การตอบสนองต่อออกซิเดชันในข้าว *Oryza sativa* L. พันธุ์ขาวดอกมะลิ 105
ทรานสเจนิกที่มียีน *OsCaM1-1* แสดงออกเกินปกติภายใต้ความเครียดจากความแห้งแล้ง

นางสาวปัญญ์ แสงไชย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2560

OXIDATIVE RESPONSES IN TRANSGENIC ‘KDML105’ RICE *Oryza sativa* L. OVEREXPRESSING *OsCaM1-1* AGAINST DROUGHT STRESS

Miss Pun Sangchai

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Biochemistry and Molecular Biology
Department of Biochemistry
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Thesis Title	OXIDATIVE RESPONSES IN TRANSGENIC 'KDML105' RICE *Oryza sativa* L. OVEREXPRESSING *OsCaM1-1* AGAINST DROUGHT STRESS
By	Miss Pun Sangchai
Field of Study	Biochemistry and Molecular Biology
Thesis Advisor	Assistant Professor Nuchanat Wutipraditkul, Ph.D.

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ปัญญ์ แสงไชย : การตอบสนองต่อออกซิเดชันในข้าว *Oryza sativa* L. พันธุ์ขาวดอกมะลิ 105 ทรานสเจนิกที่มียีน *OsCaM1-1* แสดงออกเกินปกติภายใต้ความเครียดจากความแห้งแล้ง (OXIDATIVE RESPONSES IN TRANSGENIC 'KDML105' RICE *Oryza sativa* L. OVEREXPRESSING *OsCaM1-1* AGAINST DROUGHT STRESS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นุชนาถ วุฒิประดิษฐกุล , 95 หน้า.

OsCaM1-1 คือโปรตีนแคลมอดุลินที่พบในข้าว ทำหน้าที่แปลงสัญญาณแคลเซียมอิออนไปยังโปรตีนเป้าหมายซึ่งมีความสำคัญต่อการควบคุมเซลล์และการตอบสนองทางสรีรวิทยา ภาวะเครียดจากความแห้งแล้งเป็นภาวะเครียดจากสิ่งแวดล้อมที่สำคัญอย่างหนึ่งที่ส่งผลกระทบต่อพืชผลโดยเปลี่ยนแปลงลักษณะทางสัณฐานวิทยา สรีรวิทยา และชีวเคมีของพืช ในครั้งนี้ได้ศึกษาถึงผลของภาวะเครียดจากความแห้งแล้งต่อการเจริญเติบโตและระบบต้านออกซิเดชันในข้าวขาวดอกมะลิ 105 พันธุ์ทรานสเจนิกที่มียีน *OsCaM1-1* แสดงออกเกินปกติ โดยเปรียบเทียบกับข้าวพันธุ์ควบคุมและข้าวพันธุ์ดั้งเดิม พบว่าข้าวทรานสเจนิกที่มีการแสดงออกเกินปกติของยีน *OsCaM1-1* สามารถเพิ่มความต้านทานต่อภาวะเครียดจากความแห้งแล้ง ผลการทดลองแสดงการเพิ่มขึ้นของอัตราการเจริญเติบโตสัมพัทธ์ ปริมาณน้ำสัมพัทธ์ ปริมาณรงควัตถุที่จำเป็นต่อการสังเคราะห์ด้วยแสง อัตราส่วนโพแทสเซียมอิออนต่อโซเดียมอิออน และอัตราส่วนแคลเซียมอิออนต่อโซเดียมอิออน ทั้งยังมีการเพิ่มขึ้นของอัตราการต้านอนุมูลอิสระดีพีพีเอช การสะสมสารที่สามารถป้องกันแรงดันออสโมติก (เช่น โปรลีนและน้ำตาลที่ละลายน้ำได้) และแอกติวิตีของซูเปอร์ออกไซด์ดิสมิวเทส เพอร์ออกซิเดส และคาทาเลส ขณะที่ปริมาณมาลอนไดอัลดีไฮด์และเปอร์เซ็นต์การรั่วไหลของสารอิเล็กโทรไลต์ลดลงต่ำกว่าพันธุ์ควบคุมและพันธุ์ดั้งเดิม จากการทดลองดังกล่าวแสดงให้เห็นว่าการแสดงออกเกินปกติของยีน *OsCaM1-1* อาจจะปรับระบบต้านออกซิเดชันให้ดีขึ้นภายใต้ภาวะแห้งแล้ง

ภาควิชา ชีวเคมี ลายมือชื่อนิสิต .....

สาขาวิชา ชีวเคมีและชีววิทยาโมเลกุล ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2560

5972006923 : MAJOR BIOCHEMISTRY AND MOLECULAR BIOLOGY
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PUN SANGCHAI: OXIDATIVE RESPONSES IN TRANSGENIC 'KDML105' RICE *Oryza sativa* L. OVEREXPRESSING *OsCaM1-1* AGAINST DROUGHT STRESS. ADVISOR: ASST. PROF. NUCHANAT WUTIPRADITKUL, Ph.D., 95 pp.

The OsCaM1-1 is a rice calmodulin protein which transduces Ca^{2+} signals to their target protein that is important to regulate cellular and physiological responses. Drought stress is the one of essential environmental stresses that affected the crop by changing in morphological, physiological, and biochemical direction tactics of plants. In this study, the effect of drought stress on growth and antioxidant system in the transgenic rice overexpressing *OsCaM1-1* gene was investigated and compared to the control and wild type KDML 105. The overexpression of *OsCaM1-1* in transgenic rice could increase plant tolerance to drought stress, the results showed the greater increase in the relative growth rate, the relative water content, photosynthetic pigment contents, K^+/Na^+ ratio, and Ca^{2+}/Na^+ ratio, besides, presented the better increase in DPPH scavenging rate, the osmoprotectant accumulation (such as proline and soluble sugar), and activity of superoxide dismutase, peroxidase, and catalase, while malondialdehyde content and % electrolytic leakage were lower than the control and wild type plants. These results indicated that the overexpressing *OsCaM1-1* in transgenic rice probably improved antioxidant system under drought stress.

Department:	Biochemistry	Student's Signature	.....
Field of Study:	Biochemistry and Molecular Biology	Advisor's Signature	.....
Academic Year:	2017		

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CHAPTER 1

INTRODUCTION

Khao Dawk Mali 105 rice

Rice (*Oryza sativa* L.) is an essential cuisine in Thailand. Thai rice has various types also Khao Dawk Mali 105 (KDML 105) or Jasmin rice is one of them. KDML 105 rice recognizes as aromatic rice which fragrance likes pandan. It has long grain and is most famous texture and flavor in the world. KDML 105 is high quality and most planted in Thailand. The central and north eastern regions such as Burirum, Roi Et, Sisaket, Surin, Ubon Ratchathani and Yasothon are the most appropriate geographic place in term of environment parameters for instance abundant daylight all over the year, and total precipitation so KDML 105 can be planted just only in these regions where were the principal rice exporter of Thailand. In 1945, Chonburi province is the first place in Thailand that discovered KDML rice which was called 'White Jasmin' in Thai words. Next, KDML species was selected and succeeded analyses by Ministry of Agriculture in Lopburi province, the chosen KDML was tried to plant in the northern and north eastern areas of Thailand. Until 25th May 1959, this rice species was formally declared in the name of 'KDML 105' or 'Thai Hom Mali Rice' that is the most essential consumer goods in Thailand.

An important ration of the Thai economy and labor force can be represented by a rice yield. Thai work represents 40% in cultivation and most of them are rice peasants. Rice production is an old heritage of Thailand where was the world's second largest of rice exporter. Now a day, many areas around the world farm

and produce large quantities in rice. Currently, the enlargement of the area that available for rice planting is the future project of Thailand. In 2017, the Food and Agriculture Organization of the United Nations (FAO) forecast reported that paddy rice can be produced in Thailand 33.7 million tonnes and the amount of rice exports is 10.5 million tonnes that is more than the amount average of 2012-2016 and increased from the last year's quantity (Figure 1.1).

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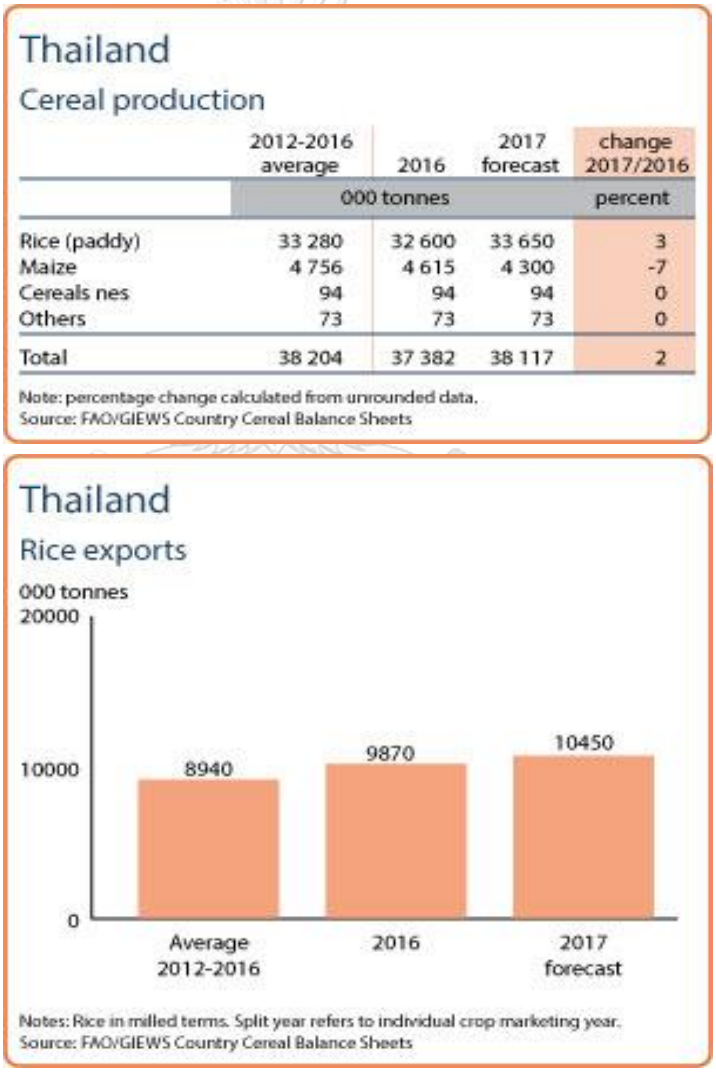


Thailand

Cereal production

	2012-2016 average	2016	2017 forecast	change 2017/2016
	000 tonnes			percent
Rice (paddy)	33 280	32 600	33 650	3
Maize	4 756	4 615	4 300	-7
Cereals nes	94	94	94	0
Others	73	73	73	0
Total	38 204	37 382	38 117	2

Note: percentage change calculated from unrounded data.
Source: FAO/GIEWS Country Cereal Balance Sheets

Figure 1.1 Thailand cereal production and rice exports (Reference Date: 31-July-2017) (http://www.fao.org/giews/countrybrief/country.jsp?code=THA)

Stress

In recent years, global warming leads to environmental stresses such as drought, heat, salinity, or cold stress that affecting in crop, living, and yield of plants (Basu, 2016; Hamanishi, 2011; Wilkins, 2009). Plants live in consistently shifting environments that are often disadvantageous or stressful for growing and developing. These prejudicial environmental conditions comprise of biotic stress such as pathogen infection and herbivore attack and abiotic stress like drought, heat, cold, nutrient default, overhang of salt, and excess of toxic metals in the soil. Drought, salt, and temperature stresses are key environmental factors that have an impact on the geographic plant division in nature, restrict crop plant productivity, and terrorize food stability. The adverse influence of these abiotic stresses is displeased by changing of climate, which results in an enhance frequency of inordinate weather (Fedoroff et al., 2010).

Drought stress

Water is a highly limited resource that is a significant factor in food and crop production. Water deficit stress results widespread losing to crop production worldwide, therefore it is a serious threat to sustainable crops. Crop yield that relies on particular climate conditions is highly affected by climate differentiation. Climate change has a large impact on water resources, and the frequency of drought problems seems to increase in future. Over the last three decades, the total rice yield was variated owing to climate variation (Ray, 2015). The changing in yield of global rice rate is shown in Figure 1.2 (Ray, 2013). The demands to remedy the rice adaptation to drought stress and to consider the breed resistance are increasing with reducing water

supplies for agriculture around the world (Serraj, 2009). Drought tolerance is a complicated determinant which combines with a function of all kind of morphological, biochemical and molecular appearances according to the mechanisms related to drought stress resistance and the regulating plant adaptation systems to drought stress have been widely studied (Pandey, 2015).

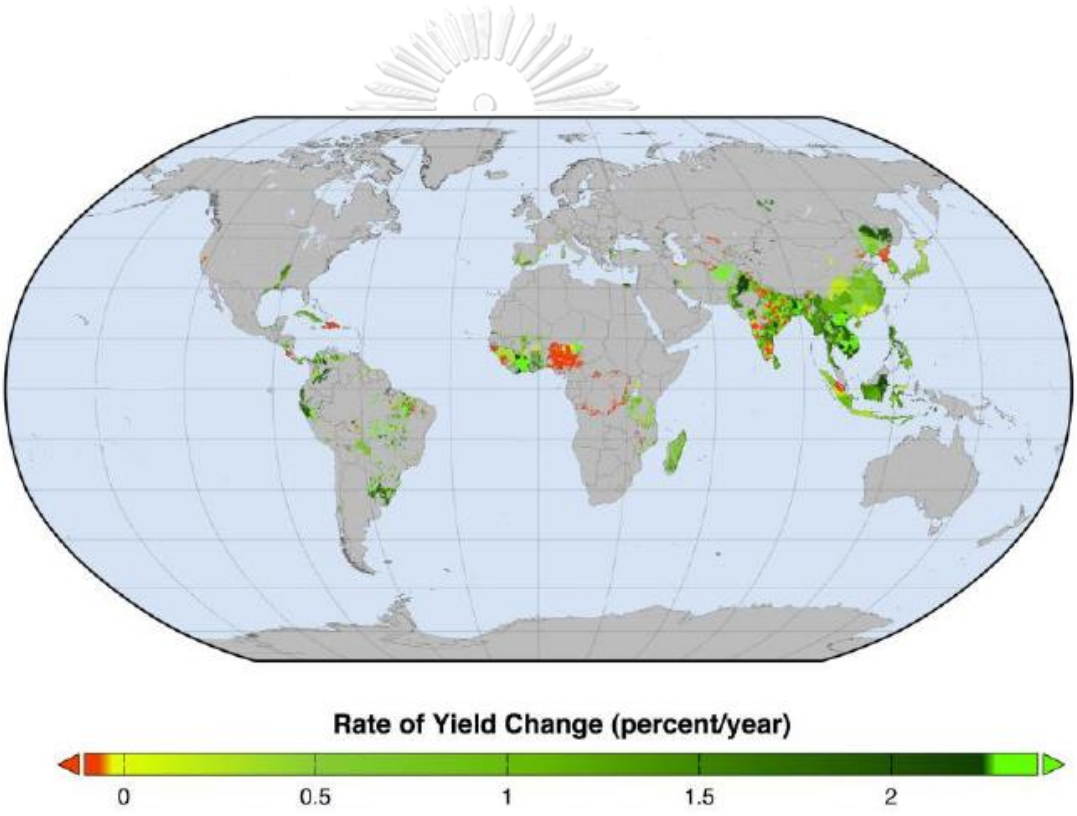


Figure 1.2 Global map expressing percentage of changing rate in rice yield. Where red zones show where yields decrease while the green zones show where rates of yields increase (Ray, 2013).

Drought stress is one of the detrimental abiotic stresses that affect the crop yields, plant growth, and development (Austin, 1989; Bottner, 1995; Pereira, 1993). It disturbs the ordinary functions of plant by impacting on water potential and turgor and affecting regular growth parameters such as fresh weight and water content (Betteridge, 2000; Jaleel, 2009). Drought causes cell membrane damage, stomatal closure which decreases the rate of photosynthesis, annoys activity of a variety of enzymes, and bears upon the nutrient transportation by water from soil to plant roots as well as introduces reactive oxygen species and free radicals which influence on antioxidant remedy. However, plants have strategies for drought tolerance such as relieve water wastage, decrease water diffusion losses, increase osmolytes as proline, glycinebetaine, organic acid which are low molecular weight compound and play important roles in supporting function of plant cell under drought condition. Moreover, plants promote various enzyme activities that display as antioxidant enzymes (Farooq, 2012; Vurukonda, 2016). Hence, drought stress is a negative effect on plant growth and it is one of the essential limitation crop yields worldwide that derogates growth and development and infests water status in plants.

Effects of drought on morphological characteristics

Plants experience drought when the demand of water supply to roots gets difficulty or when the rate of dehydration is extremely high. When water stress emerges, plants react by decelerating down or stop growing. This serves as a survival technique that is normal plant reaction to drought stress (Zhu, 2002). An expanding number of studies in term of preliminary morphological changes in rice when exposure to drought. Drought persuades reduction in plant growth and development

that has been reported in rice (Manickavelu, 2006; Tripathy, 2000). The cell growth is severely defected owing to the turgor pressure reduces in plants under drought condition. Drought has an effect on both of elongation and expansion of plants (Shao, 2008), stops cell enlargement more than cell division (Jaleel, 2009), degrades the proliferation rate of rice seedlings and reduces plant biomass production (Farooq, 2010). These studies suggest that drought significant decrease in fresh and dry weights of shoots (Centritto, 2009; Ji, 2012; Mostajeran, 2009).

Effects of drought on physiological characteristics

Drought stress influences in many ways of physiological procedures and induces in various physiological responses of plants, which encourage the plant adaptation under unfavorable environment. Under water stress, the efficiency escalation of physiological procedures is prerequisite to increase the water productivity (Serraj, 2009). The understanding of the physiological responsiveness of rice under drought condition probably contribute to continuous studies on drought tolerance in rice. An essential physiological responsiveness of plants to drought is their ability in turgor pressure maintenance by decreasing osmotic potential which is a resistant mechanism. Water deficit impacts on physiological responses with many ways such as it reduces relative water content and membrane stability index in rice (Akram, 2013; Farooq, 2010).

Effects of drought on biochemical characteristics

Under drought stress, plants assemble various types of organic and inorganic solutes lower osmotic potential in cytosol that causes keeping cell turgor (Rhodes,

1994). This biochemical process also knows as osmotic adjustment which is accomplished by accumulating in proline, sucrose, glycinebetaine and other solutes in cytosol that enlarges improving water absorption from the drought soil. Proline is the most popularly studied because it is important to drought stress tolerance. Drought also powerfully increases in the soluble sugars accumulation depending on the rate of plant water deficit. (Maisura, 2014; Shehab, 2010) as well as increases in the antioxidant activity which contributes to drought tolerance by detoxifying and scavenging reactive oxygen species.

Reactive oxygen species

Reactive oxygen species (ROS) are collection of ions, free radical compounds, and reactive molecules. ROS such as superoxide anions ($O_2^{\bullet -}$), hydroperoxyl radicals (HO_2.), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) which are reactive chemical compounds that descend from the lessening of oxygen molecule. ROS have been generated for early responding of plant cells to various stresses. The amount of 1% of oxygen they consume by plant was shifted to form ROS in different places such as peroxisomes, chloroplasts, and mitochondria. ROS play as dangerous or efficacious roles depending on their amount in plant cells. At high content of ROS, plant cells have been damaged to many levels of organism by lipid membrane peroxidation, cellular membrane degradation, protein derogation, enzyme inactivation, and gene expression alteration (Hendry, 2005; Jajic, 2015; Nair, 2008; Sgherri, 2000; Smirnoff, 1993). Thereby, ROS respond easily to lipid cellular membrane, DNA, and proteins resulting in cell aging and cell death (Beckman, 1998) (Figure 1.3). However, at down or middling concentration ROS do as secondary

messenger of intracellular signal transduction that intercede many responses in plants. ROS have a signaling capacity that can alter the gene transcription which accommodates for plant adaptation under abiotic stresses by changing in ROS production (Segal, 2018).

