# ROLE OF ENZYMES IN GLYOXYLATE CYCLE IN TRANSGENIC ‘KDML 105’ RICE *Oryza sativa* L. OVEREXPRESSING *OsCam1-1* UNDER SALT STRESS

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จุฬาลงกรณ์มหาวิทยาลัย
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# บทบาทของเอนไซม์ในวัฏจักรไกลออกซีเลตในข้าวขาวดอกมะลิ 105 *Oryza sativa* L. ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติภายใต้ภาวะเครียดต่อความเค็ม

น.ส.ธนภรณ์ อุสาหะ

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ธนภรณ์ อุสาหะ : บทบาทของเอนไซม์ในวัฏจักรไกลออกซีเลตในข้าวขาวดอกมะลิ 105 *Oryza sativa* L. ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติภายใต้ภาวะเครียดต่อความเค็ม. ( ROLE OF ENZYMES IN GLYOXYLATE CYCLE IN TRANSGENIC 'KDML 105' RICE *Oryza sativa* L. OVEREXPRESSING *OsCam1-1* UNDER SALT STRESS) อ.ที่ปรึกษาหลัก : รศ. ดร.ธีรพงษ์ บัวบูชา

จากการศึกษาก่อนหน้านี้พบว่าทรานสคริปโทมของข้าวขาวดอกมะลิ 105 ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติส่งผลต่อการแสดงออกของยีนที่เกี่ยวข้องกับหลายกระบวนการภายในเซลล์อย่างกว้างขวาง รวมทั้งยีนที่เกี่ยวข้องกับวัฏจักรไกลออกซิเลตที่มีการแสดงออกเพิ่มขึ้นในข้าวขาวดอกมะลิ 105 ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติภายใต้ภาวะเครียดต่อความเค็ม ดังนั้นในการศึกษาครั้งนี้จะมุ่งเน้นไปที่บทบาทของวัฏจักรไกลออกซิเลตในข้าวขาวดอกมะลิ 105 ภายใต้ภาวะเครียดต่อความเค็ม โดยทำการทดลองวัดค่าฟีโนไทป์ได้แก่ น้ำหนักสดและน้ำหนักแห้งของลำต้นและราก ค่าดัชนีความเขียวของใบ แอกติวิตีของไอโซซิเทรตไลเอสและมาเลตซินเทส และปริมาณน้ำตาลและแป้งรวมถึงปริมาณกรดไขมันโดยรวม ในวันที่ 0, 2, 4,และ 6 หลังจากได้รับความเครียดจากความเค็มเปรียบเทียบกับในข้าวขาวดอกมะลิ 105 พันธุ์ดั้งเดิม ผลการทดลองพบว่าข้าวขาวดอกมะลิ 105 ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติสามารถรักษาน้ำหนักสดและน้ำหนักแห้งของลำต้น รวมถึงค่าดัชนีความเขียวของใบได้ดีกว่าข้าวพันธุ์ดั้งเดิม และพบแอกติวิตีของไอโซซิเทรตไลเอสและมาเลตซินเทสของข้าวขาวดอกมะลิ 105 ที่มีการแสดงออกของยีน *OsCam1-1* เกินปกติสูงกว่าพันธุ์ดั้งเดิมในใบอ่อนสุดที่เจริญเต็มที่ในวันที่ 2 ในขณะที่ในใบที่สองที่เจริญเติบโตก่อนหน้าจะมีปริมาณการทำงานของเอนไซม์ไอโซซิเทรตไลเอสสูงกว่าพันธุ์ดั้งเดิมในวันที่ 6 นอกจากนี้ยังพบว่าข้าวขาวดอกมะลิ 105 ที่มีการแสดงออกของยีน *OsCam1-1* มีปริมาณน้ำตาล (ซูโครส กลูโคส และฟรักโทส) และแป้งต่ำกว่าข้าวพันธุ์ดั้งเดิม ซึ่งแสดงให้เห็นว่าแหล่งคาร์บอนเหล่านี้อาจถูกแมแทบอไลซ์หรือเคลื่อนย้ายออกได้ดีกว่าพันธุ์ดั้งเดิม ยิ่งไปกว่านั้นยังพบปริมาณกรดไขมันไม่อิ่มตัวมีแนวโน้มลดลงมากกว่าข้าวพันธุ์ดั้งเดิมอีกด้วย จากผลการทดลองทั้งหมดจะเห็นได้ว่าวัฏจักรไกลออกซิเลตสำคัญในวิถีถูกกระตุ้นโดยการทำงานของยีน *OsCam1-1* อาจช่วยรักษาสมดุลของแหล่งคาร์บอนและเมแทบอลิซึมพลังงานในข้าวภายใต้ภาวะเครียดจากความเค็มได้

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Tanaporn Ausaha : ROLE OF ENZYMES IN GLYOXYLATE CYCLE IN TRANSGENIC 'KDML 105' RICE *Oryza sativa* L. OVEREXPRESSING *OsCam1-1* UNDER SALT STRESS. Advisor: Assoc. Prof. TEERAPONG BUABOOCHA, Ph.D.

The previous study reported that the transcriptome profiling of the KDML 105 rice overexpressing *OsCam1-1* widely affected the expression of genes involved in several cellular processes including the genes associated with the glyoxylate cycle, which exhibited up-regulation in KDML 105 overexpressing *OsCam1-1* under salt stress. In this study, we focused on the role of the glyoxylate cycle in transgenic KDML 105 rice under salt stress. The phenotypes as follows: fresh weight and dry weight of shoot & root, SPAD value, activities of isocitrate lyase, and malate synthase; sugar, and starch contents, and total lipid content were investigated at 0, 2, 4, 6 days after salt stress treatment and compared to the wild type KDML105. The results showed that the KDML 105 rice overexpressing *OsCam1-1* better maintained fresh weight, dry weight of shoot, and SPAD value than wild type. Furthermore, the isocitrate lyase and malate synthase activities of transgenic rice overexpressing *OsCam1-1* were higher than wild type in the youngest fully expanded leaf at day 2 while the second youngest leaf has higher isocitrate lyase activity than wild type at day 6. The transgenic rice overexpressing *OsCam1-1* exhibited lower contents of sugars (sucrose, glucose, and fructose) and starch especially from the second youngest leaf than wild type suggesting that they may be metabolized or mobilized under salt stress better than the wild type. Moreover, transgenic rice also exhibited somewhat lower unsaturated fatty acid contents than wildtype. These results indicated that the glyoxylate cycle of which its key enzymes were enhanced by the overexpression of *OsCam1-1* gene, probably helps maintain and balance carbon source and energy metabolism in rice under salt stress condition.

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# CHAPTER I

# INTRODUCTION

## 1.1 Khao Dawk Mali 105 rice

Khao Dawk Mali 105 rice (KDML105) is a non-glutinous rice variety, which is sensitive to photoperiod. In 1945, Khao Dawk Mali rice was discovered in Lam Pradoo district, Panasnikom, Chonburi province by Mr. Charoon Tuntawutto. Then, Mr. Soontorn Sihanern, rice officer from Bang Kla collected 199 panicles of KDML from the farmers and sent to Rice Breeding Division. After that, all the panicles were sent to Koksamrong Rice Experiment Station, Lopburi for pure line selection. Finally, Mr. Opas Polsilp, director of this station and Mr. Mangkorn Joomthong, agricultural officer, conducted the cultivation of KDML rice for 2 years and the rows of the 105$^{th}$ rice panicle were selected. Khao Dawk Mali meaning white as jasmine flower, 105 meaning 105$^{th}$ row from 199 rows. of the average height of KDML105 mature plants is 140 centimeters with panicles of ~33 centimeters in length. The culm is erect with green leaves and leaf sheaths, and light yellow internodes. The leaf is pubescent and the leaf angle is droopy. The color of the auricle and collar is light green and the ligule shape is acute and has two white clefts. At the flowering stage, the color of the apiculi, short sterile lemma and stigma is white. At the harvesting stage, the apiculus and sterile lemma of grain will change to straw color with short hairs. The stem is averagely standing still with some lodging, the panicle is long, leaves under flag leaf are mostly senescent and the average yield is 515 kilograms per rai. Quality of grain is excellent, milled rice has transparent and good milling quality. Moreover, the cooked rice is soft and contains a natural fragrant aroma called 2-acetyl-1- pyrroline (2AP) (Figure 1), as one gram of aromatic white rice contains 0.04-0.09 micrograms of 2AP. This aroma can also be found in pandan leaves and bread flours.

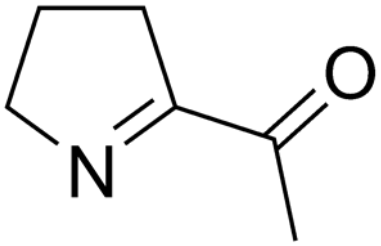



**Figure 1** Structure of 2-acetyl-1- pyrroline (2AP) (Reference Date: 8-May-2020) (http://brrd.ricethailand.go.th/library/document/E-book/brrd5301007c1.pdf)

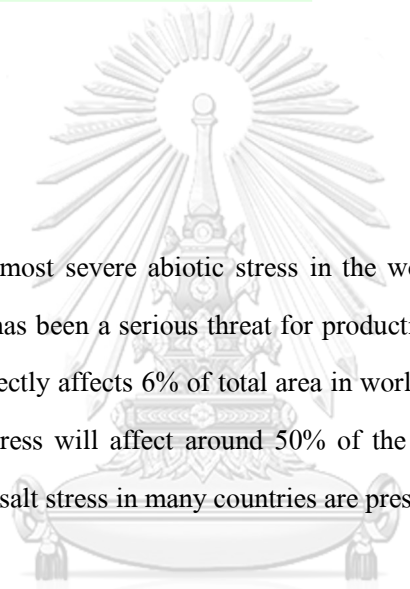
KDML105 is popular for rice consumers due to its distinctive properties so, it is the market's demand. Table 1 and Table 2 show that Thai Hom Mali Rice has export value higher than other types of rice and Thai Hom Mali rice also has higher price than popular rice from other countries. This information shows that Thai Hom Mali (trade name of KDML105) is a major export product of Thailand. However, the KDML 105 rice is not tolerant to salinity [1].

**Table 1** Thai export rice prices in 2020 (Reference Date: 8-May-2020) (http://www.thairiceexporters.or.th/default_eng.htm)

| Item | 8 Apr 20 | 15 Apr 20 | 22 Apr 20 | 29 Apr 20 | 7 May 20 |
|---|---|---|---|---|---|
| Thai Hom Mali Rice - Premium (2018/19) | 1227 | 1237 | 1208 | 1212 | 1211 |
| Thai Hom Mali Rice - Premium (2019/20) | 1165 | 1174 | 1144 | 1149 | 1132 |
| Thai Hom Mali Broken Rice A.1 Super | 491 | 495 | 496 | 498 | 497 |
| Thai Jasmine Rice (Thai Fragrant Rice) | 820 | 811 | 765 | 768 | 720 |
| White Rice 100% Grade B | 595 | 584 | 570 | 572 | 556 |
| White Rice 5% | 579 | 569 | 553 | 556 | 539 |
| White Rice 25% | 547 | 539 | 528 | 530 | 518 |
| White Broken Rice A.1 Super | 439 | 443 | 444 | 445 | 445 |
| White Glutinous Rice 10% (Major crop) | 1106 | 1115 | 1117 | 1121 | 1120 |

**Table 2** Comparison of rice prices between Thailand with other countries 2020 (Reference Date: 8-May-2020) ( http://www.thairiceexporters.or.th/default_eng.htm)

| Fragrant Rice | Price ($/MT) |
|---|---|
| Thailand Hommali 100% | 1100 |
| Vietnam Jasmine | 573-577 |
| India basmati 2% broken | 750-900 |
| Pakistan basmati 2% broken | - |
| Cambodia Pkha Malis/Rumduol | 915 |



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### 1.2 Salt stress

Salt stress is the most severe abiotic stress in the world because most crop plants are sensitive to salt stress. It has been a serious threat for production of many crop in irrigated land. salt stress directly or indirectly affects 6% of total area in world and 20% of irrigated land [2] [3] It is estimated that salt stress will affect around 50% of the cultivable land in 2050 [4]. The irrigated areas affected by salt stress in many countries are presented in Table 3 [5, 6].

**Table 3** Global estimate of salinity in irrigated lands of the world [7]

| Country | Salt-affected area of irrigated in the world | |
|---|---|---|
| | **Mha** | **%** |
| China | 6.7 | 15 |
| India | 7.0 | 17 |
| Soviet Union | 3.7 | 18 |
| United States | 4.2 | 23 |
| Pakistan | 4.2 | 26 |
| Iran | 1.7 | 30 |
| Thailand | 0.4 | 10 |
| Egypt | 0.9 | 33 |
| Australia | 0.2 | 9 |
| Argentina | 0.6 | 34 |
| South Africa | 0.1 | 9 |
| Subtotal | 29.6 | 20 |
| World | 45.4 | 20 |

mha = million hectare, % = percentage area.

Salt stress in soil is expanding worldwide in more than 100 countries and no continent is entirely free from salinity. The high sea level has impact on coastal areas as a result of climate change scenarios, effecting salinization of soil while the temperature is increased, which will certainly lead to increased evaporation. Moreover, salt stress of soils also affects ecosystems to an extent where they no longer can provide 'environmental services' to their full potential. It can be assumed that, since the earlier data collection in the 1970s to 1980s, salt stress area has expanded as recently affected areas most presumably surpass the areas strengthened through reclamation and restitution (Figure 2) [8].

**Figure 2** World map representing countries with salinity problems [8].

salt stress in soil is one of the important environmental factors that decrease yield and growth of an extensive variety of crops [9-11]. Many major field crops such as rice (*Oryza sative* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.), cotton (*Gossypium hirsutum*), sugarcane (*Saccharum officinarum*) and sorghum (*Sorghum bicolor* (L.) Moench) exhibit negative response towards salt stress [12]. Plant physiology of many crops is very sensitive to high salinity in its rhizosphere, which influences germination rate, growth stages, and ultimately yield of plants [6]. In the same way, hindering effects on plants many stages of growth due to salinity are lower $CO_2$ assimilation to many tissues in plant, enlargement of leaf area and leaf cell, relative growth, dry matter production, and poor spikelets development especially in rice, wheat and cotton [13, 14]. Normally, salt stress affects plant growth in three ways, such as ionic stress or ion imbalance, oxidative stress and osmotic stress [15]. The salt water balance was disturbed by osmotic stress, which results in a loses of water in plant tissues & cell sap and high salts concentration. This imbalance effects lead to wilting symptoms and leaf burn due to $Na^+$ and $Cl^-$ accumulation. Furthermore, high $Na^+$ concentration has inharmonious effect on potassium ($K^+$) ion, which is a major plant nutrient for development and growth [16]. Reduced nitrogen uptake has also been observed under ion imbalance [17] and this imbalance has antagonistic effect on P, K, Zn, Fe, Ca, and Mn in crop [3, 16, 18]. Ionic stress also causes disequilibrium of nutrient and results in reduced final germination percentage (FG %), decreased vegetative and reproductive growth, declined yield, and yield components of the plant under salt stress. Moreover, ionic stress in the plant due to salt stress can lead to photosynthesis reduction, oxidative stress, changes in enzymatic activities, damage of the ultrastructural cellular components, disruption of the

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biochemical membrane structure and function, and hormonal imbalance, which are the primary reasons for reduced total plant's development and growth [12, 19].

### 1.2.1 Salt stress responses in rice

Rice is classified as a salt sensitive crop in the field when compared with other cereal crop [20]. Nevertheless, salt stress is not a new problem for rice production. Usually, rice can resist a small concentration of salinity without compromising the growth and yield. However, it depends on the species and types of rice and their growth stage [10]. At early stage of growth, rice is grouped as salinity vulnerable cereal and confines its capability of production at mature stage [3, 21, 22]. Therefore, rice responses to salinity stress in many ways such as morphological responses, biochemical responses, physiological responses, which lead to the effect on crop productivity and maybe death when exposed to high concentration of salinity.

#### 1.2.1.1 Effect of salt stress on morphological responses

Salt stress induces various morphological responses of rice such as shoot height and root length, seed germination, rice growth and development. Seed germination is an important parameter of morphological characteristics [23, 24]. Shereen and co-worker investigated the effects of salt stress on seed germination of six rice varieties differing in salt tolerance by treating rice seeds with 0, 50, 75, 100, 200 mM NaCl. The results showed that germination was delayed in 3-6 days after treatments with 100 and 200 mM NaCl, supporting a strong negative relationship between seed germination with salinity and the rice cultivars exhibiting minimal leakage of solutes showed relatively higher germination rate when compared to the cultivars exhibiting higher solute leakage under concentrations of 100 and 200 mM NaCl [24]. Likewise, a study on the effects of salinity on seed germination in three different rice genotypes has found that the three different rice genotypes have different germination responses to salinity as the increase in concentration of salinity from 0 to 150 mM adversely affected the seed germination rate and significantly delayed seed germination [25]. A lot of research have reported negative effects of salt stress on shoot length and rice height. The effects of salinity on BR11 rice cultivar using different concentrations of salinity included 0, 7.81, 15.62, 23.43, and 31.25 dS/m. The results revealed severe negative effects of salt stress on rice height and shoot

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length, which constantly decreased with increasing salinity. They attributed these negative results to changes in the ability of rice to uptake nutrients and water [26-28]. In the same way, 200 mM NaCl affected the rice morphological growth in a growth chamber under controlled environmental. The results exhibited shoot length of rice was 71% reduced under salt stress conditions compared to the normal condition. [29]. Perez-Alfocea and co-worker discovered an increase in root length after salt stress. These observations also suggested an adaptable strategy of rice roots under salt stress and the ability of rice to redistribute the photosynthetic materials into roots while limiting their assimilation into biomass of shoot [30]. Rice growth and development is a fundamental plant morphological parameter. Rice seedling growth and fresh weight decreased with increased saline concentration from 5 to 7.5 dS/m [31] and high salt concentration also decreased rice stand density and production of seedling biomass [32]. Rice leaf was severely destroyed with increased salinity stress in all rice cultivars at early seedling stage [33]. Salt stress has specific effects on plant cell metabolism, particularly on leaf senescence. It can also damage the cells in transpiring leaves, and cause rice plant growth inhibition [34]. In the study on the effects of salt stress on leaf and other yield parameters of 18 rice cultivars using an artificial salt soil medium, the results found a significant decrease in leaf area of rice plants with the increase in concentration of salinity. Moreover, the reduction in the leaf area was attributed to restrained cell division and cell elongation [6] [35]. In grain development of some rice cultivars, salt stress may cause sterility of panicle especially at pollination and fertilization stages [36, 37]. Salt stress also decreases grain setting, pollen bearing capacity, and stigmatic surface [38]. The major cause of reduced grain yield under salinity stress is the lack of mobilization of carbohydrates for vegetative growth and spikelet development; furthermore, translocation of soluble sugar contents to superior and inferior spikelets was significantly reduced. These factors, which involve inhibition of starch synthetase activity during grain development are the major reasons to lower rice grain development and grain yield under salinity stress [38]. Yield components such as spikelets per panicle, panicle length, number of tillers per plant, number of florets per panicle are all critically affected due to increasing concentration of salinity. For example, Farshid and co-worker found that concentration of salinity from 2 to 8 dS/m decreased number of filled rice grains per panicle [36, 39]. All these yield components are linked to each other concerning final grain development.

