Yang and Poovaiah 2002).

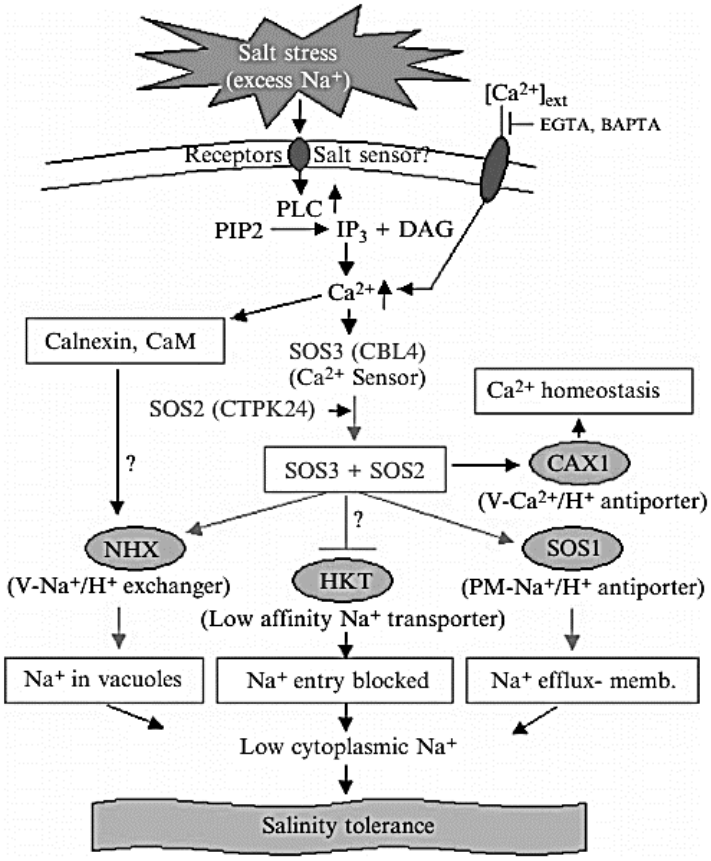

Figure 1.8 Regulation of ion (e.g., Na^+, K^+, and Ca^{2+}) homeostasis by SOS and related pathways in relation to salinity stress tolerance (Tuteja 2007).

Ca^{2+}-modulated proteins

Ca^{2+}-dependent modulation of cellular processes occurs via intracellular Ca^{2+}-binding proteins, also known as Ca^{2+} sensors, of which calmodulin (CaM) is one of the best characterized. CaM has no catalytic activity of its own but, upon binding Ca^{2+}, it

activates numerous target proteins involved in a variety of cellular processes. Recent reviews on CaM summarize the roles of CaM as a Ca^{2+} signal transducer in plants (Snedden and Fromm 1998; Zielinski 1998) and animals (Chin and Means 2000; Van Eldik and Watterson 1998). In addition, a number of recent reviews are available on the subject of other families of important Ca^{2+} sensors present in plants, such as the Ca^{2+}-dependent (CaM-independent) protein kinases (CDPKs) (Harmon et al. 2000) and annexins, whose association with membranes through binding of anionic phospholipids is Ca^{2+} dependent (Hofmann et al. 2000). A brief account of the important features of CaM as a Ca^{2+} transducer is presented below.

This study focuses on calmodulin, an essential Ca^{2+} transducer in eukaryotic cells and its functions in plants.

Calmodulin

Calmodulin is a protein that function as intracellular transducers of Ca^{2+} signals contain a common structural motif, the 'EF hand' (Strynadka and James 1989), which is a helix-loop-helix structure that binds a single Ca^{2+} ion. These motifs typically occur in closely linked pairs, interacting through antiparallel β-sheets (Strynadka and James 1989). This arrangement is the basis for cooperativity in Ca^{2+} binding. The superfamily of EF-hand proteins is divided into several classes based on differences in number and organization of EF-hand pairs, amino acid sequences within or outside the motifs, affinity to Ca^{2+} and/or selectivity and affinity to target proteins (Crivici and Ikura 1995; Strynadka and James 1989). CaM is an acidic EF-hand protein present in all eukaryotes. The CaM prototype is composed of 148 amino acids arranged in two globular domains

connected with a long flexible helix. Each globular domain contains a pair of intimately linked EF hands (Figure 1.9a).

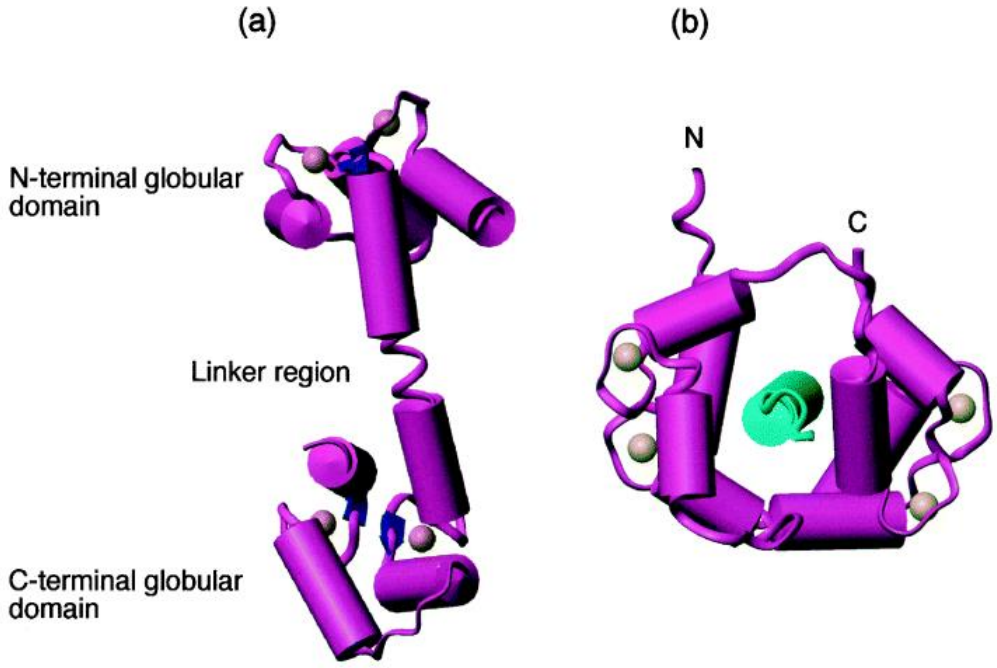

Figure 1.9 Three-dimensional structure of calmodulin (CaM). (a) Crystal structure of Ca^{2+}/CaM. (b) Solution structure of Ca^{2+}/CaM-peptide complex. α-helices are shown as cylinders (violet in CaM, light blue in the target peptide); β-sheets are indicated as deep purple arrows, and Ca^{2+} ions as spheres in light brown. The structural images were created with the Insight II software (BIOSYM Technology, San Diego, CA, USA) using the Brookhaven data base structure codes 3CLN and 2BBM, respectively. The structural images show only the backbones of CaM and the peptide (i.e. no side chains) (Snedden and Fromm 1998).

CaM and plant responses to environmental stimuli

In view of the role Ca^{2+} plays in mediating plant responses to biotic (Levine et al. 1996) and abiotic (Knight 1999) stimuli, it is not surprising that CaM, as an important cellular Ca^{2+} receptor, is involved in mediating these responses.

Calcium signals decoding elements and plant salt resistance

When Ca^{2+} is in deficit, plants are more susceptible to damage by low pH or high salt. Numerous results suggest that external and apoplastic Ca^{2+} directly alleviates symptoms produced by ion stresses or mineral toxicities, such as proton, Na^{+}, Al^{3+}, and Cl^{-} toxicities, and Ca^{2+} also helps to establish a favorable K^{+}: Na^{+} ratio under salt stress (Plieth 2005).

It is suggested that intracellular calcium signaling through a calcineurin-like pathway mediates the beneficial effect of calcium on plant salt tolerance (Liu and Zhu 1998). A salt stress induced Ca^{2+}-dependent signaling network was described and illustrated in detail to mediate Na^{+} homeostasis and salt tolerance, indicating that Ca^{2+} transporters are closely related to plant salt tolerance. It was also suggested that CaM activation might be necessary in calcium promotion of the accumulation of proline in fig calli, and the addition of calcium to media alleviated the inhibition of fig callus growth under salt stress demonstrating that CaM might act jointly with Ca^{2+} under the support of calcium signals decoding elements in plant responses to salt stress (Wang et al. 1999). It was found that in barley roots, the activation of tonoplast H^{+}-ATPase and the regulation of Na^{+} and K^{+} uptake under NaCl stress may be related to Ca^{2+}-CaM system, showing that calcium signals decoding elements may participate in the process

of plant signaling responses to salt stress through accordingly regulating cytosolic Ca^{2+} concentration (Song et al. 2008).

Metabolic regulation by CaM

CaM appears to play a signalling role in several metabolic pathways. Plants are unique among eukaryotes in producing their own carbohydrates from inorganic carbon, the synthesis of which is controlled by developmental as well as environmental signals. Studies have implicated Ca^{2+}/CaM in phototransduction pathways that control chloroplast development (Bowler and Chua 1994). This developmentally regulated process establishes the machinery for the photosynthetic activity of plants whereby carbon is fixed into sugars. In addition, CaM was also suggested to participate in sugar sensing and sugar signal transduction (Smeekens and Rook 1997).

A previous study by Saeng-ngam and workers (2012), the transgenic rice plants containing *OsCaM1-1* gene under the control of *35SCaMV promoter* (*35SCaMV-OsCaM1-1*) were constructed by Agrobacterium-mediated transformation via pCAMBIA1301 plasmid. Three transgenic rice lines harboring the 35SCaMV-OsCam1-1 and transgenic lines harboring the T-DNA from pCAMBIA1301 alone as negative controls have been produced. Therefore the aim of this study is to demonstrate that *OsCaM1-1* gene causes protection of plant cells against salt stress in the transgenic rice plants overexpressing *OsCaM1-1* gene under the control of *35SCaMV promoter* compared to the control of transgenic rice plants that harbor the T-DNA alone without the inserted gene as well as the wild-type KDML 105 plants, with special regard to changes in antioxidant enzymes like SOD, CAT and also enzymes of AsA-GSH cycle

(APX and GR), as well as ROS scavenging activity, photosynthetic pigment contents, lipid peroxidation and growth.

Confirmation of the over-expression level of *OsCaM1-1* gene in transgenic rice plants

A previous study by Takpirom, the over-expressed of *OsCaM1-1* gene regulated by 35SCaMV in the transgenic rice plants was confirmed by performing northern blot analysis of total RNA (Figure 1.10).

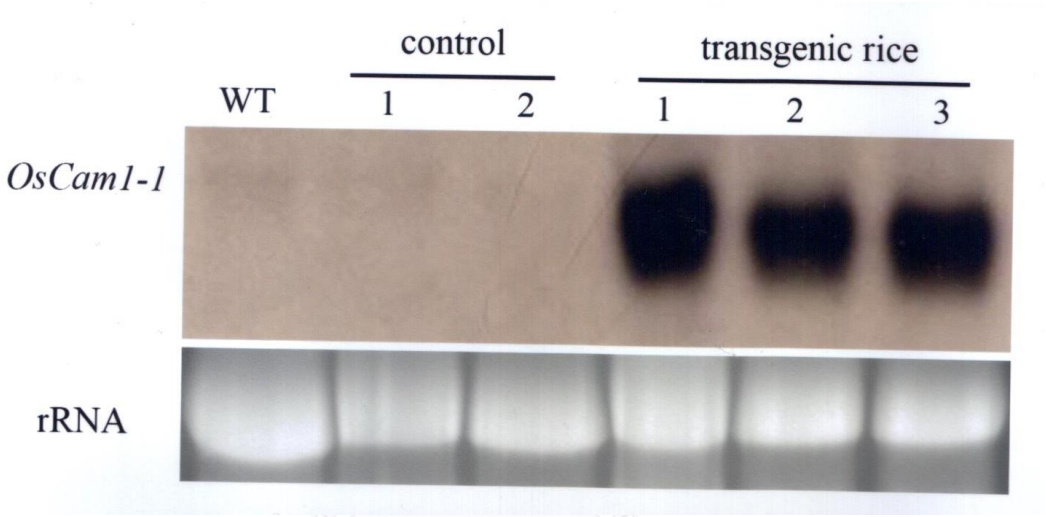

Figure 1.10 RNA blot analysis and quantitative comparison of OsCaM1-1 gene under in vitro normal growth condition of wild-type plants, two independent control rice plants lines and three transgenic rice plants. Each lane was loaded with total RNA isolated from leaves. RNA was analyzed by gel blot hybridization with a denatured ^{32}P-oligolabeled OsCaM1-1 probe. An ethidium bromide-staning gel of each analysis is shown under its corresponding autoradiography (Takpirom, 2007).