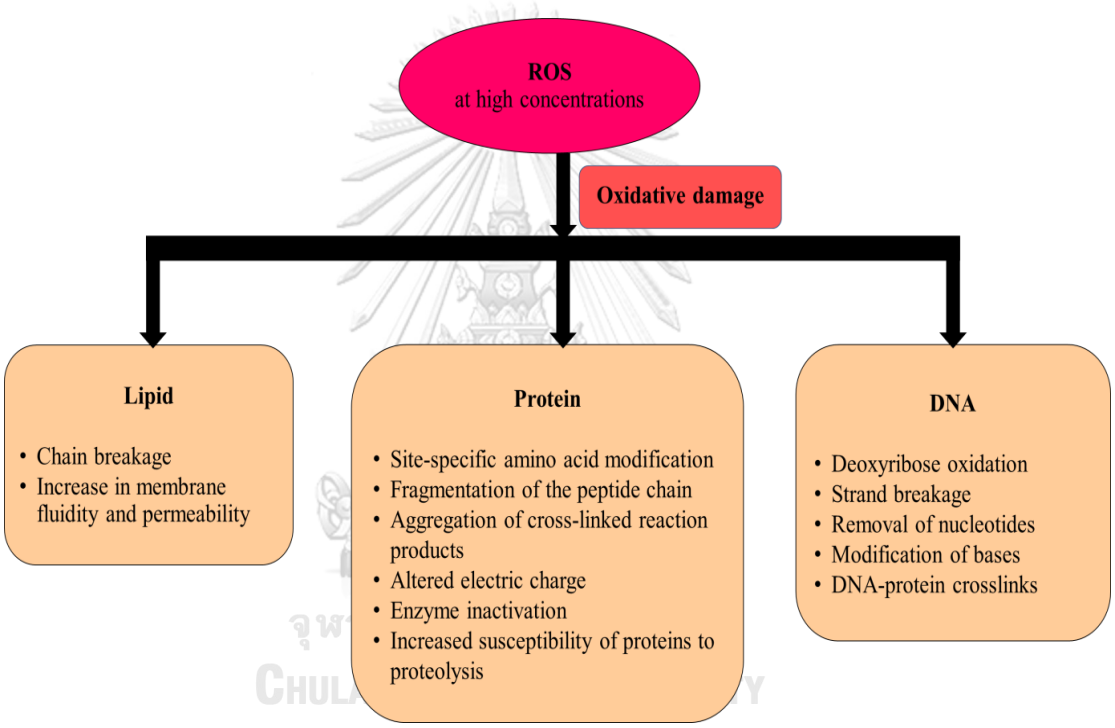


Figure 1.3 Reactive oxygen species (ROS) induced oxidative damage to lipids, proteins, and DNA (Sharma, 2012).

In all of ROS series, H_2O_2 has been believed that it accounts for the biggest problem in gene expression alteration of plant. Moreover, it has been found that H_2O_2 also trigger a various way of plant responses to abiotic stresses such as drought stress, photo-oxidative stress, and senescence inductance (Jajic, 2015). Nevertheless, H_2O_2 is shifted to $^{\bullet}OH$ which is a high reactive radical by Fenton and Haber–Weiss reactions under the attendance of the metal ion reduction (Betteridge, 2000) (Figure 1.4). Plant cells develop and sustain their redox homeostasis system for counterbalancing the oxygen radicals through the activity of various enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX) and non-enzymatic chemicals such as ascorbate (AsA) and glutathione (GSH) (Ray, 2012). In a ground state, O_2 is an innocuous molecule that has two unpaired electrons (e^-) and together with parallel spin conducts it paramagnetic. Thereby, O_2 unlikely to join the reaction with organic molecules till it is stimulated. O_2 is respectively decreased into $O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$, and $^1O_2$. Stimulation of O_2 may arise from two different devices including assimilation of enough energy for reversing the direction of one spin of unpaired e^- that leads to $^1O_2$ and $O_2^{\bullet-}$ creation which is simply reformed to H_2O_2 by catalyzing reaction of SOD and then H_2O_2 is changed into H_2O by CAT, GPX, and APX and stepwise monovalent letdown that lead to $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ (Figure 1.5) (Sharma, 2012).

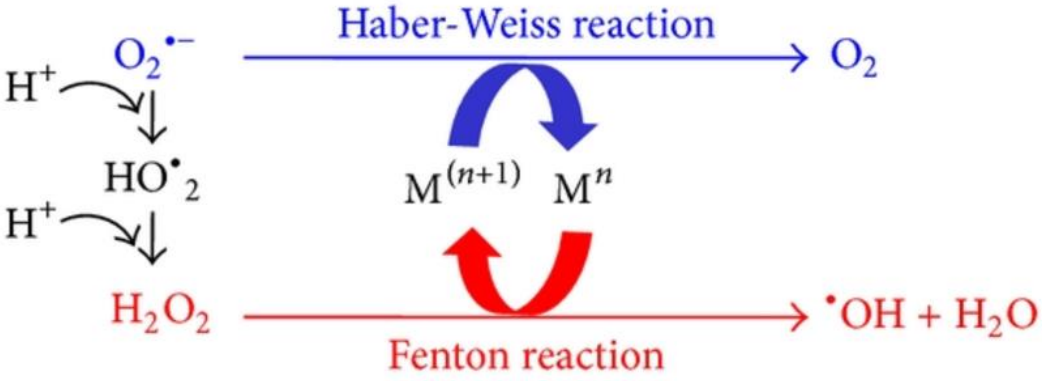


Figure 1.4 The Haber-Weiss Reaction and Fenton reaction for hydroxyl free radical generation (Ayala et al., 2014).

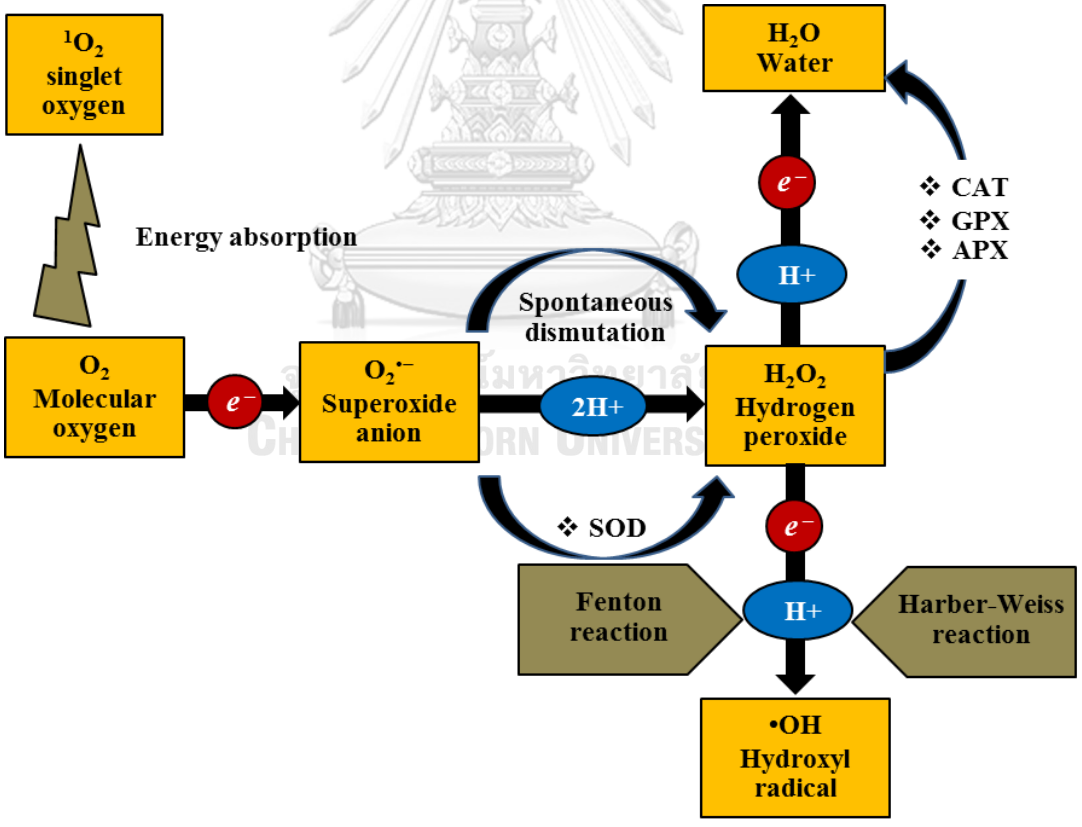


Figure 1.5 Diagram illustration of creation and dispossession of ROS in cellular of plants (Sharma, 2012).

Role of antioxidants under drought

An ordinary effect of drought is the interference between the generation and scavenging of reactive oxygen species (ROS) (Faize, 2011; Smirnoff, 1998). Plant cells are prevented against the destructive effects of ROS by a complicated antioxidant system includes the non-enzymatic (such as AsA and GSH) and enzymatic antioxidants include SOD, CAT, GPX, APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Noctor, 1998). These antioxidants are crucial constituents of the ROS scavenging system in plants, and their exhibitions improve drought endurance in rice. The activities of AsA, GSH, APX (Selote and Khanna-Chopra 2004), SOD, MDHAR, DHAR, GR (Sharma, 2005) and CAT (Shehab, 2010) constantly increase with enhancing rate of drought stress in rice. The enhancement in the antioxidants activities in response to drought stress presents the preventive activity to resist the oxidative derogation in rice. The activities of SOD, POD and CAT efficiently decrease the ROS level as well as reduce the negative impact of drought (Lum, 2014; Yang, 2014). Hence, increase in the commonly generating antioxidants like enzymatic and non-enzymatic compounds probably one strategy for decreasing or protecting oxidative injury and improving the drought tolerance of plants (Hasanuzzaman, 2014).

Places of generation of ROS

ROS are generated at various areas in chloroplasts, peroxisomes, mitochondria, cytosol, cell membrane, apoplast, and cell walls (Blokhina, 2010; Das, 2014; Hossain, 2011; Mhamdi, 2010) (Figure 1.6). ROS are regularly generated by leaking of e^- onto O_2 through the system of e^- transportation at chloroplasts, mitochondria, and plasma membrane, besides ROS are produced as a by-product of metabolic pathways that occur in various cellular compartments.

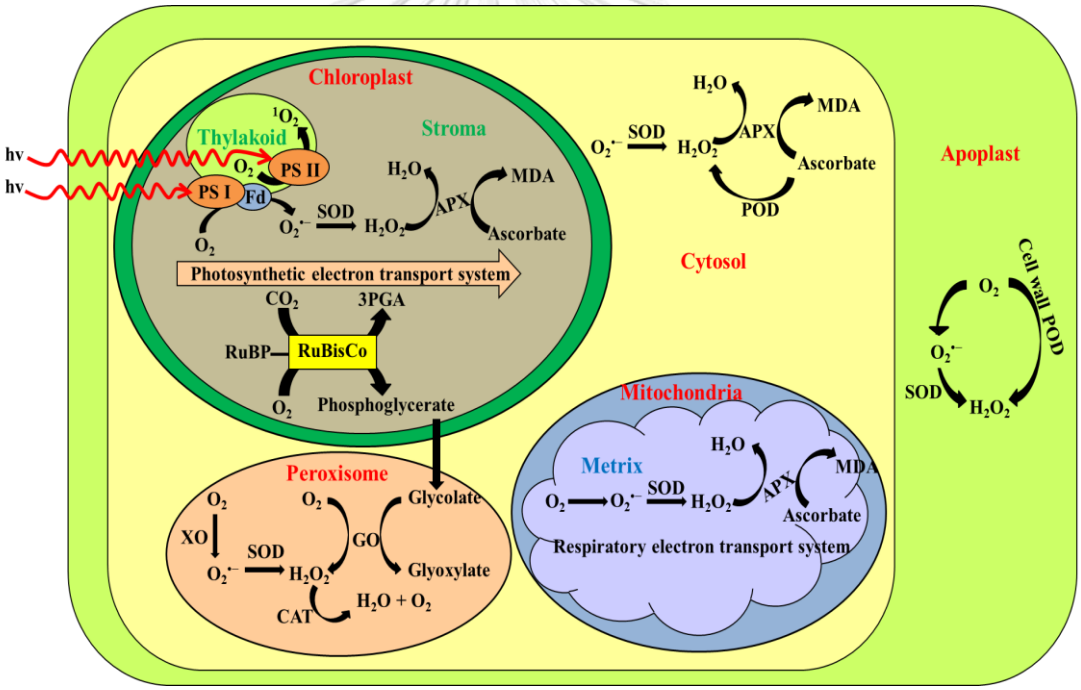


Figure 1.6 Various places of generation of ROS in plant cells (Blokhina, 2010; Das, 2014; Hossain, 2011; Mhamdi, 2010).

Calmodulin

Calcium ion (Ca^{2+}) acts as an important part in many characters of stress responses because it is one of the secondary messengers in plant intracellular that transduce information from the environmental stimulation to cellular tools liable on biological responsiveness. Moreover, Ca^{2+} functions like a significant nutrient of plants and like a component of the plant cell wall structure (Kulaz, 2017). Abiotic and biotic stresses urge Ca^{2+} signals that ordinarily happen in the temporary increase of cytosolic Ca^{2+} concentration. The free Ca^{2+} concentration in cytosol changes by the dynamic modification from a regulation of Ca^{2+} movements; between cytosol where has to keep Ca^{2+} at low level, and neighboring parts where accumulate high Ca^{2+} concentration like the vacuole and apoplast, pass through channels and pumps (Figure. 1.7). Calmodulin (CaM) is one of the Ca^{2+} sensors that transduce Ca^{2+} signals to their aim proteins. CaM is an acidic protein which composes of 148 amino acid residues and four EF-hand motifs or high- relevance Ca^{2+} binding sites. Normally, CaM has been generated in all eukaryotic organisms. CaM acts as a crucial matter in calcium signaling pathway and regulates the major physiological characters of plant namely metabolism, growth, and division of cell (Wang, 2016). While Ca^{2+} concentration is rising close to 1μM in intracellular in response to some stimulator, Ca^{2+} is binding to CaM that drives to change in the protein conformation and modulate its activities. Not only CaM relates to various proteins but also it is an essential subunit of the Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases I–IV) collection. When bound to Ca^{2+}, CaM changes in conformation and activities of the CaM kinase (Figure 1.8) that can regulate the activities by phosphorylating in

various goal enzymes. This is known that varieties of enzymes are adjusted by Ca^{2+} through CaM (Cooper, 2000; Karp, 1999).

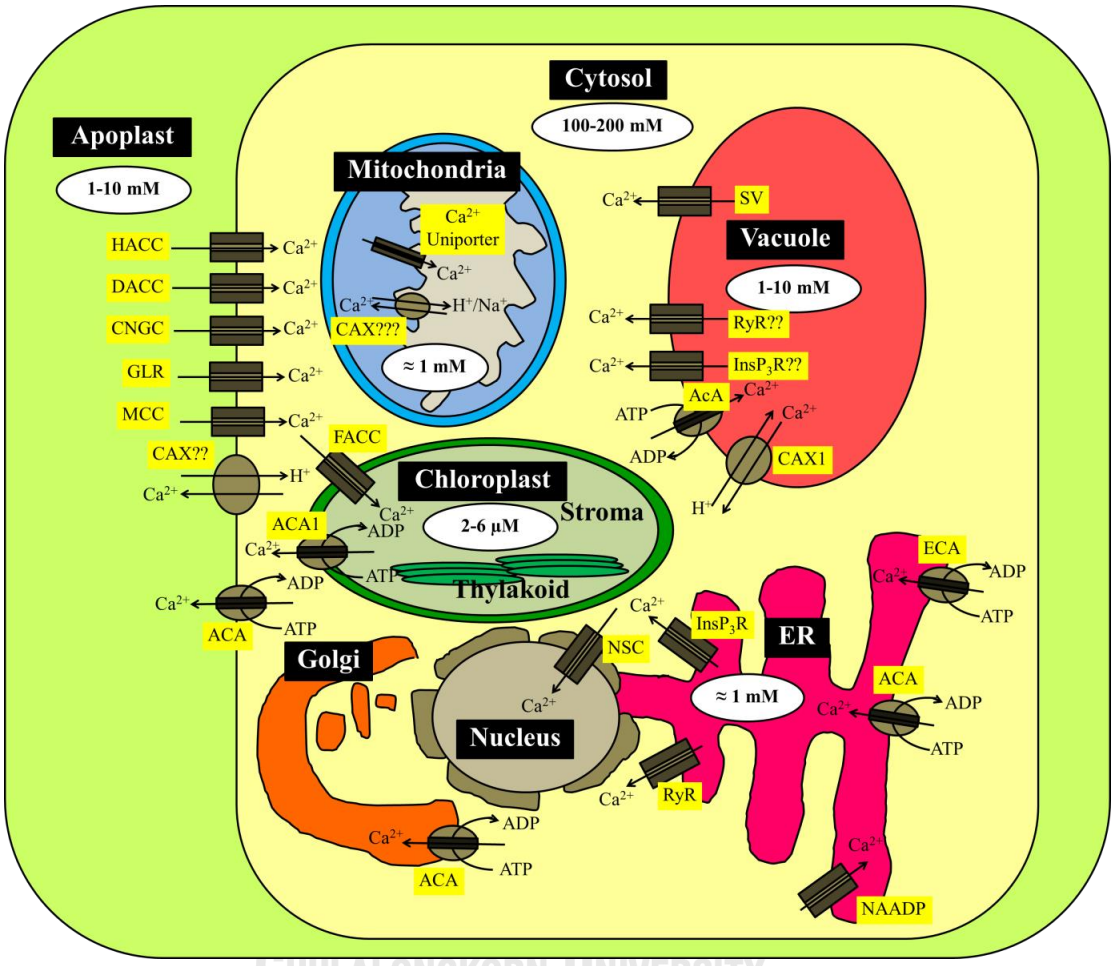


Figure 1.7 Schematic chart of Ca^{2+} transporters participated in Ca^{2+} equilibrium storage in the internal plant cellular. Where ACA is autoinhibited calcium ATPase, CAX is calcium exchanger, CNGC is cyclic nucleotide gated channel, DACC is depolarization activated cation channel, ECA is ER-type calcium ATPase, FACC is fast-activating cation channel, GLR is glutamate receptor-like channel, ACC is hyperpolarization activated cation channel, InsP3R is inositol 1,4,5-trisphosphate receptor-like channel, MCC is mechanosensitive cation channel, NSC is non-selective

cation channel, RyR is cyclic ADP-ribose (cADPR)-activator ryanodine receptor-like channel, and SV is slow-activating vacuolar channel (Bose, 2011).

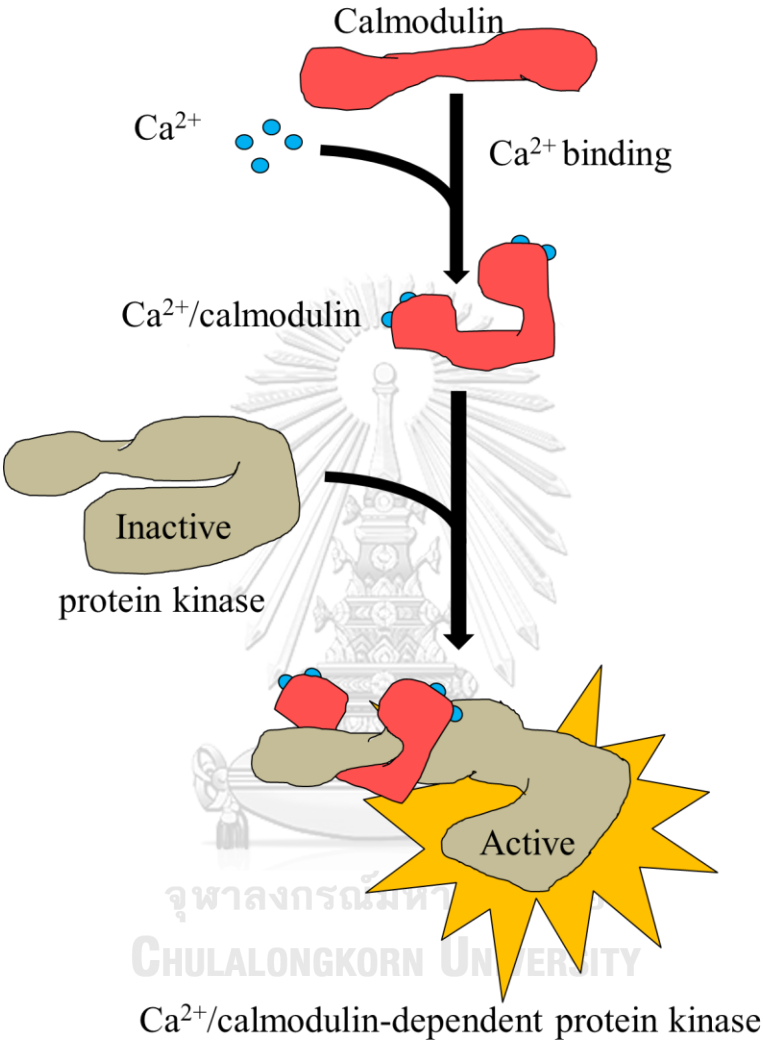


Figure 1.8 Diagram of the binding of Ca^{2+} to calmodulin (Ca^{2+}/calmodulin) and the binding of Ca^{2+}/calmodulin to protein kinases (Ca^{2+}/calmodulin-dependent protein kinases) (Cooper, 2000; Karp, 1999)

Postulation of OsCaM1-1 gene overexpressing level in transgenic rice plants

In previous study, the overexpression of *OsCaM1-1* gene controlled by 35SCaMV promoter in the transgenic KDML 105 rice was testified the total RNA with northern blot analysis (Figure 1.9) and studied the role of the *OsCaM1-1* in salt tolerance in rice. The transgenic plants overexpressing *OsCaM1-1* shows the better tolerance to salt stress than the control plants (Figure 1.10) by exhibiting a better ability to store their shoot and root dry weight throughout salt stress. The data remarks that *OsCaM1-1* signaling is likely to play an essential role in the level of *OsCaM1-1* gene expression possibly promotes to salt resistance in rice has been reported by Sang-ngam and coworkers (Saeng-ngam, 2012). Nevertheless, the role of *OsCaM1-1* has not been reported in the condition of drought, heat, or cold stress. Here, the morphological, biochemical, and physiological changes were determined in response to drought stress in the transgenic rice overexpressing *OsCaM1-1* gene compared to the wild type and control plants.