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#### 1.2.1.2 Effect of salt stress on physiological responses

Salt stress induces various physiological responses on rice plants such as reduced photosynthesis rate, lower stomatal conductance ($g_s$), higher amount of osmolyte and decreased water potential. Photosynthesis is a complicate process depending on photosynthetic pigments, photosystems, components of electron transport system, gas-exchange characteristics and activities of different enzymes involved in carbon metabolism [3, 40, 41]. Rate of photosynthesis decreases under salt stress condition firstly due to osmotic stress, which results in stomatal closure and secondly by higher $Na^+$ and $Cl^-$ accumulation, which can destroy thylakoid membrane in the chloroplast [10]. High concentration of saline induces limitation of $CO_2$ diffusion, which causes inactivation of RuBisCo. Moradi and co-worker demonstrated the effects of salt stress on three rice genotypes differing in salt stress tolerance at seedling and reproductive stages under greenhouse conditions. The results reported that the salt-sensitive genotype IR29 showed significantly decreased photosynthetic $CO_2$ fixation, stomatal conductance ($g_s$) and transpiration when compared to the salt-tolerant genotypes IR652 and IR632 [42]. Photosynthetic pigments, chlorophyll a and chlorophyll b, are considerably affected by salt stress. The contents of precursor of chlorophyll biosynthesis such as glutamate and 5-aminolevullinic acid were reduced by accumulation of high $Na^+$ concentration under salt stress [41]. Amirjani conducted experiments to study the effect of salinity including 0, 25, 50, 100 and 200 mM NaCl on chlorophyll content. Forty four and 27% of chlorophyll *a* and chlorophyll *b* were decreased respectively at 200 mM NaCl when compared to normal condition [14]. In another study, Rahman and co-worker revealed that 12-day-old rice seedlings was treated with 150 mM NaCl for 3 days caused 23 and 19% of chlorophyll a and chlorophyll b were reduced respectively when compared to normal condition. At 3 days after salt stress treatment chlorophyll *a* and chlorophyll *b* contents decreased by 46 and 48% respectively when compared to normal condition [43]. In the same way Kibria and co-worker found that both chlorophyll *a* and chlorophyll *b* contents were reduced with an increased in salinity concentration of 40 and 60 mM NaCl. However, salt-sensitive and salt-tolerant rice cultivars have different patterns of reduction of total chlorophyll contents [44]. Moreover, photosynthesis reduction caused by stomatal closure and $CO_2$ deficiency affects source to sink mobilization of photosynthates and carbohydrate metabolism in leaves. Photosynthates may provide plants with salt stress tolerance ability by accumulation of osmolytes [3]. Pattanagul

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and Thitisaksakul studied carbohydrate metabolism in salt-tolerant and salt-sensitive rice genotypes. The result showed that starch accumulation increased in salt-tolerant genotype while total soluble sugar and sucrose content increased in salt-sensitive rice genotype. From these results, they explained that starch accumulation may play a role in salt-tolerant rice genotype, it is possible that adjusting carbon partitioning could have an important implication on plant growth under salt stress while salt-sensitive rice genotype could not use carbohydrate for plant growth and accumulation of these sugars in salt-sensitive rice genotype maybe the result of reduction of sink demand due to growth limitation [45]. Another important physiological parameter is water potential because it is used for determining the water status of the plants [46]. Khan and co-worker did the experiments in *Cucumis sativa,* the result showed that the water potential decreased linearly with increasing salinity levels [47]. In another experiment, Romero and co-worker demonstrated water uptake and water relationships under saline growing conditions in the tomato (*Lycopersicon esculentum*) cultivars. They found that leaf water potential and many plant processes were affected by increased concentration of salts in the root. Plant's ability to extract water from the soil and maintain turgor pressure was disturbed at very low soil water potentials. However, at low or moderate salt concentration or higher soil water potential, plants adjust osmotically by accumulating osmolyte to preserve a gradient of potential for the influx of water [48].

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#### 1.2.1.3 Effect of salt stress on biochemical responses

Salt stress influences biochemical procedures in many ways causing nutrient imbalance and oxidative stress. Nutrient imbalance is a result of competitive absorption, translocation and distribution between salinity ($Na^+$ and $Cl^-$) and the essential nutrient elements such as N, P, K, and Ca resulting in the reduction of quantitative and qualitative yield [49]. Several reports showed that accumulation of $Na^+$ and $Cl^-$ ions reduces the uptake of nutrients and nutrient accumulation in the plant cell [30, 50-53]. $K^+$ is one of the primary macronutrients for plants with concentrations of 100-200 mM required for optimum metabolic functions [34, 54-56]. $K^+$ activates more than 50 enzymes because $K^+$ is a cofactor in cytosol, however, these enzymes are susceptible to high cytosolic $Na^+$ concentrations and high $Na^+/K^+$ ratios; consequently, high levels $Na^+$ concentrations in cytosolic or higher $Na^+/K^+$ ratios, result in toxicity of metabolic

processes caused by competition for the binding sites of many enzymes [9, 34, 57, 58]. Another primary macronutrient is nitrogen (N), many studies in plants have indicated that salt stress could decrease accumulation of nitrogen in plants and nitrogen uptake under salinity conditions occurs due to interaction between $Cl^-$ and $NO_3^-$ and/or between $Na^+$ and $NH_4^+$ that eventually decreases the growth and yield of the crop [59, 60]. Moreover, P is one of the essential nutrients for plants, the availability of phosphorus is also decreased in high salinity concentration due to ionic strength effects that reduced the activity of $PO_4^{3-}$ as phosphate concentration in saline soil was strictly controlled by absorption processes and low solubility of Ca-P minerals [60, 61]. Concentrations of secondary macronutrients such as $Ca^{2+}$ and $Mg^{2+}$ of all plant organs transiently declined in response to external NaCl [60, 62]; furthermore, $Ca^{2+}$ was replaced by $Na^+$ from the plasma membrane causing a change in plasma membrane permeability and integrity, which can lead to $K^+$ leaking from plant cells [34, 63]. In rice, Azarin and co-worker found that salt stress affects accumulations of $Na^+$, $Na^+/K^+$ ratio, and $Cl^-$ in root and shoot while $K^+$, and $NO_3^-$ contents were decreased in both shoot and root under 1.2% salt stress condition. They also reported that $Cl^-$ ions negatively affected the biomass and survival of rice [64]. Another study in rice demonstrated that increase of $Na^+$ content and reduction of $K^+$ content interfered ion homeostasis in both of shoots and roots of rice seedlings, which might be due to the entrance of higher concentration of $Na^+$ into plant via nonselective cation channel that caused $K^+$ efflux and guard cell nonselective cation channel outward rectifying potassium channels. They also reported that higher accumulation of $Na^+$, which resulted in a higher $Na^+/K^+$ ratio, led to the decrease in Mg, Mn and Zn contents [65]. In addition, Munns and Zhang reported that tolerant rice genotypes sustain higher ratios of $K^+/Na^+$ and $Ca^{2+}/Na^+$ and tended to accumulate less $Na^+$ in rice under salinity stress [6, 66]. Another important biochemical response under salt stress is oxidative stress, a common consequence of salt stress, which is the induction of overabundant accumulation of reactive oxygen species (ROS). Oxidative stress can cause peroxidation of lipids, oxidation of protein, DNA production, inactivation of enzymes, senescence, expansion, and physiologically less active green foliage [60, 67]. Moreover, under salt stress condition plants change their metabolic pathways to manage with the photo-inhibitory effects such as heat dissolution by the xanthophyll pigments and transfer of electrons to $O_2$ acceptors, which produce ROS [68]. The cellular organelles consisting of mitochondria and chloroplasts in plants are the important intracellular components where

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production of ROS occurs. Leaking of electrons during transport reacts with $O_2$ under normal aerobic metabolic conditions to produce ROS, such as, hydrogen peroxide ($H_2O_2$), superoxide ($O_2^-$) and the hydroxyl radical (OH•). These ROS are toxic compounds in the plant cell [69]. Salt stress can significantly diminish the removal of ROS through the antioxidant mechanism [70-72]. Antioxidant systems are separated into two categories: 1) non-enzymatic compositions such as glutathione (GSH) and ascorbate acid (AsA) 2) enzymatic compositions such as superoxide dismutase (SOD), catalases (CAT), peroxidases (POX), ascorbate peroxidase (APX) [34, 73-75]. In rice, under salt stress condition (150 mM NaCl), lipid peroxidation increased by 80% and 203%, LOX activity increased by 69% and 95% and $H_2O_2$ content increased by 74% and 92% after salinity treatment 3 and 6 days, respectively, and the accumulation of ROS such as $O_2•^-$ and $H_2O_2$ in leaves was much higher than normal condition, especially after 6 days of salt stress treatment. They suggested that the high activities of SOD, CAT, DHAR and MDHAR under salt stress in rice led to the salt stress tolerance [43]. Many studies in rice indicated that salt-tolerant cultivar can exhibit higher antioxidant and lower ROS generation rate than salt-sensitive cultivar. For example, Vaidyanathan and co-worker studied ROS species in salinity stress in two rice cultivars, namely Pokkali (PK), a salt tolerant cultivar and Pusa Basmati 1 (PB), a salt sensitive cultivar. They founded that PK cultivar has lower the $H_2O_2$ production and lipid peroxidation than PB cultivar with concurrent improvement of the activities of ROS-detoxifying enzymes as well as high levels of GSH and AsA [76]. Kibria and co-worker investigated the effects of salt stress on antioxidant enzymes in four rice salt sensitive cultivars and three rice salt tolerant cultivars by treating 30-day old rice seedlings with four concentrations of 0, 20, 40, and 60 mM NaCl. The result revealed that the increase in antioxidant enzyme activity, as the CAT and APX activities were increased under salt stress in the rice salt tolerant genotypes with increasing salinity concentration. However, the activity of POX enzyme followed the opposite trend in all rice genotypes while the CAT and APX activities were decreased in salt sensitive rice cultivar under high concentration of salt stress [44]. In another study, oxidative stress was induced by salt stress in two rice varieties, Swarna, a salt sensitive rice cultivar and Nonabokr, a salt tolerant rice cultivar treated with 200 mM NaCl. The result showed accumulation of ROS and lipid peroxidation in the two rice varieties, however, the damage of biomolecules including protein, enzymes, lipid and membranes was reduced in the tolerant rice variety with accumulation of

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protective phenolic compounds significantly higher than the sensitive rice variety [77]. Moreover, Hasanuzzaman and co-worker studied the effect of salt stress in two rice genotypes by treating with 150 and 300 mM NaCl for 48 hr and found increased lipid peroxidation (76% and 159%) and $H_2O_2$ (35% and 69%) levels in the salt sensitive rice varieties at 150 and 300 mM NaCl, respectively compared to the salt tolerant rice varieties and the activities of APX, DHAR, GR, CAT, MDHAR, GPX, and glyoxalase I were increased in the tolerant variety more than the sensitive variety [78].

#### 1.2.1.4 Effect of salt stress on crop productivity

Salt stress induced damages effecting various growth and yield parameters of rice such as number of spikelets per panicle, number of tillers per plant, number of primary branches per panicle, 1000-grain weight and percentage of fertility [34, 37, 38, 79, 80]. Especially, salinization between 3-leaf and panicle initiation stages is the most susceptible in terms of reduction in seed yield and for saline EC levels >2 dS/m resulting in a yield loss of up to 1 t $ha^{-1}$ per unit EC (dS/m) for salt stress around the panicle initiation stage [79]. In contrast, shoot weight decreased at maturity was important when plants were salinized before booting but not after that [81]. Moreover, the recovery from salt stress imposed at panicle development stage is difficult compared to that at vegetative stages [34, 79]. There are many studies on the effect of salt stress on rice productivity. For example, Zheng and Shanon found that reproductive stage of rice was affected by salt stress. They observed that salt stress decreased growth and survival percentage of seedlings and reduced grain yield by decreasing fertility percentage, pollen viability, tiller number and 1000-grain weight where reducing the level of yield to a greater extent with higher level of salinity [80]. Saleethong and co-worker reported that grain yield was reduced by salt stress in the tolerant cultivars (32%) and the sensitive cultivars (56%) and they also reported that grain quality was decreased by salt stress as less accumulation of major nutrient content in the grain was found [82]. Chunthaburee and co-worked conducted the experiment with four different cultivars (Pokkali, KKU-LLR-012, Niewdam Gs.no.00621 are salt-tolerant cultivars and KKU-LLR-039 is salt-sensitive cultivar) and found that yield and harvest index including 1000-grain weight, panicle fertility and filled grain percentage were reduced under salt stress [83]. Kumar and Khare studied the effect of salt stress on differential growth and yield

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responses in two rice cultivars (Panvel-3 (tolerant) and Sahyadri-3 (sensitive)) to 100 mM NaCl and founded that 1000-grain weight, the number of grain per panicle, filled grain percentage, and grain yield were reduced under salt stress both in tolerant and sensitive cultivars but, yield reduction was higher in the sensitive cultivar when compared to the tolerant cultivar. They also found that grain quality of rice was lessened by salt stress through reduction of starch and protein content of rice grain [84]. Furthermore, Aref and co-worker conducted the experiment in a greenhouse with 2–8 dS/m of salinity, which was given to the plant at different developmental stages (tillering, panicle initiation, panicle emergence and ripening). They reported that biomass yield, grain yield and harvest index were reduced under salt stress at tillering and panicle initiation stage and increasing yield reduction was found with increasing level of salinity [85].

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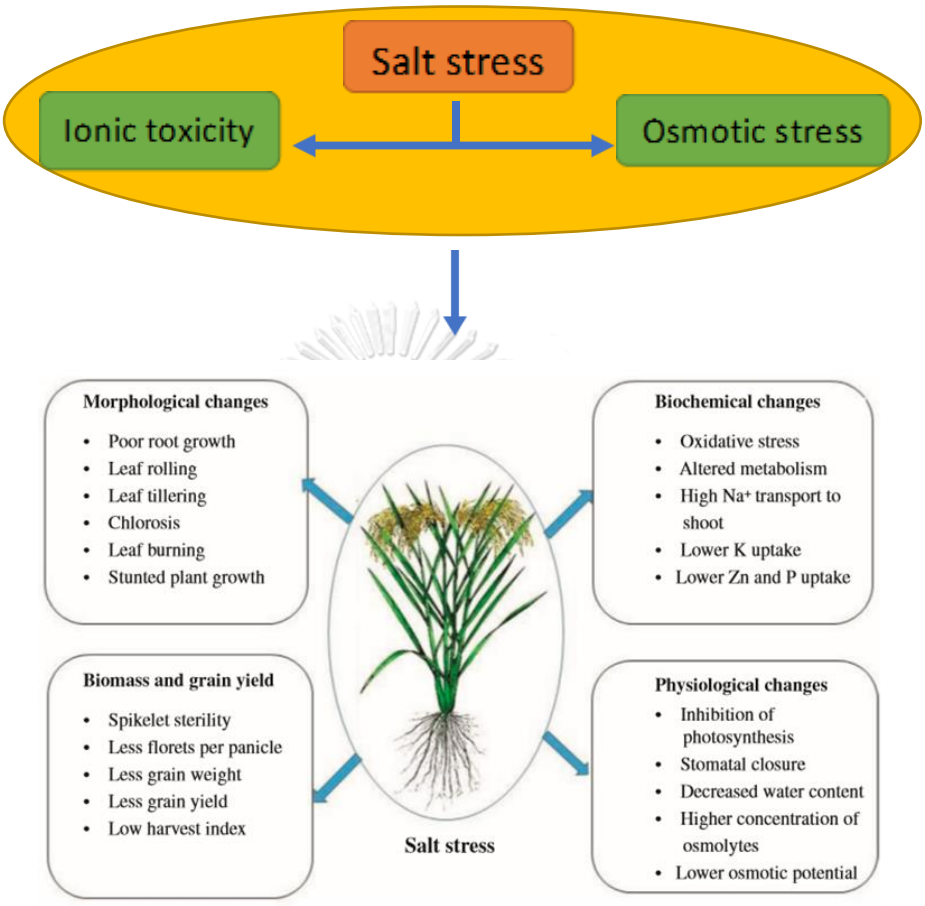


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**Figure 3** Salinity-induced major responses in rice plants [34]

### 1.3 Calmodulin

Calcium ion ($Ca^{2+}$) signaling appeared very early during evolution as an important secondary messenger and became a fundamental intracellular signaling component [86]. The role of $Ca^{2+}$ in cell signaling has been extensively studied [87, 88]. Many evidences reveal that various abiotic stresses such as light, cold, gravity, drought, heat, salt, wind, wounding, pathogen and touch, attacks can rapidly induce height in cytosolic $Ca^{2+}$ concentration [89-94] and high levels of cytosolic $Ca^{2+}$ are toxic to plant cells [91, 94, 95]. $Ca^{2+}$ gradient opposite the inner membrane system as well as the plasma membrane are implicated in cell signaling process controlled by stimulus-responsive $Ca^{2+}$ penetrable channels, $Ca^{2+}/H^{+}$ exchangers and $Ca^{2+}$ pumps [91, 94, 96] Furthermore, the transient changes of $Ca^{2+}$ concentration in intracellular triggered by various stimuli differ from each other in terms of frequency, duration, amplitude, spatial distribution inside the plant cell and these stimulus-specific $Ca^{2+}$ transients are named calcium signatures [94, 97]. Stimulus-specific signals are decrypted by downstream effector proteins to generate particular responses [94, 98]. These effectors include $Ca^{2+}$ sensor proteins, which are separated by three major group in plants, via calmodulin (CaM) and CaM-like proteins (CML), calcineurin B like (CBL) and calcium-dependent protein kinases (CDPKs) proteins [94, 99]. Calmodulin (CaM) is a pervasive $Ca^{2+}$-binding protein, which exists in eukaryotes [94, 95, 100-103]. It is a small acidic, heat stable and multifunctional protein composed of two globular domains. domain I is located near the N-terminus and domain II is located near the C-terminus. Each domain consists of 2 regions binding with $Ca^{2+}$, called EF-hand motifs [88, 94, 104, 105]. Although CaM lacks of catalytic activity, binding to or chelating of $Ca^{2+}$ causes conformational changes in the globular domains leading to interaction with the target proteins [88, 92, 106]. Multiple forms of calmodulin are reported in plants [88, 101, 107]. For example, A seven genes encoding four different CaMs are CaM1/CaM4, CaM2/CaM3/CaM5, CaM6, and CaM7, which differ only in one to five amino acid residues and share a at least 97% identity at the primary sequence level are indicated in *Arabidopsis thaliana* [88, 94, 106, 108-110]. Comparably, wheat (*Triticum aestivum*) reported 10 cDNAs encoding three CaM proteins [88, 111] Tomato (*Lycopersicon esculentum*) tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*), reported multiple genes for CaM such as four, six, and seven respectively [88, 112]. Moreover, rice (*Oryza sativa*)

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genome includes five *CaM* genes encoding two sets of CaM proteins consist of the OsCaM1 encoded by *OsCaM1-1*, *OsCaM1-2*, and *OsCaM1-3* in rice differs by two amino acid residues from the CaM encoded by *OsCaM2* and *OsCaM3* [88, 113]. Targets of CaMs is called the CaM-binding proteins (CBPs). Various results founded that CaM bind to a variety of CBPs in plants, which include phosphatases, kinases, metabolic enzymes, receptors, transcription factors, cytoskeletal proteins and ion channels and pumps [92, 94, 102, 103, 114, 115]. Therefore, CaMs play as multifunctional regulatory proteins, and their functional significance is materialized through the actions of their downstream target proteins [94]. As mentioned above, calmodulin structure and its activation mechanisms are represented in Figure 4.