Objectives of the thesis

Evaluate the antioxidative systems, growth, photosynthetic pigments, lipid peroxidation and scavenging activity in transgenic rice *Oryza sativa* L. 'KDML105' overexpressing *OsCaM1-1* to comparison with the control transgenic lines and wild-type plants.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Rice seeds

Rice *Oryza sativa* L. cultivar Khao Dok Ma Li 105 (KDML 105) and transgenic rice plants overexpressing *OsCaM1-1* gene.

2.1.3 Instruments

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

Automatic micropipette: Pipetman P2, P20, P100, P200, P1000 (Gilson Medical Electronics S.A., France)

Balance: Sartorius CP423s (Scientific Promotion Co. USA)

Centrifuge 5804R (Eppendorf, Germany)

Centrifuge Sorvall Legend XTR (Thermo Scientific, USA)

CentriVap Concentrator (Labconco, USA)

-20 ºC Freezer (Sharp, Japan)

Forma -86C ULT Freezer (Thermo Electron Corporation, USA)

Forma ClassII, A2 Biological safety cabinet (Thermo Electron Corporation, USA)

Microwave oven (Panasonic, Japan)

Mixer Mill MM400 (Retsch®, Germany)

60 ºC Oven (Memmert, Germany)

Oven Series8000 (Contherm, New Zealand)

Plant Growth Chamber (Human Lab, South Korea)

pH meter: pH900 (Precisa, Germany)

Spectrophotometer: DU®640 (Beckman Coulter, USA)

-80 ºC Ultra low temperature freezer (New Brunswick Scientific, England)

Vibro shaker (Labinco BV, Netherlands)

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

2.1.4 Inventory supplies

Cryo kit for cooling the grinding jars with liquid nitrogen (Retsch®, Germany)

Filter paper: Whatman No.1 (Whatman International Ltd., England)

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

Grinding jar, PTFE 1.5 ml (Retsch®, Germany)

Microcentrifuge tube 1.5 ml (Axygen Heyward, USA)

Pipette tips 100, 1000 µl (Axygen Heyward, USA)

2.1.5 Chemicals and reagents

Agar (Bacteriological grade) (Criterion Chemical LLC, USA)

Albumin, Bovine serum (Sigma Chemical Co., USA)

Ammonium dihydrogen orthophosphate (BDH laboratory reagent, England)

Ammonium sulfate (Carlo Erba Reagenti, Italy)

L-ascorbic acid, 99% (Aldrich, Germany)

Boric acid (Univar, Australia)

Calcium chloride dihydrate (for analysis) (Carlo Erba Reagenti, Italy)

Calcium nitrate-4-hydrate, 98% (Riedel-deHaën®, Germany)

Casein hydrolysate (Himedia laboratories Pvt, Ltd., India)

Cobaltous chloride hexahydrate (Fluka, Switzerland)

Copper(II) sulfate (Carlo Erba Reagenti, Italy)

Coomassie® Brilliant Blue G-250 (Fluka, Switzerland)

N,N-Dimethylformamide (Carlo Erba Reagenti, Italy)

2,2-diphenyl-1-picryl-hydrazyl-hydrate (Sigma-Aldrich, Germany)

Dithiothreitol (Bio Basic Inc., Canada)

Ethanol, absolute (BDH, England)

Etylenediaminetetraacetic acid disodium salt (EDTA) (Carlo Erba Reagenti, Italy)

Gallic acid (Sigma Chemical Company Co., USA)

L-glutamine (Phyto Technology Laboratories™, USA)

Guaiacol (Sigma-Aldrich, Germany)

Hydrochloric acid (Merck, Germany)

Hydrogen peroxide (Sigma Chemical Co., USA)

Iron(II) sulphate (Carlo Erba Reagenti, Italy)

Magnesium chloride (Carlo Erba Reagenti, Italy)

Magnesium sulfate (Sigma Chemical Co., USA)

Manganese(II) sulfate (Sigma Chemical Co., USA)

Methanol (Merck, Germany)

L-methionine (Sigma-Aldrich, Germany)

Myo-inositol (Sigma-Aldrich, Germany)

Nicotinic acid (Sigma-Aldrich, Germany)

Nitroblue tetrazolium (NBT) (Fermentas, Inc., USA)

Phosphoric acid, 85% (Lab Scan, Ireland)

Potassium chloride (Carlo Erba Reagenti, Italy)

Potassium dihydrogen phosphate (Carlo Erba Reagenti, Italy)

di-Potassium hydrogen phosphate (Carlo Erba Reagenti, Italy)

Potassium iodide (Sigma Chemical Co., USA)

Potassium nitrate (Carlo Erba Reagenti, Italy)

L-proline (Phyto Technology Laboratories™, USA)

Protease inhibitor mix (GE Healthcare, England)

Pyridoxine (Sigma Chemical Co., USA)

Riboflavin, 98% (Sigma Chemical Co., USA)

Sodium carbonate, anhydrous (Carlo Erba Reagenti, Italy)

Sodium chloride (Carlo Erba Reagenti, Italy)

Sodium dihydrogen phosphate (Carlo Erba Reagenti, Italy)

di-Sodium hydrogen phosphate (Carlo Erba Reagenti, Italy)

Sodium hypochlorite (Haiter, Thailand)

Sodium molybdate (Sigma Chemical Co., USA)

Sucrose (Carlo Erba Reagenti, Italy)

Thiamine (Sigma Chemical Co., USA)

2-Thiobarbituric acid (Sigma-Aldrich, Germany)

Titanium(IV) chloride (Merck, Germany)

Trichloroacetic acid (Carlo Erba Reagenti, Italy)

Zinc sulfate (Sigma Chemical Co., USA)

2.2 Growth conditions and treatments

The KDML 105 cultivar rice seeds were obtained from Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand). Transgenic rice plants containing *OsCaM1-1* gene under the control of 35SCaMV promoter (35SCaMV-*OsCaM1-1*) were constructed by Agrobacterium-mediated transformation via pCAMBIA1301 plasmid. Three putative transgenic rice lines harboring the 35SCaMV-*OsCaM1-1* and the putative transgenic line harboring the T-DNA from pCAMBIA1301 alone as negative control were kindly provided by the research group "Special Task Force for Activating Research (STAR): Biochemical and Molecular Mechanisms of Rice in Changing Environments" by the Ratchadaphisek-sompot Endowment Fund, Chulalongkorn University. Seeds of the transgenic rice plants overexpressing *OsCaM1-1*, the control (negative control transgenic line), and wild-type KDML105 were generated as described below.

Rice seeds were dehusked and sterilized with 70% (v/v) ethanol for one minute and then with 35% (w/v) sodium hypochlorite for 20 minutes. The seed were rinsed three times with sterile water and germinated in nutrient broth medium (NB) (Li et al. 1993) containing 0.8% (w/v) agar at 25-28 °C under 16 h light (200 μmole m^{-2} sec^{-1})/ 8 h dark photo period. After 7 days, germinated seeds were transferred to and grown in

Limpinuntana's nutrient solution (Limpinuntana 1978) (Appendix A) for 35 days. After 42 days, the seedlings were transferred to pots filled with sandy loam consisting about 10% clay soil and nitrogen/ phosphorus/potassium fertilizer (15: 15: 15) and grown for 4 months in the greenhouse. The seeds were harvested and dried at 60°C for 3 days in the incubator in order to decrease the moisture content. Refrigerated the seeds at 4 °C to use in experiments.

For the experiments, rice seeds were dehusked and sterilized with 70% (v/v) ethanol for one minute and then with 35% (w/v) sodium hypochlorite for 20 minutes. The seed were rinsed three times with sterile water and germinated in NB medium containing 0.8% (w/v) agar at 25-28 °C under 16 h light (200 µmole m^{-2} sec^{-1})/ 8 h dark photo period. After 7 days, germinated seeds were transferred to and grown in Limpinuntana's nutrient solution for 14 days. For the exposure to salt stress, the 3-week-old seedlings were supplemented with 0, 50, 100, 150 or 200 mM NaCl. The leaves of 3-week-old seedlings were harvested after 3 days of salt stress and then stored at -80 °C until biochemical analyses (H_2O_2 content, the relative growth rate (RGR) of shoot and root, relative water content (RWC), the leaf chlorophyll *a*, *b* and carotenoid contents, lipid peroxidation, DPPH radical scavenging activity and antioxidant enzyme activities) were done.

2.3 Measurement of H_2O_2 content

The H_2O_2 level was modified from method those described by Patterson (1984). For H_2O_2 extraction, 0.05 g of leaves were ground to fine powder by Mixer Mill MM400 at a frequency of 35 Hz for one minute, two times. Then, 1 ml of 50 mM potassium phosphate buffer, pH 7.0 was added, and centrifuged (6,000 xg, 25°C, 15

min). 0.9 ml of supernatant was mixed with 0.3 ml of 1% v/v $TiCl_4$ in conc. HCl, then incubated at room temperature for 15 min. The content of H_2O_2 was measured by monitoring the A_{410} of the titanium-peroxide complex. Absorbance values were calibrated to a standard curved generated with known concentrations of H_2O_2.

2.4 Antioxidant enzyme extracts and assay

Enzyme extracts for evaluating the superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) activities were prepared by first freezing 0.1 g leaf samples were ground to fine powder by Mixer Mill MM400 and extracted with 1 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.5, containing 0.5 mM ethylenediamine- tetraacetic acid (EDTA), 5 mM dithiothreitol, and 1 µl/ml protease inhibitor, with the addition of 5 mM ascorbic acid in the case of APX and GR assay. The homogenates were centrifuged (15,000 xg, 4 ºC, 20 min) prior to harvesting the supernatant as the enzyme extract.

2.4.1 Superoxide dismutase activity

SOD activity was assayed according to the method of Beauchamp and Fridovich (1971) by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) to formazan. The 1.2 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 50 mM sodium carbonate, 75 µM NBT, 2µM riboflavin, 0.1 mM EDTA, and 50 µg enzyme. The reaction mixtures were illuminated for 15 min at a light. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT monitored at 560 nm.

2.4.2 Catalase activity

CAT activity was measured following Aebi (1984) by monitoring the decline of absorbance at 240 nm due to decomposition of H_2O_2. The 1.2 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 50 µg enzyme, and 17 mM H_2O_2 was added to start reaction. The extinction coefficient value of 39.4 $mM^{-1}cm^{-1}$ was used for calculations.

2.4.3 Ascorbate peroxidase activity

APX activity was assayed according to the method Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. The 1.2 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 50 µg enzyme, and 0.1 mM H_2O_2 was added to start reaction. The extinction coefficient value of 2.8 $mM^{-1}cm^{-1}$ was used for calculations.

2.4.4 Glutathione reductase activity

The method of GR activity assay were modified from those described by Sherwin and Farrant (1998). GR activity was assayed by monitoring the decrease of absorbance at 340 nm due to reduction of GSSG to GSH. The 1.2 ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 3 mM $MgCl_2$, 0.1 mM EDTA, 0.15 mM NADPH, and 50 µg enzyme. The reaction was initiated by addition of 10 mM GSSG. The extinction coefficient value of 6.22 $mM^{-1}cm^{-1}$ was used for calculations.