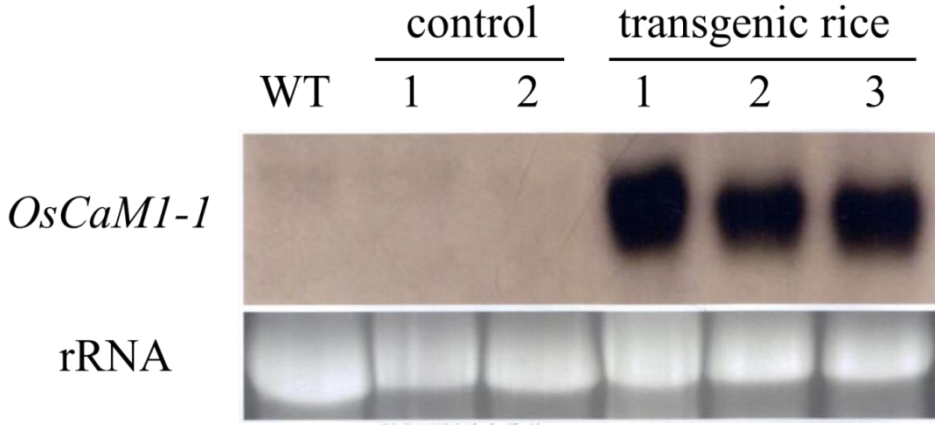


Figure 1.9 Analysis of RNA gel blot and quantitative comparison of OsCaM1-1 of wild type (WT) plants, two vector control lines, and three transgenic lines. Total RNA which isolated from rice leaves was determined by using a denatured ^{32}P-oligolabeled OsCaM1-1 probe with gel blot hybridization method and analyzing of an ethidium bromide staining gel is presented below its consistent autoradiography (Saeng-ngam, 2012).

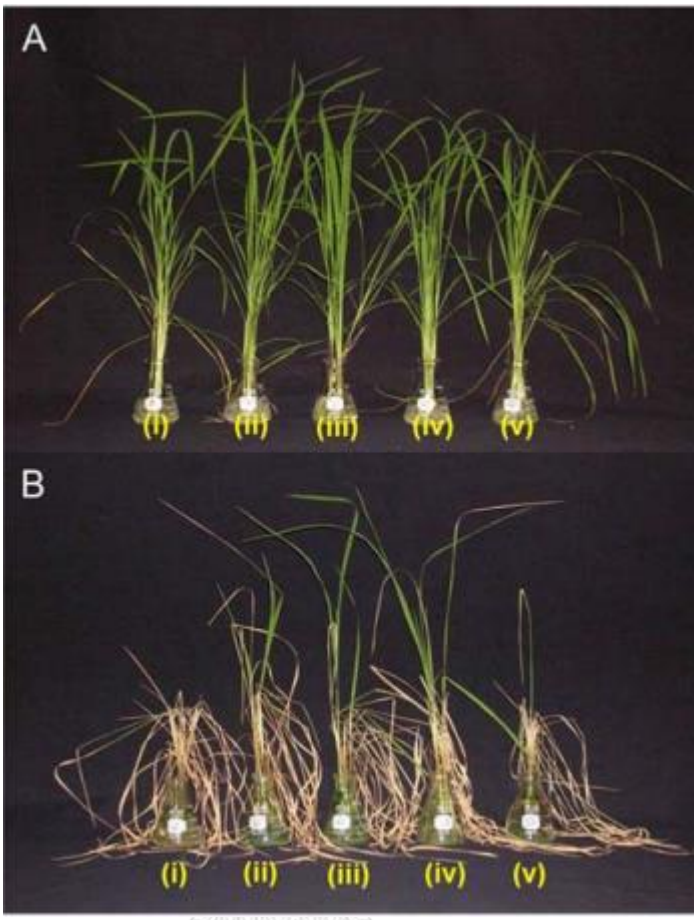


Figure 1.10 The phenotype comparison of rice cultivars of (i) the wild type line, the three transgenic rice lines, (ii) CaM1-1T1, (iii) CaM1-1T2 and (iv) CaM1-1T3, and (v) the control line all grown under (A) non stress or (B) salt stress conditions for 15 d (Saeng-ngam, 2012)

Objective of the thesis

To investigate the effect of overexpressing *OsCaM1-1* gene in 21-day-old transgenic rice and compare with WT and control under drought stress condition by determining physiological and biochemical parameters including H_2O_2 content, relative growth rate, DPPH scavenging rate, lipid peroxidation, cell membrane stability, relative water content, photosynthetic pigment contents, antioxidant enzyme activity; SOD, CAT, and POD, proline content, soluble sugar content, and Na^+, K^+, and Ca^{2+} contents.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Plant materials

Seeds of KDML 105 rice cultivar were provided by Department of Agriculture, Ministry of Agriculture and Cooperatives (Bangkok, Thailand). Rice seeds of the three transgenic KDML 105 lines overexpressing *OsCaM1-1* (CaM1-1T1, CaM1-1T2, and CaM1-1T3) and negative control transgenic line (transformed by only pCAMBIA1301 vector) (control)) were previously constructed by Sang-ngam and coworkers (Saeng-ngam, 2012) (Figure 2.1).

Figure 2.1 The KDML 105 paddies rice of WT, Control, CaM1-1T1, CaM1-1T2, and CaM1-1T3.

Seeds harvesting

In August, KDML 105 seeds were steeped in 6% (v/v) sodium hypochlorite for 3 min and rinsed completely with deionized water for several times, and incubated under dark for 5 d until it germinated. The germinated seeds were set on the basket that was nourished with deionized water, and incubated under 16 hr light/8 hr dark photoperiod, at 25 ± 2 °C, and relative humidity of 70%, for 7 d. After that seedlings were moved into the glass bottle and changed the deionized water nourishment to Limpinuntana's nutrient solution (Wutipraditkul, 2015), and further incubated for 14 d. Onwards, 21-day-old rice seedlings were planted in the pot that contained the completely mixed soil which composed of 3 parts of clay/1 part of mold (be careful the decreasing of water level and filling water 2-3 times/week). After planting a week, the urea fertilizer (46-0-0) was applied for increasing the number of leaves (applied fertilizer every 2 weeks). In late November, rice burgeoned and the nutrigrow fertilizer (16-16-16) was applied to nourish the rice (applied fertilizer every 1 week). In December, spikes were harvested and incubated at 60°C, for 3 d. Eventually, paddies were further used as plant materials.

Instruments

- Autoclave (Labo Autocalve MLS-3020, Sanyo Electric Co., Ltd., Japan)
- Automatic micropipette (Pipetman P2, P20, P100, P200, P1000, Gilson Medical Electronics S.A., France)
- Atomic absorption spectrometer (AA280FS, Agilent Technologies, USA)
- Balance (Sartorius CP423s, Scientific Promotion Co. USA)
- Centrifuge 5804R (Eppendrof, Germany)
- Centrifuge Sorvall Legend XTR (Thermo Scientific, USA)
- -20 °C freezer (Sharp, Japan)
- Microwave oven (Panasonic, Japan)
- Mixer Mill MM400 (Retsch®, Germany)
- 60 °C 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 °C Ultra low temperature freezer (New Brunswick Scientific, England)
- Vibro shaker (Labinco BV, Netherlands)
- Vortex mixer (Model K 550-GE, Scientific Inc., USA)

Inventory supplies

- Cryo kit for cooling the jars with liquid nitrogen (Retsch®, Germany)
- Filter paper (Whatman Interantional Ltd., England)
- Grinding balls, 2 mm Ø, stainless steel (Retsch®, Germany)
- Microcentrifuge tube 1.5 mL (Axygen Heyward, USA)
- Pipett tips 100, 1000 µL (Axygen Heyward, USA)

Chemicals and reagents

- Ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$) (BDH laboratory reagent, England)
- Ammonium sulfate ($(NH_4)_2SO_4$) (Carlo Erba Reagenti, Italy)
- L-ascorbic acid ($C_6H_8O_6$), 99% (Aldrich, Germany)
- Boric acid (H_3BO_3) (Sigma-Aldrich, Germany)
- Bovine serum albumin (BSA) (Sigma Chemical Co., USA)
- Calcium chloride dihydrate ($CaCl_2.2H_2O$) (Carlo Erba Reagenti, Italy)
- 98% Calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$) (*Riedel-de Haën*®, Germany)
- Cobaltous chloride hexahydrate ($CoCl_2 \cdot 6H_2O$) (Fluka, Switzerland)
- Copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$) (Sigma Chemical Co., USA)
- Coomassie® Brilliant Blue G-250 (CBBG) (Fluka, Switzerland)
- *N*,*N*-Dimethylformamide (DMF) (Carlo Erba Reagenti, Italy)
- 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) (Sigma Chemical Co., USA)
- Dithiothreitol (DTT) (Bio Basic Inc., Canada)
- Ethanol (C_2H_5OH), absolute (BDH laboratory reagent, England)
- Etylenediamineetraacetic acid disidium salt (EDTA) (Carlo Erba Reagenti, Italy)
- Gallic acid ($C_6H_2(OH)_3COOH$) (Sigma Chemical Company Co., USA)
- Guaiacol ($C_7H_8O_2$) ($C_7H_8O_2$) (Sigma-Aldrich, Germany)
- 37% Hydrochloric acid (HCl) (Merck, Germany)
- 30% Hydrogen peroxide (H_2O_2) (Sigma Chemical Co., USA)

- Iron (II) sulphate heptahydrate ($FeSO_4.7H_2O$) (Carlo Erba Reagenti, Italy)
- Magnesium chloride tetrahydrate ($MnCl_2.4H_2O$) (Carlo Erba Reagenti, Italy)
- Magnesium sulphate hepahydrate ($MgSO_4.7H_2O$) (Carlo Erba Reagenti, Italy)
- Methanol (CH_3OH) (Merck, Germany)
- 98% L-methionine ($C_5H_{11}NO_2S$) (Sigma-Aldrich, Germany)
- Nitroblue tetrazolium (NBT) (Fermentas, Inc., USA)
- Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, Germany)
- Potassium chloride (KCl) (Carlo Erba Reagenti, Italy)
- Potassium dihydrogen phosphate (KH_2PO_4) (Carlo Erba Reagenti, Italy)
- di-Potassium hydrogen phosphate trihydrate ($K_2HPO_4.3H_2O$) (Carlo Erba Reagenti, Italy)
- Potassium iodide (KI) (Sigma Chemical Co., USA)
- Potassium nitrate (KNO_3) (Carlo Erba Reagenti, Italy)
- L-proline ($C_5H_9NO_2$) (Sigma-Aldrich, Germany)
- 98% Riboflavin ($C_{17}H_{20}N_4O_6$) (Sigma Chemical Co., USA)
- Sodium carbonate anhydrous (Na_2CO_3) (Carlo Erba Reagenti, Italy)
- Sodium chloride (NaCl) (Carlo Erba Reagenti, Italy)
- Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.\ H_2O$) (Carlo Erba Reagenti, Italy)
- di-Sodium hydrogen phosphate dihydrate ($Na_2HPO_4.\ 2H_2O$) (Carlo Erba Reagenti, Italy)
- Sodium hypochlorite (NaClO) (Haiter, Thailand)
- Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$) (Sigma Chemical Co., USA)

- 2-Thiobarbituric acid (TBA) (Sigma-Aldrich, Germany)
- Titanium (IV) chloride (TiCl4) (Merck, Germany)
- Trichloroacetic acid (TCA) (Carlo Erba Reagenti, Italy)
- Zinc sulphateheptahydrate ($ZnSO_4.7H_2O$) (Sigma Chemical Co., USA

2.2. Drought treatment

Seeds were soaked in 6% (v/v) sodium hypochlorite for 3-5 min and rinsed entirely with deionized water. Then, they were inseminated on deionized water in darkness. After 5 d, the proliferating seeds were immersed into deionized water and moved into a plant growth chamber (Human Lab, South Korea) under managed situations (temperature of 25 ± 2 °C, 16 h light (200 µmol/ m^{2}·s)/8 h dark photo period, and relative humidity of 70%) for 7 d. Next, proliferating seeds were matured in Limpinuntana's nutrient solution for 14 d. For drought treatment, the 21-day-old seedlings were separated into two groups: 1) exposed to 20% (w/v) polyethylene glycol-4000 (PEG-4000) (Chinpongpanich, 2012; Wu, 2015) with various treatment times at 0, 6, 12, 24 and 48 h treated with various PEG-4000 concentration (0, 10, 15, 20, and 25% (w/v)) for 24 h. Finally, the leaves were collected and maintained at -80 °C Ultra low temperature freezer (New Brunswick Scientific, England) until biochemical analysis.

2.3. Measurement of hydrogen peroxide content

Hydrogen peroxide (H_2O_2) was analyzed following the explained method of Lin and Kao (Lin, 1996) with some modification. Leaves (0.1 g) were pounded in liquid nitrogen, after that the sample was added with 1 mL of 50 mM potassium phosphate buffer (pH 7.0) and centrifuged (Eppendorf model 5804R, Germany) at 6,000 ×g, 25 °C for 25 min. Then, supernatant was added with 1% (v/v) titanium tetrachloride in conc. hydrochloric. After that the solution was incubated for 15 min and centrifuged at 6,000 ×g, 25 °C for 15 min. Lastly, the solution absorbance was recorded at 410 nm

(Spectrophotometer: DU® 640, Beckman Coulter, USA). H_2O_2 content was then calculated using a standard curve of known H_2O_2 concentration.

2.4. Measurement of Na^+, K^+, and Ca^{2+} concentration

Fresh leaves (0.02 g) were dried at 60 °C for 3 d in an electric oven (Memmert, Germany). Next, the pounded dry sample was mixed with 10 mL of 0.5 M nitric acid (HNO_3); prepared from 65% (v/v) of HNO_3 (Sigma-Aldrich, Germany). After rested in normal condition for 2 d., the sample tubes were incubated at 80 °C for 1 h in an electric oven. The concentration of Na^+, K^+, and Ca^{2+} were recorded by using the atomic absorption spectrometer (AA280FS, Agilent Technologies, USA) with some modification according to the method of Munns (Munns, 2016).

2.5. Determination of lipid peroxidation

Lipid peroxidation was observed by determining the malondialdehyde (MDA) content, a breakdown product of lipid peroxidation, using 2-thiobabituric acid (TBA). Fresh leaves (0.05 g) were homogenized in liquid nitrogen. Then 1 mL of 0.1% trichloroacetric acid (TCA) was added and the samples were centrifuge at 5,000 ×g for 15 min. Next, 0.3 mL of the supernatant was mixed with 0.9 mL of 0.5% Thiobarbituric acid (TBA) in 10% TCA and incubated in 95 °C for 25 min. The absorbance was read at 532 and 600 nm. The MDA content was determined by subtracting absorbance of supernatant at 60 nm from that of 532 nm using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed on µmol per g fresh weight of sample (Heath, 1968).

2.6. Determination of cell membrane stability (electrolytic leakage)

The electrolytic leakage was determined as described by Sullivan and Ross (Sullivan, 1979) with some modification. The fresh leaves (0.05 g) were cut into small pieces and soaked in 5 mL of deionized water for 2 h. The electrical conductivity (EC_1) was measured using electric conductivity detector: Cyberscan con 510 (Thermo scientific, Germany) before and after autoclaved at 121 °C for 20 min. The percentage of electrolytic leakage was calculated following the equation (Blum, 1981):

Electrolytic leakage (%) = $100 – [(EC_1/EC_2) \times 100]$

2.7. Measurement of relative growth rate

Twenty-one -day-old seedlings were randomly selected and dried at 60 °C for 3 d in an electric oven (Memmert, Germany). Then, the dry weight was recorded using Balance (Sartorius CP423S, Scientific Promotion Co. USA). The relative growth rate (RGR) was calculated using the following equation (Gardner, 1985):

$RGR = (\ln W_2 – \ln W_1)/ (t_2 – t_1)$

Where W_1 is initial weight, W_2 is final weight, t is the time intervals of drying and subscripts denote initial (1: before drought treatment) and final (2: after 24 h drought treatment) sampling.

2.8. Measurement of relative water content

The seedlings were randomly selected, and then leaf was cut into pieces of 1 cm^2 and weighted to determine initial weight (FW). After that leave samples were soaked in deionized water for 24 h and the turgid weight (TW) was recorded. The

samples were dried at 60 °C for 72 h and measured the dry weight (DW). The relative water content (RWC) was calculated using the equation (Michael, 1985):

RWC (%) = (FW – DW) × 100/ (TW – DW)

2.9. Measurement of proline content

Proline content was measured as described by method of Bates and coworkers (Bates, 1973) with some modification. The pounded fresh leaves (0.05 g) were extracted with 3% (w/v) sulfosalicylic acid and centrifuged at 6,000×g, 25 °C for 15 min. Then, the 0.4 mL of supernatant was mixed with 0.3 mL of ninhydrin and 0.3 mL of acetic. Next, the solutions were boiled at 100 °C for 1 h and suddenly incubated on ice for 5 min. After that, the 0.5 mL of the solution mixture was added with 0.5 mL of toluene. The absorbance was recorded at 520 nm. Proline was estimated from standard curve.

2.10. Measurement of soluble sugar content

Pounded dry leaves (0.05 g) were added with deionized water. Next, the samples were mixed with 1% (w/v) phenol and 98% (v/v) sulfuric acid, and then samples were incubated for 1 h. The absorbance was read at 485 nm (DuBois, 1956).

2.11. Measurement of photosynthetic pigments

Leaves (0.03 g) were homogenized in liquid nitrogen. The sample was merged with *N*,*N*-dimethylformamide (DMF) and centrifuged at 5,000 ×g, 4 °C for 10 min.

The absorbance was read at 461, 647, and 664 nm. The photosynthetic pigment contents were calculated following the equations (Inskeep, 1985):

Chlorophyll *a* (μg/mL) = [12.7(A_{664})] – [2.79(A_{647})]

Chlorophyll *b* (μg/mL) = [20.70(A_{647})] – [4.62(A_{664})]

Total chlorophyll (μg/mL) = [17.90(A_{647})] + [8.08(A_{664})]

Carotenoid content (μg/mL) = 4 × [(A_{461}) + (0.46 × A_{664})]

2.11. Measurement of DPPH radical scavenging rate

DPPH radical scavenging rate was measured following the method of Szabo and coworkers (Szabo, 2007) with some modification. 0.05 g fresh leaves were pounded in liquid nitrogen, after that added 95% methanol in the sample tube. Then, the sample solutions were shaken at 3,000 ×g, 10 °C for 5 min using a Vibro shaker (Labinco BV, Netherlands) and centrifuged at 13,000 ×g, 25 °C for 5 min. The solution mixture of the samples and DPPH reagent were incubated under dark for 30 min and the absorbance was measured at 515 nm. The percentage of DPPH radical scavenging rate was calculated following the formula:

DPPH radical scavenging rate (%) = ($A_{control}$ - A_{sample}) × 100/$A_{control}$

2.12. Determination of protein content

Protein was extracted as described by Bradford (Bradford, 1976) with some modification. Fresh leaves (0.1 g) were homogenized in liquid nitrogen. Then, the sample was mixed with 1 mL of extraction buffer composed of 1 mM dithiothreitol, 1 mM Ethylenediaminetetraacetic acid (EDTA) and 100 mM phenylmethylsulfonyl fluoride (PMSF) in 0.1 M potassium phosphate buffer (pH 7.5) and centrifuged at

14,000 ×g, 4 °C for 25 min. Supernatants were used for antioxidant enzyme activity assay.

2.13. Determination of antioxidant enzyme activity

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed following the method as described by Agarwal and coworkers (Agarwal, 2005). The reaction mixtures of 1.2 mL contained of 0.1 mM EDTA, 50 mM sodium carbonate, 13 mM methionine, 75 µM Nitro blue tetrasolium (NBT), and 50 µg enzyme in 50 mM potassium phosphate buffer (pH 7.8). The reaction was started by adding 2 µM riboflavin and incubated under two 15 W fluorescent lamps cat in light for 15 min. The absorbance was recorded at 560 nm. One unit of SOD activity was determined as the amount of enzyme required to cause 50% inhibition of the decrease of NBT monitored at 560 nm.

Catalase (CAT) (EC 1.11.1.6) activity was determined following the method of Hugo and coworkers (Hugo, 1974). The reaction mixtures of 1.2 mL containing 50 µg enzyme and 1 M H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0). H_2O_2 was lastly added to start the reaction, the quickly recorded the absorbance at 240 nm for 0-3 min and H_2O_2 consumption was calculated using the extinction coefficient, 39.4 mM^{-1} cm^{-1}.

Peroxidase (POD) (EC 1.11.1.7) activity was measured according to the method described Putter (Putter, 1974). The reaction mixtures of 1.2 mL compose of 30 mM guaiacol, 50 µg enzyme in 50 mM potassium phosphate buffer (pH 7.0), and 0.1 mM H_2O_2 was added to start the reaction. The absorbance was read at 436 nm.

POD activity was determined by monitoring of the formation of tetra-guaiacol (extinction coefficient, 6.39 mM^{-1} cm^{-1}).

2.14. Statistical analysis

Statistical analysis was managed by demonstration with a one-way analysis of variance (ANOVA) following by the Duncan's new multiple range test ($P < 0.05$) using SPSS software version 16.0.