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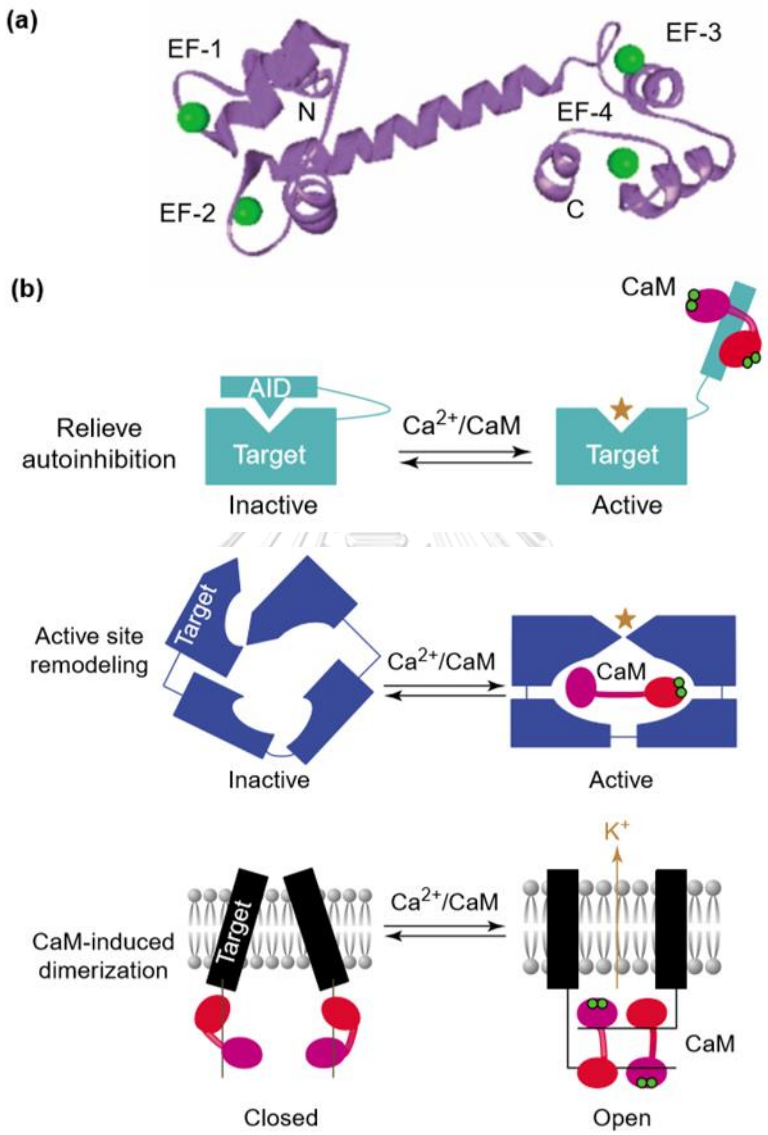


**Figure 4** (a) Crystal structure of $Ca^{2+}$-bound CaM and the green ball represented $Ca^{2+}$ binding with four regions of EF-hand motifs. (b) The mechanism of protein activation by CaM [95, 116].

### 1.3.1 CaM-mediated regulation of salt stress in plants

High concentration of saline is one of the main environmental stresses regularly experienced by plants, which results in osmotic stress on plant cells. Osmotic stress induces a primary event that is an increase in the concentration of cytosolic $Ca^{2+}$ and following transduction of $Ca^{2+}$ signals that supports appropriate cellular responses in an endeavor to relieve potential damages [94, 117, 118]. signaling of CaM-mediated is lively involved in osmotic stress of plant response [94, 114]. Various studies of role of CaM under salt stress, for example, Abe and Yoo studied overexpression of a salt-induced CaM gene from soybean. The result showed that *GmCaM4* in *Arabidopsis* impels salt stress tolerance via the up-regulation of DNA-binding activity of a MYB transcription factor MYB2. Moreover, MYB2 also interacted with CaM in a $Ca^{2+}$-dependent manner and regulate salt responsive genes [94, 119, 120]. Yamagushi and co-worker reported that *AtCaM15* interacts with the C-terminus of AtNHX1 and localized the vacuolar lumen. $Ca^{2+}$ and pH affected the interaction between AtNHX1and *AtCaM15* and the binding of AtCaM15 to AtNHX1 alters the $Na^{+}/K^{+}$ selectivity of the exchanger by reducing its $Na^{+}/H^{+}$ exchange speed. The presence of $Ca^{2+}$-pH-dependent signaling components in the vacuole was suggested by the interaction between AtNHX1 and *AtCaM15*, which are involved in mediating plant responses to salinity stress [94, 121]. The mechanism of interpretation and generation of $Ca^{2+}$ signals, which are regulated by CaM during abiotic stresses of plant responses include salt stress were represented in Figure 5.

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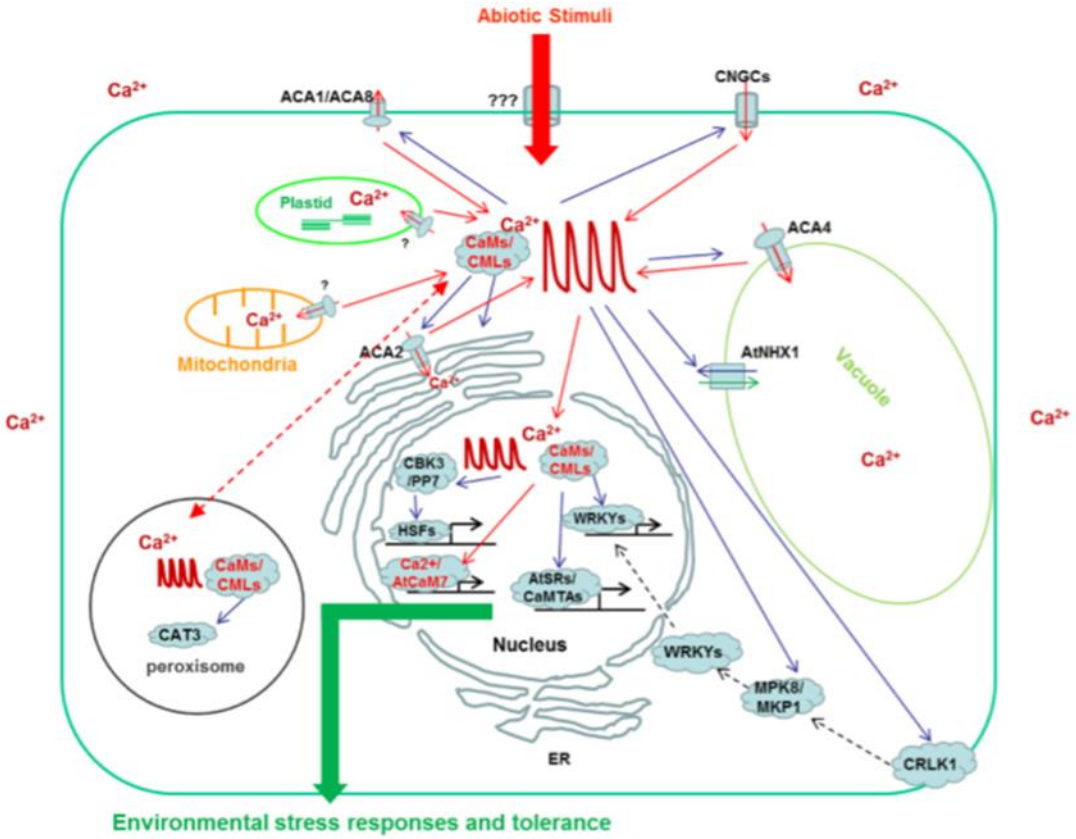


**Figure 5** CaM /CML and their target proteins regulating various cell precesses through $Ca^{2+}$ signaling under abiotic stress. The red arrows presented actions modifying $Ca^{2+}$ transients or CaM/CMLs and the blue arrows presented actions regulated by $Ca^{2+}$/CaMs or $Ca^{2+}$/CMLs [94].

### 1.4 Glyoxylate cycle

Glyoxylate cycle is a modified metabolic pathway form of the tricarboxylic acid (TCA) cycle, which occurs in fungi, bacteria and plants. It starts after fatty acids are converted to acetyl CoA by β-oxidation, which then enters the cycle. Glyoxylate cycle synthesizes carbohydrates from fatty acids by converting acetyl-CoA (2-carbon atom) , which may come from acetate, and acetyl-CoA is then metabolited in the glyoxylate cycle to synthesize succinate and malate [122, 123] (Figure 6).

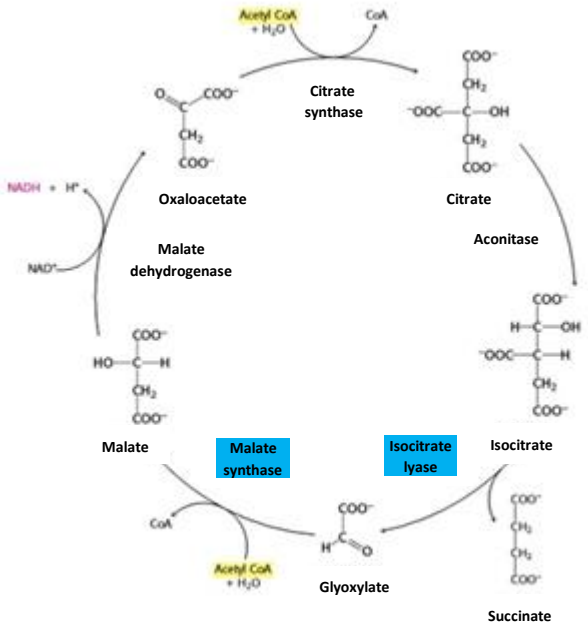


**Figure 6** Glyoxylate cycle [124]

The glyoxylate cycle has two marker enzymes viz isocitrate lyase (ICL) (EC 4.1.3.1) and malate synthase (MS) (EC 4.1.3.2). Isocitrate lyase catalyzes the conversion of isocitrate to glyoxylate and succinate (Figure 7). Glyoxylate can react with phenylhydrazine, so this chemical reaction can be used to measure isocitrate lyase activity.

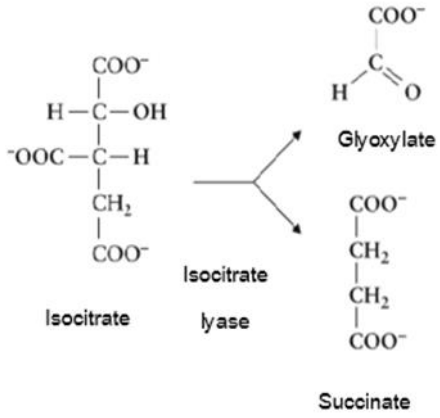


**Figure 7** Isocitrate is converted to glyoxylate and succinate by isocitrate lyase [125].

Glyoxylate + Phenylhydrazine ⟶ Glyoxylate phenylhydrazine

The other key enzyme, malate synthase catalyzes the conversion of glyoxylate and acetyl CoA to malate (Figure 8) [126]. Malate and coenzyme A can react with DNTB (5,5'-dithiobis-(2-nitrobenzoic acid)), producing TNB and coenzyme A derivative, which can be used to detect malate synthase activity.

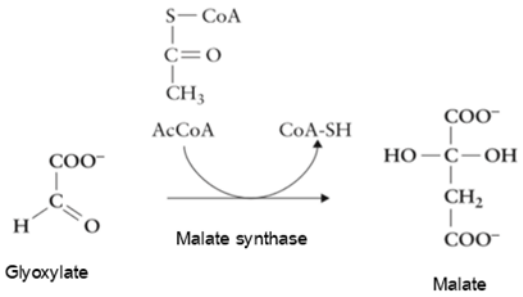


**Figure 8** Glyoxylate was converted to malate by malate synthase [125].

Malate + Coenzyme A $\xrightarrow{\text{DNTB}}$ TNB + Coenzyme A derivative

In previous research, role of the key enzymes in glyoxylate cycle via isocitrate lyase and malate synthase were studied in many plant species. For example, Eastmond and co-worker studied the role of glyoxylate cycle in lipid catabolism and post germinative growth in Arabidopsis mutants *icl-1* and *icl-2*, which lacked the glyoxylate cycle because of the absence of the key enzyme isocitrate lyase. The result showed that the glyoxylate cycle is regarded as essential for post germinative growth in Arabidopsis because, the glyoxylate cycle is important for seedling survival recovery after prolonged dark condition and seedling survival that approximate growth in nature. They also found that lipid catabolism in Arabidopsis mutants were severely inhibited in contrast, in the presence of exogenous sugars, the rate of lipid breakdown is substantially increased and approximates that of wild-type seedlings [127]. In another experiment, Cornah and co-worker demonstrated lipid utilization, gluconeogenesis, and seedling growth in *Arabidopsis* mutants lacking the glyoxylate cycle enzyme malate synthase *(mls*) compared to wild type and Arabidopsis mutants (*icl-2*) which lack the glyoxylate cycle enzyme isocitrate lyase from the experiment of Eastmond [127]. The results revealed that *Arabidopsis* sp. mutants (*mls*) seedling grow faster, used their lipid to generate more carbohydrate than *icl* seedlings and Arabidopsis mutants (*mls*) also contained isocitrate lyase enzyme activity at the same level as in wild type seedlings [128]. Furthermore, Lu and co-worker studied the important metabolic role of the glyoxylate cycle in submerged rice seedlings (anaerobic condition). They reported that the activity assay of pyruvate decarboxylase, isocitrate lyase and acetyl-coenzyme A synthetase in the submerged seedlings showed an 8.8-fold, 3.5-fold and 3-fold increase in the unsubmerged seedlings, respectively [123].

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### 1.5 Previous studied of overexpressing *OsCaM1-1* gene under salt stress

In previous study, Saeng-ngam and co-worker examined the role of *OsCaM1-1* in ABA accumulation under salt stress. The *OsCaM1-1* gene encoding 149 amino acid-long polypeptide sequence was overexpressed in KDML105 rice cultivar under the control of a 35SCaMV promoter. The result of this studied showed that the overexpression of *OsCaM1-1* gene up-regulated genes that involved in ABA biosynthesis via ABA aldehyde oxidase and 9-cis-expoxycarotenoid dioxygenas*e* as the transgenic rice lines have higher ABA content than the wild type and the control line. Moreover, they also reported that the transgenic KDML105 rice lines overexpressing *OsCaM1-1* were tolerant to salt stress (Figure 9) [129]. Next, Yuenyong and co-worker studied downstream components of the calmodulin signaling pathway in the rice salt stress response revealed by transcriptome profiling and target identification. They performed an experiment in transgenic 'Khao Dawk Mali 105' rice over-expressing *OsCam1–1* and wild type rice and founded that overexpression of *OsCam1–1* widely affected the expression of genes involved in several cellular processes under salt stress, including hormone-mediated regulation, signaling, transcription, secondary metabolism, lipid metabolism, carbohydrate metabolism, photosynthesis, glycolysis, tricarboxylic acid (TCA) cycle and glyoxylate cycle. In addition, the transgenic rice lines had higher sucrose and starch contents than wild type whereas, the photosynthesis rate in the transgenic rice was slightly lower than in wild type under salt stress. So, they suggested that carbon metabolism and energy were affected by *OsCam1–1* overexpression [130]; furthermore, Yuenyong and co-worker also studied role of isocitrate lyase under salt stress in plant. From transcriptome analysis result, they founded that genes associated with the glyoxylate cycle exhibited positive gene expression in KDML105 overexpressing *OsCam1-1* under salt stress (Figure 10). They constructed transgenic Arabidopsis lines: the *Aticl* mutant expressing *OsICL* driven by the native *AtICL* promoter, the *Aticl* mutant overexpressing *OsICL* driven by the 35SCaMV promoter, and WT overexpressing *OsICL* driven by the 35SCaMV promoter. The result showed that glucose and fructose contents of the *Aticl* mutant were highest, whereas those of *OsICL*-expressing lines were similar to or lower than those of the WT under salt

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stress and the germination rate, seedling fresh and dry weights of those of the *Aticl* mutant were lower than the *OsICL*-expressing lines, and the two lines with the *icl* mutant background were similar to the WT. In addition, The Fv/Fm and temperature of rosette leaves in the *OsICL* expressing lines were less affected by salt stress than the *Aticl* mutant [131].