2.5 Measurement of photosynthetic pigments

Total chlorophyll, chlorophyll *a*, *b* and carotenoids were extracted in 1 ml of *N*,*N*-dimethylformamide (DMF) by grinding 0.05 g of leaves to fine powder with Mixer Mill MM400 at a frequency of 35 Hz for one minute, two times. The homogenate was centrifuged (5,000 xg, 10 min, 4 ºC) and the absorbance of the resulting supernatant was taken at 461, 647 and 664 nm. Total chlorophyll, chlorophyll *a*, *b* and carotenoid contents were calculated according to modified method of Arnon (1949):

Total chlorophyll (a and b) (mg/l) = $17.90 (A_{647}) + 8.08 (A_{664})$

Chlorophyll a (mg/l) = $12.70 (A_{664}) - 2.79 (A_{647})$

Chlorophyll b (mg/l) = $20.70 (A_{647}) – 4.62 (A_{664})$

Carotenoids (mg/l) = A_{461} x 200

The pigment concentrations were calculated in mg/g FW of sample.

2.6 Measurement of lipid peroxidation

Lipid peroxidation was estimated measuring the formation of malondialdehyde (MDA), a breakdown product of lipid peroxidation, with 2-thiobarbituric acid (TBA) according to De Vos and workers (1989). 0.1 g of leaves were ground to fine powder by Mixer Mill MM400 at a frequency of 35 Hz for one minute, two times. Then, 1 ml of 0.1% trichloroacetic acid (TCA) was added and centrifuged (14,000 rpm, 25 °C, 15 min). After centrifugation, 0.3 ml of the supernatant was mixed with 0.75 ml 0.25% TBA in 10% TCA. The absorbance of resulting supernatant was taken at 532 and 600

nm. MDA content was determined by subtracting absorbance of supernatant at 600 nm from that of 532 nm and using absorbance coefficient of 155 $mM^{-1}cm^{-1}$ and was expressed as μmol per g fresh weight of sample.

2.7 Measurement of DPPH radical scavenging activity

The measurement of the DPPH radical scavenging activity was performed according to methodology described by Brand-Williams and workers (1995). 0.1 g of leaves were ground to fine powder by Mixer Mill MM400 at a frequency of 35 Hz for one minute, two times. Then, 1.0 ml of absolute methanol was added and evaporated by centrivap concentrator. The samples were reacted with the stable DPPH radical in an ethanol solution. The reaction mixture consisted of adding 0.5 ml of sample, 3 ml of absolute ethanol and 0.3 ml of DPPH radical solution 0.5 mM in ethanol. When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in color (from deep violet to light yellow) were read at 517 nm after 30 min of reaction using a spectrophotometer. The mixture of ethanol and sample without DPPH radical solution serve as blank. The control solution was prepared by mixing ethanol and DPPH radical solution. The scavenging activity percentage (AA%) was calculated follows:

$$AA\% = \frac{(A_{control} - A_{test})}{A_{control}} \times 100$$

2.8 Growth measurement

Growth measurements, plants removed from the nutrient solutions, and shoots were separated from the roots and washed carefully with deionized water (Maiti et al. 1996). Shoot and root fresh weight (FW) and shoot and root dry weight (DW) of stressed and non stressed plants in saline conditions were determined. Shoot and root samples dried in an oven at 60°C for 72 h. to determine dry mater.

2.9 Measurement of relative growth rate (RGR)

The samples were randomly selected and oven-dried at 60 °C for 72 h for determination of dry weight. RGR was determined following the method outlined by Gardner (1985). RGR was calculated from the increase in dry weight of plants at the beginning and end of salt treatment, using the equation:

$$RGR = (\ln W_f - \ln W_i) / (t_f - t_i)$$

where, W_f and W_i are the shoot and root dry weight of plant, t_f and t_i are the times and subscripts denote initial and final sampling that is, day 0 (before salt stress treatment) and day 3 (after 3 days salt stress treatment).

2.10 Measurement of relative water content (RWC)

To determine relative water content (RWC), plants from each treatment conditions were randomly selected and the method described by Whetherley and Barrs (1962) was followed. About 0.1 g leaf sample was cut into smaller pieces and weighed to determine initial weight (W_i). The leaf samples were then floated in freshly de-ionized water for 12 h and weighed thereafter to determine fully turgid weight (W_f).

The sample was oven-dried at 60˚C for 3 days and the dry weight was obtained (W_d). The relative water content (RWC) was determined using the following formula:

$$RWC = [(W_i - W_d)/(W_f - W_d)] \times 100$$

2.11 Protein content

Protein concentrations in enzyme extract were measured according to the method of Bradford (1976), using different known concentrations of bovine serum albumin as the standard protein for calibration.

2.12 Statistical analysis

The significance of differences between mean values was compared by Duncan's test. Differences at $p < 0.05$ were considered significant.

CHAPTER III

RESULTS

3.1 Effect of salt stress on H_2O_2 content

The effects of salt stress on H_2O_2 content in the transgenic rice plants overexpressing *OsCaM1-1* gene under the control of *35SCaMV promoter*, the control (negative control transgenic line) and wild-type KDML105 were determined. Figure 3.1A showed H_2O_2 contents of all plants determined on various treatment time levels (0, 1, 2, 3, 4 days). At different treatment time levels, all plants with 150 mM NaCl treatment showed an increase in H_2O_2 content with increasing time except on day 4. The highest value was observed on day 3. Based on these results, duration time of 3 days was selected for further evaluation of the plant's responses to high salinity. Figure 3.1B shows H_2O_2 content of all plants were determined on various concentrations of NaCl (0, 50, 100, 150 and 200 mM) for 3 days. The H_2O_2 contents in all plants increased with increasing salinity levels. They showed the highest value at 150 mM NaCl. However in all plants, they were decreased rapidly under 200 mM NaCl. Significantly decrease H_2O_2 content on day 4 after treated with 150 mM NaCl and 200 mM NaCl for 3 days, maybe due to salt stress-induced cell death.

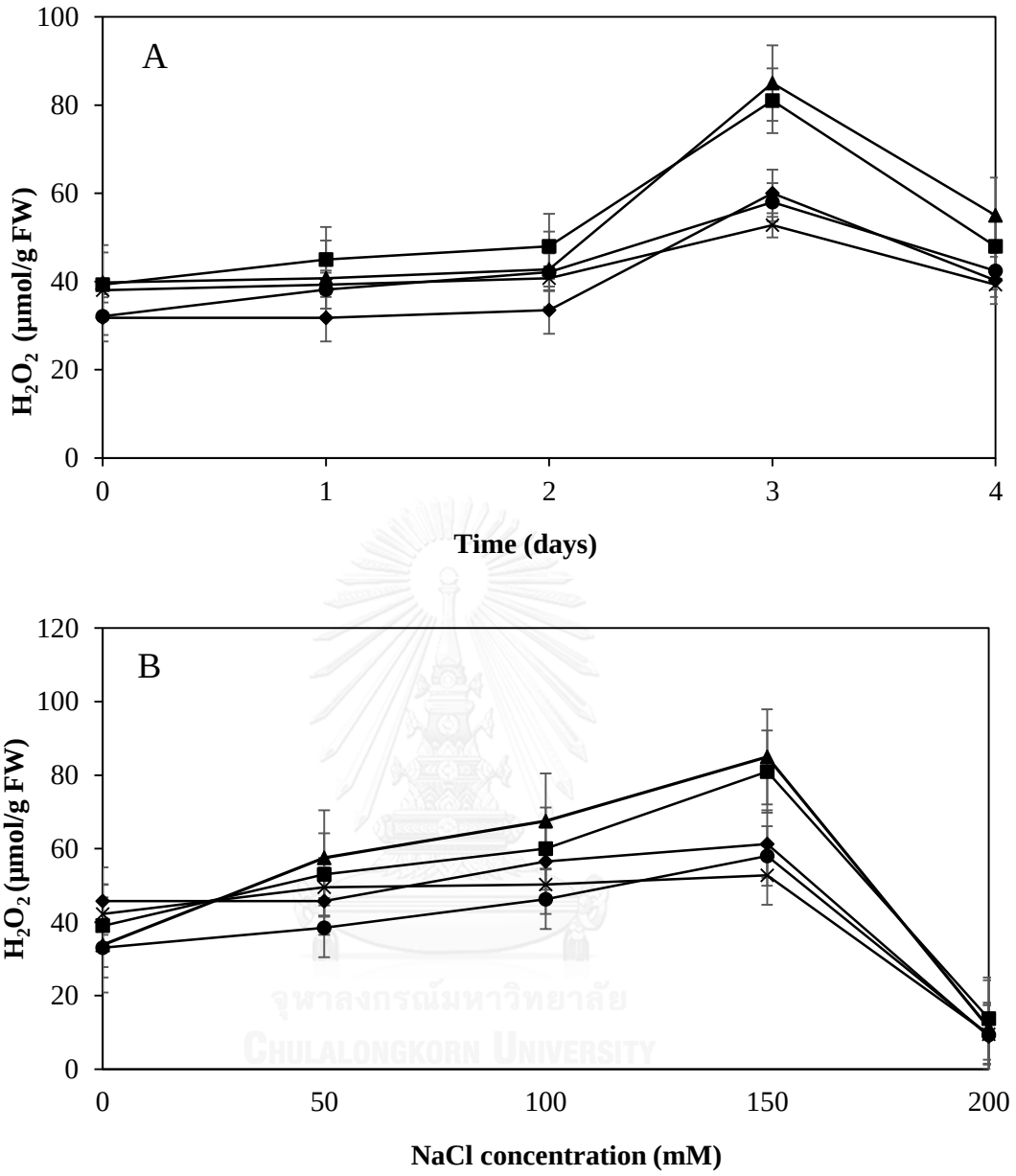

Figure 3.1 Effect of high salinity on H_2O_2 content in leaves of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene (—●—, —×—, —◆—) compare to the control transgenic (—▲—) and wild-type plants (—■—) after expose to salt stress (150 mM NaCl) at different treatment time (0, 1, 2, 3 and 4 days)(A) and to different treatment solutions (0, 50, 100, 150 and 200 mM NaCl) for 3 days (B).

3.2 Effect of salt stress on antioxidant enzymes

Antioxidative enzymes are one of the response mechanisms against environmental stresses. As such, their activity profiles are important in the evaluation of tolerance mechanisms. To determine the response of transgenic rice plants overexpressing the *OsCaM1*-gene, the control transgenic and wild-type plants to salt induced oxidative stress, SOD, CAT, APX and GR activities were measured in leaves of seedlings grown either in normal growing conditions (0 mM NaCl treatment) or under salt stress (150 mM NaCl treatment).

SOD activities of the *OsCaM1-1* overexpressing rice plants, control transgenic and wild-type KDML 105 under the effect of salt stress and normal growing conditions are shown in Figure 3.2A. The SOD activities were increased by high salinity. Under salt stress, the SOD was increased significantly in the *OsCaM1-1* overexpressing rice plants (42.2, 39.7 and 40.8 µmole/min/mg protein, respectively). These transgenic plants had increased SOD activity about 53% when compared to plants in normal growing conditions. While the control transgenic and wild-type plants, the SOD was increased slightly about 9%.

Salt treatment also increased significantly CAT activity in the three transgenic *OsCaM1-1* overexpressing rice plants. These transgenic plants had increased CAT activity about 33%. On the other hand, the control transgenic and wild-type plants exhibited significant decrease in CAT activity under salt stress about 24% in comparison with that of the non-salinized plants (Figure 3.2B).

The analysis of the activity of APX in transgenic rice plants overexpressing the *OsCaM1-1* gene showed significantly high activity under high salinity when compared to those of the control transgenic and wild-type plants (Figure 3.2C). In addition, APX

activities of the transgenic plants under salt stress were increased significantly about 70-85% when compared to without salt stress, while that of the control transgenic and wild-type plants were increased about 20 and 38%, respectively.