CHAPTER 3

RESULTS

3.1 H_2O_2 content

The H_2O_2 acts as an essential role of the signaling molecule, the swing of the H_2O_2 level alters following the environmental stress. To know the effect of *OsCaM1-1* in the H_2O_2 content dominated on effects of drought stress condition, leaves of the three transgenic lines overexpressing *OsCaM1-1* gene, wild type, and control were measured. Figure 3.1A presented the H_2O_2 content of all lines scaled on various treatment times (0, 6, 12, 24, and 48 h) under drought stress (treated with 20% (w/v) PEG-4000). At 24 h, the H_2O_2 content of the three transgenic plants, wild type, and control plants (22.7, 21.9, 26.6, 31.7, and 29.1 µmol/g FW, respectively) was exhibited the highest level under drought stress condition but it reduced rapidly when treatment time of drought stress at 48 h (Figure 3.1A). As a result, period of time at 24 h was used for further experiments of the drought stress responses of plants. Figure 3.1B showed the H_2O_2 content of all plants measured on various PEG-4000 concentration treatments at 0, 10, 15, 20, and 25% (w/v) PEG-4000 for 24 h. The results showed the highest H_2O_2 value at 20% PEG-4000 (Figure 3.1B) which was selected for further experiments. The H_2O_2 content of the three transgenic plants under drought stress showed increase about 163-222% that was lower increased than the H_2O_2 content of wild type and control plants (290 and 783%, respectively) when compared with the non stress condition (Figure 3.2).

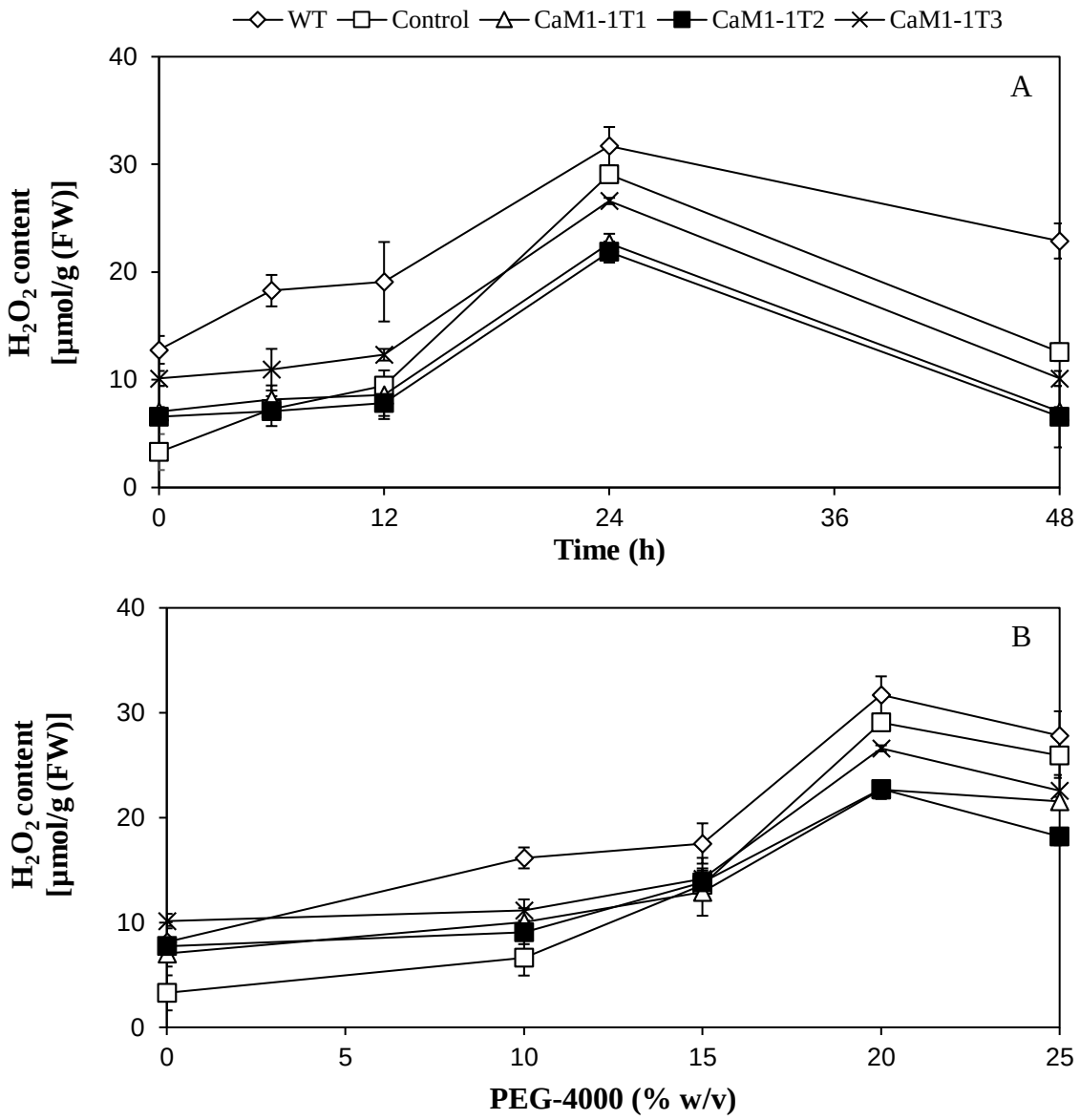


Figure 3.1 Effect of drought stress on H_2O_2 content in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (20% (w/v) PEG) A) at different treatment time (0, 6, 12, 24, and 48h) and to B) different PEG concentration (0, 10, 15, 20, and 25% (w/v)). Values are Mean ± SD (n=5).

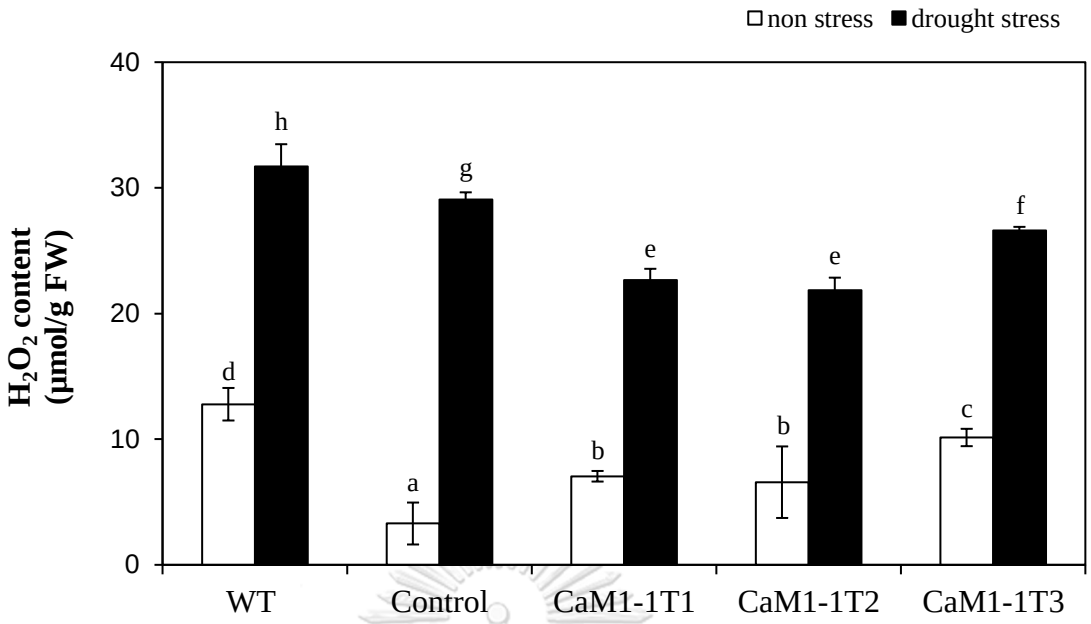


Figure 3.2 Effect of drought stress on H_2O_2 content in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.2 Na^+, K^+, and Ca^{2+} concentrations

In order to evaluate the capacity of drought resistance of *OsCaM1-1* gene on the ion collection under drought stress, Na^+, K^+, and Ca^{2+} contents were distilled and measured in leave of the three transgenic plants overexpressing *OsCaM1-1* gene, wild type, and control plants. The results showed that drought stress increased Na^+ content when comparison to non stress condition, however, the three transgenic plants overexpressing *OsCaM1-1* gene increased slightly in Na^+ content about 18-38%, while the wild type and control plants showed significant increased about 109 and 101%, respectively (Figure 3.3A). However, there was no difference in K^+ content of all plants under both of drought and non stress condition (Figure 3.3B). Under drought treatment, the three transgenic plants overexpressing *OsCaM1-1* gene exhibited the significant increase of the Ca^{2+} content about 52-84%, while the wild type and control plants exhibited a slight increase when compared to non stress condition (Figure 3.3C). Accordingly, the K^+/Na^+ and Ca^{2+}/Na^+ ratios of the three transgenic plants under drought stress condition showed slight reduction about 11-16% and 19-27%, respectively, while that of the wild type (about 60 and 72%, respectively) and control (46 and 63%, respectively) plants exhibited significant decrease under drought stress (Figure 3.4).

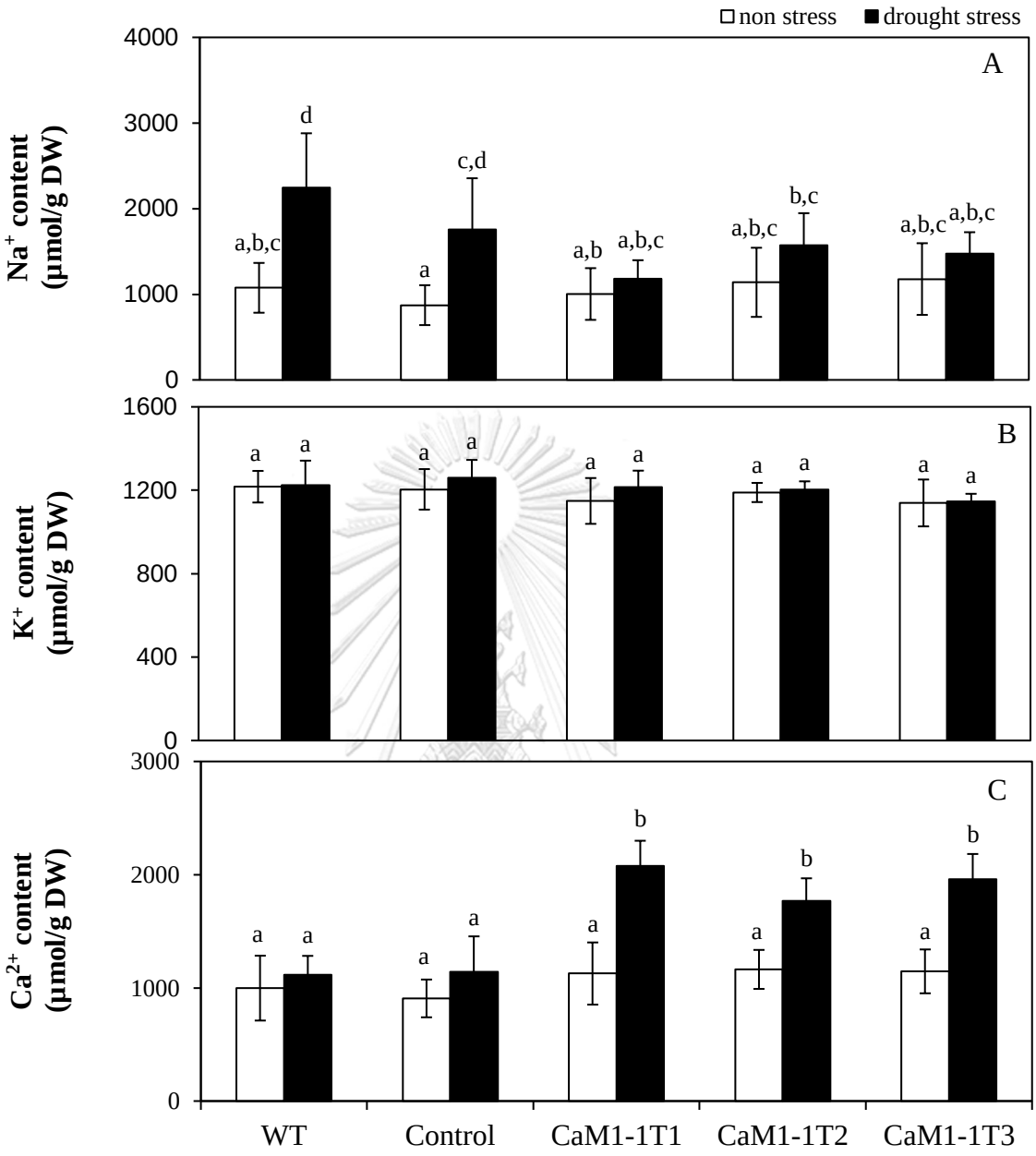


Figure 3.3 Effect of drought stress on Na^+, K^+, and Ca^{2+} contents in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

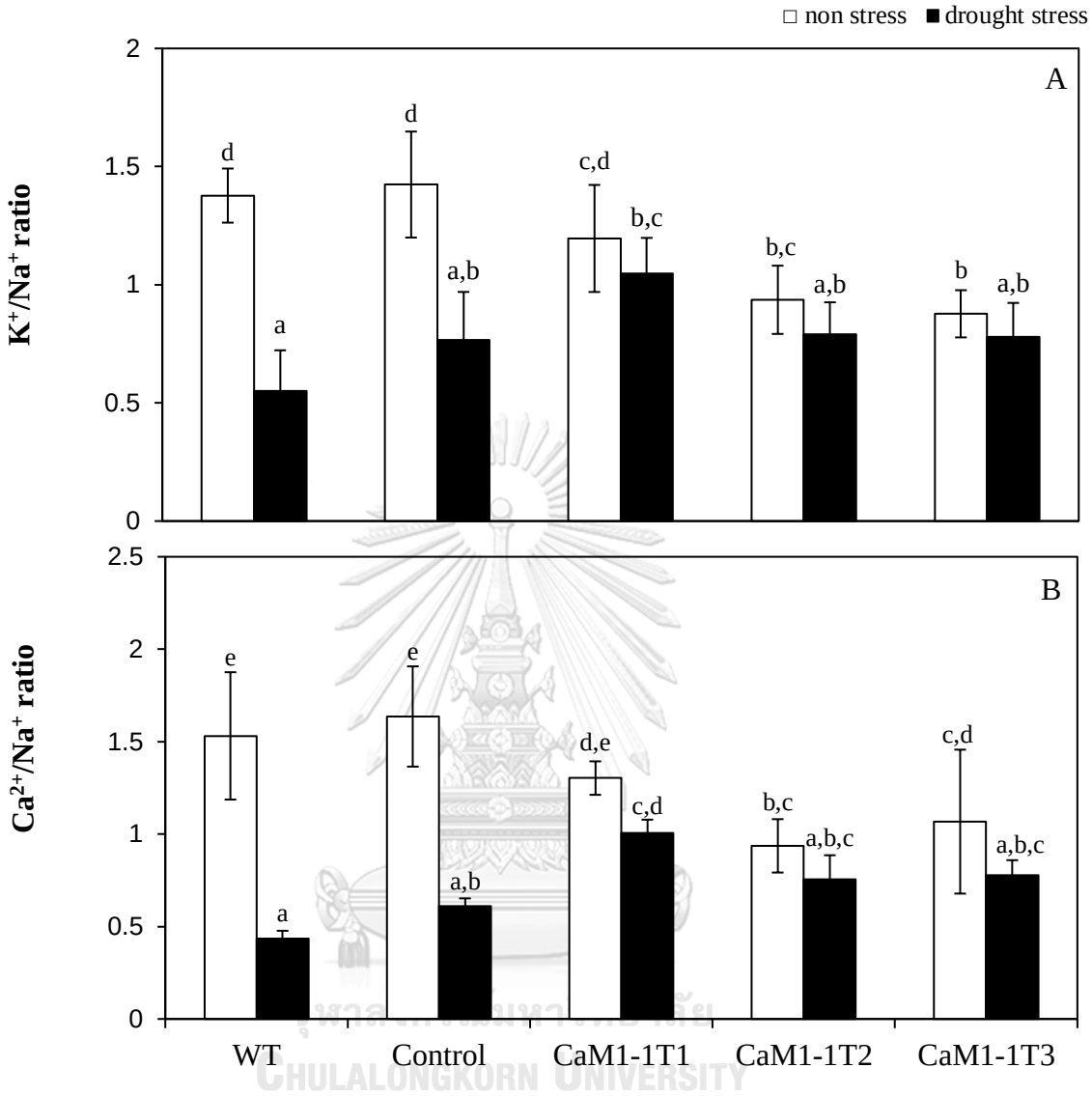


Figure 3.4 Effect of drought stress on K^+/Na^+ and Ca^{2+}/Na^+ ratio in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.3. Lipid peroxidation

The MDA is the end product of the lipid peroxidation which is an oxidative degradation of lipid membrane. To estimate the role of *OsCaM1-1* in lipid peroxidation under drought treatment, the MDA content of the three transgenic plants overexpressing *OsCaM1-1* gene, wild type, and control plant was extracted from the leaves. Drought stress was found to increase the MDA accumulation when compared with non stress (Figure 3.5). The result demonstrated that MDA content of the three transgenic plants showed the lower increase about 22-50% than that of the wild type and control plants that were highly increased about 262 and 201%, respectively.

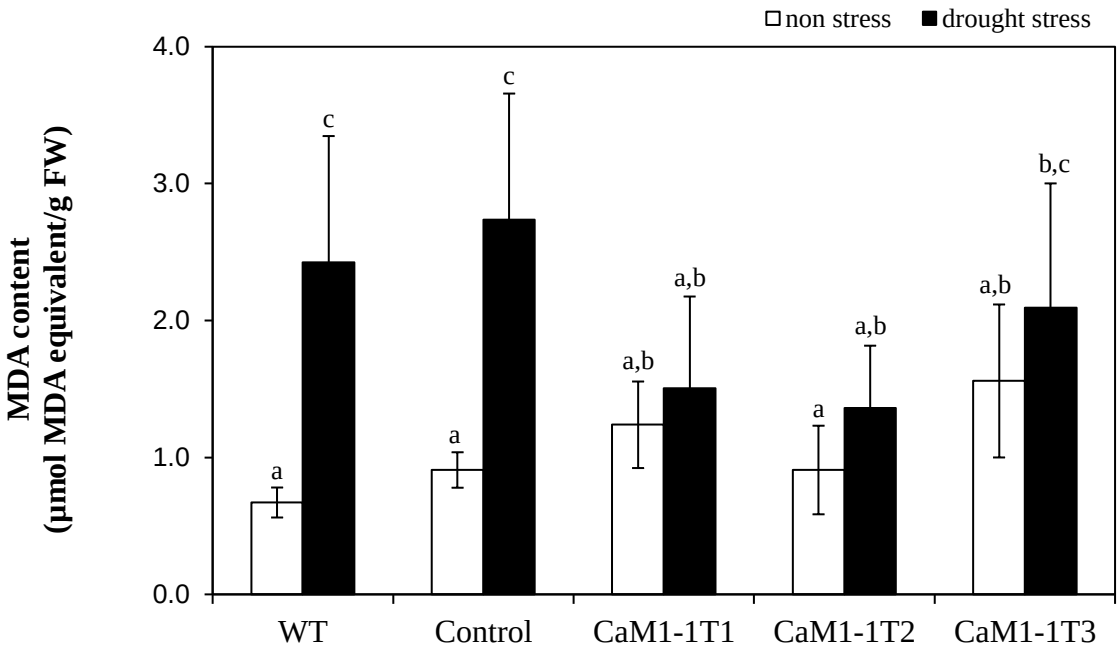


Figure 3.5 Effect of drought stress on MDA content in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.4. Cell membrane stability

To understand the role of *OsCaM1-1* gene in the cell membrane stability under drought stress, the leave electrolytic leakage of the three transgenic plants, wild type, and control plants was determined after exposed to drought stress. Drought stress condition led to significant increase of the electrolytic leakage in the wild type and control plants (about 29 and 23%, respectively) while in the transgenic plants, it did not increase significantly about 0-3% (Figure 3.6).