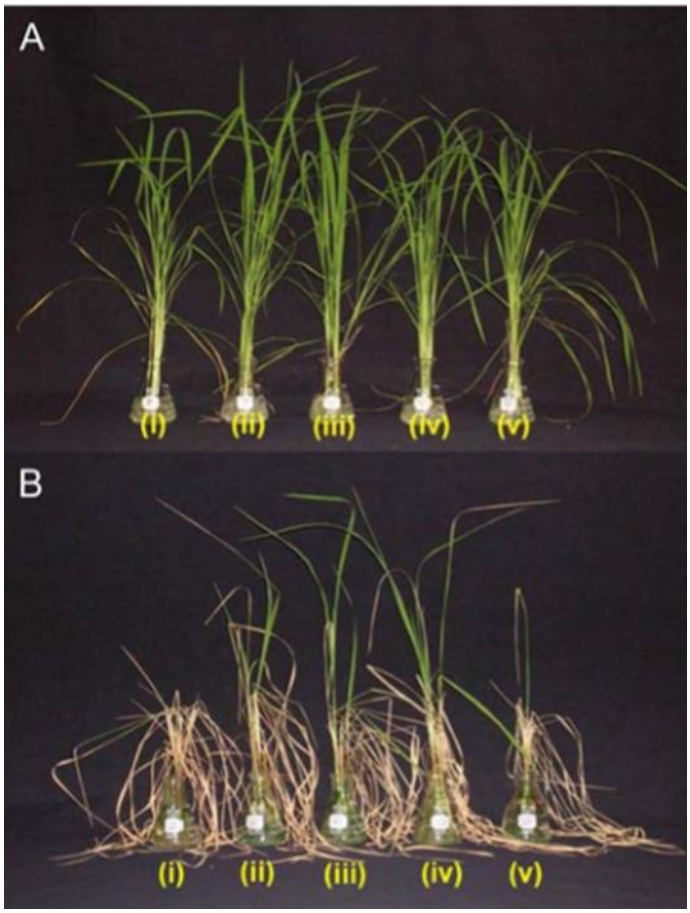


**Figure 9** The phenotype comparison of (i) wild type (KDML-wt) rice cultivar, the three transgenic rice lines, (ii) CaM1-1T1, (iii) CaM1-1T2 and (iv) CaM1-1T3, and (v) the control KDML-vector (T1) line, all grown under (A) normal or (B) salt-stress conditions for 15 day [129].

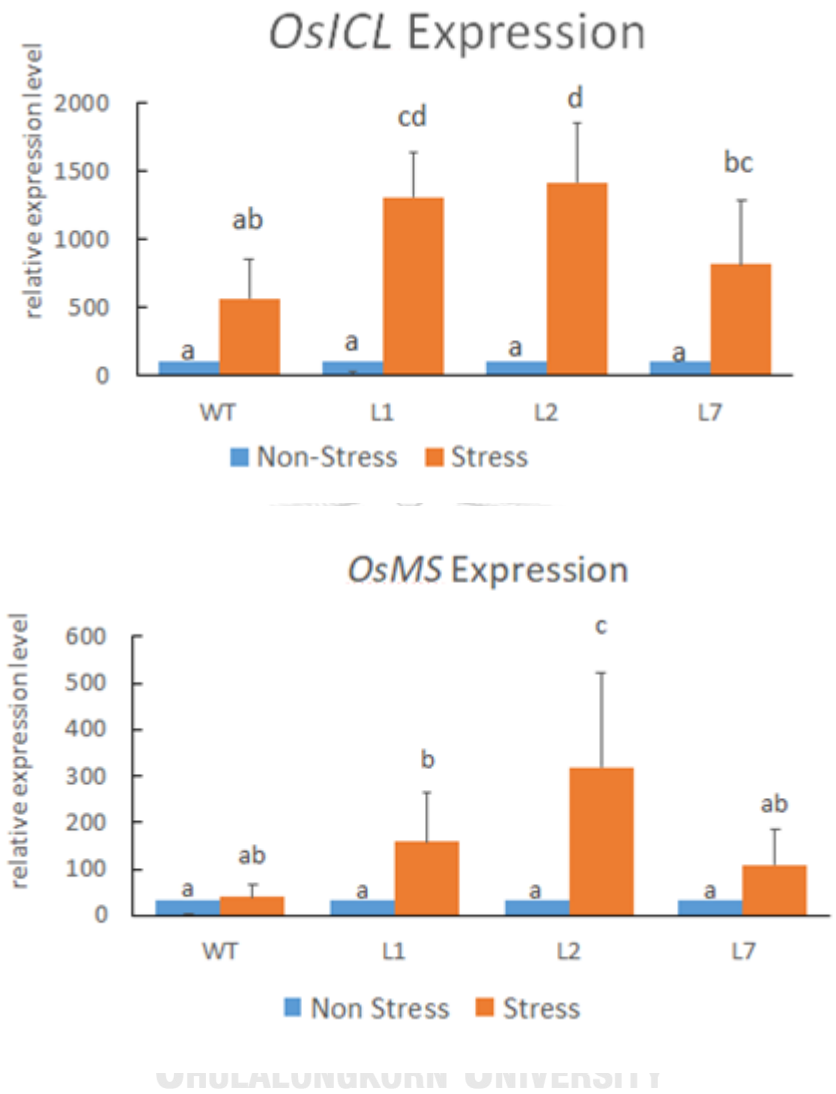


**Figure 10** qPR-PCR confirming expression level of glyoxylate enzyme genes (*OsICL*: Isocitrate lyase, *OsMS*: Malate synthase) in the transgenic rice lines were higher than wild type under salt stress [131].

**1.6 Objective of this thesis**

To investigate the role of the glyoxylate cycle in KDML 105 rice over-expressing *OsCam1-1* under salt stress by measuring the amount of starch, sugar, unsaturated fatty acid and activity of isocitrate lyase and malate synthase in various days after salt stress and leaf positions of KDML 105 rice over-expressing *OsCam1-1*.



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# CHAPTER II

# MATERIALS AND METHODS

## 2.1. Materials

### 2.1.1. Plants materials

Seed of Khao Dawk Mali 105 rice cultivar (*Oryza sativa* CV. KDML105) and seed of the three transgenic KDML105 rice lines overexpressing *OsCam1-1*: L1, L2, and L7 previously constructed by Sang-ngam and colleagues [132] were used.

### 2.1.2. Chemicals, enzymes and reagents

- Acetyl-CoA (Roche, Switzerland)
- Ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo7O_{24}\cdot 4H_2O$) (Carlo Erbra Reagent, France)
- Ammonium nitrate ($NH_4NO_3$) (Carlo Erbra Reagent, France)
- Amyloglucosidase (Sigma, USA)
- Boric acid ($H_3BO_3$) (Carlo Erba Reagent, France)
- Bovine Serum Albumen (BSA) (Sigma, USA)
- Calcium chloride dihydrate ($CaCl_2\cdot 2H_2O$) (Carlo Erba Reagent, France)
- Canola oil (Commercial)
- Chlorofrom (VWR Chemicals, USA)
- Citric acid (monohydrate) (Carlo Erba Reagent, France)
- Coenzyme A (Sigma, USA)
- Coomassie® brilliant blue G-250 (Fluka, Switzerland)
- Copper (II) sulfate ($CuSO_4\cdot 5H_2O$) (Carlo Erba Reagent, France)
- D,L-isocitric acid ($C_6H_5Na_3O_7$) (Sigma, USA)
- Dithiothreitol (DTT) (Bio Basic Inc., Canada
- DTNB (Ellman's Reagent) (5,5'-dithio-bis-[2-nitrobenzoic acid]) (Sigma-

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Aldrich, USA)

- Ethyl alcohol 99.99% (Quality Reagent Chemical, New Zealand)
- Ethylenediaminetetraacetic acid (EDTA) (Carlo Erba Reagent, France)
- Fructose (Carlo Erba Reagent, France)
- Glacial acetic acid (Carlo Erba Reagent, France)
- Glucose (Carlo Erba Reagent, France)
- HEPES (Vetec™, Singapore)
- Iron (II) sulphate ($FeSO_4 \cdot 7H_2O$) (Carlo Erba Reagent, France)
- Liquid nitrogen (Linde, Ireland)
- Magnesium chloride ($MgCl_2$) (Carlo Erba Reagent, France)
- Magnesium sulfate ($MgSO_4 \cdot 7H_2O$) (Carlo Erba Reagent, France)
- Manganese chloride ($MnCl_2 \cdot 4H_2O$) (Carlo Erba Reagent, France)
- Methanol (Macron Fine Chemicals™, USA)
- O-phosphoric acid 85% (Merck, Germany)
- Phenylhydrazine (Tokyo Chemical Industry Co., Ltd., Japan)
- Potassium chloride (KCl) (Carlo Erba Reagent, France)
- Potassium hydroxide (KOH) (Carlo Erba Reagent, France)
- Potassium nitrate ($KNO_3$) (Carlo Erba Reagent, France)
- Potato starch (Sigma, USA)
- Sodium acetate ($CH_2COONa$) (Carlo Erba Reagent, France)
- Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) (Carlo Erba Reagent, France)
- Sodium glyoxylate monohydrate ($HC(O)COONa \cdot H_2O$) (Sigma, USA)
- Sodium hydroxide (NaOH) (Carlo Erba Reagent, France)
- Sucrose (Carlo Erba Reagent, France)
- Sulfuric acid (Merck, Germany)
- Tricine (Sigma, USA)
- Vanillin (Fluka, Switzerland)
- Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Carlo Erba Reagent, France)

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- α-Amylase (Sigma, USA)

**2.1.3. Instruments, glassware and plasticware**

- -80 ˚C ultralow temperature freezer (New Brunswick Scientific, England)
- -20 ˚C freezer (Hitachi, Japan)
- 0.1-2 µl micropipette (Axygen, USA)
- 1 ml syringe (Nipro, Japan)
- 1.5 ml microcentrifuge tube (Axygen, USA)
- 15 ml plastic centrifuge tube (Nest®, USA)
- 2 ml microcentrifuge tube (Axygen, USA)
- 2 ml clear glass vial (Vertical Chromatography Co.,Ltd., Thailand)
- 250 µl glass insert vial (Agilent Technologies, USA)
- 30-300 µl multichannel pipette (Finnpipette® Thermo Scientific, USA)
- 5-50 µl multichannel pipette (Finnpipette® Thermo Scientific, USA)
- 50 ml plastic centrifuge tube (Nest®, USA)
- 60 ˚C oven (Memmert, Germany)
- 9 mm rib-sided open-top cap, PP(Vertical Chromatography Co., Ltd., Thailand)
- 96 deep-well plate (Nest®, USA)
- 96 well plate (Corning, USA)
- Agilent Hi-Plex Ca (Duo) ligand exchange column (Agilent Technologies, USA)
- Autoclave (Sanyo, Japan)
- CentriVap benchtop vacuum concentrators (Lanconco, USA)
- Chlorophyll Meter SPAD-502 Plus (Konica Minolta, Japan)
- CP224S Competence Analytical Balance, 220 g x 0.1 mg (Sartorius, Germany)
- CP423S Precision Balance, 420 g x 0.001 g (Sartorius, Germany)
- C-MAG HS 7 magnetic stirrers (IKA®, USA)
- Duran bottle (Duran, Germany)
- FE20 FiveEasy™ benchtop pH meter (Mettler Toledo™, USA)
- Fume Hood
- Grinding balls stainless steel 2mm Ø (Retsch® Germany)

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- High Performance Liquid Chromatography (HPLC) prominence LC-20 series (Shimadzu, Japan)
- Lyophilizer (Labconco, USA)
- Microcentrifuge (Hettich, Germany)
- Micropipette P20 P100 P200 P1000 (Gilson, France)
- Mixer Mill MM 400 (Retsch, Germany)
- Plant growth chamber (Humanlab, South Korea)
- Plastic pipette tip (Axygen, USA)
- Red PTFE/white silicone septa 8.9*1mm Ø (Agilent Technologies, USA)
- Refrigerated centrifuge 5418 R (Eppendorf, Germany)
- Synergy H1 microplate reader (BioTek®, USA)
- ThermoMixer® C (Eppendorf, Germany)
- UV-STAR® microplate 96 well (Greiner bio-one, Austria)
- VertiClean™ NYLON syringe filters, 13mm, 0.2µm (Vertical Chromatography Co., Ltd., Thailand)
- Vortex-Genie 2 (Scientific Industries, Inc., USA)

**2.1.4. Planting materials**

- Clay soil
- Loam soil
- Urea fertilizer (46-0-0)
- Fertilizer (16-16-16)

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## 2.2 Method

### 2.2.1 Rice cultivation for seed collection

In August 2018, both of Khao Dawk Mali 105 rice seeds and its three transgenic Khao Dawk Mali 105 rice lines overexpressing *OsCam1-1* (L1, L2, and L7) seeds were soaked in 2% (w/w) of sodium hypochlorite for 10 min and rinsed with distilled water three times, and soaked in distilled water and incubated under darkness for five to seven days until it germinated. Then, the germinating seeds were transferred onto the basket that contained Yoshida's solution [133] and grown for 2 weeks in the growth chamber under 16 hr light/8 hr dark photoperiod at 25 ˚C, and 70% humidity. After seedlings were nourished with Yoshida's solution, they were removed to natural light in the green house at the Faculty of Science, Chulalongkorn University, and seedlings were transplanted to large pots that were added with completely mixed clay soil : mold soil (2:1 w/w) and filled with water 2-3 times per week. After planting for 1 week, the urea fertilizer was added every 2 weeks. When rice seedlings entered the vegetative stage, the fertilizer (16-16-16) was applied every week until entering the flowering stage. Finally, spikes were harvested in December 2018 and dried at 60 °C for 3 days. Then, rice seeds were further used as plant materials.

### 2.2.2 Rice cultivation, salt stress treatment and sample collection

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The seed of Khao Dawk Mali 105 rice and its three transgenic Khao Dawk Mali 105 rice lines overexpressing *OsCam1-1* (L1, L2, and L7) were soaked in 2% (w/w) of sodium hypochlorite for 10 min and washed completely with distilled water three times, and soaked in distilled water and incubated under darkness for five to seven days until it germinated. Then, the germinated seeds were transferred onto the basket that contained Yoshida's solution and grown for 1 week in the growth chamber under 16 hr light/8 hr dark photoperiod at 25 °C, and 70% humidity. After seedlings were nourished with Yoshida's solution, they were moved to natural light in the green house at the Faculty of Science, Chulalongkorn University, and seedlings were transplanted to pots that were added with clay soil from field submerged with 10 L of water in

tray for fourteen days. The 21-day old seedlings of the 4 rice lines were divided into two groups, control and stress conditions, and treated as follows;

Stress condition: seedling was treated with 115 mM NaCl (electrical conductivity $(EC)_{1:5}$ of 2 $dS/m^{-1}$ [134]) for 0, 2, 4, 6 days

Control condition: seedling was treated with water for 0, 2, 4, 6 days

The experiments were performed in five biological replicates using Randomized Complete Block Design (RCBD) experimental plan. Next, rice leaves were collected from two positions: (1) the youngest fully expanded leaf and (2) the second youngest leaf, which has matured just before the youngest fully expanded leaf by snap freezing in liquid nitrogen and maintained in the -80 ˚C ultralow temperature freezer.

### 2.2.3. Measurement of fresh weight and dry weight

Khao Dawk Mali 105 rice and its three transgenic Khao Dawk Mali 105 rice lines overexpressing *OsCam1-1* (L1, L2 and L7) were grown for 21 days. Then, the rice seedlings were treated with 115 mM NaCl for 0, 2, 4, 6 days. Fresh weight values of root and shoot were separately collected. After that, root and shoot tissues were completely dried by hot air oven at 60 °C for 3 days and weighted to collect the dry weight values.

### 2.2.4. Measurement of green index

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The green index of plant leaves was measured by Chlorophyll Meter SPAD-502 Plus. The measurement was done in the top, the middle and the lower part of the leaf, then the values were averaged. In theory of chlorophyll Meter SPAD-502 Plus, SPAD value from chlorophyll Meter SPAD-502 Plus was related with the amount of chlorophyll content by measuring and calculating the absorbance of the leaf in two wavelength regions such as (400-500 nm) and (600-700 nm).

### 2.2.5. Activity assay of isocitrate lyase

#### 2.2.5.1. Preparation of crude protein extracts

Preparation of crude protein extracts was performed using the method described by [135] and [136]. Approximately 100 milligrams of rice leave tissues were collected, frozen in liquid nitrogen and grounded by Mixer Mill MM 400 until became fine powder. Then, tissue powders were homogenized at 4 ˚C with 1 ml of extraction buffer that contained 170 mM Tricine pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA and 2 mM DTT; and mixed by vortex. The homogenate was centrifuged at 12,000xg for 20 min at 4 °C and the supernatant was transferred to a new microtube for using as a crude protein in the enzymatic activity assay and the pellet was discarded.

#### 2.2.5.2. Assay of isocitrate lyase activity

Isocitrate lyase activity was determined using the method described by (Franzisket & Gerhardt, 1980) and the method modified from that described by (Lu, Wu, & Han, 2005). The reaction buffer consisted of 170 mM Tricine buffer pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, and 6 mM phenylhydrazine. Twenty-five microliters of crude protein extracts were added into 100 µl of reaction buffer, then mixed using pipette in a 96-well UV microplate at 25 ˚C. $A_{324}$ of the reaction mixture was measured by Synergy H1 microplate reader until it was stable, then 25 µl of 4 mM D-L isocitric acid was added to the mixture and homogeneously mixed. After that, $A_{324}$ of the reaction mixture was further measured for 10 min. The difference of $A_{324}$ between 0 min and 10 min was used to calculating the amount of glyoxylate production per 10 min using the glyoxylate standard curve. The protein content of the aliquoted crude protein extract was determined using Bradford method (Bradford, 1976) in a 96-well plate. $A_{595}$ was measured using Synergy H1 microplate reader, and bovine serum albumin (BSA) was used for constructing a standard curve. The principle chemical reaction of the isocitrate lyase assay is shown in Figure 11, and the equation for calculating the activity of isocitrate lyase is shown in Figure 12.