An increase in GR activity was observed in all plants under salt stress (Figure 3.2D). The transgenic rice plants overexpressing the *OsCaM1-1* gene were higher GR activity under saline conditions than the control transgenic and wild-type plants. GR activities of the transgenic plants under salt stress were increased about 65-82% when compared to non-salinized plants, while that of the control transgenic and wild-type plants were increased about 46 and 36%, respectively.

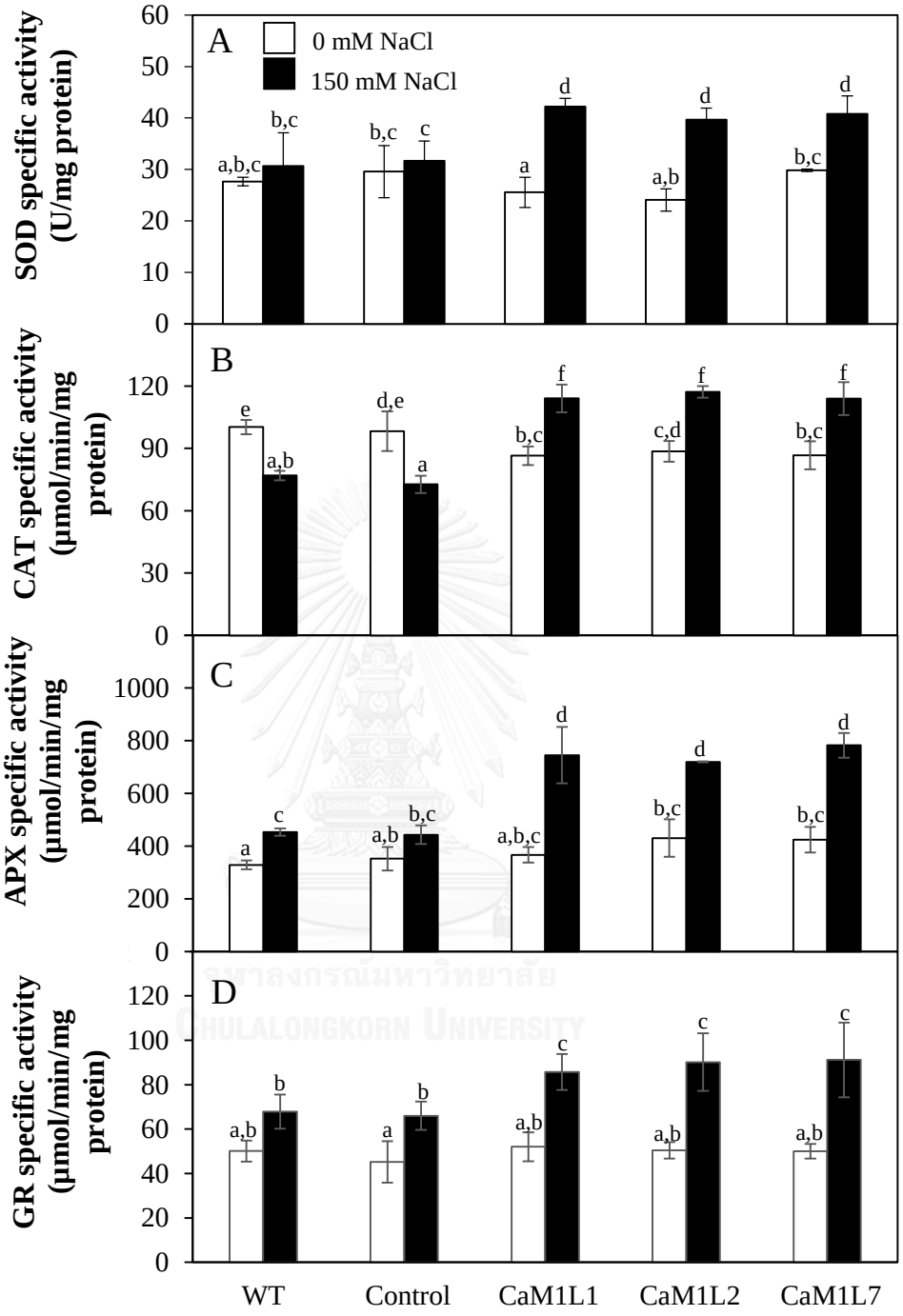

Figure 3.2 Effect of high salinity on activity levels of SOD (A), CAT (B), APX (C) and GR (D) in leaves of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to the control transgenic

and wild-type plants after expose to salt stress (0, 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

3.3 Effect of salt stress on photosynthetic pigments

Photosynthetic pigment contents (chlorophyll *a*, *b* and carotenoids) were extracted and determined from transgenic rice plants overexpressing the *OsCaM1-1* gene, the control and wild-type KDML105 plants, which grown either in normal growing conditions (0 mM NaCl treatment) or under salt stress (150 mM NaCl treatment). Salinity were had an effect on total chlorophyll, chlorophyll *a* and *b* and carotenoid contents. All photosynthetic pigments were higher in the *OsCaM1-1* overexpressing rice plants, grown under salt stress than in the control and wild-type plants (Figure 3.3). The results showed total chlorophyll (Figure 3.3A), chlorophyll *a* (Figure 3.3B) and *b* (Figure 3.3C) and carotenoid contents (Figure 3.3D) under salt stress decreased about 5, 9, 16 and 3% in transgenic plants respectively, in the control about 24, 33, 40 and 20%, in the wild-type about 27, 40, 40 and 19% when comparison with non-salinized plants.

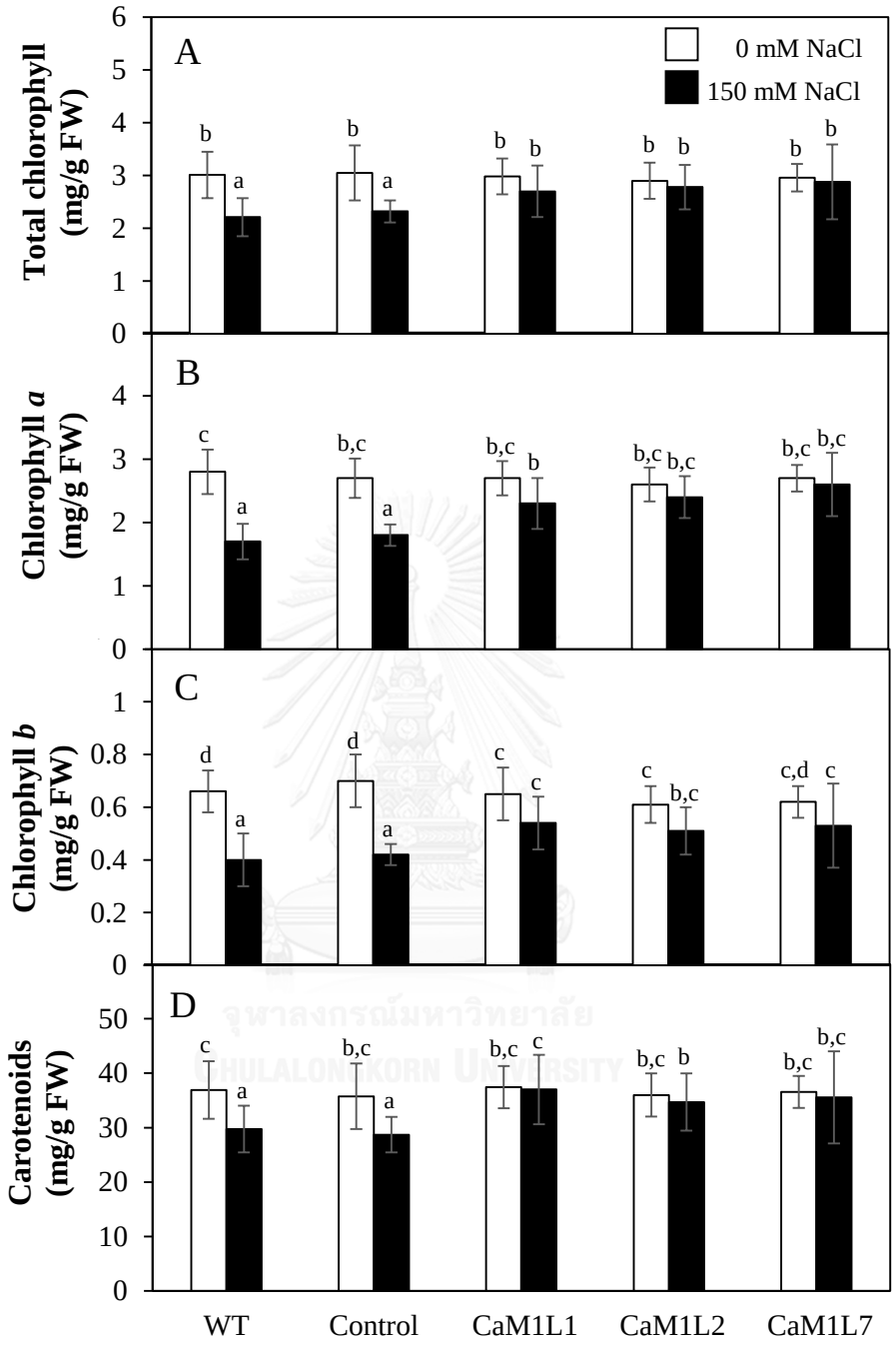

Figure 3.3 Effect of high salinity on total chlorophyll (A), chlorophyll a (B), chlorophyll b (C) and carotenoid (D) contents of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to

the control transgenic and wild-type plants after expose to salt stress (0, 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

3.4 Effect of salt stress on lipid peroxidation

Malonaldehyde (MDA) content was an important indicator to measure lipid peroxidation. MDA content were extracted and determined from the *OsCaM1-1* overexpressing rice plants, the control and wild-type KDML105 plants, which grown under without and with high salinity (150 mM NaCl) condition. Salt stress affected MDA content, they were significantly higher in the control transgenic and wild-type (0.046 and 0.048 μmole/g FW, respectively) than the *OsCaM1-1* overexpressing rice plants (0.031-0.035 μmole/g FW). Under high salinity, MDA contents in transgenic plants increased about 49% while that of the control transgenic and wild-type plants were increased about 97% as compared to non-salinized plants (Figure 3.4).

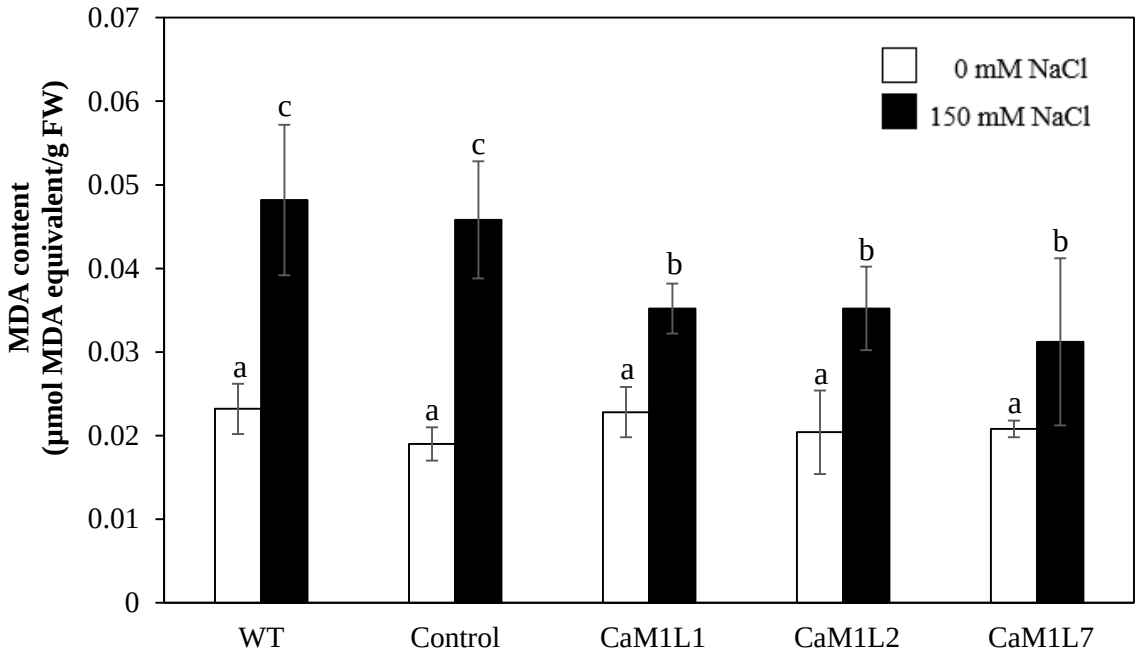

Figure 3.4 Effect of high salinity on lipid peroxidation of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to the control transgenic and wild-type plants after expose to salt stress (0, 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

3.5 Effect of salt stress on DPPH radical scavenging activity

DPPH assay is rapid and sensitive way to survey the antioxidant activity of a specific compounds or plant extracts. The effect of salt stress on DPPH radical scavenging activity in the transgenic rice plants overexpressing *OsCaM1-1* gene under the control of *35SCaMV promoter*, the control and wild-type plants were determined. Under salt stress, statistically significant increase of DPPH scavenging activity was observed in the *OsCaM1-1* overexpressing rice plants (about 70-77%) while it increased slightly in the control transgenic (about 13%) and wild-type plants (about 12%) in comparison with the without salt (Figure 3.5).