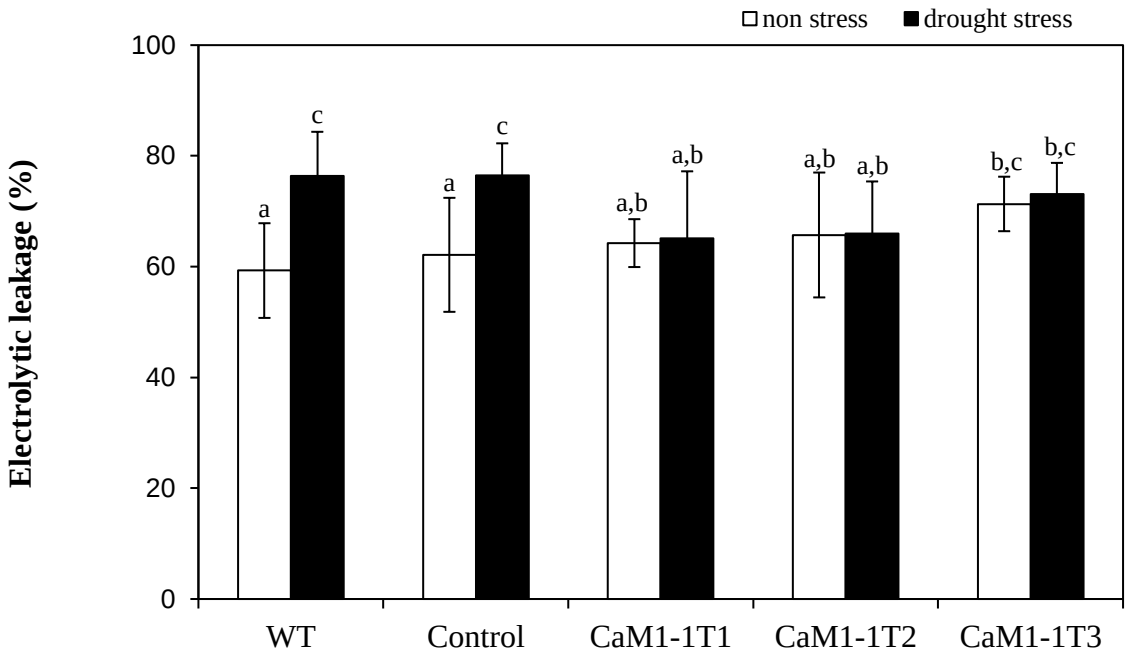


Figure 3.6 Effect of drought stress on electrolytic leakage in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.5. Relative growth rate

To estimate the drought tolerance ability of *OsCaM1-1* gene, 21-day-old rice seedling of the three transgenic plants overexpressing *OsCaM1-1*, wild type, and control plants were collected randomly and estimated. The phenotype responses were remarked in terms of the relative growth rate of shoot and root. The result showed no difference in the phenotype of all plants under normal condition (Figure 3.7A) but the three transgenic rice plants showed more drought tolerance than the wild type and control plants under drought stress condition (Figure 3.7B). The relative growth rate of shoot of the three transgenic plants overexpressing *OsCaM1-1* gene had lower decreased about 8-9% than that of the wild type and control plants that showed reduction about 57 and 62%, respectively under drought stress (Figure 3.8A). In the same way, the relative growth rate in root of the three transgenic rice plants presented insignificant decrease about 11-15% under drought stress, while the wild type and control plants showed significant decrease about 63 and 60%, respectively (Figure 3.8B) when compared with non stress condition.

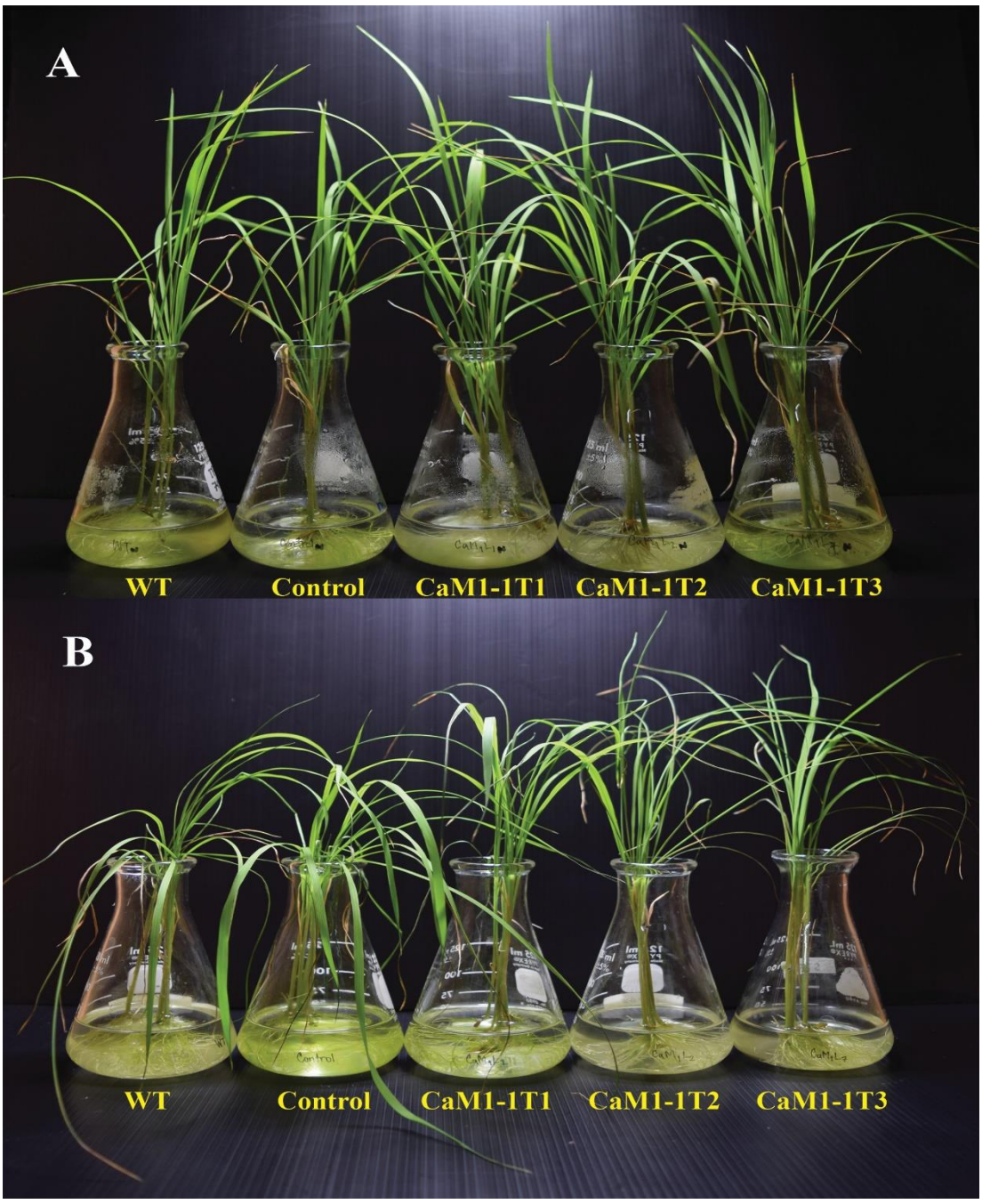


Figure 3.7 The phenotype of the three transgenic rice seedlings, the wild type (WT) and control (negative control transgenic line) seedlings under (A) non stress and (B) drought stress (20% PEG-4000) for 24 h.

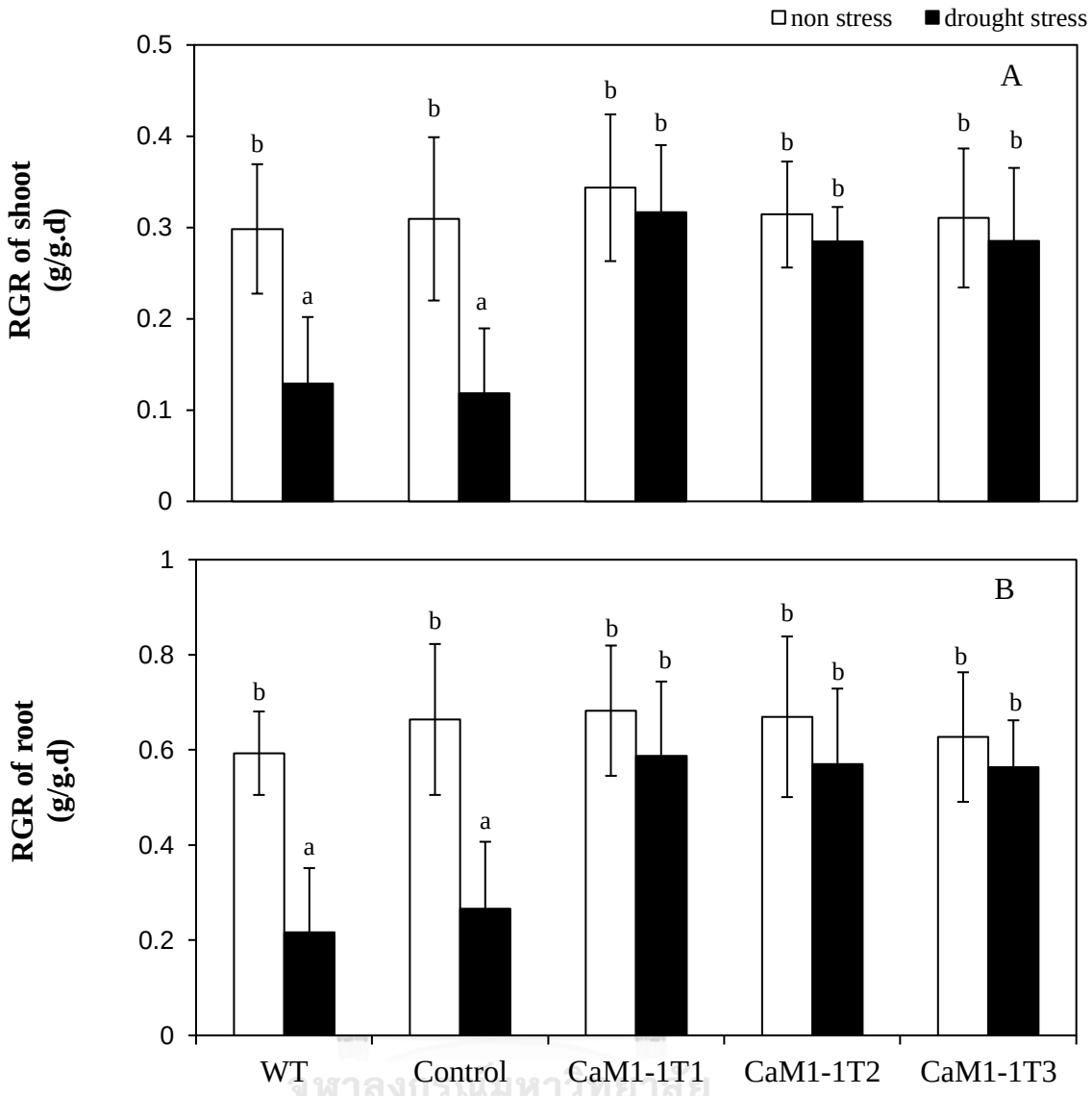


Figure 3.8 Effect of drought stress on RGR of (A) shoot and (B) root of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=10) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.6. Relative water content

To explore the role of *OsCaM1-1* gene on the ability of the plant water retention under drought treatment, the relative water content (RWC) was determined in leaves of the three transgenic plants overexpressing *OsCaM1-1* gene, wild type, and control plants. There were indifference in RWC level between the three transgenic rice plants, wild type, and control plants under non stress condition (Figure 3.9). However, drought stress caused decrease in the RWC level when compared to non stress condition. The three transgenic plants overexpressing *OsCaM1-1* exhibited the slight decrease in the RWC about 4-8% that was lesser than the wild type and control plants which were highly decreased about 22 and 28%, respectively.

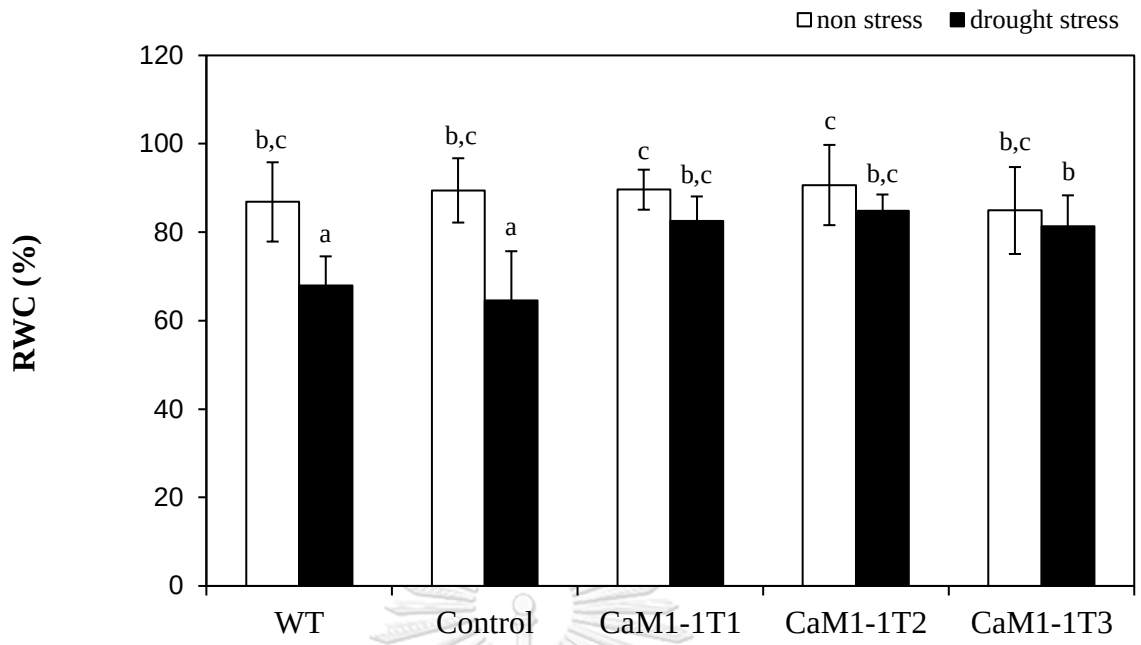


Figure 3.9 Effect of drought stress on RWC in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.7. Proline content

To realize the role of *OsCaM1-1* in the proline accumulation under drought stress, the proline content in leaves of the three transgenic plants overexpressing *OsCaM1-1* gene, wild type, and control plants was extracted. The results have revealed that drought stress increased significantly in the proline content when compared with non stress condition (Figure 3.10), however, its content on the three transgenic plants was higher (about 359-714%) than that in the wild type and control plants (about 188 and 244%, respectively).

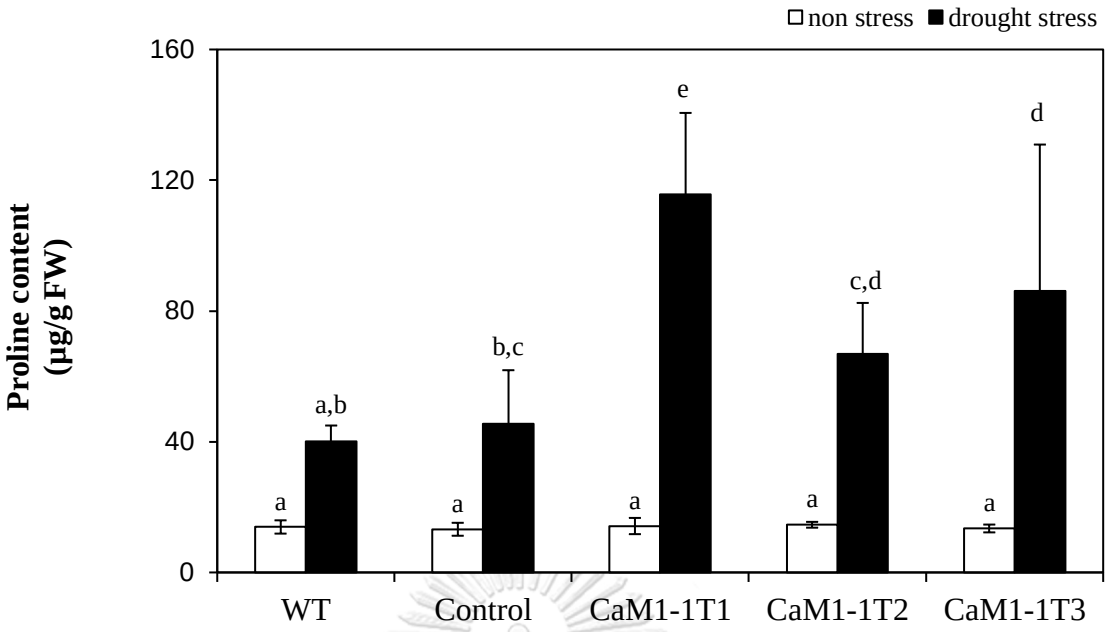


Figure 3.10 Effect of drought stress on proline content in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.8. Soluble sugar content

To know the role of *OsCaM1-1* on the effect of drought stress on the osmoprotectant gathering, the soluble sugar content was extracted in leaves of the three transgenic plants overexpressing *OsCaM1-1* gene, wild type, and control plants. The results showed that drought stress increased significantly the soluble sugar content when compared with non stress condition (Figure 3.11), however, the soluble sugar accumulation was higher level in the three transgenic plants (14.6, 13.1, and 13.1 µg/g FW, respectively) than that of the wild type and control plants (9.8 and 10.9 µg/g FW, respectively).

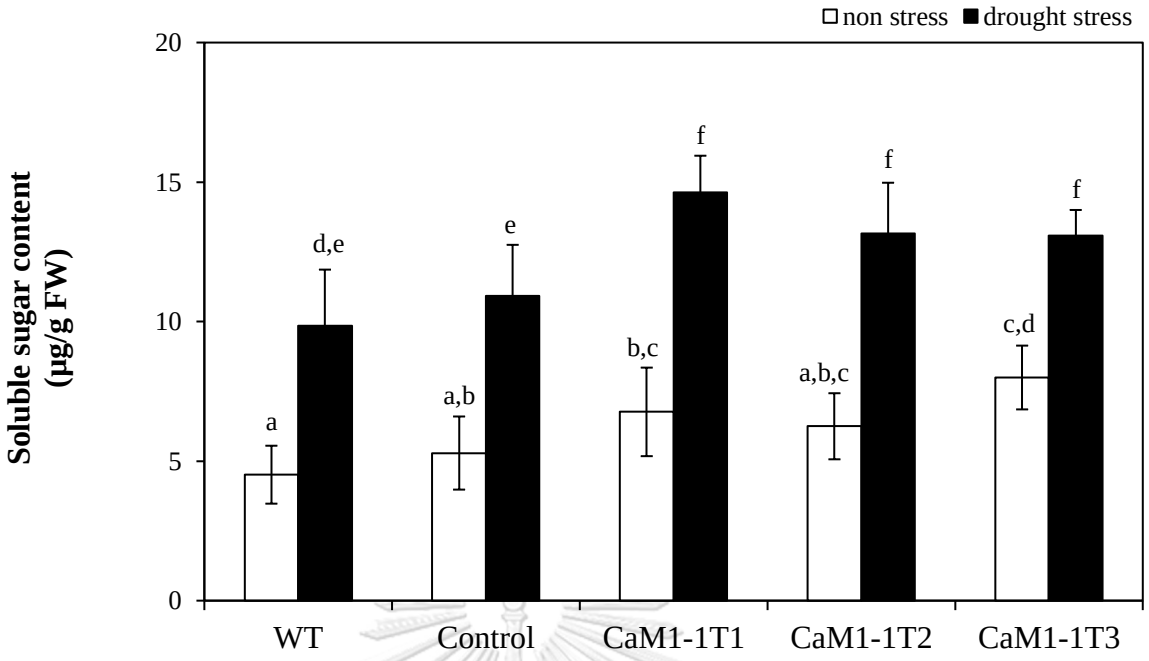


Figure 3.11 Effect of drought stress on soluble sugar content in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.9. Photosynthetic pigment contents

To observe the role of *OsCaM1-1* on the effects of drought stress on photosynthetic pigment contents (chlorophyll *a*, *b*, total chlorophyll, and carotenoid), after treated with and without 20% PEG-4000 leaves of the three transgenic rice overexpressing *OsCaM1-1* gene, wild type, and control plants were extracted and analyzed. Drought stress caused reduces the concentration of chlorophyll *a*, *b*, total chlorophyll, and carotenoid (Figure 3.12). The photosynthetic pigment contents were considerably diminished under the effect of drought stress, nevertheless, the reduction in the three transgenic plants were lower than the wild type and control plants. The results showed that the chlorophyll *a*, b, total chlorophyll, and carotenoid contents of the three transgenic plants decreased about 3-5%, 20-31%, 9-12%, and 4%, respectively, while that of wild type (16, 52, 27, and 16%, respectively) and control (17, 62, 30, and 19%, respectively) plants were highly decreased under drought treatment.