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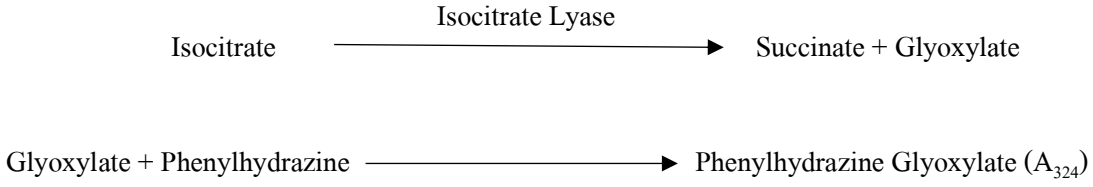


**Figure 11** The principle chemical reaction of isocitrate lyase assay

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

**Figure 12** The equation applied for calculating isocitrate lyase activity

**2.2.6. Activity assay of malate synthase**

Crude protein extracts were determined following step 2.2.5.1. The malate synthase activity was determined using the method described by [137] and method modified from that described by [138]**.** The reaction buffer contained 0.1 M HEPES buffer pH 7.8, 6 mM $MgCl_2$, 2.5 mM acetyl CoA, and 2 mM 5,5'-Dithio-bis (2-Nitrobenzoic Acid) (DTNB). Twenty-five microliters of crude protein extracts were added into 100 µl of reaction buffer and mixed using pipette in a 96-well UV microplate at 25 ˚C. $A_{412}$ of the reaction mixture was measured by Synergy H1 microplate reader until it was stable. Then, the reaction was initiated by the addition of 25 µl of 5 mM sodium glyoxylate. The increase in $A_{412}$ nm of the reaction mixture was recorded for approximately 10 minutes. The difference of $A_{412}$ between 0 min and 10 min was used to calculate the amount of free coenzyme A produced per 10 min using the coenzyme A standard curve. The protein content of the aliquoted crude protein was determined using Bradford method in a 96-well plate. $A_{595}$ was measured using Synergy H1 microplate reader, and bovine serum albumin (BSA) was used for constructing a standard curve. The principle chemical reaction

of malate synthase assay is shown in Figure 13, and the equation for calculating the activity of malate synthase is shown in Figure 14.

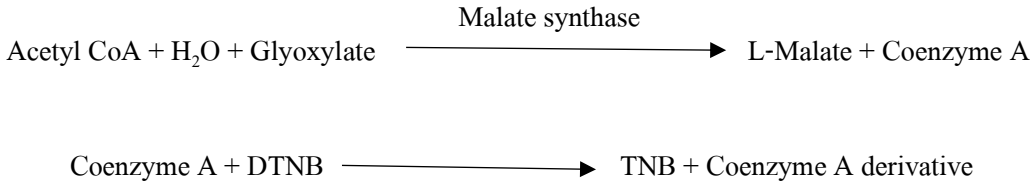


**Figure 13** The principle chemical reaction of malate synthase assay

$$\text{nmol coenzyme A / mg protein / min} = \frac{((A_{412}\ \text{min}\ 10) - (A_{412}\ \text{min}\ 0)) \times (\text{coefficient of coenzyme A standard curve})}{(\text{mg protein}) \times (10\ \text{mins})}$$

**Figure 14** The equation used for calculating malate synthase activity

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### 2.2.7. Determination of sugar and starch contents

#### 2.2.7.1 Sugar and starch extraction

Sugar extraction was conducted using the method modified from that described by [139] and [140]. While starch extraction was conducted using the method modified from that described by [141]. Rice leaf samples were collected by snap-freezing in liquid nitrogen and were lyophilized. After that, samples were ground by mixer mill until tissues became fine powder. Thirty milligrams of tissue powder were weighted and 600 µl of 80% ethanol v/v were added, and the mixtures were incubated at 80 ˚C for 10 min. Then, the mixtures were centrifuged at 4,000xg for 15 min and supernatants were transferred to new clean microtubes. The extraction procedure was repeated twice more and the supernatants were mixed to the previous extract. The supernatants and pellets were dried by CentriVap at 60 °C for 5 hr. The dried supernatants were used for sugar determination and the dried pellets were used for starch determination.

#### 2.2.7.2. Sugar content measurement

In sugar content measurement, the dried supernatants were re-suspended in 1 ml ultra-pure water and completely mixed by vortex. Then, the supernatants were drawn by 1 ml syringe without needle and filtered by Syringe Filters into 2 ml clear glass vials. After that, glucose, sucrose and fructose contents were determined using High Performance Liquid Chromatography (HPLC) with Agilent Hi-Plex Ca (Duo) ligand exchange column. The HPLC flow rate was 0.4 ml/min, 20 min per sample and the column temperature was 85 ˚C. 100% ultra-pure water was used as the mobile phase, and a refractive index detector (RID) was used to detect the sugars. The sugar contents were calculated by used sucrose, glucose, and fructose standard curves.

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#### 2.2.7.3. Starch content measurement

In starch content measurement, the dried pellets were resuspended in 1 ml of 50 mM acetate buffer pH 4.5 and completely mixed by vortex. They were heated in boiled water for 30 min until the pellets were broken and homogeneously mixed into the buffer. Then, 4 µl each of the enzymes: α-amylase and amyloglucosidase (30mg/ml) were added to the resuspended pellets to convert starch to glucose. After that, the mixtures were incubated under 37 °C for 2 hours and centrifuged at 4,000xg for 15 min. Two-hundred microliters of supernatants were transferred to new clean microtubes and added with 800 µl of absolute ethanol. Then, the supernatants were mixed by vortex, incubated at 80 °C for 10 min and dried by CentriVap at 60 °C for 5 hr. After that, the dried supernatants were resuspended in 1 ml ultra-pure water and completely mixed by vortex. Finally, glucose contents were determined using High Performance Liquid Chromatography (HPLC) with Agilent Hi-Plex Ca (Duo) ligand exchange column with the same condition used for sugar content measurement and the starch content was calculate by glucose standard curve.

### 2.2.8. Determination of unsaturated fatty acid content

First, 30 mg of rice leave was lyophilized and ground with Mixer Mill until tissues became fine powder. Unsaturated fatty acid was extracted by the method modified from that described by [142]. Six-hundred µl of chloroform : methanol (2:1, v/v) was added and mixed for 5 min on the incubator shaker. Next, the mixture was centrifuged at 2,000 rpm for 5 min and the supernatants were moved to new clean microtubes. Then, the procedure was repeated twice more and the supernatants were combined. Unsaturated fatty acid content was determined using the method described by [143] and the method modified from that described by [144]. Twenty microliters of sample solution were added in a 96-well microplate and evaporated in fume hood at 90 ˚C for 10 min, and 180 µl of concentrated sulfuric acid ($H_2SO_4$) was added. The mixture was incubated in oven at 90 ˚C for 20 min and the microplate was cooled to room temperature on ice water for approximately 2 min. Then, 50 microliters of vanillin–phosphoric acid reagent were added to each well and incubated at 37 ˚C for 15 min. Finally, the microplate was stored for 45 min in dark box for color development. $A_{530}$ was measured with Synergy H1

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microplate reader and canola oil (commercial oil) was used for constructing a standard curve. the principle chemical reaction of sulfo-phospho-vanillin (SPV) is shown in Figure 15.

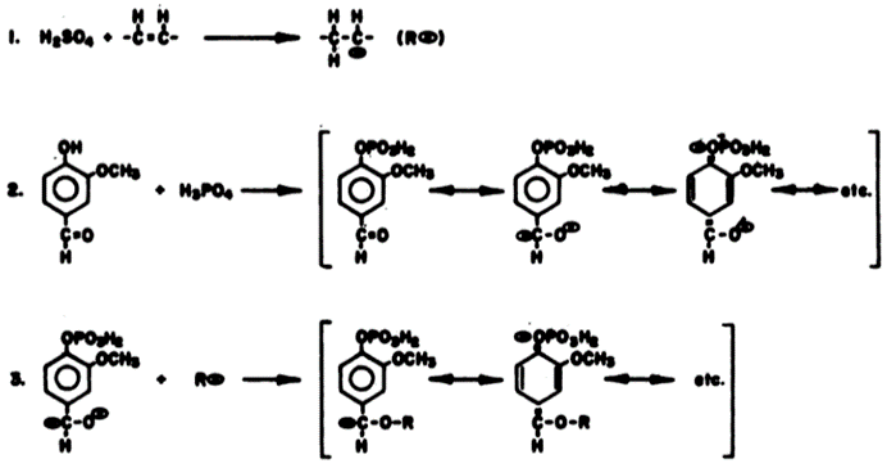

**Figure 15** The principle chemical reaction of sulfo-phospho-vanillin (SPV). (1) unsaturated compounds react with sulfuric acid to produce a carbonium ion, (2) vanillin reacts with phosphoric acid to produce an aromatic phosphate ester, and (3) the carbonium ion reacts with the activated carbonyl group of phospho-vanilin to produce a charged colored complex that is stabilized by resonance and absorbs miximally at about 530 nm [145].

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**2.2.9. Statistical analysis**

In every experiment, rice seedlings were grown using Randomized Complete Block Design (RCBD), and for statistical analysis, analysis of variance (ANOVA) followed by comparing the means with Duncan's multiple range test with significance set as $p<0.05$ using SPSS software version 22.0 was conducted.

# CHAPTER III

# RESULTS

From the previous studies, the transgenic Khao Dawk Mali 105 (KDML 105) rice lines overexpressing a calmodulin (*OsCam1-1*) gene were shown to be more tolerant to salt stress. By RNA seq, *OsCam1-1* overexpression was found to affect the expression of several genes that regulate a wide range of processes. Here, we aimed to investigate the role of glyoxylate cycle in the gained salt tolerance ability of the rice lines overexpressing *OsCam1-1* gene. Firstly, growth of the transgenic plants grown in soil under salt stress was examined.

## 3.1 Fresh weight and dry weight

Fresh weight and dry weight of shoot and root were measured to evaluate the salt tolerance ability of the three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, and L7) compared with wild type. The result showed that the shoot fresh weight of the wild type decreased significantly at day 4 and day 6 of salt stress (115 mM NaCl) while all three lines of transgenic rice overexpressing *OsCam1-1* did not exhibit significant decreases in shoot fresh weight (Figure 16). On the other hand, the result of the root fresh weight showed significant decreases at day 4 and day 6 of salt stress and no clear difference between the wild type and the transgenic lines under both salt stress and normal conditions (Figure 16). Furthermore, the result of the shoot dry weight showed that the transgenic lines could maintain shoot dry weight better than the wild type at day 4 and day 6 when the plants were treated with salinity whereas no significant difference of the root dry weights were found between the wild type and the transgenic line under both normal and salt stress conditions (Figure 17). The appearance of the three transgenic rice overexpressing *OsCam1-1* and the wild type under normal and salt stress conditions showed that no difference among them at day 0 and day 2 (Figure 18 and Figure 19). However at day 4, the color of leaf was starting to change to yellow in all lines under salt stress (Figure 20) and at day 6, the wild type had more rolled and dried leaves than the transgenic plants under salt stress (Figure 21).

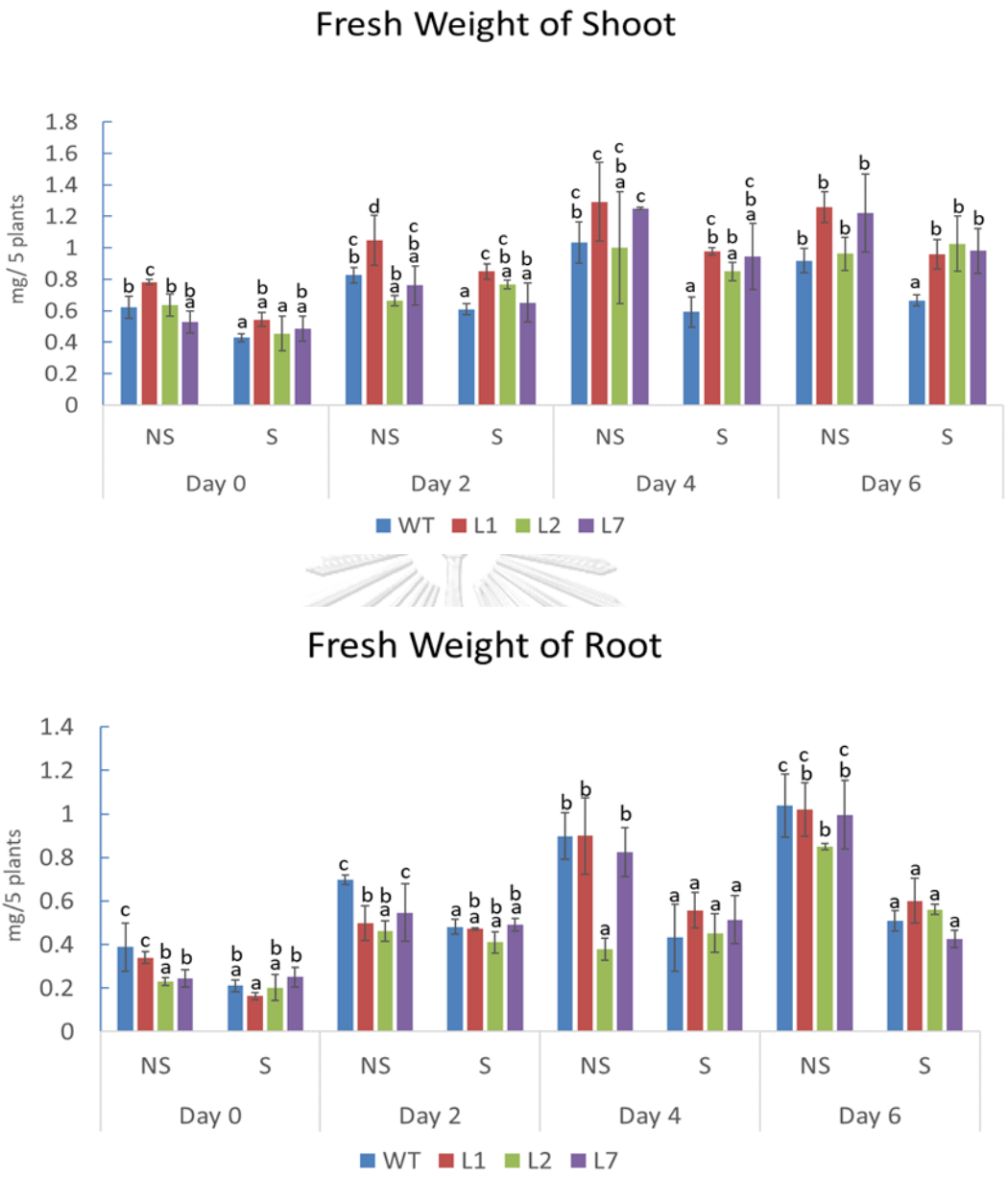


**Figure 16** Fresh weight of shoot and root of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) when exposed to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress (S) and non-stress (NS) conditions.

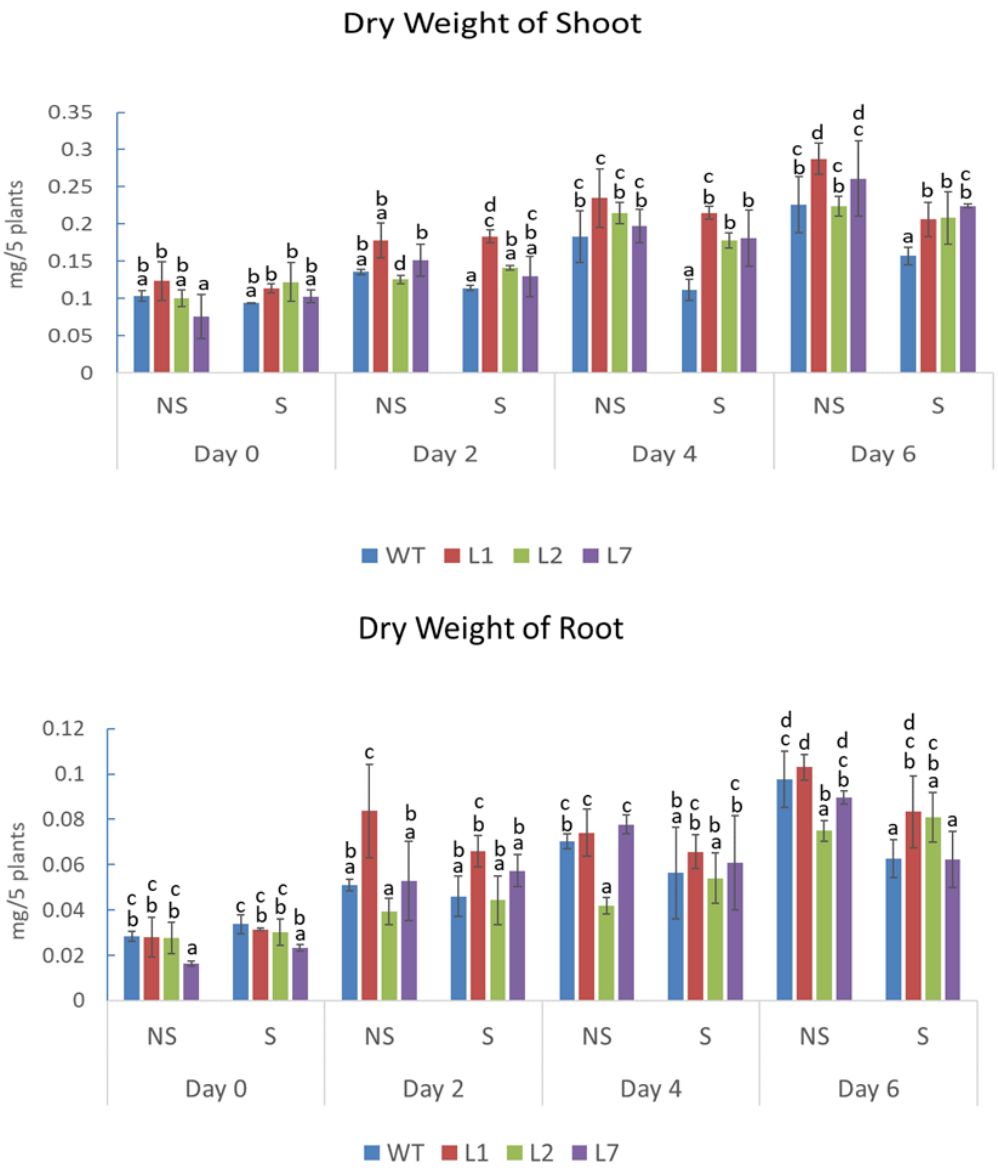


**Figure 17** Dry weight of shoot and root of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) when exposed to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress (S) and non-stress (NS) conditions.