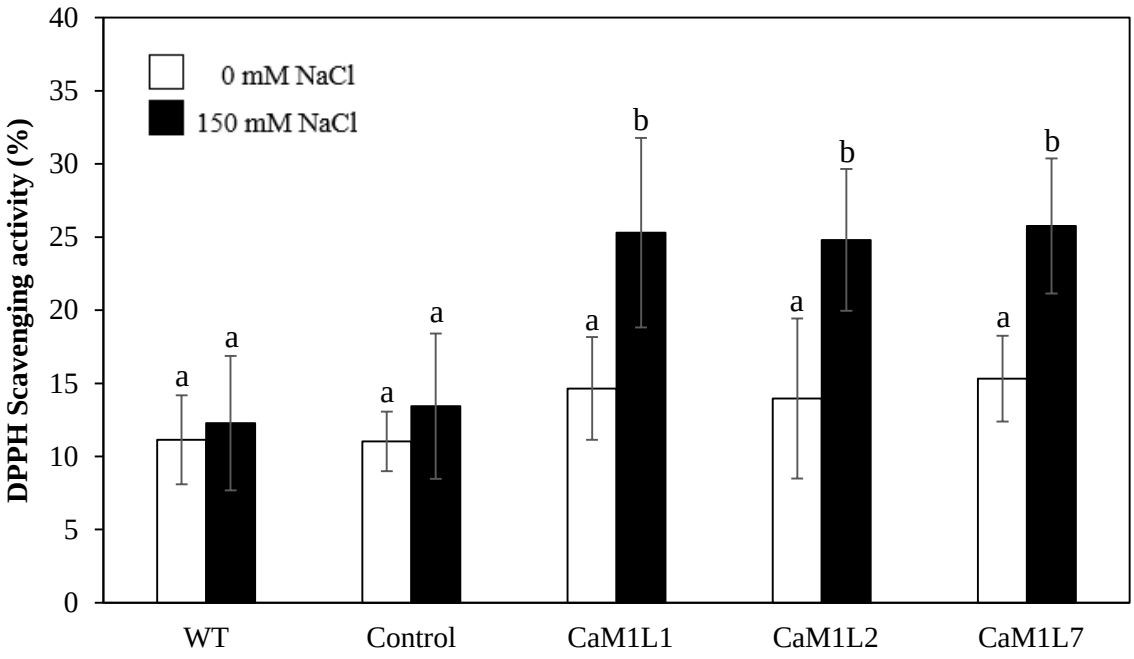

Figure 3.5 Effect of high salinity on DPPH radical scavenging activity of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to the control transgenic and wild-type plants after expose to salt stress (0, 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

3.6 Effect of salt stress on growth

Salt stress (150 mM NaCl) significantly decreased shoot fresh and dry weight of the transgenic rice plants overexpressing *OsCaM1-1* gene under the control of *35SCaMV* promoter, the control and wild-type in comparison with the control without salt. The transgenic rice plants protected their growth performances under saline stress, while the control and wild-type had high reductions in their shoot fresh and dry weight. Salt stress caused about 31% reductions in shoot fresh weight in the transgenic rice plants and about 62% reductions in control and wild-type. The weight of dry shoot was reduced about 12% in the transgenic rice plants and about 34% in the control and wild-type. However, there is no statistically significant difference between the two conditions in fresh and dry weight of root in all plants (Figure 3.6).

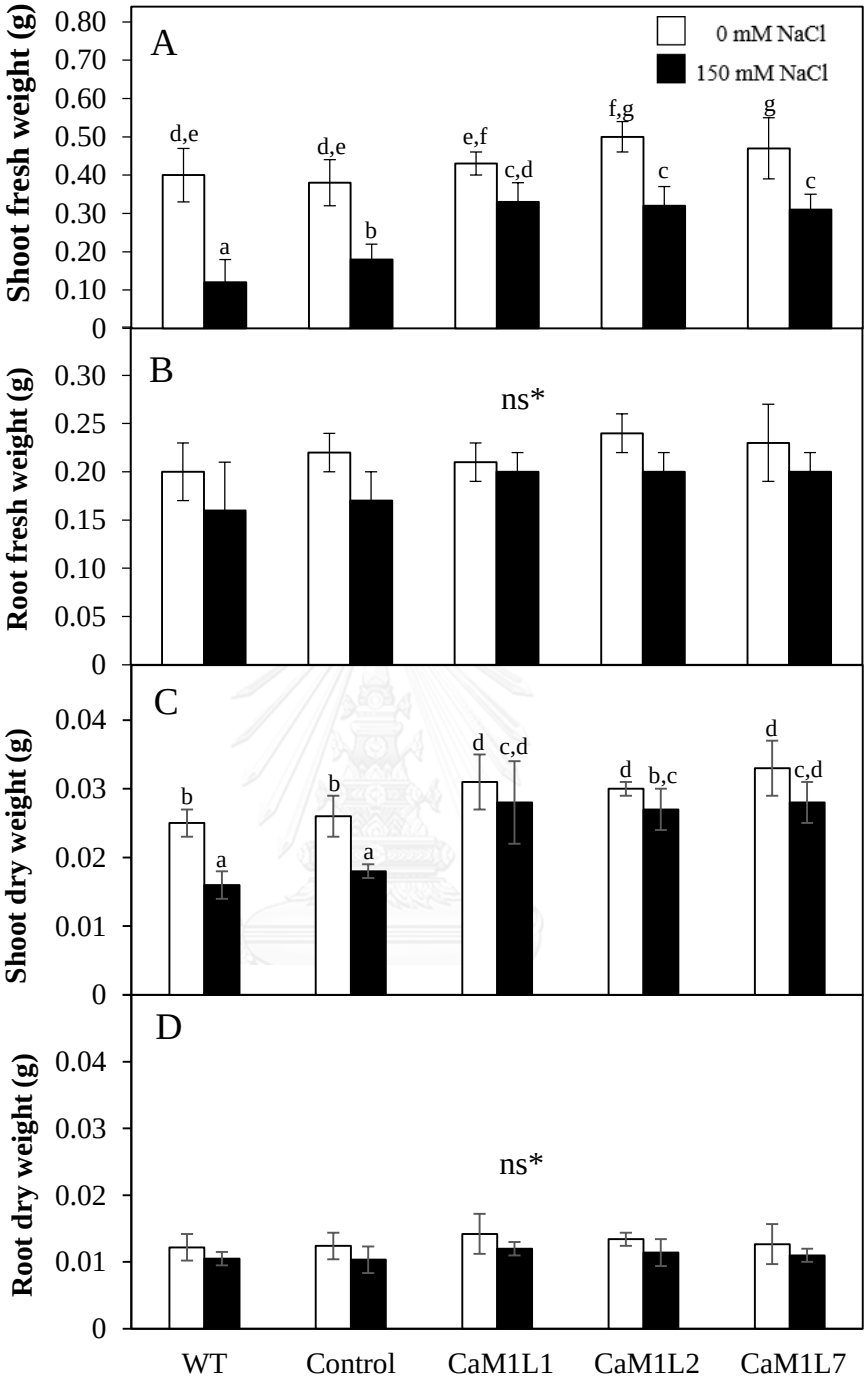

Figure 3.6 Effect of high salinity on shoot fresh weight (A), root fresh weight (B), including shoot dry weight (C) and root dry weight (D) of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene

compare to the control transgenic and wild-type plants after expose to salt stress (0, 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

3.7 Effect of salt stress on relative growth rate

Relative growth rate (RGR) of shoots and roots in the transgenic rice plants overexpressing *OsCaM1-1* gene under the control of *35SCaMV* promoter, the control and wild-type were determined under 0 mM NaCl treatment and salt stress (150 mM NaCl) condition. The RGR of shoots and roots of all plants decreased significantly, except in transgenic rice plants, decreased slightly under high salinity. Moreover, the RGR of shoot in transgenic rice plants were higher than the control and wild-type under salt stress (Figure 3.7A).

Besides, results also showed that overexpression of *OsCaM1-1* gene in the KDML105 rice cultivar was found to grow and enhance the plant adaptation to salt stress better than the control and wild-type. The three transgenic lines overexpressing *OsCaM1-1* gene show less differences in growth and development than the control and wild-type. The transgenic plants increased vigour, whereas the control and wild-type displayed reduced vigour with many wilting leaves (Figure 3.8).

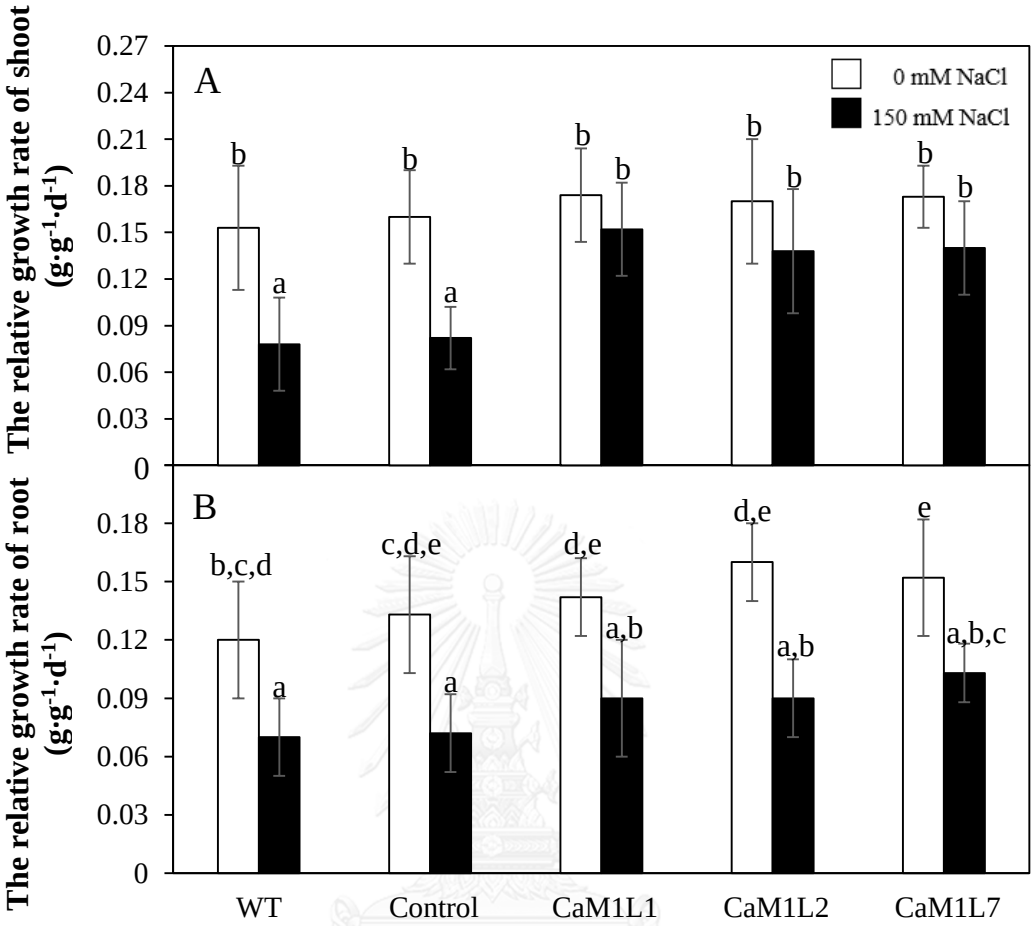

Figure 3.7 Effect of high salinity on the relative growth rate (RGR) in shoot (A) and root (B) of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to the control transgenic and wild-type plants after expose to salt stress (0 and 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