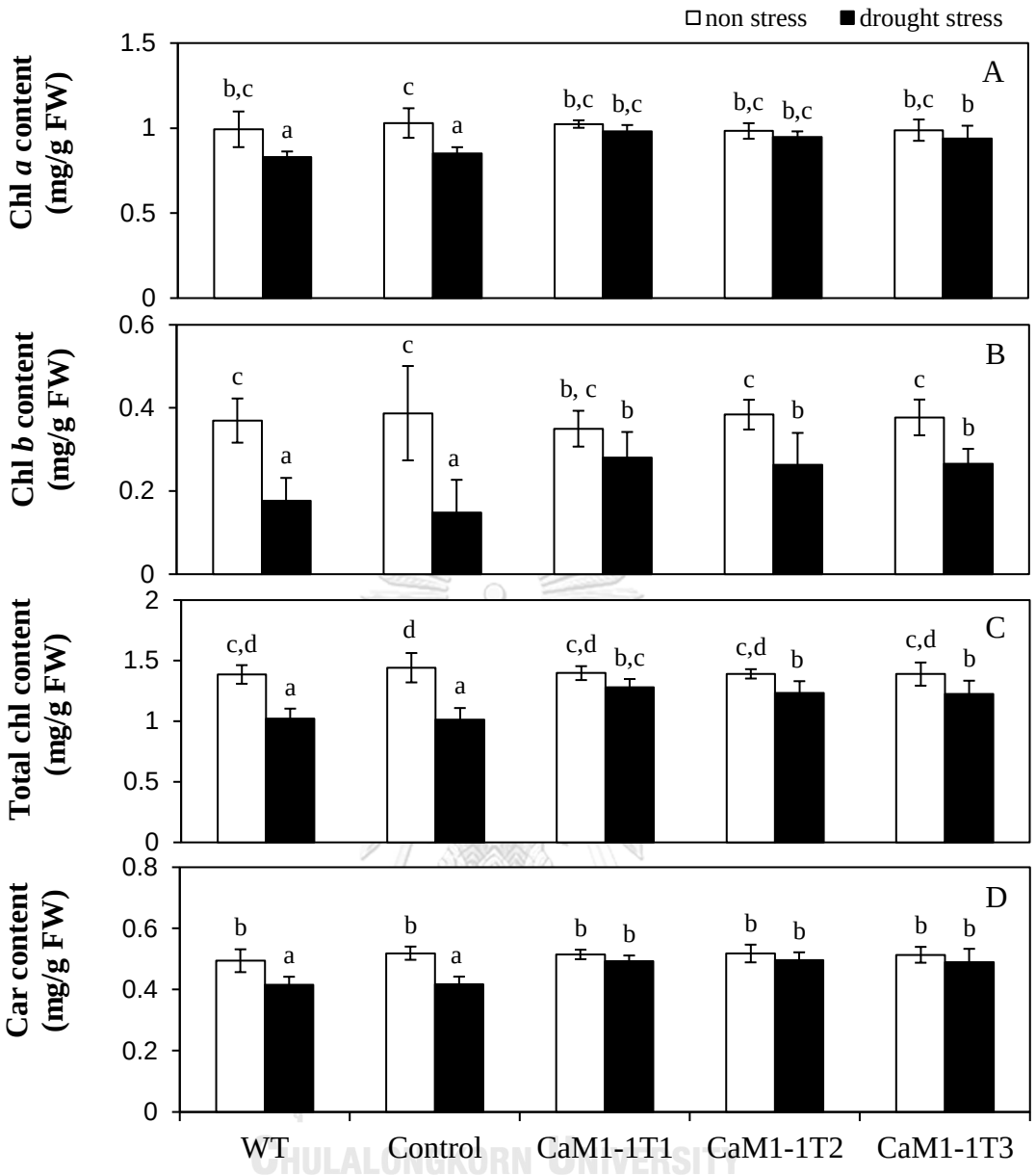


Figure 3.12 Effect of drought stress on photosynthetic pigment contents ((A) chlorophyll *a* (Chl *a*), (B) chlorophyll *b* (Chl *b*), (C) total chlorophyll (Total chl), (D) carotenoid (Car)) in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.10. DPPH scavenging rate

To realize the role of *OsCaM1-1* in DPPH scavenging rate subjected to drought stress, leaves of the three transgenic plants overexpressing *OsCaM1-1* gene, wild type, and control were analyzed under with and without drought stress. Comparison to non stress condition, the DPPH scavenging rate was up-regulated under drought stress condition (Figure 3.13). It was significantly up-regulated in the three transgenic plants about 27-90%, while in wild type and control about 13 and 25%, respectively.

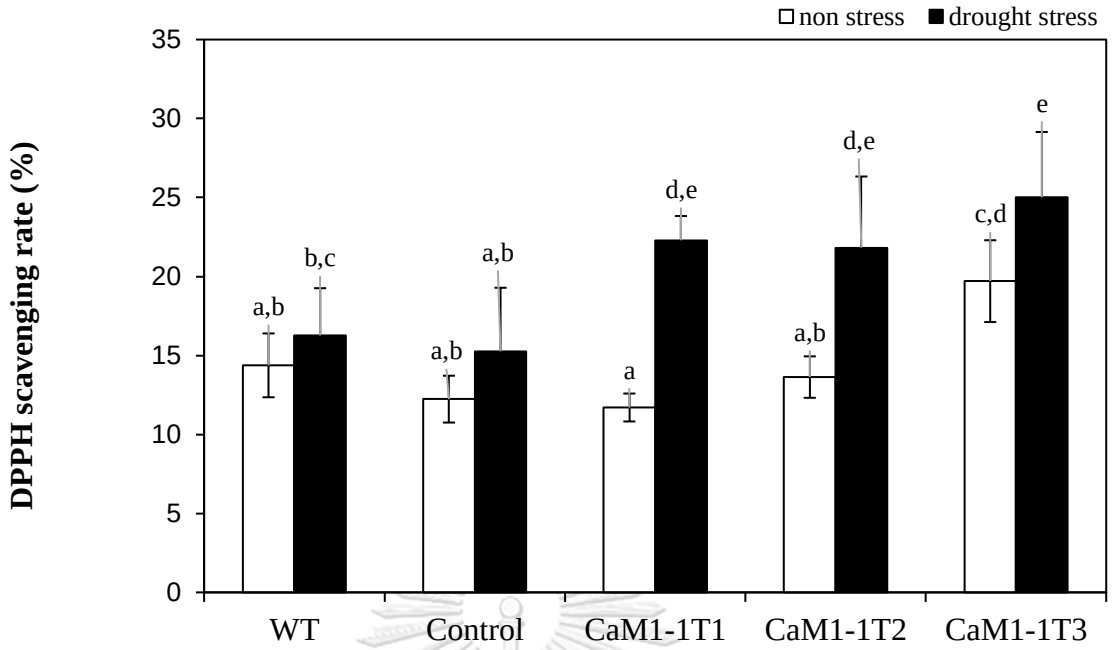


Figure 3.13 Effect of drought stress on DPPH scavenging rate in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

3.11. Antioxidant enzyme activity

To comprehend the role of *OsCaM1-1* in cell prevention controlled to drought induced oxidative stress, activities of antioxidant enzymes such as SOD, CAT, and POD were assayed in the transgenic plant compared with the wild type and control plants. Under non stress, the activity of antioxidant enzymes are similarity in all type plants (Figure 3.14). But under drought stress condition, the activities of SOD (Figure 3.14A) and POD (Figure 3.14C) were slightly increased in the wild type (about 9 and 14%, respectively) and control plants (about 13 and 17%, respectively), while they were significantly increased in the transgenic plants (about 27-32% and 36-68%, respectively). In contrast, drought stress led to decrease of the CAT activities in the wild type and control plants, but not in the three transgenic plants, a significant increase activities (about 27-54%) were detected (Figure 3.14B).

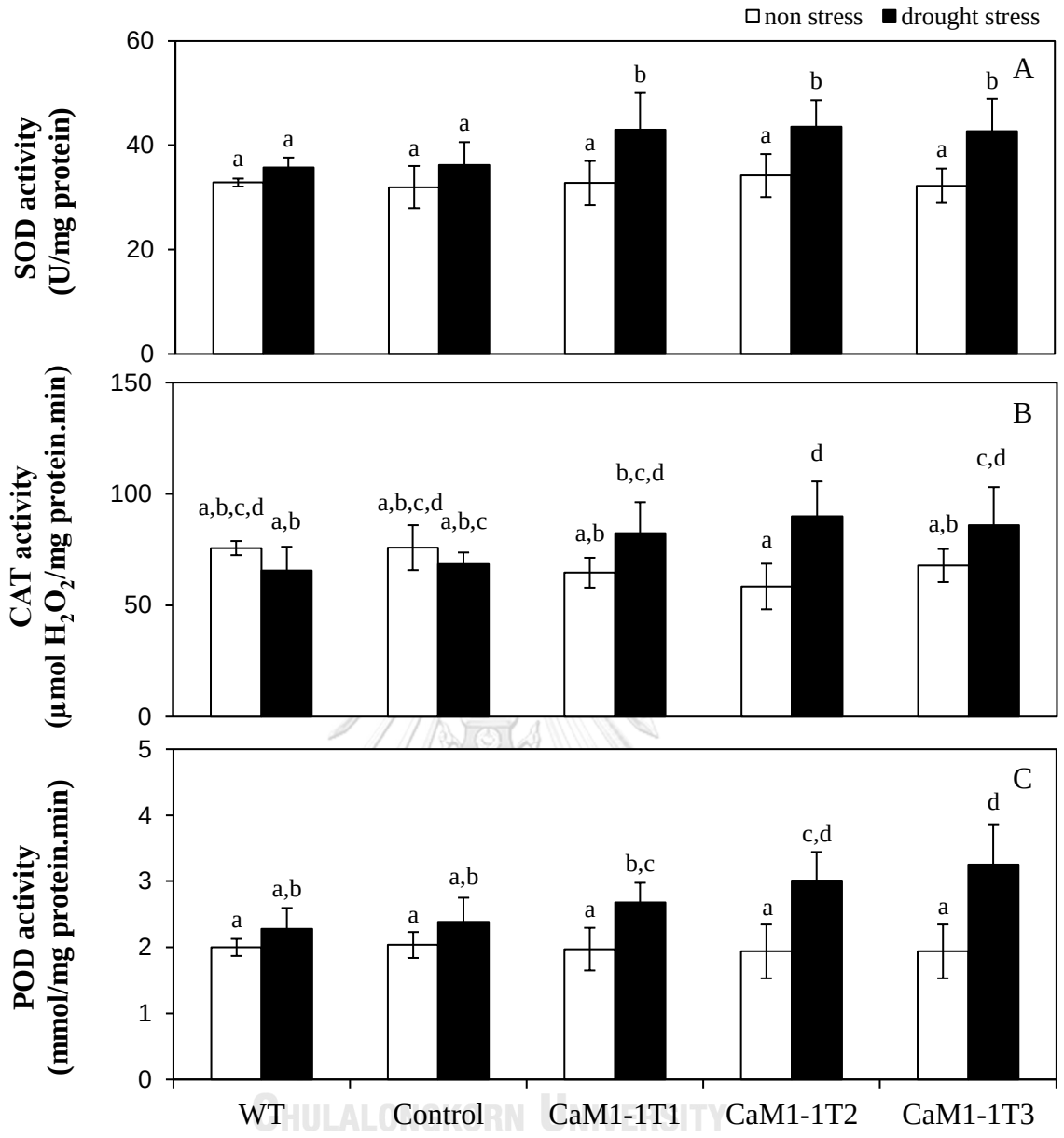


Figure 3.14 Effect of drought stress on the activity of SOD, CAT, and POD in leaves of the three transgenic rice overexpressing *OsCaM1-1* gene compare to wild type (WT) and control (negative control transgenic line) plants after expose to drought stress (0 and 20% PEG-4000) for 24 h. Values are Mean ± SD (n=5) and difference letters above the bars indicate significant differences ($P < 0.05$, Duncan's multiple comparison tests).

CHAPTER 4

DISCUSSIONS

To investigate the effect of the overexpressing *OsCaM1-1* in transgenic rice and participation in drought tolerance devices, the three transgenic plants with the *35SCaMV-OsCaM1-1* insertion and the vector control transgenic line (control) have been investigated the effect of drought stress compared to wild type plants. The results showed that the H_2O_2 content, ion content (Na^+, K^+, and Ca^{2+}), lipid peroxidation, electrolytic leakage, DPPH radical scavenging rate, RGR, and activities of SOD and POD, osmoprotectant accumulation (proline and soluble sugar) in all plants and CAT activity of the three transgenic plants were increased, while RWC, photosynthetic pigments, and CAT activity of the wild type and control were decreased under drought stress. H_2O_2 content in all plants increased with increasing level of the PEG-4000 treatment and exposure time, the findings of this study indicated that exposure time and drought level were two important factors affecting the plant development under drought stress condition.

The reactive oxygen species (ROS) is the by-product that ordinarily generates from the physiological metabolisms (You, 2015). In many plant (such as *Cleome gynandra* (Uzilday, 2012), sorghum (Nxele, 2017), Iranian Perennial ryegrass (Mohammadi, 2017), peppermint (Rahimi, 2018), purslane (Jin, 2015), and rice (Guo, 2006; Wang, 2010) reports, it was remarked that accumulation of ROS enhanced under drought stress when compared to without stress condition. The cation translocation presents in many essential duties including plant nourishment, signal transduction, plant growth and plant development (Pardo, 2006). The Na^+ uptake

presents to make an osmotic status for the higher water absorption and the higher turgor retention, nevertheless the excess Na^+ uptake is toxic to plants (Pardo, 2002). The K^+ plays an essential role in water equilibrium storage in plant cells. It is one of the major essences of cell osmotic potential in many plants, and it acts as a key role in osmotic adjustment under water stress (Shabala, 2002). Plants need a mechanism for identifying and responding to abiotic stresses, and the mechanisms are associated with intracellular Ca^{2+} concentration. The Ca^{2+} represents as a secondary messenger in molecular mechanisms and essential effector enzymes such as kinases and phosphatases, and then changes in cytosolic Ca^{2+} concentrations from extracellular and intracellular storages (endoplasmic reticulum and vacuoles) to cytosol conduct signal related to remedy and adaptations to abiotic stresses through participation of calmodulin, Ca^{2+} dependent kinases, and other Ca^{2+} dependent proteins (Sadiqov, 2002). In previous reports, drought stress increased in K^+ and Na^+ contents in *Atriplex halimus* L. (Martìnez, 2003), and increased root Ca^{2+} content in Norway spruce seedlings (Zlobin, 2018), on the other hand, drought did not change in Na^+ content in Sakha 102 rice (Hazman, 2016), unaffected the amount of Na^+ and K^+ balanced in Atlas mastic tree (Fayyaz, 2013), and also decreased in Ca^{2+} content in barley (Feng, 2016) when compared with without stress condition. The lipid peroxidation of the cellular membrane is persuaded by ROS (Apel, 2004) and the malondialdehyde (MDA) is a product of the lipid peroxidation and uses as an indicator to indicate the plant membrane degradation (Ma, 2015). Under drought stress, MDA was increased in many plants like peppermint (Rahimi, 2018), purslane (Jin, 2015), wolfberry (Zhao, 2018) , hot pepper (Anjum, 2012), stevia (Karimi, 2015), dendrobium (Xiaolu, 2016), *Cleome gynandra* (Uzilday, 2012), and rice (Hazman, 2016; Wang, 2010; Zu, 2017).

The electrolytic leakage determination as the impact on the cell membrane stability which is affected by abiotic stresses (Bajji, 2002) and drought stress increased in electrolytic leakage in various plants such as Iranian Perennial ryegrass (Mohammadi, 2017), purslane (Jin, 2015), black pepper (Anjum, 2012), rice (Chutipaijit, 2016; Swapna, 2017), maize, and sorghum (Takele, 2010). In this study, we observed that the overexpression of *OsCaM1-1* in the three transgenic plants showed the less enhancement in the H_2O_2 level and Na^+ content (Figure 3.2 and 3.3A) and the higher increase in the Ca^{2+} content (Figure 3.3C) resulting in the less reduction in the K^+/Na^+ and Ca^{2+}/Na^+ ratio (Figure 3.4), and the three transgenic plants also presented the less increase in the MDA content and the electrolytic leakage (Figure 3.5 and 3.6) than that of the wild type and control plants under drought stress condition. These results correspond to previous studies that the overexpressing *ShCML44* and *OsDSR-1*, which are a calmodulin-like gene, in transgenic tomato (Munir, 2016) and transgenic rice (Yin, 2017), respectively, reduced in ROS and MDA accumulations and the membrane damage under drought stress when compared to the wild type plants.

The relative growth rate (RGR) is one essential parameter to indicate the plant growth (Hoffmann, 2002). There are many plant (such as sorghum (Nxele, 2017), and stevia (Karimi, 2015)) reports, it was observed that biomass and growth of plant were reduced under drought stress. In this study, the three transgenic plants overexpressing of *OsCaM1-1* gene showed the better retention in the RGR in both shoot and root than the wild type and control plants under drought stress (Figure 3.8A and 3.8B). These results are consistent with previous reports such as in the overexpression of *OsDSR-1* in the transgenic rice, which are a calmodulin-like gene, showed the better plant

growth and better survival under drought stress condition than the wild type (Yin, 2017).

The RWC or the relative turgidity, is an index of the leave water status (Smart, 1974) and drought stress also led to loss of cell turgor by reducing water availability in various plants such as sorghum (Nxele, 2017), Iranian Perennial ryegrass (Mohammadi, 2017), purslane (Jin, 2015), and dendrobium (Xiaolu, 2016). Under drought condition, the osmoprotectant such as proline and soluble sugar is a robust scavenger of ROS and represents like an osmolyte in plants for reducing the cellular osmotic potential and defending to retain in the turgor pressure by represents like a consistent solute (Chen, 2005; Delauney, 1993; Yamada, 2005). In previous reports, it was observed that the proline and soluble sugar accumulations were enhanced in many plants such as Iranian Perennial ryegrass (Mohammadi, 2017) , black pepper (Anjum, 2012), purslane (Jin, 2015), peanut (Zhang et al., 2017), stevia (Karimi, 2015), and rice (Mishra, 2017; Swapna, 2017; Zu, 2017) under drought stress. In this study, the three transgenic plants overexpressing of *OsCam1-1* gene appeared the less reduction in the RWC (Figure 3.9) and showed the higher accumulation in both of proline and soluble sugar content (Figure 3.10 and 3.11) more than that of the wild type and control plants under drought stress. These results are harmonized with the overexpression of *OsCPK9* and *ShCML44* which a calcium sensor in transgenic rice (Wei, 2014) and transgenic tomato (Munir, 2016), respectively, the osmolyte accumulation was higher increased and the RWC was less decreased than the wild type plants under drought stress.

The physiological function of plant leaves significantly related to the photosynthetic pigments such as chlorophyll *a*, *b*, and carotenoid (Sims, 2002). In

earlier studies have been reported that drought stress decreased in pigment contents in rice (Swapna, 2017; Wang, 2010; Zu, 2017), purslane (Jin, 2015), sorghum (Nxele, 2017), and maize (Takele, 2010) when compared with without stress condition. However, the three transgenic plants overexpressing of *OsCam1-1* gene exhibited the higher retention in the photosynthetic pigment level than the wild type and control plants under drought stress (Figure 3.12). These results are consistent with the previous reports in the overexpression of *PeCBL* and *OsCPK9*, which are a calcium sensor like calmodulin, in transgenic triploid white poplar (Li, 2012) and transgenic rice (Wei, 2014), respectively, showed the higher retention in the chlorophyll content than the wild type plants under drought treatment.

Abiotic stress such as drought stress leads to the oxidative damage and the ROS accumulation, however, ROS can be eliminated by the enzymatic and non-enzymatic antioxidant processes in cells for decreasing and preventing the damage of the oxidative stress (Basu, 2009; Pandey, 2015). The increased activities of DPPH scavenging rate, SOD (superoxide dismutase), CAT (catalase), POD (peroxidase), APX (ascorbate peroxidase), and GPX (guaiacol peroxidase) activities under drought stress condition are consistent with the prior studies showed that drought stress increased in the H_2O_2 content, the activities of SOD, CAT, and POD in wolfberry (Zhao, 2018), hot pepper (Anjum, 2012), stevia (Karimi, 2015), *Cleome gynandra* (Uzilday, 2012), and rice (Mishra, 2017; Swapna, 2017) when compared with without stress condition. Furthermore, drought stress also increased in the DPPH scavenging rate in rice (Chutipaijit, 2016). In this study, the overexpression of *OsCam1-1* in transgenic lines showed the less increase in the H_2O_2 content under drought stress by the motive of the upper increase in the DPPH-radical scavenging rate (Figure 3.11)

and the higher promotion in the enzymatic antioxidant activities (Figure 3.12) than that of the wild type and control plants under drought stress condition. However, the CAT activities of the wild type and control showed increase under drought stress (Figure 3.12B). This corresponds to the report of Munir and coworkers (Munir, 2016) and Yin and coworkers (Yin, 2017), the overexpression of *ShCML44*, a calcium sensor like calmodulin, in transgenic tomato and the overexpression of *OsDSR-1*, a calmodulin like gene, in transgenic rice, respectively, show the better promotion in the antioxidant enzyme activities more that wild type plants under drought condition.

Thus, the overexpressing of *OsCam1-1* is one of positive regulator to the plant growth rate and the photosynthetic pigment content, it improved in the modulation of the non-enzymatic (osmoprotectant accumulation) and enzymatic antioxidant activities, enhanced in the water retaining, and diminished in the cell membrane lipid peroxidation defended from the membrane damage by the reason of the maintained lipids inside their membrane that accommodated for gathering of the cell membrane stability leading to promoted in the retention of RGR in transgenic rice under drought stress.

CHAPTER 5

CONCLUSION

In conclusion, the overexpressing *OsCam1-1* gene increased drought tolerance in transgenic plants by the better promotion in the ability of antioxidant enzyme activity, DPPH scavenging rate, and the retention of K^+/Na^+ ratio and Ca^{2+}/Na^+ ratio of plants. Besides, the overexpressing *OsCam1-1* also improved the osmoprotectant accumulation and the water retention ability as well as decreased the cell membrane damage in response to drought stress. These results suggested that the overexpression of *OsCam1-1* is a positive indicator to the antioxidant system leading to drought resistance in rice.