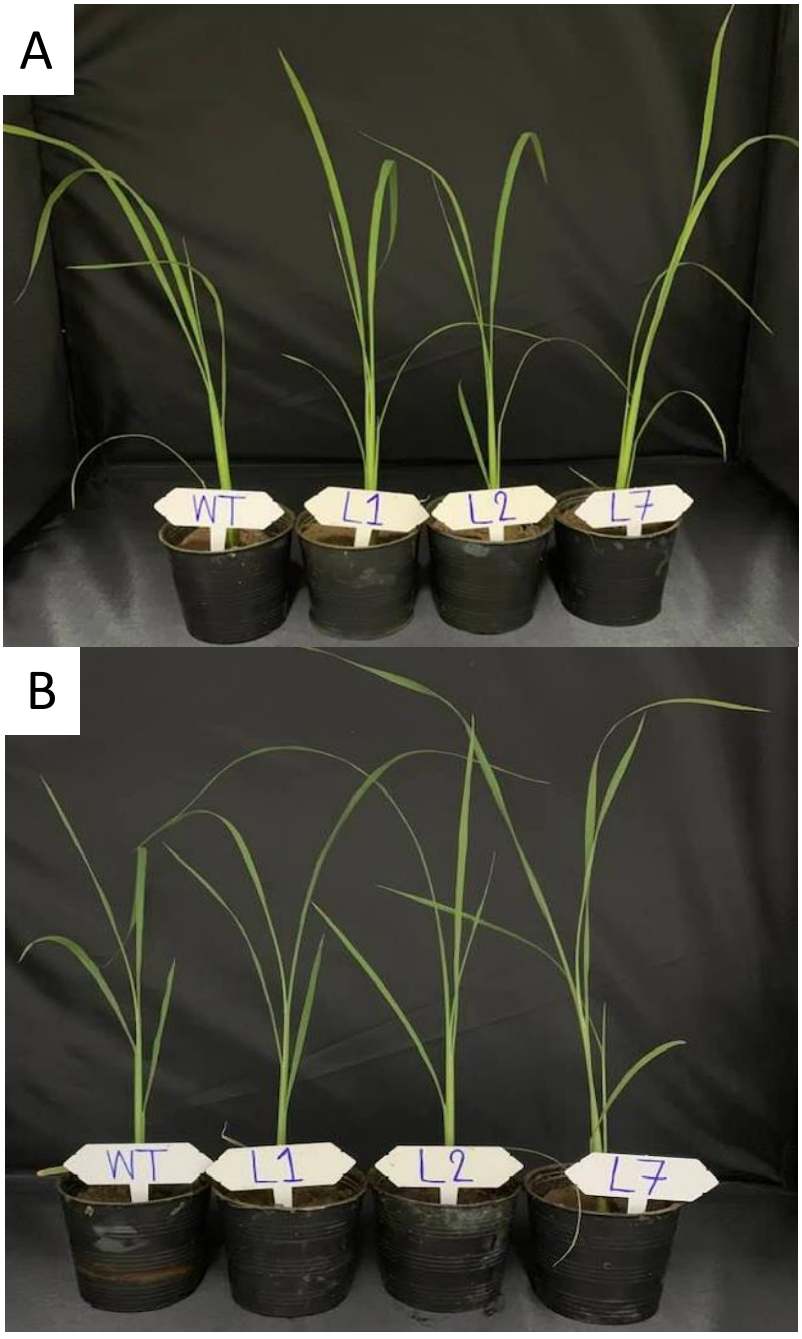


**Figure 18** The phenotype of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at day 0 under (A) non-stress and (B) salt stress (115 mM NaCl) conditions.


**Figure 19** The phenotype of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at day 2 under (A) non-stress and (B) salt stress (115 mM NaCl) conditions.

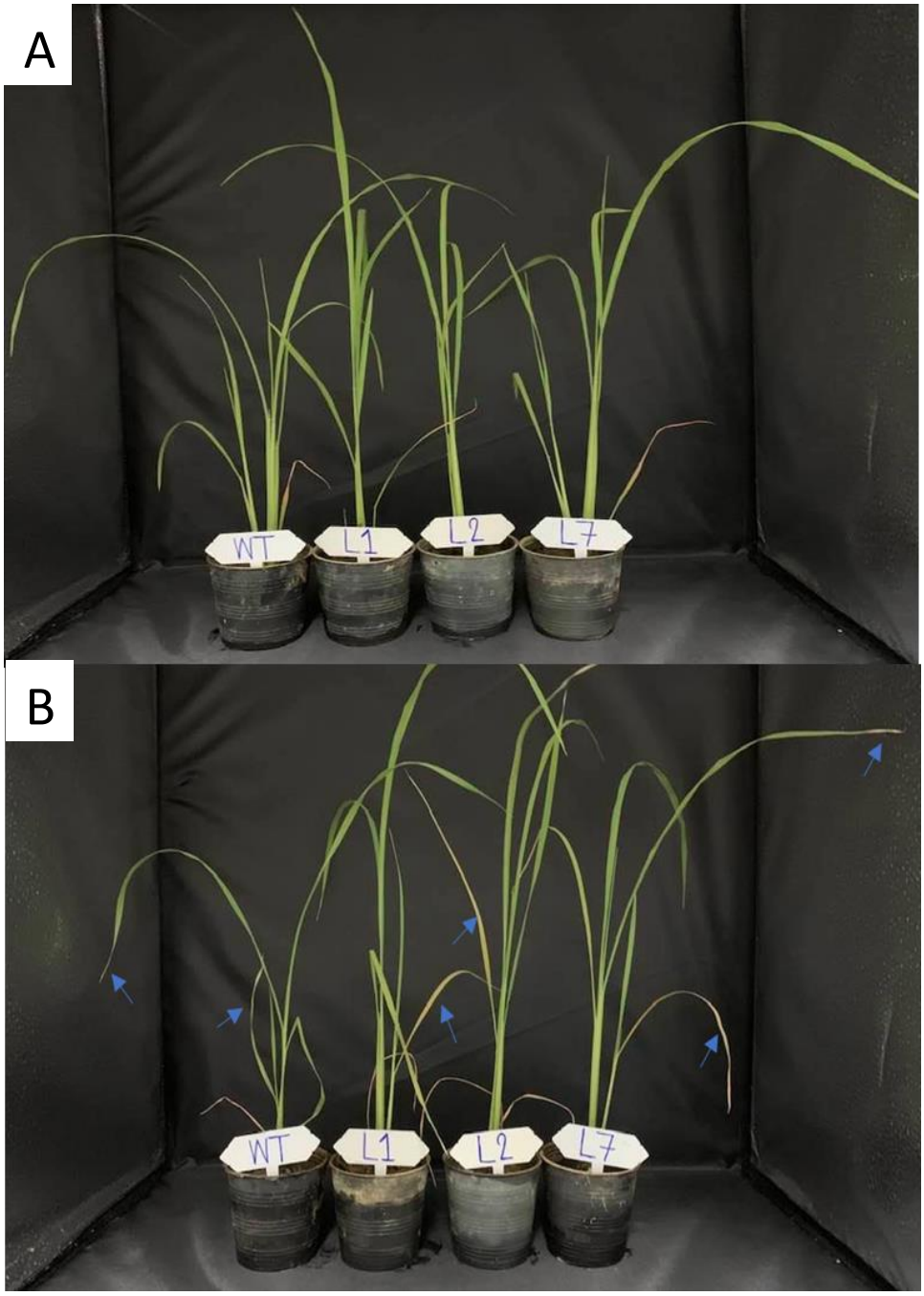


**Figure 20** The phenotype of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at day 4 under (A) non-stress and (B) salt stress (115 mM NaCl) conditions.

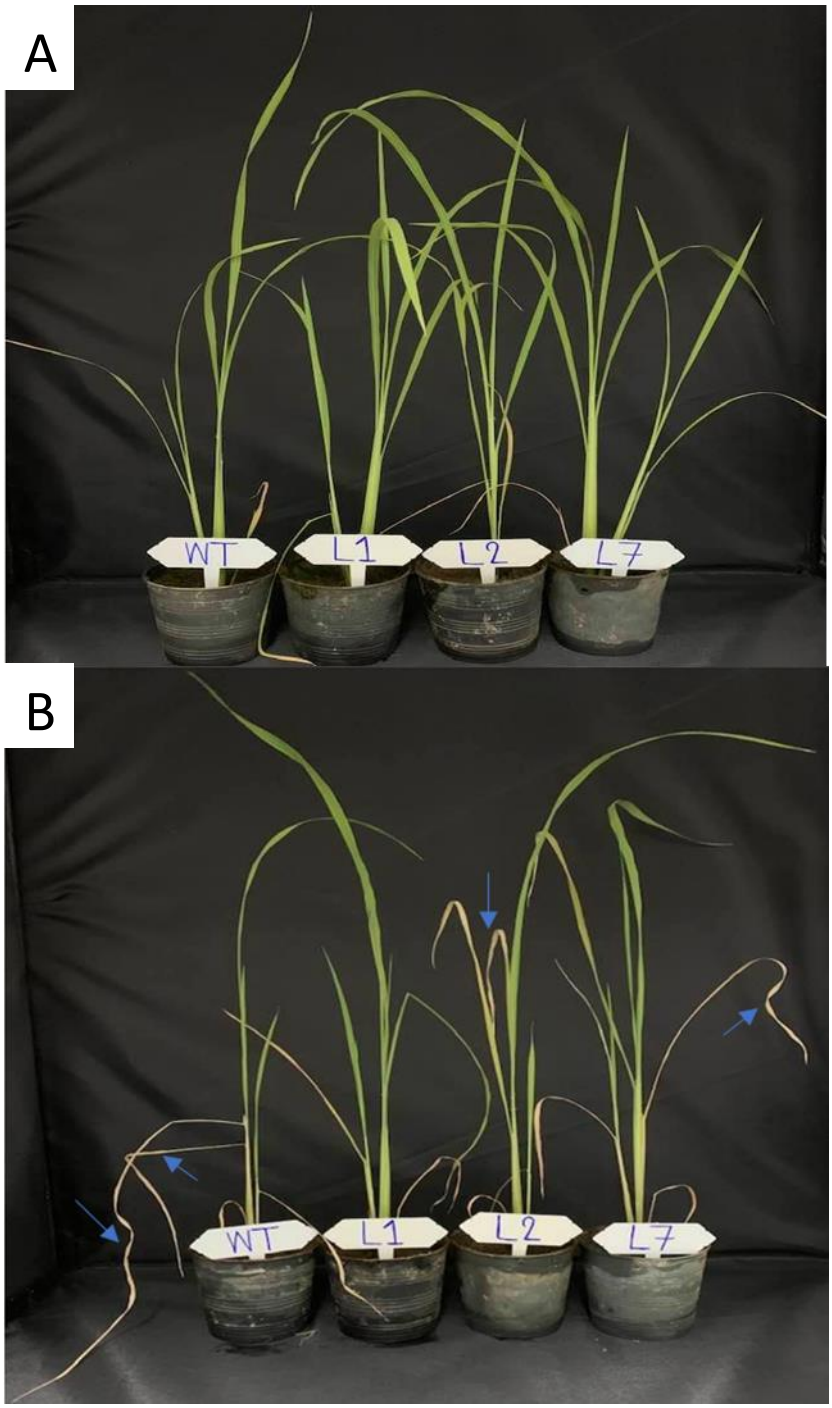


**Figure 21** The phenotype of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at day 6 under (A) non-stress and (B) salt stress (115 mM NaCl) conditions.

**3.2 The green index of plant leaves**

To estimate the green index of the three transgenic rice overexpressing *OsCam1-1* and the wild type grown under salt stress condition (115 mM NaCl), SPAD value was determined. The results show that the SPAD values at day 0 and day 2 were not different between wild type and transgenic rice lines overexpressing *OsCaM1-1* both in the youngest fully expanded leaf and the second youngest leaf (Figure 22 and Figure 23). Salt stress decreased the SPAD value of all rice lines in second youngest leaf after salt stress treatment at day 4 and day 6 (Figure 24 and Figure 25). At day 6 under salt stress treatment, SPAD values in the second youngest leaf of both wild type and the transgenic rice lines appeared to decrease significantly. Corresponding to its appearance, the wild type had noticeably lower SPAD values than the three transgenic rice overexpressing *OsCam1-1*.

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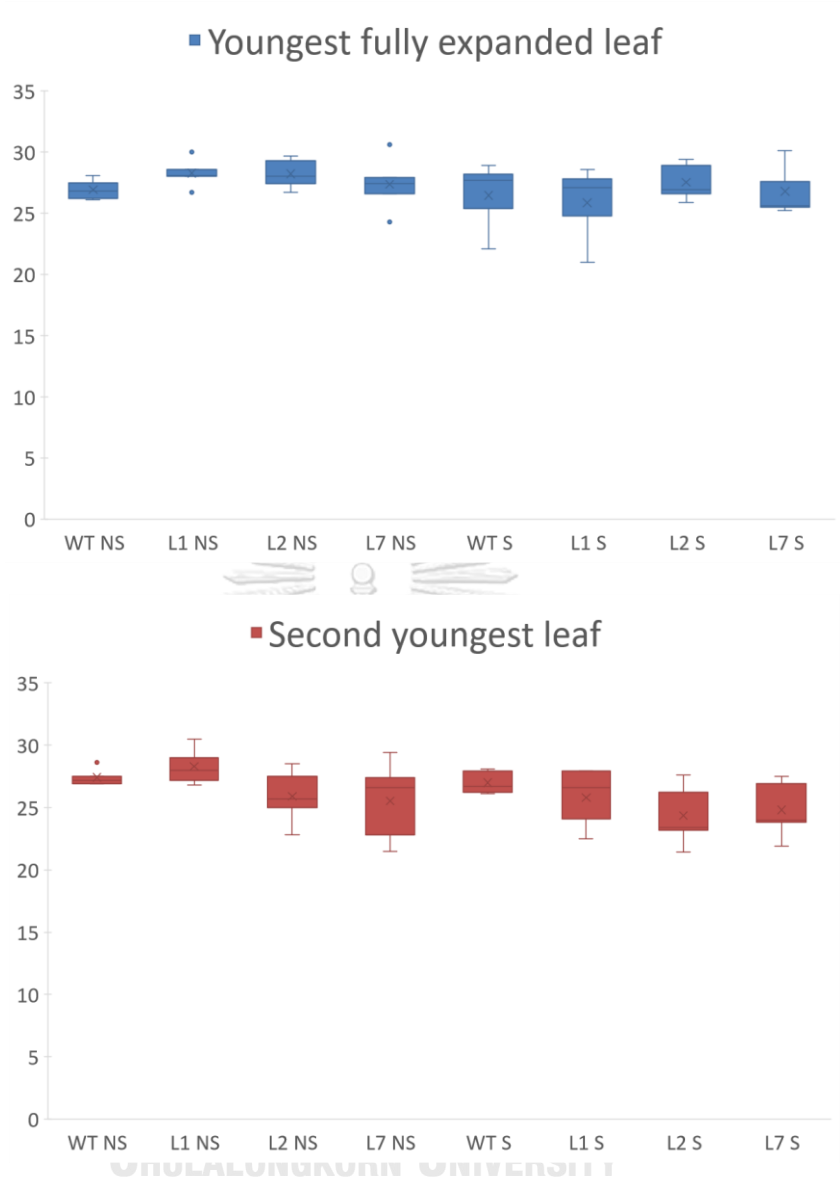


**Figure 22** SPAD value of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 0 under non-stress (NS) and salt stress (115 mM NaCl) (S) treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$).

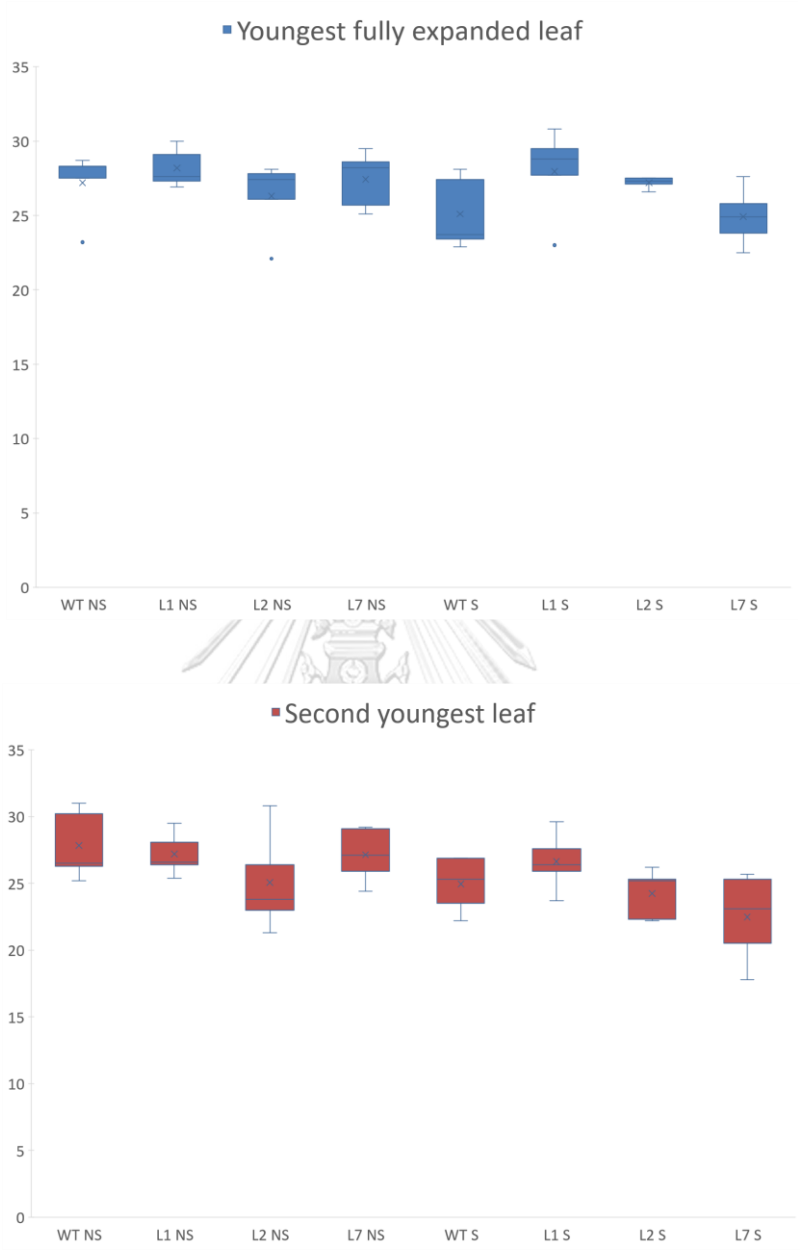


**Figure 23** SPAD value of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 2 under non-stress (NS) and salt stress (115 mM NaCl) (S) treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$).