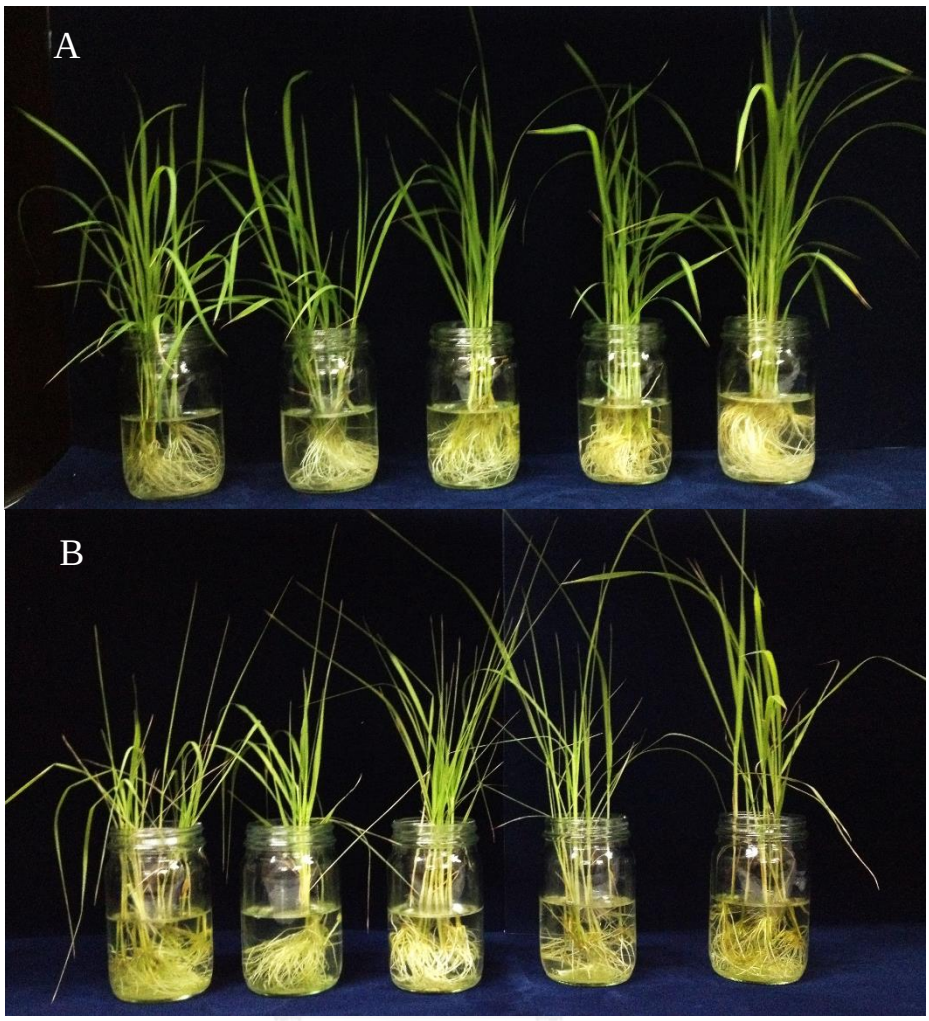

Figure 3.8 The phenotype comparison of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to the control transgenic and wild-type plants under normal (0 mM NaCl) (A) and salt-stress conditions (150 mM NaCl) (B) for 3 days.

3.8 Effect of salt stress on relative water content

Relative water content in leaves of transgenic rice plants overexpressing *OsCaM1-1* gene, the control and wild-type plants were determined. Fresh leaf was measured from plant grown either in normal growing condition (0 mM NaCl treatment) or under salt stress (150 mM NaCl treatment). Relative water content in the leaves of the control and wild-type plants grown under salt stress decreased significantly (about 46%) while RWC in the *OsCaM1-1* overexpressing rice plants decreased slightly (about 17%) (Figure 3.9) when compared to without salt stress.

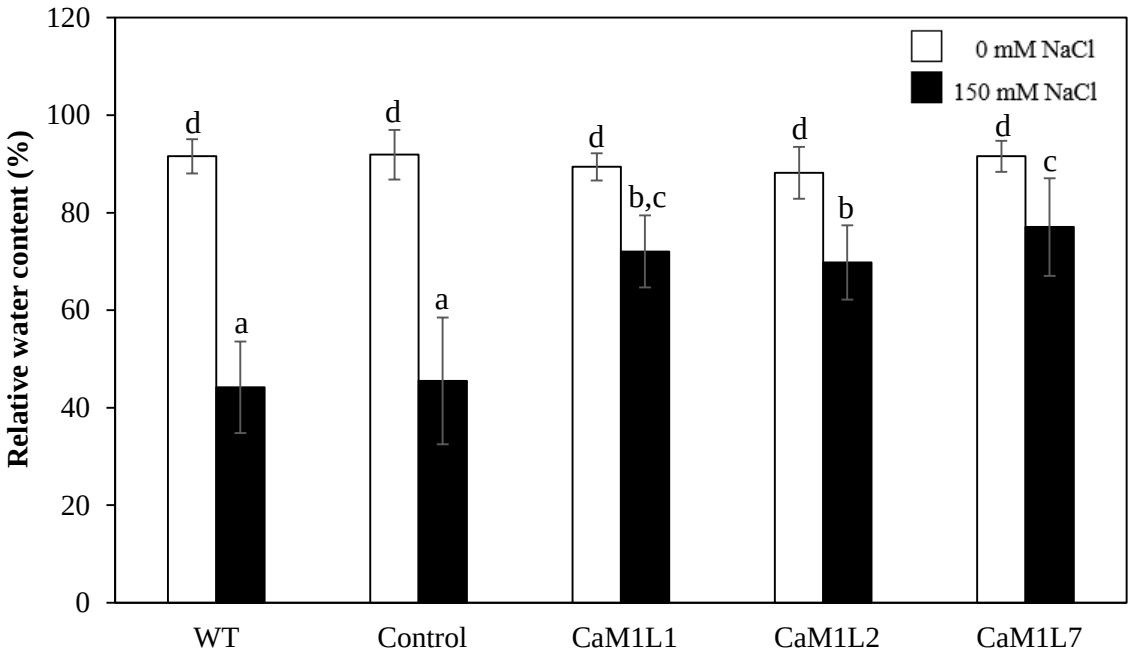

Figure 3.9 Effect of high salinity on relative water content (RWC) of the three transgenic 'KDML 105' rice *Oryza sativa* L. overexpressing *OsCaM1-1* gene compare to the control transgenic and wild-type plants after expose to salt stress (0, 150 mM NaCl) for 3 days. Data are shown as the ± SD, and were derived from five replicates. Means with a different letter are significantly different at $p < 0.05$ according to Duncan's test.

CHAPTER IV

DISCUSSION

To examine the effect of *OsCaM1-1* overexpression in transgenic rice and involved in salt tolerance mechanisms, three transgenic rice harboring the *35SCaMV-OsCaM1-1* and the transgenic line harboring T-DNA from pCAMBIA1301 alone as negative control have been examined the effect of salt stress compared to wild-type plants. The results showed that the H_2O_2 content, lipid peroxidation, DPPH radical scavenging activity, RGR, and activities of antioxidant enzymes (SOD, APX and GR) in all plants were increased, while the CAT activity (except in the control and wild-type plants), RWC and photosynthetic pigments were decreased under salt stress. H_2O_2 content in all plants increased with increasing level of the NaCl treatments and exposure time, the findings of this study indicated that exposure time salinity level were two important factors affecting the plant development under salinity condition.

In many plant (such as tobacco, citrus, wheat and soybean) reports, it was observed that production of ROS increased under saline conditions (Hasegawa et al. 2000) and ROS-mediated membrane damage has been demonstrated to be a major cause of the cellular toxicity by salinity in different crop plants such as rice, tomato, citrus, pea and mustard (Ahmed 2009; Dionisio-Sese and Tobita 1998; Gueta-Dahan et al. 1997; Mittova et al. 2004). Salt stress produced ion leakage, indicating injury to membrane integrity, which could be affected by ROS formed during leaf photosynthesis or respiration, enhancing lipid peroxidation of the membranes (Lechno et al. 1997; Savoure´ A 1999). Lipid peroxidation measured as MDA content is

considered to be indicator of oxidative damage from stress. Here, lipid peroxidation (MDA content) was increased by salinity in all plants, which is consistent with previous reports in cotton (Meloni et al. 2003) and *N.plumbaginifolia* (Savoure´ A 1999).

Most crops do not grow well on soils that contain salts. One reason is that salt causes a reduction in rate and amount of water that plant roots can take up from the soil. Excess salt concentration also enhances the osmotic potential of soil matrix which restricts the water uptake by plants.

H_2O_2 has important role in redox signaling in regulating normal processes, including oxidative stress (Rhee 2006). H_2O_2, a product of SOD reaction was a strong oxidant and it initiates localized oxidative damage leading to disruption of metabolic function and losses of cellular integrity at sites where it accumulates. Excessive levels of H_2O_2 could be minimized through the activities of CAT and different peroxidases. While mitochondria and plastids are the major sources of H_2O_2 in the cells, peroxisomes and glyoxisomes also contain SOD, CAT and APX, which are responsible for its production and scavenging (Jimenez et al. 1997; Yamaguchi et al. 1995). CAT and APX, two potential scavengers of H_2O_2, maintain its level and prevented uncontrolled export of this toxic species from organelles to cytosol and competed to remove H_2O_2 (Valyova et al. 2012). GR, one of the important enzymes in ascorbate-glutathione cycle, catalyzes the NADPH-dependent reduction of oxidized glutathione and is important in protecting many plants from oxidative stress caused by salt stress (Foyer et al. 1991). SOD activity level was found to correlate with the change in the H_2O_2 content. These results suggest that H_2O_2 increase probably results from the association of increased SOD (which produces H_2O_2), APX (which remove H_2O_2) and GR activities but not CAT activity, in addition to other possible mechanisms not investigated in the present

experiment. Increased SOD, GR and APX activities agree with previous reports in moderately salinity tolerant wheat genotype (Sairam et al. 2005) and in pea leaves (Hernández and Almansa 2002), whereas in tobacco cultivar By-2, where salt stress had no effect on SOD activity (Hoque et al. 2007). Some reports showed that salt stresses results in an increase in the CAT activity, such as in the wild salt-tolerance tomato (*Lycopersicon pennellii*) (Mittova et al. 2004) and sea plantain (*Plantago maritima*) (Sekmen et al. 2007). But some reports agree with results showing, a decreased CAT activity in the rice cultivar *Dongjin* seedlings under drought stress (Lee et al. 2001), mung bean (*Vigna radiata*) (Hossain et al. 2011) and tobacco cultivar By-2 (Hoque et al. 2007) under salt stress (Hasanuzzaman et al. 2011). So an increased H_2O_2 generation in the leaves of salt-stressed rice plants may function in the signaling of oxidative stress, which leads to induction of some enzymes.