REFERENCES

Agarwal, S., Sairam, R.K., Srivastava, G.C.,Tyagi, A., Meena, R.C. 2005. Role of ABA, salicylic acid, calcium and hydrogen peroxide on antioxidant enzymes induction in wheat seedlings. *Plant Science*. 169:559-570.

Akram, H.M., Ali, A., Sattar, A.,Saeed-Ur-Rehman, H., Bibi, A. 2013. Impact of water deficit stress on various physiological and agronomic traits of three Basmati rice (*Oryza sativa* L.) cultivars. *Journal of Animal and Plant Sciences*. 23:1415-1142.

Anjum, S., Farooq, M., Xie, X.Y., Liu, X.J., Ijaz, M. 2012. Antioxidant defense system and proline accumulation enables hot pepper to perform better under drought. *Scientia Horticulturae*. 140:66-73.

Apel, K., Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*. 55:373-399.

Austin, R.B. 1989. Prospects for improving crop production in stressful environments. Cambridge University Press, Cambridge. 235-248 pp.

Ayala, A., M.F. Muñoz, and S. Argüelles. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*. 2014:31.

Bajji, M., Kinet, J.M., Lutts, S. 2002. The use of the electrolyte leakage method for assessing cell membrane stability as a water stress tolerance test in durum wheat. *Plant Growth Regulation*. 36:61-70.

Basu, S., Ramegowda, V., Kumar, A., Pereira, A. 2016. Plant adaptation to drought stress. *F1000Research*. 5:1-10.

Basu, S., Roychoudhury, A., Paromita, S. P., Sengupta, D. 2009. Differential oxidative responses of indica rice cultivars to drought stress. *Plant Growth Regulation*. 60:51-59.

Bates, L.S., Waldren, R.P., Teare, I.D. 1973. Rapid determination of free proline for water-stress studies. *Plant and Soil*. 39:205-207.

Beckman, K.B., Ames, B.N. 1998. The free radical theory of aging matures. *Physiological Reviews*. 78:547-581.

Betteridge, D.J. 2000. What is oxidative stress?. *Metabolism - Clinical and Experimental*. 49:3-8.

Blokhina, O., Fagerstedt, K.V. 2010. Reactive oxygen species and nitric oxide in plant mitochondria: origin and redundant regulatory systems. *Physiologia Plantarum*. 138:447-462.

Blum, A., Ebercon, A. 1981. Cell membrane stability as a measure of drought and heat tolerance in wheat1. *Crop Science*. 21:43-47.

Bose, J., Pottosin, I.I., Shabala, S.S., Palmgren, M.G., Shabala, S. 2011. Calcium efflux systems in stress signaling and adaptation in plants. *Frontiers in plant science*. 2:85.

Bottner, P., CoÛteaux, M.M., Vallejo, R. 1995. Soil Organic matter in mediterranean-type ecosystems and global climatic changes: a case study—the soils of the mediterranean basin. *Global Change and Mediterranean-Type Ecosystems.*:306-325.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*. 72:248-254.

Centritto, M., Lauteri, M., Monteverdi, M.C., Serraj, R. 2009. Leaf gas exchange, carbon isotope discrimination, and grain yield in contrasting rice genotypes subjected to water deficits during the reproductive stage. *Journal of Experimental Botany*. 60:2325-2339.

Chen, C., Dickman, M.B. 2005. Proline suppresses apoptosis in the fungal pathogen *Colletotrichum trifolii*. *Proceedings of the National Academy of Sciences of the United States of America*. 102:3459.

Chinpongpanich, A., Limruengroj, K., Phean-o-pas, S., Limpaseni, T., Buaboocha, T. 2012. Expression analysis of calmodulin and calmodulin-like genes from rice, *Oryza sativa* L. *BMC Research Notes*. 5:625.

Chutipaijit, S. 2016. Changes in physiological and antioxidant activity of indica rice seedlings in response to mannitol-induced osmotic stress. *Chilean journal of agricultural research*. 76:455–462.

Cooper, G.M. 2000. The cell: a molecular approach, Sunderland (MA).

Das, K., Roychoudhury, A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Frontiers in Environmental Science*. 2:1-13.

Delauney, A.J., Verma, D.P.S. 1993. Proline biosynthesis and osmoregulation in plants. *The Plant Journal*. 4:215-223.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*. 28:350-356.

Faize, M., Burgos, L., Faize, L., Piqueras, A., Nicolas, E., Barba, E.G., Clemente-Moreno, M.J., Alcobendas, R., Artlip, T., Hernandez, J.A. 2011. Involvement

of cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved tolerance against drought stress. *Journal of Experimental Botany*. 62:2599-2613.

Farooq, M., Hussain, M., Wahid, A., Siddique, K. 2012. Drought stress in plants: an overview. Springer, Berlin, Heidelberg. 1-33 pp.

Farooq, M., Kobayashi, N., Ito, O., Wahid, A., Serraj, R. 2010. Broader leaves result in better performance of indica rice under drought stress. *Journal of Plant Physiology*. 167:1066-1075.

Fayyaz, P., Etemadi, E., Julaiee, M.N., Zolfaghari, R. 2013. Sodium and potassium allocation under drought stress in Atlas mastic tree (Pistacia atlantica subsp. Mutica). 90-94 pp.

Fedoroff, N.V., D.S. Battisti, R.N. Beachy, P.J.M. Cooper, D.A. Fischhoff, C.N. Hodges, V.C. Knauf, D. Lobell, B.J. Mazur, D. Molden, M.P. Reynolds, P.C. Ronald, M.W. Rosegrant, P.A. Sanchez, A. Vonshak, and J.K. Zhu. 2010. Radically rethinking agriculture for the 21st Century. *Science (New York, N.Y.)*. 327:833-834.

Feng, X., Liu, W., Zeng, F., Chen, Z., Zhang, G., Wu, Feibo. 2016. K^+ Uptake, H^+-ATPase pumping activity and Ca^{2+} efflux mechanism are involved in drought tolerance of barley. *Environmental and Experimental Botany*. 129:57-66.

Gardner, F.P., Pearce, R.B., Mitchell, R.L. 1985. Physiology of crop plants. Iowa State University Press, Ames, IA, USA. 31-46 pp.

Guo, Z., Ou, W., Lu, S., Zhong, Q. 2006. Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. *Plant Physiology and Biochemistry*. 44:828-836.

Hamanishi, E.T., Campbell, M.M. 2011. Genome-wide responses to drought in forest trees. *Forestry: An International Journal of Forest Research*. 84:273-283.

Hasanuzzaman, M., Alam, M.M., Rahman, A., Hasanuzzaman, M., Nahar, K., Fujita, M. 2014. Exogenous proline and glycine betaine mediated upregulation of antioxidant defense and glyoxalase systems provides better protection against salt-induced oxidative stress in two rice (*Oryza sativa* L.) varieties. *BioMed Research International*. 2014:17.

Hazman, M., Hause, B., Eiche, E., Riemann, M., Nick, P. 2016. Different forms of osmotic stress evoke qualitatively different responses in rice. *Journal of Plant Physiology*. 202:45-56.

Heath, R.L., Packer, L. 1968. Photoperoxidation in isolated chloroplasts: I. kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*. 125:189-198.

Hendry, G.A. 2005. Oxygen free radical process and seed longevity. *Seed Science Research*. 3:141-147.

Hoffmann, W.A., Poorter, H. 2002. Avoiding bias in calculations of relative growth rate. *Annals of Botany*. 90:37-42.

Hossain, M.A., Hasanuzzaman, M., Fujita, Mas. 2011. Coordinate induction of antioxidant defense and glyoxalase system by exogenous proline and glycinebetaine is correlated with salt tolerance in mung bean.

Hugo, A., Wyss S.R., Bernhard, S., František, S. 1974. Heterogeneity of erythrocyte Catalase II. *European Journal of Biochemistry*. 48:137-145.

Inskeep, W.P., Bloom, P.R. 1985. Extinction coefficients of chlorophyll *a* and *b* in *N,N*-dimethylformamide and 80% acetone. *Plant Physiology*. 77:483-485.

Jajic, I., Sarna, T., Strzalka, K. 2015. Senescence, stress, and reactive oxygen species. *Plants*. 4.

Jaleel, C.A., Manivannan, P., Wahid, A., Farooq, M., Al-Juburi, H. J., Somasundaram, R., Panneerselvam, R. 2009. Drought stress in plants: A review on morphological characteristics and pigments composition. *International Journal of Agriculture and Biology*. 11:100-105.

Ji, K., Wang, Y., Sun, W., Lou, Q., Mei, H., Shen, S., Chen, H. 2012. Drought-responsive mechanisms in rice genotypes with contrasting drought tolerance during reproductive stage. *Journal of Plant Physiology*. 169:336-344.

Jin, R., Wang, Y., Liu, R., Gou, J., Chan, Z. 2015. Physiological and metabolic changes of purslane (*Portulaca oleracea* L.) in response to drought, heat, and combined stresses. *Frontiers in Plant Science*. 6:1123.

Karimi, M., Ahmadi, A., Hashemi, J., Abbasi, A., Tavarini, S., Guglielminetti, L., Angelini, L. 2015. The effect of soil moisture depletion on Stevia (*Stevia rebaudiana* Bertoni) grown in greenhouse conditions: growth, steviol glycosides content, soluble sugars and total antioxidant capacity. *Scientia Horticulturae*. 183:93–99.

Karp, G. 1999. Cell and molecular biology: concept and experiment. *In* chapter-15: Cell signaling and signal transduction. Vol. 6th edition.

Kulaz, H., Tuncturk, R., Tunçtürk, Mu. 2017. Effect of zinc applications on some nutrient concentrations of soybean (*Glycine max* L.) cultivars. 520-527 pp.

Li, D., Song, S., Xia, X., Yin, W. 2012. Two CBL genes from *Populus euphratica* confer multiple stress tolerance in transgenic triploid white poplar. *Plant Cell, Tissue and Organ Culture (PCTOC)*. 109:477-489.

Lin, C.C., Kao, C.H. 1996. Proline accumulation is associated with inhibition of rice seedling root growth caused by NaCl. *Plant science (Limerick)*. 114:121-128.

Lum, J.A.G., Conti, R.G., Morgan, A.T., Ullman, M.T. 2014. Procedural learning deficits in specific language impairment (SLI): a meta-analysis of serial reaction time task performance. *Cortex; a Journal Devoted to the Study of the Nervous System and Behavior*. 51:1-10.

Ma, J., Du, G., Li, X., Zhang, C., Guo, J. 2015. A major locus controlling malondialdehyde content under water stress is associated with Fusarium crown rot resistance in wheat. *Molecular Genetics and Genomics*. 290:1955-1962.

Maisura, M.A.C., Iskandar, L., Ahmad, J., Hiroshi, E. 2014. Some physiological character responses of rice under drought conditions in a paddy system *Journal of the International Society of Southeast Asian Agricultural Sciences* 20:104-114.

Manickavelu, A., Nadarajan, N., Ganesh, S.K., Gnanamalar, R.P., Chandra, B.R. 2006. Drought tolerance in rice: morphological and molecular genetic consideration. *Plant Growth Regulation*. 50:121-138.

Martìnez, J.P., Ledent, J. F., Bajji, M., Kinet, J.M., Lutts, S. 2003. Effect of water stress on growth, Na^+ and K^+ accumulation and water use efficiency in relation to osmotic adjustment in two populations of *Atriplex halimus* L. *Plant Growth Regulation*. 41:63-73.

Mhamdi, A., Queval, G., Chaouch, S., Vanderauwera, S., Van, B.F., Noctor, G. 2010. Catalase function in plants: a focus on Arabidopsis mutants as stress-mimic models. *Journal of Experimental Botany*. 61:4197-4220.

Michael, S.R., Barko, J.W. 1985. Laboratory culture of submersed freshwater macrophytes on natural sediments. *Aquatic Botany*. 21:251-263.

Mishra, S.S., Panda, D. 2017. Leaf traits and antioxidant defense for drought tolerance during early growth stage in some popular traditional rice landraces from Koraput, India. *Rice Science*. 24:207-217.

Mohammadi, S., Hossein, M., Nematollah, E., Mehdi, A.M., Mostafa, A., Mohammad, P. 2017. Molecular and physiological responses of Iranian Perennial ryegrass as affected by trinexapac ethyl, paclobutrazol and abscisic acid under drought stress. *Plant Physiology and Biochemistry*. 111:129-143.

Mostajeran, A., Rahimi, E.V. 2009. Effects of drought stress on growth and yield of rice (*Oryza sativa* L.) cultivars and accumulation of proline and soluble sugars in sheath and blades of their different ages leaves. *American-Eurasian Journal Of Agricultural & Environmental Sciences*. 5:264-272.

Munir, S., Liu, H., Xing, Y., Hussain, S., Ouyang, B., Zhang, Y., Li, H.,Ye, Z. 2016. Overexpression of calmodulin-like (*ShCML44*) stress-responsive gene from *Solanum habrochaites* enhances tolerance to multiple abiotic stresses. *Scientific Reports*. 6:31772.

Munns, C.F., Shaw, N., Kiely, Mairead, Specker, Bonny L., Thacher, T.D., Ozono, K., Michigami, T., Tiosano, D., Mughal, M.Z., Mäkitie, O., Ramos, A.L., Ward, L., DiMeglio, L.A., Atapattu, N., Cassinelli, H., Braegger, C., Pettifor, J.M., Seth, A., Idris, H.W., Bhatia, V., Fu, J., Goldberg, G., Sävendahl, L., Khadgawat, R., Pludowski, P., Maddock, J., Hyppönen, E., Oduwole, A., Frew, E., Aguiar, M., Tulchinsky, T., Butler, G., Högler, W. 2016. Global

consensus recommendations on prevention and management of nutritional rickets. *The Journal of Clinical Endocrinology & Metabolism*. 101:394-415.

Nair, A.S., Abraham, T. K., Jaya, D.S. 2008. Studies on the changes in lipid peroxidation and antioxidants in drought stress induced cowpea (*Vigna unguiculata* L.) varieties. *Journal of Environmental Biology*. 29:689-691.

Noctor, G., Foyer, C.H. 1998. Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*. 49:249-279.

Nxele, X., Klein, A., Ndimba, B.K. 2017. Drought and salinity stress alters ROS accumulation, water retention, and osmolyte content in sorghum plants. *South African Journal of Botany*. 108:261-266.

Pandey, V., Shukla, A. 2015. Acclimation and tolerance strategies of rice under drought stress. *Rice Science*. 22:147-161.

Pardo, B., Contreras, L., Serrano, A., Ramos, M., Kobayashi, K., Iijima, M., Saheki, T., Satrústegui, J. 2006. Essential role of aralar in the transduction of small Ca^{2+} signals to neuronal mitochondria. *Journal of Biological Chemistry*. 281:1039-1047.

Pardo, J.M., Quintero, F.J. 2002. Plants and sodium ions: keeping company with the enemy. *Genome Biology*. 3:reviews1017.1011-reviews1017.1014.

Pereira, J.S., Chaves, M.M. 1993. Plant water deficits in mediteranean ecosystem. *Water Deficit and Growth*. 4:237-251.

Putter, J. 1974. Methods of enzymatic analysis. Verlag Chemie, Weinhan. 685-690 pp.

Rahimi, Y., Taleei, A., Ranjbar, M. 2018. Long-term water deficit modulates antioxidant capacity of peppermint (*Mentha piperita* L.).

Ray, D.K., Gerber, J.S., Mac, D., Graham, K., West, P.C. 2015. Climate variation explains a third of global crop yield variability. *Nature Communications*. 6:1-9.

Ray, D.K., Mueller, Nathaniel D., West, P.C., Foley, J.A. 2013. Yield trends are insufficient to double global crop production by 2050. *PLOS ONE*. 8:1-8.

Ray, D.K., Ramankutty, N., Mueller, N.D., West, P.C., Foley, J.A. 2012. Recent patterns of crop yield growth and stagnation. *Nature Communications*. 3:1293.

Rhodes, D., Samaras, Y. 1994. Cellular and molecular physiology of cell volume regulation. CRC Press, Boca Raton. 347-361 pp.

Sadiqov, S.T., Akbulut, M., Ehmedov, V. 2002. Role of Ca^{2+} in drought stress signaling in wheat seedlings. *Biochemistry (Moscow)*. 67:491-497.

Saeng-ngam, S., Takpirom, W., Buaboocha, T., Chadchawan, S. 2012. The role of the *OsCam1-1* salt stress sensor in ABA accumulation and salt tolerance in rice. *Journal of Plant Biology*. 55:198-208.

Segal, L.M., Wilson, R.A. 2018. Reactive oxygen species metabolism and plant-fungal interactions. *Fungal Genetics and Biology*. 110:1-9.

Serraj, R., Kumar, A., McNally, K.L., Slamet L.I., Bruskiewich, R., Mauleon, R., Cairns, J., Hijmans, R.J. 2009. Chapter 2 - Improvement of drought resistance in rice. Academic Press. 41-99 pp.

Sgherri, C.L.M., Maffei, M., Navari, I.F. 2000. Antioxidative enzymes in wheat subjected to increasing water deficit and rewatering. *Journal of Plant Physiology*. 157:273-279.

Shabala, S.N., Lew, R.R. 2002. Turgor regulation in osmotically stressed arabidopsis epidermal root cells. Direct support for the role of inorganic ion uptake as revealed by concurrent flux and cell turgor measurements. *Plant Physiology*. 129:290-299.

Shao, H.B., Chu, L.Y., Shao, M.A., Jaleel, C.A., Hong, M.M. 2008. Higher plant antioxidants and redox signaling under environmental stresses. *Comptes Rendus Biologies*. 331:433-441.

Sharma, P., Dubey, R.S. 2005. Lead toxicity in plants. *Brazilian Journal of Plant Physiology*. 17:35-52.

Sharma, P., Dubey, R.S., Jha, A., Shanker, D.R., Pessarakli, M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*. 2012:1-26.

Shehab, G.G., Ahmed, O.K., El, B.H.S. 2010. Effects of various chemical agents for alleviation of drought stress in rice plants (*Oryza sativa* L.). *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*. 38:139-148.

Sims, D.A., Gamon, J.A. 2002. Relationships between leaf pigment content and spectral reflectance across a wide range of species, leaf structures and developmental stages. *Remote Sensing of Environment*. 81:337-354.

Smart, R.E., Bingham, G.E. 1974. Rapid estimates of relative water content. *Plant Physiology*. 53:258.

Smirnoff, N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytologist*. 125:27-58.

Smirnoff, N. 1998. Plant resistance to environmental stress. *Current Opinion in Biotechnology*. 9:214-219.

Sullivan, C.Y., Ross, W.M. 1979. Stress Physiology in Crop Plants. John Wiley and Sons, New York. 263-281 pp.

Swapna, S., Shylaraj, K.S. 2017. Screening for osmotic stress responses in rice varieties under drought condition. *Rice Science*. 24:253-263.

Szabo, M., Idiţoiu, C., Chambre, D., Lupea, A. 2007. Improved DPPH determination for antioxidant activity spectrophotometric assay. *chempap*. 61:214–216.

Takele, A. 2010. Differential responses of electrolyte leakage and pigment compositions in maize and sorghum after exposure to and recovery from pre- and post-flowering dehydration. *Agricultural Sciences in China*. 9:813-824.

Tripathy, J.N., Zhang, J., Robin, S., Nguyen, T.T., Nguyen, H.T. 2000. QTLs for cell-membrane stability mapped in rice (*Oryza sativa* L.) under drought stress. *Theoretical and Applied Genetics*. 100:1197-1202.

Uzilday, B., Turkan, I., Ozgur, R., Karakaya, H. 2012. Comparison of ROS formation and antioxidant enzymes in *Cleome gynandra* (C-4) and Cleome spinosa (C-3) under drought stress. *Plant Science*. 182:59-70.

Vurukonda, S.S.K.P., Vardharajula, S., Shrivastava, M., SkZ, A. 2016. Enhancement of drought stress tolerance in crops by plant growth promoting rhizobacteria. *Microbiological Research*. 184:13-24.

Wang, H.Z., Zhang, L.H., Ma, J., Li, X.Y., Li, Y., Zhang, R.P., Wang, R.Q. 2010. Effects of water stress on reactive oxygen species generation and protection system in rice during grain-filling stage. *Agricultural Sciences in China*. 9:633-641.

Wang, X., Putkey, J.A. 2016. PEP-19 modulates calcium binding to calmodulin by electrostatic steering. *Nature Communications*. 7:13583.

Wei, S., Hu, W., Deng, X., Zhang, Y., Liu, X., Zhao, X., Luo, Q., Jin, Z., Li, Y., Zhou, S., Sun, T., Wang, L., Yang, G., He, G. 2014. A rice calcium-dependent protein kinase *OsCPK9* positively regulates drought stress tolerance and spikelet fertility. *BMC Plant Biology*. 14:133.

Wilkins, O., Waldron, L., Nahal, H., Provart, N.J., Campbell, M.M. 2009. Genotype and time of day shape the Populus drought response. *The Plant Journal*. 60:703-715.

Wu, J., Zhang, Z., Zhang, Q., Han, X., Gu, X., Lu, T. 2015. The molecular cloning and clarification of a photorespiratory mutant, *OSCDM1*, using enhancer trapping. *Frontiers in Genetics*. 6:226.