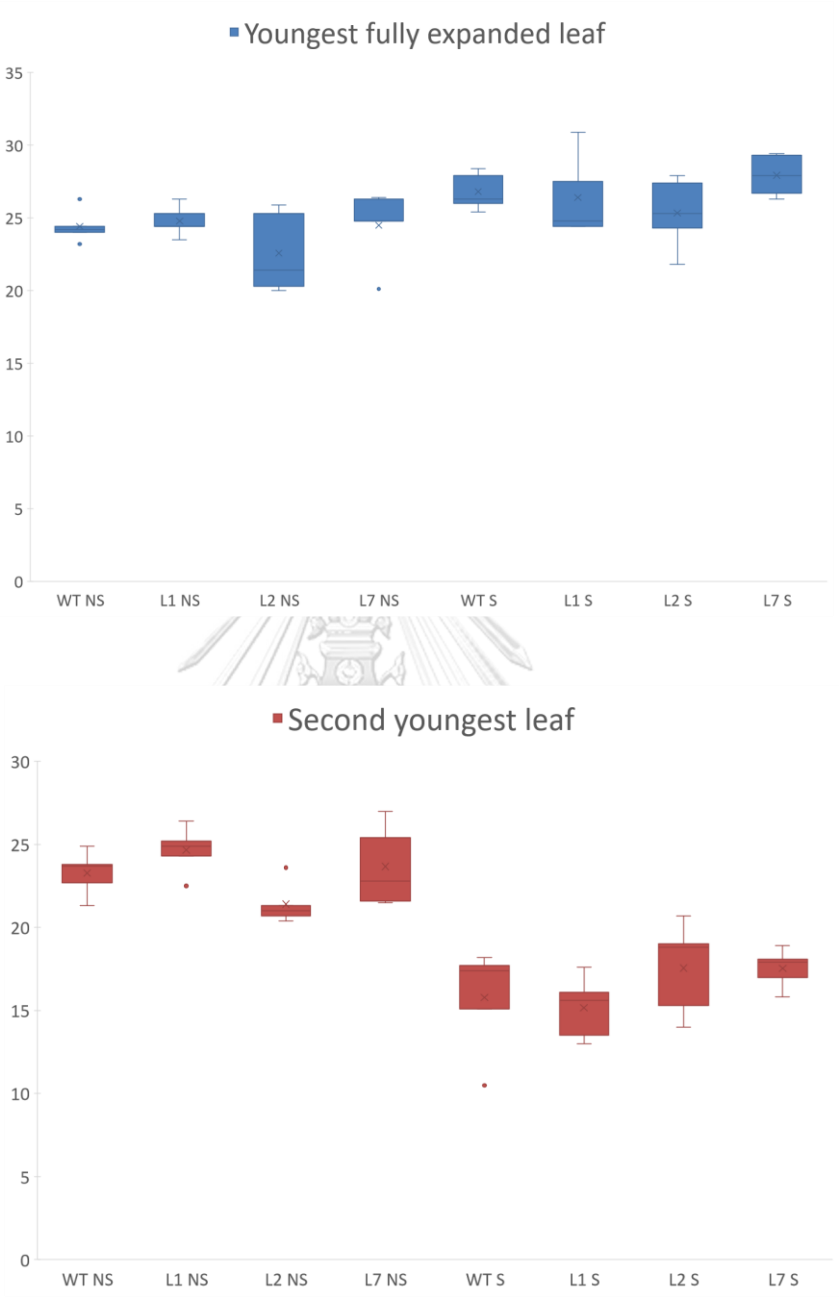


**Figure 24** SPAD value of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 4 under non-stress (NS) and salt stress (115 mM NaCl) (S) treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$).

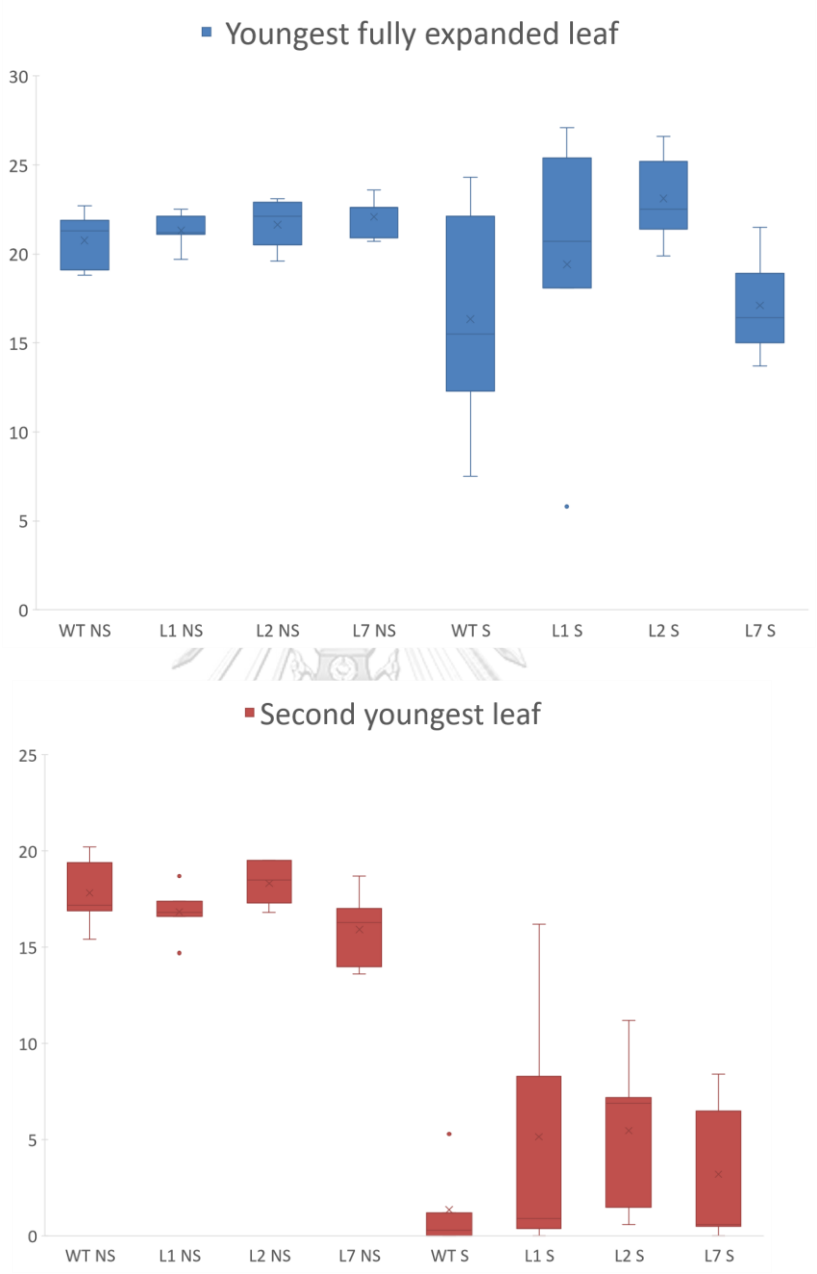


**Figure 25** SPAD value of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compare with wild type (WT) at days 6 under 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$).

### 3.3 Enzymes activity of glyoxylate cycle

The glyoxylate cycle is a variation of the tricarboxylic acid (TCA) cycle that microorganisms and plants use to convert acetyl-CoA to succinate. The glyoxylate cycle has two key enzymes: isocitrate lyase and malate synthase. To study the role of glyoxylate cycle in the KDML105 rice overexpressing *OsCam1-1* under salt stress, isocitrate lyase and malate synthase activities were measured. The results of the experiment were shown as follows.

#### 3.3.1 Isocitrate lyase activity

Isocitrate lyase is a one of key enzyme in glyoxylate cycle which cleavage of isocitrate to succinate and glyoxylate. To explore the role of glyoxylate cycle in salt tolerance, the isocitrate lyase activity was measured in the youngest fully expanded and the second youngest seedling leaves of the wild type rice and the three transgenic rice lines overexpressing *OsCam1-1* (L1, L2 and L7) 0, 2, 4 and 6 days after growing under normal and salt stress (115 mM NaCl) conditions. The result reveal that the youngest fully expanded leaf of the three transgenic lines had significantly higher isocitrate lyase activity than wild type after salt stress treatment at day 2 and day 4. After that, the isocitrate lyase exhibited no significant differences at day 6 between stress and non-stress condition among all plant examined (Figure 26A). The isocitrate lyase activity in second youngest leaf showed no significant differences at day 0, day 2 and day 4 between stress and non-stress condition among all plant examined. In contrast, the second youngest leaf at day 6 had higher isocitrate lyase activity after salt stress treatment both in the three transgenic rice lines overexpressing *OsCam1-1* and wild type. Moreover, the three transgenic rice lines had higher isocitrate lyase activity than wild type both under non-stress and salt stress treatment (Figure 26B).

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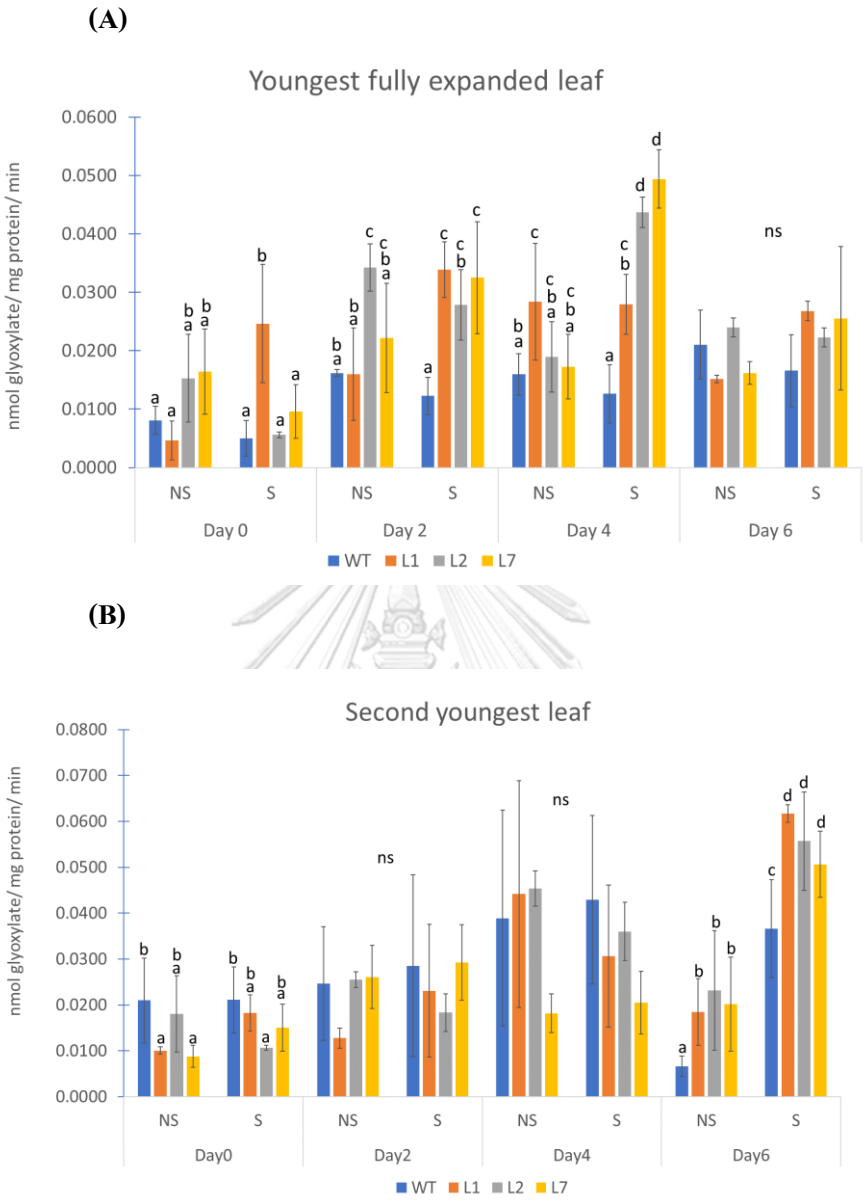


**Figure 26** Isocitrate lyase activity in the youngest fully expanded leaf (A) and the second youngest leaf (B) of the three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) comparing with the wild type (WT) at days 0, 2, 4, and 6 after exposure to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

### 3.3.2 Malate synthase activity

Malate synthase is another key enzyme in glyoxylate cycle, which catalyzes the conversion of glyoxylate to malate. To understand the role of glyoxylate cycle in salt tolerance, the malate synthase activity was measured in the youngest fully expanded and the second youngest seedling leaves of the KDML105 wild type rice and the three transgenic rice lines overexpressing *OsCam1-1* (L1, L2 and L7) 0, 2, 4 and 6 days after growing under normal and salt stress (115 mM NaCl) conditions. Figure 27A indicated that the youngest fully expanded leaf of the three transgenic lines had significantly higher malate synthase activity than the wild type after salt stress treatment time of 2 days. After that the malate synthase activity had significantly dropped after salt stress treatment at day 6 in both of wild type and the three transgenic lines. On a similar trend, the malate synthase activity in second youngest leaf at day 2 of the transgenic lines exhibited higher malate synthase activity than wild type either under normal or salt stress condition. At day 4 and day 6, no clear significant differences was observed between the transgenic lines and wild type (Figured 27B).

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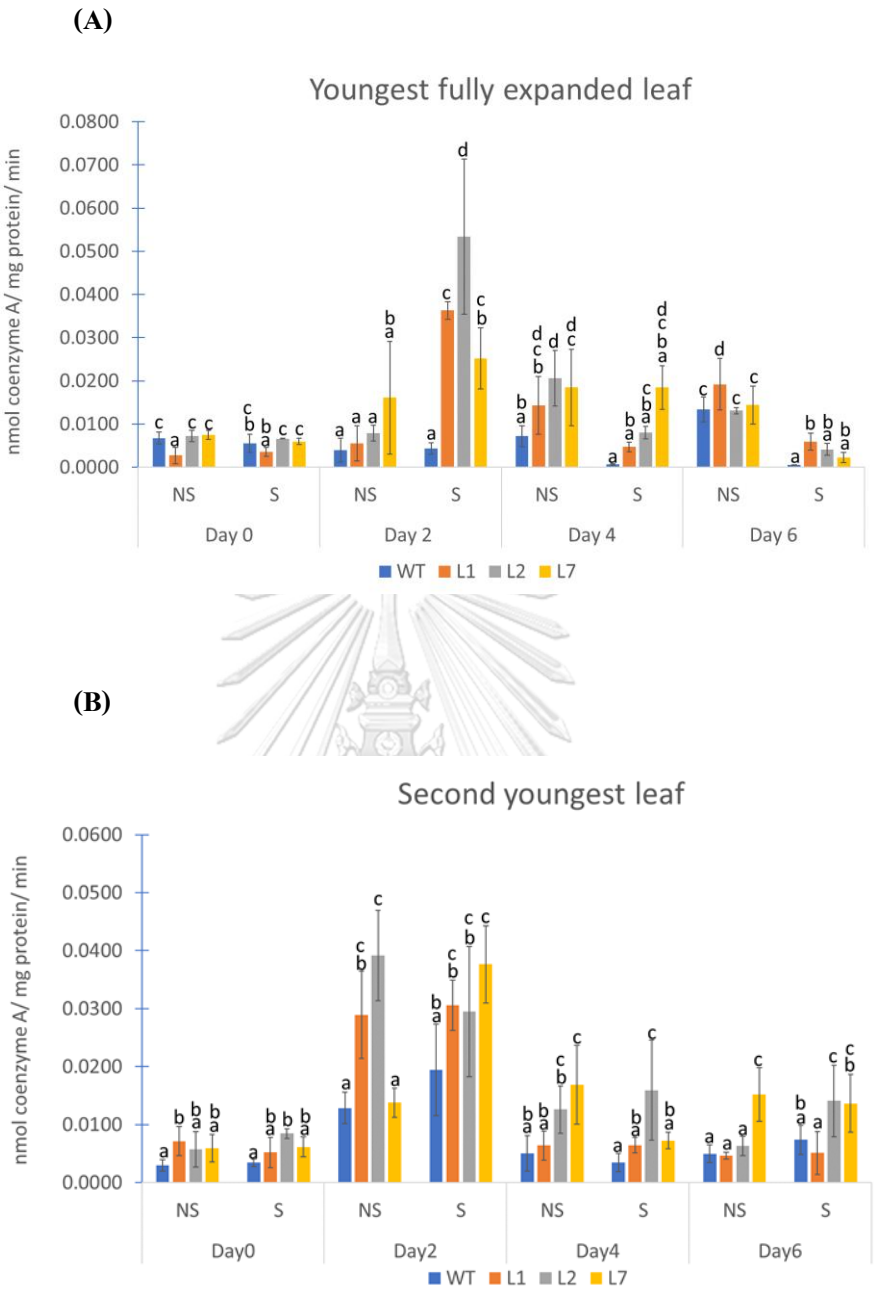


**Figure 27** Malate synthase activity in the youngest fully expanded leaf (A) and the second youngest leaf (B) of the three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) comparing with the wild type (WT) at days 0, 2, 4, and 6 after exposure to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

### 3.4 Sugars and starch contents

To examine the effect of *OsCam1-1* overexpression on the accumulation of sugars and starch, which are the end products of photosynthesis under salt stress, contents of sucrose, glucose, fructose and starch extracted from leaves of wild type and the three transgenic rice overexpressing *OsCaM1-1* gene (L1, L2, L7) under normal and salt stress conditions (115 mM NaCl) were measured.