Although salinity treatments caused significant increase in H_2O_2 content and lipid peroxidation in plants, but the transgenic rice improved growth under salinity by alleviating oxidative stress, H_2O_2 content and lipid peroxidation decreased while DPPH radical scavenging activity, RGR, photosynthetic pigments and activities of antioxidant enzymes associated with the H_2O_2 scavenging system increased when compared to the control transgenic and wild-type plants. This suggests that *OsCaM1-1* could protect the plants from toxic effects of NaCl by reducing the H_2O_2 content. Overexpression of *OsCaM1-1* gene in transgenic rice plants has showed improvement in protection against oxidative stress. The results show that enzyme activities include SOD, APX and GR in all plants were increased by salinity and were higher in transgenic plants than the control transgenic and wild-type KDML105 plants. CAT was also increased in transgenic plants under salinity but it decreased in control and wild-type. The

decreasing of CAT by salt stress in the control and wild-type plants may indicate that the plant is not able to maintain protection against active oxygen under salt stress particularly at high salt concentrations and may be due to salt stress-induced damage to the enzyme. Further, overexpression of *OsCaM1-1* gene may induce cooperation of protective enzymes such as SOD, CAT, and APX to eliminate ROS which increased by salt stress and keep a homeostasis between producing and cleaning of ROS and reduce the level of free radicals. This way, injury to cells could be decreased or avoided increasing ROS.

In this study, GR activity was also increased in all plants, particularly higher in transgenic plants when compared to control and wild-type under salt stress. This result suggests that *OsCaM1-1* gene may help transgenic plants more active in exhibiting ascorbate-glutathione cycle and reducing H_2O_2.

Photosynthetic pigments, chlorophyll content such as total chl, chl *a* and chl *b* and also carotenoid contents were decreased in all plants due to salt stress, but no significant changes in the transgenic plants when compared to the control and wild-type plants. The effect of salinity on photosynthetic pigment contents as depletion may be considered to be a result of the increasing activity of the chlorophyll-degrading enzyme chlorophyllase (Reddy 1986) leading to degradation of β-carotene and formation of zeaxanthins, which are apparently involved in protection against photoinhibition (Sharma and Hall 1991). Rice plants overexpressing the *OsCaM1-1* gene show enhanced tolerance to salinity stress, as indicated by the higher chlorophyll and carotenoid contents. These results may suggest that *OsCaM1-1* gene protect photosynthetic apparatus from salt induced oxidative stress and helps the transgenic plants maintain their photosystem better than the control and wild-type. The chlorophyll

content was protected probably because of the high antioxidant enzyme activities that prevented degradation of leaf chlorophyll (Sevengor 2011).

Furthermore, the loss of chlorophylls under salt stress could be related to photoinhibition or ROS formation, as demonstrated by the increased lipid peroxidation (Kato and Shimizu 1985). The significant raise in MDA content in the control and wild-type may be due to decrease of salt dependant up regulation of its antioxidant enzyme system under salinity stress. However, the three transgenic plants overexpressing *OsCaM1-1* gene showed lesser MDA levels than the control and wild-type plants. This result shows that overexpression of *OsCaM1-1* gene had higher capacity for the scavenging of ROS generated by salinity than the control and wild-type. Scavenging of DPPH free radical is the basis of a common antioxidant assay (Sharma and Bhat 2009). Here, DPPH radical scavenging activity showed a capacity of plants to fight stress, especially the *OsCaM1-1* overexpressing plants. Overexpression of *OsCaM1-1* gene may promote the transgenic plants by induced scavenging activity to increase the antioxidant capacity to detoxify ROS. The RGR of the *OsCaM1-1* overexpressing plants was found to be maintained better than that of the control and wild-type plants when grown under salt stress. These results indicate that overexpression of the *OsCaM1-1* gene also helps improve growth and salt stress tolerant in transgenic plants.

Besides, one of the early symptoms of salinity stress in plant tissue is the decrease of RWC. Extent of salt-induced effects on RWC has been used as one the vital water relation parameters for assessing degree of salt tolerance in plants (Noreen and Ashraf 2008; Siddiqi et al. 2009). RWC in the leaves of plants grown under salinity stress decreased significantly in all plants compared to those grown in non-saline conditions. This reduction of RWC in stressed plants may be associated with a decrease

in plant vigor and was observed in many plant species such as rice and kidney bean (Halder and Burrage 2003; Lopez et al. 2002). However, the three transgenic plants overexpressing *OsCaM1-1* showed less decline in RWC than the control and wild-type plants. These results suggest that plants overexpression of the *OsCaM1-1* helps plants maintain ability to absorb more water from the nutrient.

Previous report has shown that *OsCaM1-1* mRNA levels strongly increased in response to salt treatment (Phean-o-pas et al. 2005). And Saeng-ngam and workers (2012) also showed that *OsCaM1*signaling is likely to play an important role in ABA biosynthesis (ABA levels have been reported to be key messengers in salt-stress responses (Ghassemian et al. 2008; Guo et al. 2008; Hong-Bo et al. 2008; Mahajan et al. 2008)) and the level of *OsCaM1-1* gene expression, presumably contribute to salt resistance in rice. These results suggest that the *OsCaM1-1* gene product functions as a sensor for salt stress-induced calcium signals that helps the plant to cope with salt stress. The results showed that at high salinity, the three transgenic plants overexpressing *OsCaM1-1* gene had significantly higher scavenging activity than the control and wild-type, indicating that the transgenic plants still have more capacity to cope with salt stress.

CHAPTER V

CONCLUSIONS

Calcium signaling has been implicated in transducing signals from environmental changes into adaptive responses in plants. However, the mechanisms of how calcium signals are used to mediate stress responses have not been fully understood. Here, another possible roles of the *OsCaM1-1* gene which its expression is highly induced were investigated the antioxidative system. Transgenic rice plants overexpressing *OsCaM1-1* gene exhibited higher in antioxidant enzyme activities, photosynthetic pigments, ROS scavenging activity including growth rate and RWC than the control and wild-type KDML105 plants under salinity. The results obtained here suggested that the *OsCaM1-1* gene product may function as sensor for salt stress-induced calcium signals that lead to increasing the activity of antioxidant system, which in turn helps the plant has a better protection against salt stress.

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APPENDICES

APPENDIX A

Chemical solution

1. Extraction buffer

H_2O_2 extraction buffer

0.05 M Potassium phosphate buffer, pH 7.0

KH_2PO_4	0.68	g
K_2HPO_4	0.87	g

Adjust pH utilizing both solutions, and make up the volume to 100 ml with distilled water.

Antioxidant enzyme extraction buffer

0.5 M Sodium phosphate buffer, pH 7.5

NaH_2PO_4	6.0	g
Na_2HPO_4	7.1	g

Adjust pH utilizing both solutions, and make up the volume to 100 ml with distilled water.

1 mM EDTA

EDTA	0.04	g

Dissolve in distilled water and make up the volume to 100 ml.

1 M DTT

DTT	0.15	g

Dissolve in distilled water and make up the volume to 1 ml.

5 mM Ascorbic acid

Ascorbic acid	0.09	g

Dissolve in distilled water and make up the volume to 100 ml.

Working solution:

0.5 M Sodium phosphate buffer, pH 7.5	20	ml
1 mM EDTA	50	ml
1 M DTT	50	µl
5 mM Ascorbic acid	20	ml
Protease inhibitor	100	µl

Make up the volume to 100 ml with distilled water and store at 4 °C.

2. Measurement of H_2O_2 content

1% $TiCl_4$ in conc. HCl

$TiCl_4$	0.58	ml
conc. HCl	99.42	ml

Prepare on ice in the fume hood and store at 4 °C in a dark place.

3. Antioxidant enzyme assay

0.5 M Potassium phosphate buffer, pH 7.0

KH_2PO_4	6.8	g
K_2HPO_4	8.7	g

Adjust pH utilizing both solutions, and make up the volume to 100 ml with distilled water.

0.5 M Potassium phosphate buffer, pH 7.8

KH_2PO_4	6.8	g
K_2HPO_4	8.7	g

Adjust pH utilizing both solutions, and make up the volume to 100 ml with distilled water.

10 mM GSSG

GSSG	0.006	g

Dissolve in distilled water and make up the volume to 1 ml and store at 4 °C in a dark place.

1 M H_2O_2

30% H_2O_2	1.01	ml

Dissolve in distilled water and make up the volume to 10 ml and store at 4 °C in a dark place.

133.3 mM Methionine

L-Methionine	2.0	g

Dissolve in distilled water and make up the volume to 100 ml.

30 mM $MgCl_2$

$MgCl_2$	0.3	g

Dissolve in distilled water and make up the volume to 100 ml.

1.5 mM NADPH

NADPH	0.001	g

Dissolve in distilled water and make up the volume to 1 ml and store at 4 °C in a dark place.

7.5 mM NBT

NBT	0.006	g

Dissolve in distilled water and make up the volume to 1 ml.

0.5 M Na_2CO_3

Na_2CO_3	5.3	g

Dissolve in distilled water and make up the volume to 100 ml.

0.2 mM Riboflavin

Riboflavin	0.008	g

Dissolve in distilled water and make up the volume to 100 ml and in a dark place.

4. Measurement of DPPH radical scavenging activity

0.5 mM DPPH

DPPH	0.02	g

Dissolve in absolute ethanol and make up the volume to 100 ml and store at 4 °C in a dark bottle.

5. Preparation for protein determination by Bradford (1976)

Bradford solution

Coomassie Brilliant Blue (G250)	0.02	g
Absolute ethanol	10	ml

Stir the solution in Erlenmeyer flask protected from light for 2 hours then add 20 ml of 85% phosphoric acid. Bring the volume to 200 ml with distilled water and filter through Whatman filter paper. Store the solution in a brown glass bottle (usable for several weeks).

6. Preparation of NB medium

Reagents:

			Final concentrations (1X)
100X NB Nitrate stock 1 L			
$(NH_4)_2SO_4$	46.3	g	(463 mg/l)
KNO_3	283.0	g	(2,830 mg/l)
100X NB Sulfate stock 1 L			
$MgSO_4.7H_2O$	18.5	g	(185 mg/l)
$MnSO_4.H_2O$	1.0	g	(10 mg/l)
$ZnSO_4.7H_2O$	200	mg	(2 mg/l)
$CuSO_4.5H_2O$	2.5	mg	(0.025 mg/l)
100X NB Halide stock 1 L			
$CaCl_2.2H_2O$	16.6	g	(166 mg/l)
KI	75.0	mg	(0.75 mg/l)
$CoCl_2.6H_2O$	2.5	mg	(0.025 mg/l)

100X NB PBMO stock 1 L

KH_2PO_4	46.0	g	(460 mg/l)
H_3BO_3	300	mg	(3 mg/l)
Na_2MoO_4	25.0	mg	(0.25 mg/l)

100X NB NaFeEDTA stock 1 L

$FeSO_4.7H_2O$	2.78	g	(27.8 mg/l)
Na_2EDTA	3.78	g	(37.8 mg/l)

Nicotinic acid stock (1 mg/ml)

Dissolve 100 mg of nicotinic acid in sterilized deionized H2O, adjust volume to 100 ml. Filter sterilize the solution.

Pyridoxine stock (1 mg/ml)

Dissolve 100 mg of pyridoxine in sterilized deionized H2O, adjust volume to 100 ml. Filter sterilize the solution.

Thiamine stock (1 mg/ml)

Dissolve 1 g of thiamine in sterilized deionized H2O, adjust volume to 100 ml. Filter sterilize the solution.

Mix the followings:

			Final concentrations (1X)
100X NB Nitrate stock	10	ml	
100X NB Sulfate stock	10	ml	
100X NB Halide stock	10	ml	
100X NB PBMO stock	10	ml	
100X NB NaFeEDTA stock	10	ml	
Myo-inositol	100	mg	(100 mg/l)
Nicotinic acid stock (1 mg/ml)	1	ml	(1 mg/l)
Pyridoxine stock (1 mg/ml)	1	ml	(1 mg/l)
Thiamine stock (1mg/ml)	1	ml	(10 mg/l)
Casein hydrolysate	300	mg	(300 mg/l)
L-Proline	500	mg	(500 mg/l)
L-Glutamine	500	mg	(500 mg/l)
Sucrose	30	g	(30 g/l)

Adjust to pH 5.8 and add 8.0 g of agar, then make up the volume to 1 L with distilled water. Finally, autoclave for 20 minutes at 121 °C.