Wutipraditkul, N., Wongwean, P., Buaboocha, T. 2015. Alleviation of salt-induced oxidative stress in rice seedlings by proline and/or glycinebetaine. *Biologia Plantarum*. 59:547–553.

Xiaolu, W., Jie, Y., Aoxue, L., Yu, C., Yijun, F. 2016. Drought stress and re-watering increase secondary metabolites and enzyme activity in dendrobium moniliforme. *Industrial Crops and Products*. 94:385-393.

Yamada, M., Morishita, H., Urano, K., Shiozaki, N., Yamaguchi, S.K., Shinozaki, K.,Yoshiba, Y. 2005. Effects of free proline accumulation in petunias under drought stress. *Journal of Experimental Botany*. 56:1975-1981.

Yang, P.M., Huang, Q.C., Qin, G.Y., Zhao, S.P., Zhou, J.G. 2014. Different drought-stress responses in photosynthesis and reactive oxygen metabolism between autotetraploid and diploid rice. *Photosynthetica*. 52:193-202.

Yin, X., Huang, L., Wang, M., Cui, Y., Xia, X. 2017. *OsDSR-1*, a calmodulin-like gene, improves drought tolerance through scavenging of reactive oxygen species in rice (Oryza sativa L.). 37:75.

You, J., Chan, Z. 2015. ROS regulation during abiotic stress responses in crop plants. *Frontiers in Plant Science*. 6:1092.

Zhang, C., N.J. Selvaraj, Q. Yang, and Y. Liu. 2017. A survey of aflatoxin-producing *Aspergillus* sp. from peanut field soils in four agroecological zones of China. *Toxins*. 9.

Zhao, J.H., Li, H.X., Zhang, C.Z., An, W., Yin, Y., Wang, Y.J., Cao, Y.L. 2018. Physiological response of four wolfberry (*Lycium Linn*.) species under drought stress. *Journal of Integrative Agriculture*. 17:603-612.

Zhu, J.K. 2002. Salt and drought stress signal transduction in plants. *Annual review of plant biology*. 53:247-273.

Zlobin, I.E., Ivanov, Y.V., Kartashov, A.V., Kuznetsov, V.V. 2018. Impact of drought stress induced by polyethylene glycol on growth, water relations and cell viability of Norway spruce seedlings. *Environmental Science and Pollution Research*. 25:8951-8962.

Zu, X., Lu, Y., Wang, Q., Chu, P., Miao, W., Wang, H., La, H. 2017. A new method for evaluating the drought tolerance of upland rice cultivars. *The Crop Journal*. 5:488-498.

APPENDIX

APPENDIX A

CHEMICAL SOLUTION PREPARATION

1. Limpinuntana's nutrient solution (300X)

Reagents:			Final concentration (1X)
• **Solution A (300 mL)**			
KNO_3	9.100	g	(0.10 g/L)
$Ca(NO_3)_2.4H_2O$	14.169	g	(0.16 g/L)
• **Solution B (300 mL)**			
$NH_4H_2PO_4$	3.4506	g	(41 mg/L)
$MgSO_4.7H_2O$	3.6972	g	(38 mg/L)
NaCl	4.973	g	(55 mg/L)
• **Solution C (300 mL)**			
$FeSO_4.7H_2O$	0.834	g	(20 mg/L)
NaEDTA	1.134	g	(27 mg/L)
• **Solution D (300 mL)**			
$MnCl_2.4H_2O$	1.134	g	(1.44 mg/L)
H_3BO_3	0.1297	g	(1.14 mg/L)
$Na_2MoO_4.2H_2O$	0.1026	g	(0.025 mg/L)
$ZnSO_4.7H_2O$	0.0079	g	(0.088 mg/L)
$CuSO_4.5H_2O$	0.00351	g	(0.039 mg/L)

Limpinuntana's nutrient solution (1X, 300 mL)

Solution A-D were mixed together by adding 1 mL/each, after that make up the volume to 300 mL with deionized water and autoclave at 121 °C, 20 minutes.

2. 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 deionized water.

Antioxidant enzyme extraction buffer

- **0.5 M Sodium phosphate buffer, pH 7.5**

NaH_2PO_4	0.68	g
Na_2HPO_4	0.87	g

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

- **1mM EDTA**

EDTA	0.04	g

Dissolve in deionized water and make up the volume to 100 mL.

- **1M DTT**

DTT	0.15	g

Dissolve in deionized water and make up the volume to 100 mL.

- **5mM Ascorbic acid**

Ascorbic acid	0.09	g

Dissolve in deionized water and make up the volume to 100 mL.

- **1µL/mL PMSF**

Working solution:

0.5 M Sodium phosphate buffer, pH 7.5	20	mL
1mM EDTA	50	mL
1M DTT	50	µL
5mM Ascorbic acid	20	mL
1µL/mL PMSF	100	µL

Make up the volume to 100 mL with deionized water and store at 4 °C.

3. **Measurement of hydrogen peroxide 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. The solution must store in the brown glass bottle.

4. **Measurement of DPPH radical scavenging rate**

- **6 mM DPPH (10 mL)**

DPPH	0.237	g

Dissolve in 95% methanol and make up the volume to 10 mL and store at 4 °C. The solution must store in the brown glass bottle.

5. **Determination of protein**

- **Bradford reagent**

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

The solution is stirred under dark condition for 2 h, after that 20 mL of 85% phosphoric acid is added in the solution. Then, make up the volume to 200 mL with deionized water and filter the solution using Whatman filter paper. The solution must store in the brown glass bottle (available for several weeks.).

6. **Determination of antioxidant enzyme activity**

- **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 deionized water.

- **0.05 M Potassium phosphate buffer, pH 7.8**

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 deionized water.

- **1 M H_2O_2**

30% H_2O_2	1.01	mL

Dissolve in deionized water and make up the volume to 10 mL and store at 4 °C. The solution must store in the brown glass bottle.

- **133.3 mM Methionine**

L-Methionine	2.0	g

Dissolve in deionized water and make up the volume to 100 mL.

- **7.5 mM NBT**

NBT	0.006	g

Dissolve in deionized water and make up the volume to 1 mL.

- **0.5 M Na_2CO_3**

Na_2CO_3	5.3	g

Dissolve in deionized water and make up the volume to 100 mL.

- **0.2 mM Riboflavin**

Riboflavin	0.008	g

Dissolve in deionized water and make up the volume to 100 mL. The solution must store in the brown glass bottle.

APPENDIX B

CALCULATIONS

1. Determination of lipid peroxidation

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

V_t = the total volume of extract solution

V_r = the total volume of reaction mixture

V_s = the volume of the extract solution contain in the reaction mixture

m = the weight of sample

ε = the extinction coefficient (155 $mM^{-1}mL^{-1}$)

For example:

MDA content of WT plants under drought treatment (treated with 20% PEG) for 24h.

A_{532} = 0.065, A_{600} = 0.022

$$\text{TBARS content (µmol MDA equivalents g FW)} = \frac{(0.065 - 0.022) \times 1\ mL \times 1.2\ mL \times 100}{155 \times 0.3\ mL \times 0.05\ g}$$

$$= 2.2193548\ umol/gFW$$

2. Determination of cell membrane stability

$$\text{Electrolytic leakage (\%)} = 100 - [(EC_1/EC_2) \times 100]$$

Where, EC_1 is the first electro conductivity and EC_2 is the second electro conductivity.

For example:

Electrolytic leakage of WT plants under drought treatment (treated with 20% PEG) for 24h.

EC_1 = 5.06 µs/cm, EC_2 = 12.48 µs/cm

Electrolytic leakage (%) = 100 – [(5.06/12.48) × 100]

= 59.46%

3. Measurement of relative growth rate

$$RGR = \frac{\ln W_2 - \ln W_1}{t_2 - t_1}$$

W_1 = Initial weight, W_2 = Final weight, and t_1 and t_2 are initial and final treatment times, respectively (t_1 = day 0, before drought treatment and t_2 = day 1 or 24 h, drought treatment).

For example:

RGR of shoot of WT plants under drought treatment (treated with 20% PEG) for 24h.

W_1 = 0.0198 g, W_2 = 0.0199 g

$$RGR = \frac{\ln 0.0199 - \ln 0.0198}{1 - 0}$$

= 0.0050378 g/g.d

4. Measurement of relative water content

$$RWC = \frac{(FW - DW) \times 100}{(TW - DW)}$$

FW = fresh weight, DW = dry weight, and TW = turgid weight

For example:

RWC of WT plants under drought treatment (treated with 20% PEG) for 24h.

FW = 0.0027 g, TW_2 = 0.004 g, DW= 0.0000 g

$$\text{RWC (\%)} = \frac{(0.0027 - 0.0000)}{(0.004 - 0.0000)} \times 100$$

$$= 67.50\%$$

5. Measurement of photosynthetic pigments

Chlorophyll *a* (μg/mL) = $[12.7(A_{664})] - [2.79(A_{647})]$

Chlorophyll *b* (μg/mL) = $[20.70(A_{647})] - [4.62(A_{664})]$

Total chlorophyll (μg/mL) = $[17.90(A_{647})] + [8.08(A_{664})]$

Carotenoid content (μg/mL) = $4 \times [(A_{461}) + (0.46 \times A_{664})]$

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

For example:

Photosynthetic pigments of WT plants under drought treatment (treated with 20% PEG) for 24h.

A_{461} = 1.936, A_{647} = 0.774, A_{664} = 2.227

Chlorophyll *a* (μg/mL) = $[12.7(2.227)] - [2.79(0.774)]$

= 26.12344 μg/mL

= 26.12344 μg/0.03 g sample

= 0.87 μg/g sample

Chlorophyll *b* (μg/mL) = $[20.70(0.774)] - [4.62(2.227)]$

= 5.24544 μg/0.03 g sample

= 0.17 μg/g sample

Total chlorophyll (μg/mL) = [17.90(0.774)] + [8.08(2.227)]

= 11.84168 μg/0.03 g sample

= 1.06 μg/g sample

Carotenoid content (μg/mL) = 4 × [(1.936) + (0.46 × 2.227)]

= 31.84876 μg/0.03 g sample

= 0.39 μg/g sample

6. Measurement of DPPH radical scavenging rate

$$\text{DPPH radical scavenging rate (\%)} = \frac{(A_{control} - A_{sample}) \times 100}{A_{control}}$$

For example:

DPPH radical scavenging rate content of WT plants under drought treatment (treated with 20% PEG) for 24h.

$A_{515\ (control)} = 0.861$, $A_{515\ (sample)} = 0.702$

$$\text{DPPH radical scavenging rate (\%)} = \frac{(0.861 - 0.702) \times 100}{0.861}$$

= 18.45588%

7. SOD activity

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

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

For example:

SOD activity of WT plants under drought treatment (treated with 20% PEG) for 24h.

A_{560} control = 0.384, A_{560} sample = 0.342

$$\text{SOD activity} = \frac{1}{0.384/2} \times 0.342$$

$$= 1.78125 \text{ U/0.05 mg protein}$$

SOD activity for 1 mg protein is 35.625 U/mg protein

8. 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 1 µmol of H_2O_2 per min at 240 nm ($\text{Ɛ} = 39.4\ mM^{-1}cm^{-1}$)

For example:

CAT activity of WT plants under drought treatment (treated with 20% PEG) for 24h.

A_{240}/min = 0.1186

$$\text{Specific activity} = \frac{0.1186 \times 1.2 \text{ mL} \times 1{,}000}{39.4\ mM^{-1}mL^{-1} \times 0.05 \text{ mg}}$$

$$= 72.243655 \text{ µmol/min/mg protein}$$

9. POD activity

$$\text{Specific activity} = \frac{A_{436}/\text{min} \times \text{total volume} \times 1{,}000}{\varepsilon \times \text{protein content (0.05 mg)}}$$

One unit of POD activity was defined as the amount of enzyme to reduce 1 µmol of guaiacol per min (ε = 6.39 $mM^{-1}cm^{-1}$).

For example:

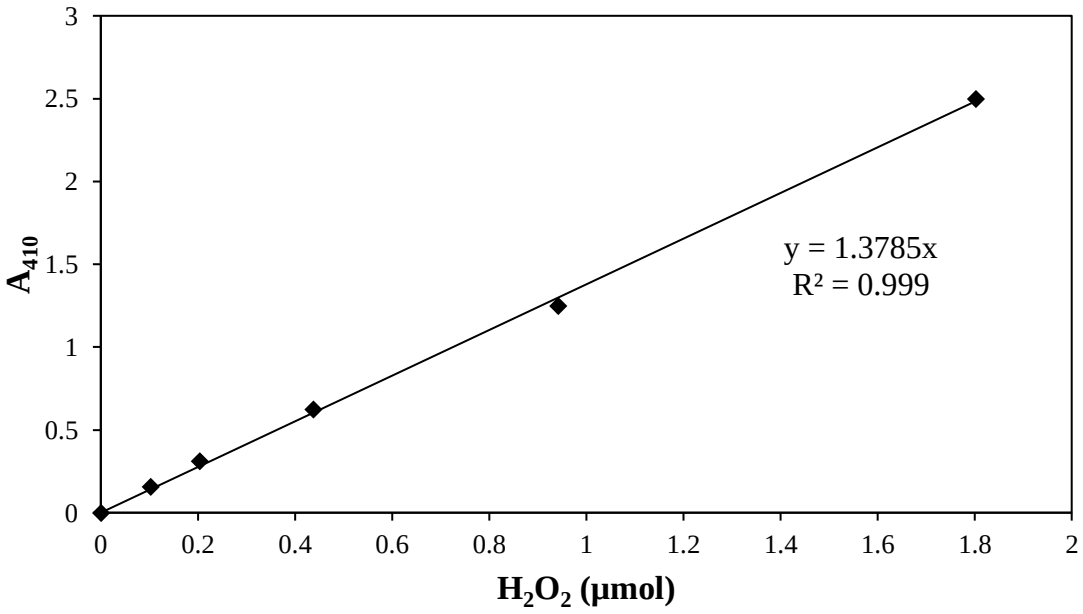
POD activity of WT plants under drought treatment (treated with 20% PEG) for 24h.

A_{436}/min = 0.6476

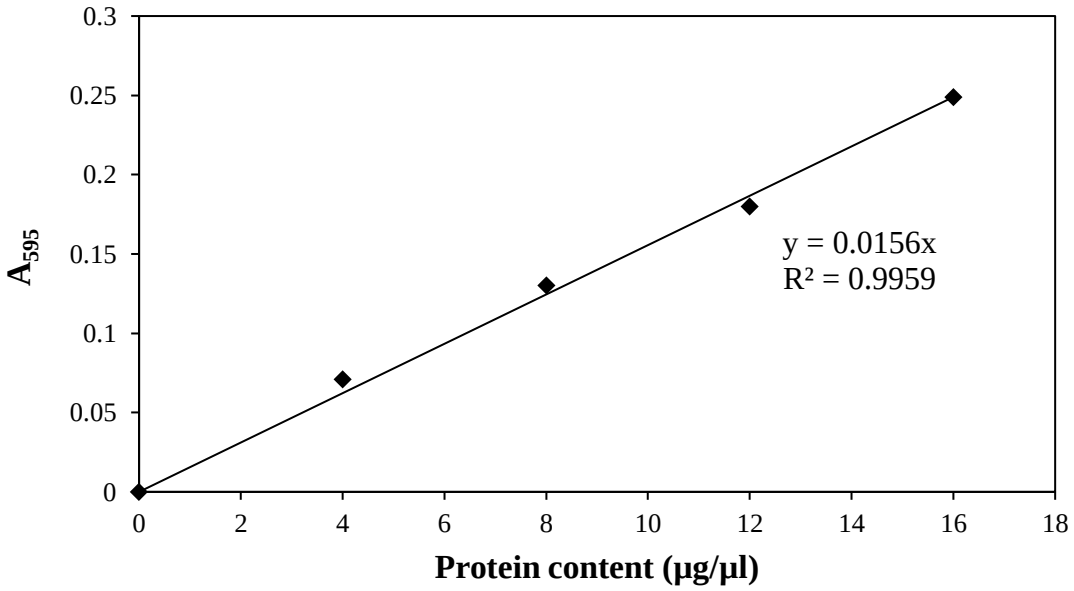
$$\text{Specific activity} = \frac{0.6476 \times 1.2 \text{ mL} \times 1{,}000}{6.39 \text{ mM}^{-1}\text{cm}^{-1} \times 0.05 \text{ mg}}$$

$$= 2432.3 \text{ µmol/min/mg protein}$$

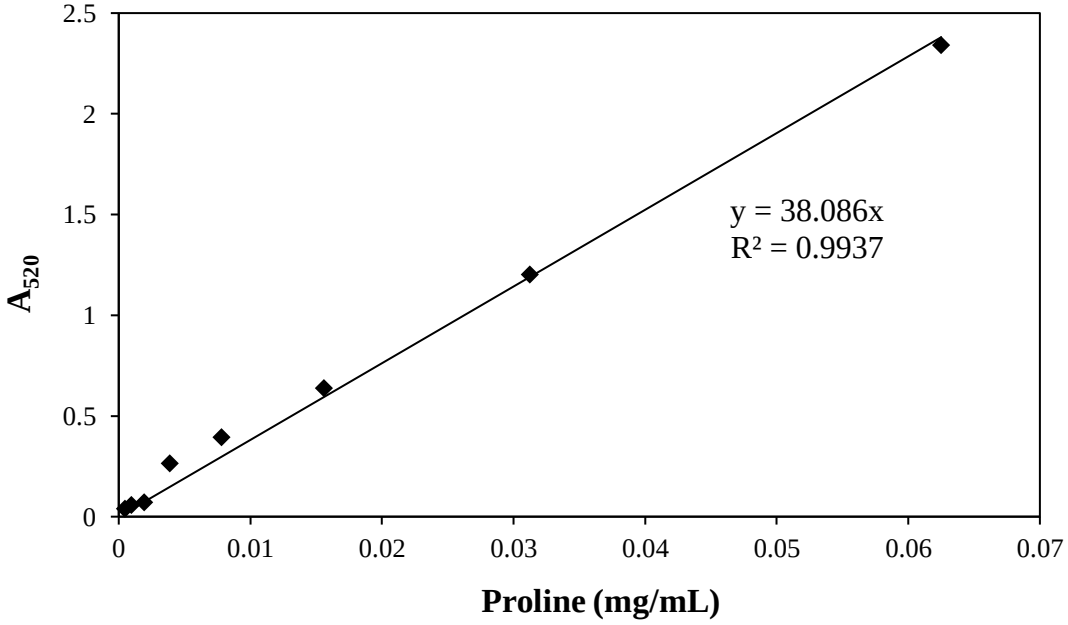
H_2O_2 standard curve



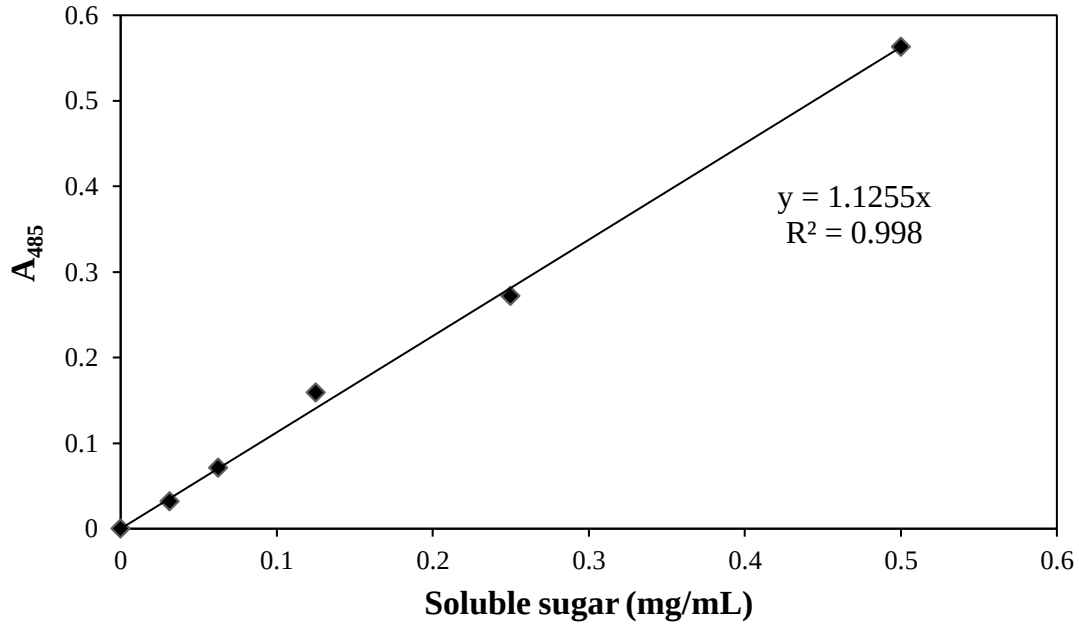
BSA standard



Proline standard



Soluble sugar standard



VITA

Miss Pun Sangchai was born on November 15, 1993 in Bangkok. She graduated with the Bachelor degree of Science from department of Biochemistry, Faculty of Science, Chulalongkorn University in 2016. She continues to study for Master degree of Science at the Biochemistry and Molecular biology program, Faculty of Science, Chulalongkorn University.