7. Limpinuntana's nutrient solution

Reagents:

		Final concentrations (1X)
300X Solution A (1 L)		
KNO_3	30.333 g	(0.10 g/l)
$Ca(NO_3)_2.4H_2O$	47.230 g	(0.16 g/l)
300X Solution B (1 L)		
$MgSO_4.7H_2O$	12.324 g	(41 mg/l)
$NH_4H_2PO_4$	11.502 g	(38 mg/l)
NaCl	16.577 g	(55 mg/l)
300X Solution C (1 L)		
$FeSO_4.7H_2O$	6 g	(20 mg/l)
Na_2EDTA	8 g	(27 mg/l)
300X Solution D (1 L)		
$MnCl_2.4H_2O$	0.4323 g	(1.44 mg/l)
H_3BO_3	0.3420 g	(1.14 mg/l)
$Na_2MoO_4.2H_2O$	0.0075 g	(0.025 mg/l)

$ZnSO_4.7H_2O$	0.0264 g	(0.088 mg/l)
$CuSO_4.5H_2O$	0.0117 g	(0.039 mg/l)

1X Limpinuntana's solution (300 ml)

Mix solution A, B, C and D together (1 ml each), then add deionized water to 300 ml and autoclave for 20 minutes at 121 °C.

APPENDIX B

Calculations

1. SOD activity

$$\text{Specific activity} = \frac{1}{A_{560}\,(\text{control})\,/2} \times A_{560}\,(\text{sample})$$

One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT monitored at 560 nm.

For example:

SOD activity of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

$A_{560\ control} = 0.9473$, $A_{560\ sample} = 0.6554$

$$\text{Specific activity} = \frac{1}{0.9473\,/2} \times 0.6554$$

$$= 1.38 \text{ U}/ 0.05 \text{ mg protein}$$

SOD specific activity for 1 mg protein is 27.66 U/mg protein

2. CAT activity

$$\text{Specific activity} = \frac{A_{240}/\text{min} \times \text{total volume} \times 1{,}000}{\varepsilon \times \text{protein content (0.05 mg)}}$$

One unit of CAT activity was defined as the amount of enzyme to reduce 1µmol of H_2O_2 per min at 240 nm (ε = 39.4 $mM^{-1}cm^{-1}$).

For example:

CAT activity of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

A_{240}/min = 0.1692

$$\text{Specific activity} = \frac{0.1692 \times 1.2 \text{ ml} \times 1{,}000}{39.4\ mM^{-1}cm^{-1} \times 0.05 \text{ mg}}$$

$$= 103.07\ \mu\text{mol/min/mg protein}$$

3. APX activity

$$\text{Specific activity} = \frac{A_{290}/\text{min} \times \text{total volume} \times 1{,}000}{\varepsilon \times \text{protein content (0.05 mg)}}$$

One unit of APX activity was defined as the amount of enzyme to oxidize 1 μmol of ascorbate per min at 290 nm ($\varepsilon = 2.8\ mM^{-1}cm^{-1}$).

For example:

APX activity of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

$A_{290}/\text{min} = 0.0383$

$$\text{Specific activity} = \frac{0.0383 \times 1.2\ \text{ml} \times 1{,}000}{2.8\ mM^{-1}cm^{-1} \times 0.05\ \text{mg}}$$

$$= 328.28\ \mu\text{mol/min/mg protein}$$

4. GR activity

$$\text{Specific activity} = \frac{A_{340}/\text{min} \times \text{total volume} \times 1{,}000}{\varepsilon \times \text{protein content (0.05 mg)}}$$

One unit of GR activity was defined as the amount of enzyme to reduce GSSG to GSH per min at 340 nm (ε = 6.22 $mM^{-1}cm^{-1}$).

For example:

GR activity of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

A_{340}/min = 0.0129

$$\text{Specific activity} = \frac{0.0129 \times 1.2 \text{ ml} \times 1{,}000}{6.22 \text{ mM}^{-1}\text{cm}^{-1} \times 0.05 \text{ mg}}$$

$$= 50.16 \text{ µmol/min/mg protein}$$

5. Measurement of RGR

$$RGR = (\ln W_f - \ln W_i) / (t_f - t_i)$$

where, W_f and W_i are the shoot and root dry weight of plant, t_f and t_i are the times and subscripts denote initial and final sampling that is, day 0 (before salt stress treatment) and day 3 (after 3 days salt stress treatment).

For example:

RGR of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

$W_i = 0.022$ g, $W_f = 0.025$ g

$$RGR = (\ln 0.026 - \ln 0.021) / (3 - 0)$$

$$= 0.07 \text{ g} \cdot \text{g}^{-1} \cdot \text{d}^{-1}$$

6. Measurement of RWC

$$RWC = [(W_i - W_d)/(W_f - W_d)] \times 100$$

where, W_i is the leaf initial weight, W_f is the leaf fully turgid weight, and W_d is the leaf dry weight.

For example:

RWC of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

W_i = 0.0040 g, W_f = 0.0043 g, W_d = 0.0003 g

$$RWC = [(0.0040 - 0.0003)/(0.0043 - 0.0003)] \times 100$$

$$= 92.5\ \%$$

7. Measurement of photosynthetic pigments

Total chlorophyll (a and b) (mg/l) = 17.90 (A_{647}) + 8.08 (A_{664})

Chlorophyll a (mg/l) = 12.70 (A_{664}) - 2.79 (A_{647})

Chlorophyll b (mg/l) = 20.70 (A_{647}) – 4.62 (A_{664})

Carotenoids (mg/l) = A_{461} x 200

The pigment concentrations were calculated in mg/g FW of sample.

For example:

Photosynthetic pigments of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

A_{461} = 1.1106, A_{647} = 0.4251, A_{664} = 1.2101

Total chlorophyll (a and b) (mg/l) =17.90 (0.4251) + 8.08 (1.2101) = 17.39 mg/l

Chlorophyll a (mg/l) =12.70 (1.2101) - 2.79 (0.4251) = 14.18 mg/l

Chlorophyll b (mg/l) =20.70 (0.4251) – 4.62 1.2101) = 3.37 mg/l

Carotenoids (mg/l) =1.1106 x 200 = 222.12 mg/l

Total chl 17.39 mg/l = 17.39 µg/ml

= 17.39 µg/0.05 g sample

= 347.8 µg/g sample

The total volume of extraction is 8 ml

Total chl = 347.8 x 8

= 2782.4 µg/g

= 2.78 mg/g FW

***For chl *a*, chl *b* and carotenoids, the results are calculated using the same method.

8. Measurement of lipid peroxidation

$$\text{TBARS content (µmol MDA equivalents g FW)} = \frac{(A_{532}-A_{600}) \times Vt \times Vr \times 100}{\varepsilon \times Vs \times m}$$

where, Vt is the total volume of extract solution

Vr is the total volume of reaction mixture

Vs is the volume of the extract solution contain in the reaction mixture

m is the weight of sample

ε is the extinction coefficient (155 $mM^{-1}cm^{-1}$)

For example:

MDA content of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

A_{532} = 0.1547, A_{600} = 0.1013

$$\text{TBARS content (µmol MDA equivalents g FW)} = \frac{(0.1547-0.1013) \times 1 \text{ ml} \times 3 \text{ ml} \times 100}{155 \times 0.075 \text{ ml} \times 0.05 \text{ g}}$$

$$= 0.028 \text{ µmol/g FW}$$

9. Measurement of DPPH radical scavenging activity

$$AA\% = \frac{(A_{control} - A_{test})}{A_{control}} \times 100$$

For example:

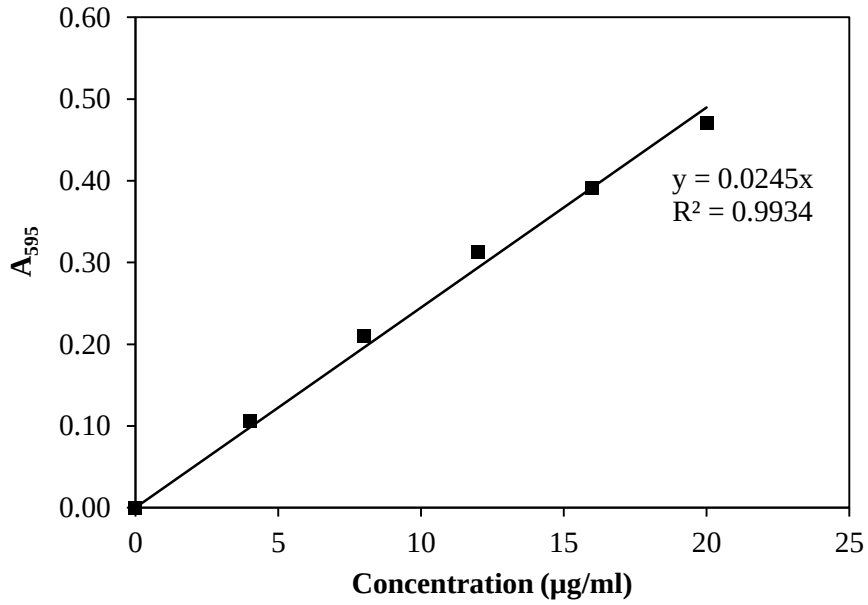
DPPH content of wild-type KDML105 plants under without salt (0 mM NaCl) for 3 days

A_{517} control = 0.7596, A_{517} test = 0.6705

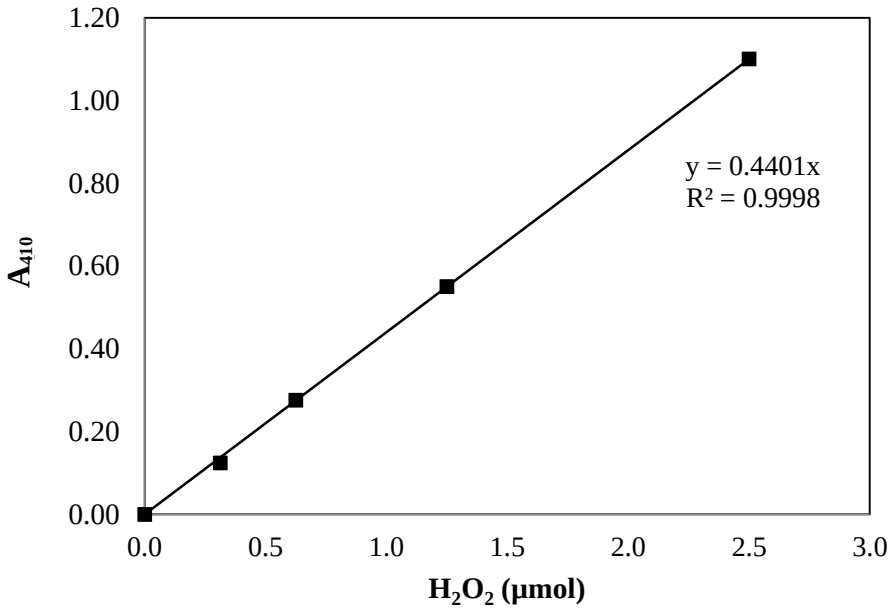
$$AA\% = \frac{(0.7596 - 0.6705)}{0.7596} \times 100$$

$$= 11.73\ \%$$

10. Calibration curve for protein content of standard BSA



11. Calibration curve of standard H_2O_2



VITA

Miss Taviporn Kaewneramit was born on December 29, 1988 in Samutsongkhram. She graduated with the degree of Bachelor of Science from Department of Chemistry, Faculty of Science, Silpakorn University in 2011. She keeps on studying for Master degree of Science at the Biochemistry Program, Faculty of Science at Chulalongkorn University.