#### 3.4.1 Sugars content

The result showed that sucrose contents in both of the youngest fully expanded leaf and second youngest leaf decreased upon salt stress. The decreases are especially pronounced after 4 and 6 days of treatment (Figure 28), which is also true for the glucose content in the second youngest leaf (Figure 29B). Compared with wild type, the transgenic lines appeared to have lower sucrose and glucose contents under salt stress treatment in the second youngest leaf at day 2 (Figure 28B and Figure 29B). For glucose, the only induction under salt stress found was in the second youngest leaf of the wild type at day 2, which led to the lower levels of glucose in the transgenic rice lines, in another word, the glucose level was better maintained under salt stress in the transgenic lines. For fructose, salt stress increased its content in the youngest fully expanded leaf had increased in the wild type and some transgenic lines under salt stress in day 2, and in the second youngest leaf of wild type after salt stress treatment at day 2, day 4, and day 6. While those in the transgenic rice lines did not increased, which led to the statistically significantly lower levels than the wild type, especially at day 6, in which about 2-fold lower fructose contents were found (Figure 30).

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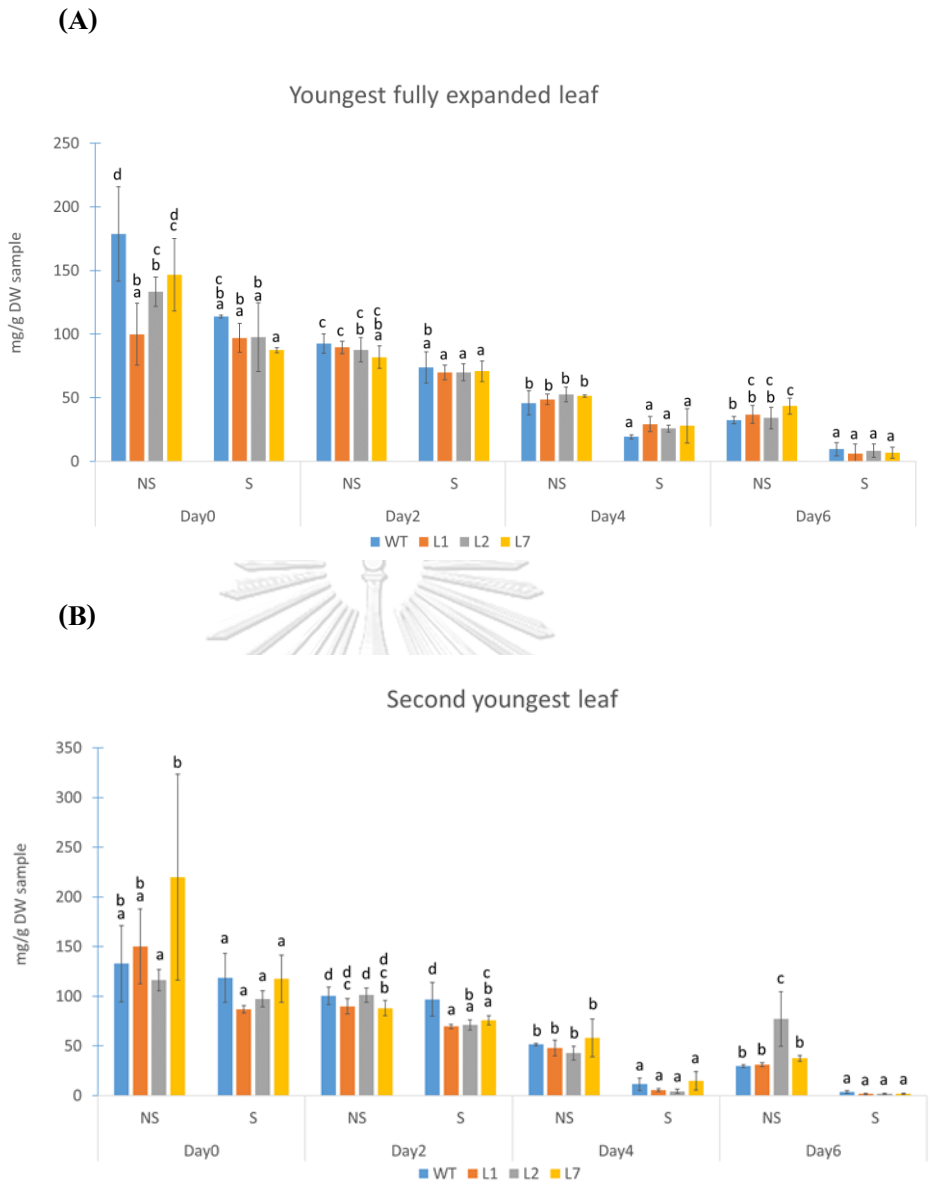



**Figure 28** Sucrose content in the youngest fully expanded leaf (A) and second youngest leaf (B) of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 0, 2, 4, and 6 after exposed to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

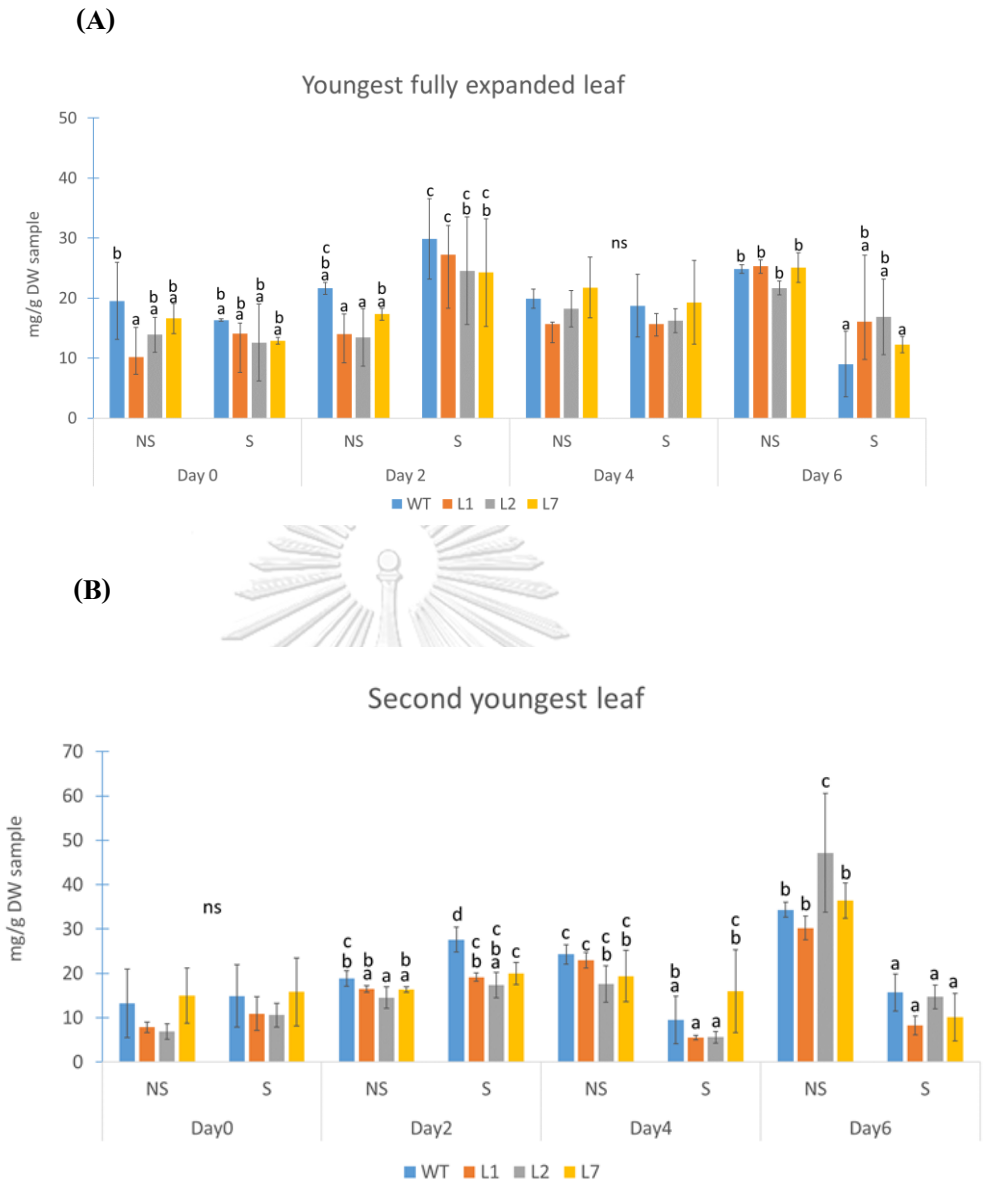
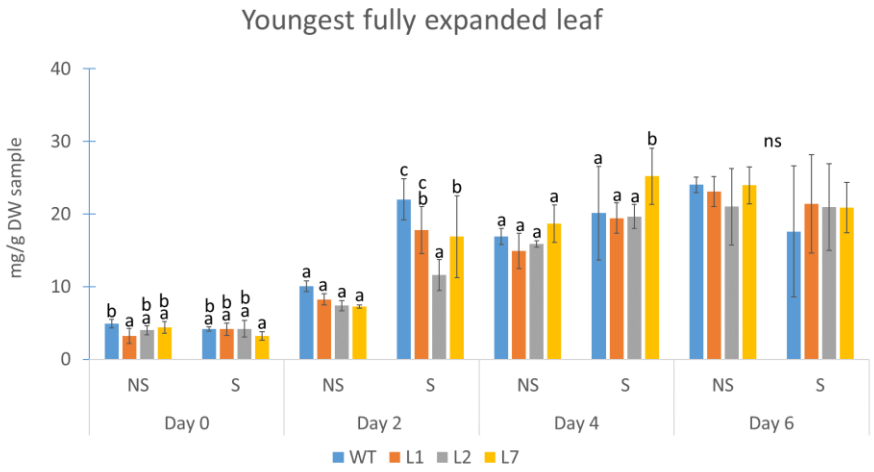



**Figure 29** Glucose content in the youngest fully expanded leaf (A) and second youngest leaf (B) of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 0, 2, 4, and 6 after exposed to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

**(A)**

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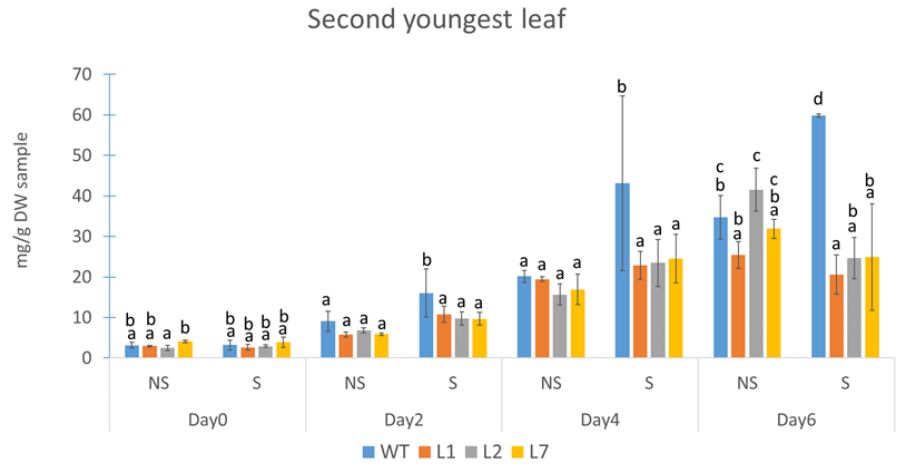


**Figure 30** Fructose content in the youngest fully expanded leaf (A) and second youngest leaf (B) of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 0, 2, 4, and 6 after exposed to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

### 3.4.2 Starch content

The starch content had decreased in both of the youngest fully expanded leaf and the second youngest leaf after salt stress treatment at days 2, 4, and 6. Furthermore, starch content of the wild type significantly decreased while those in some transgenic line were slightly better maintained after salt stress treatment in the youngest fully expanded leaf at day 6. On the contrary, the second youngest leaf had lower starch content than wild type after 6 days of salt tress treatment (Figure 31)



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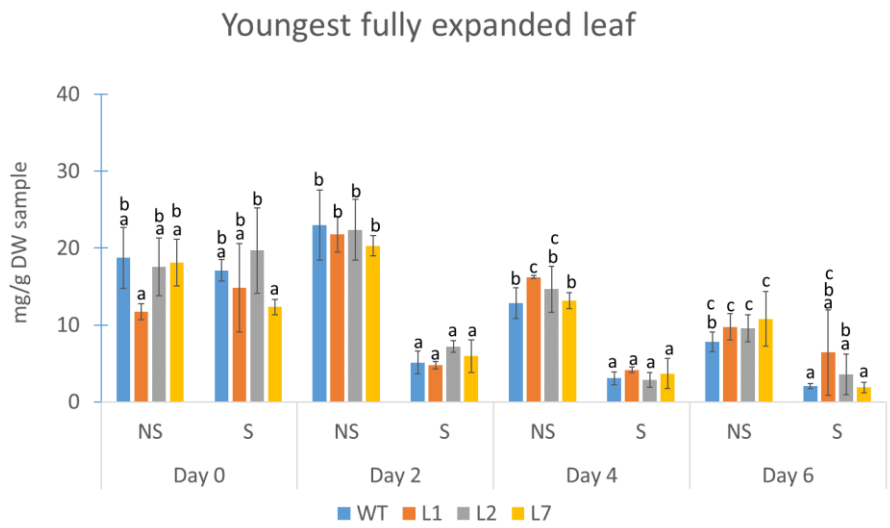


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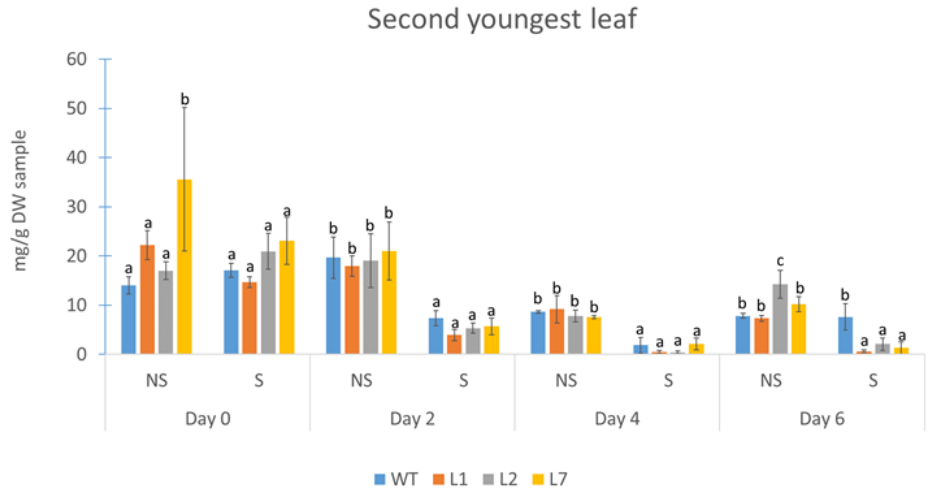


**Figure 31** Starch content in the youngest fully expanded leaf (A) and the second youngest leaf (B) of three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) compared with wild type (WT) at days 0, 2, 4, and 6 after exposed to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

### 3.5 Unsaturated fatty acid content

To examine the effect of *OsCam1-1* overexpression on unsaturated fatty acid content in rice leaf under salt stress condition, unsaturated fatty acid content of the youngest fully expanded and the second youngest seedling leaves of the KDML105 wild type rice and the three transgenic rice lines overexpressing *OsCam1-1* (L1, L2 and L7) was measured 0, 2, 4 and 6 days after growing under normal and salt stress (115 mM NaCl) conditions. Figure 32A showed that the youngest fully expanded leaf of the three transgenic line had significantly lower unsaturated fatty acid content than the wild type after salt stress treatment time of 2 days. The unsaturated fatty acid content exhibited no significant differences at day 4 and day 6 between stress and non-stress conditions among all plant examined. Figure 32B presented the unsaturated fatty acid content in the second youngest leaf of the three transgenic line compared with the wild type. The result revealed that the unsaturated fatty acid contents of the transgenic lines had a tendency to be lower than the wild type in day 4 and day 6 under normal condition while under salt stress condition line 2 in day 2 and line 1 in day 6 exhibited significantly lower unsaturated fatty acid content than the wild type.

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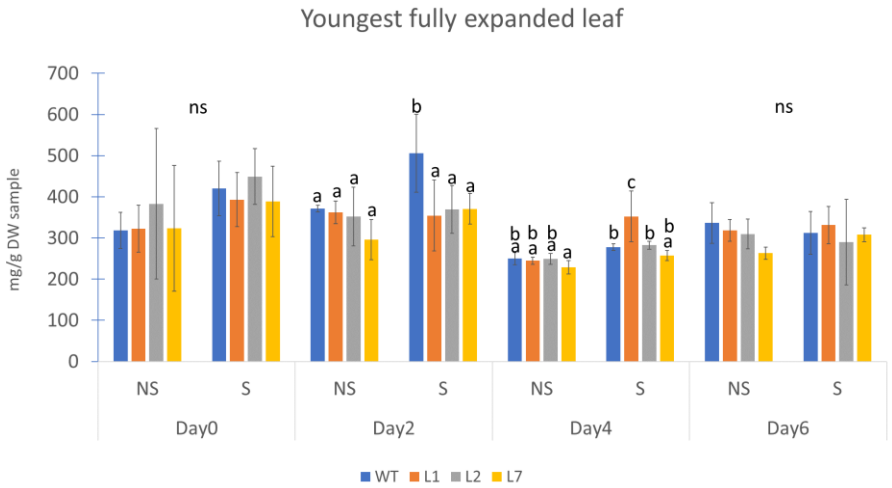


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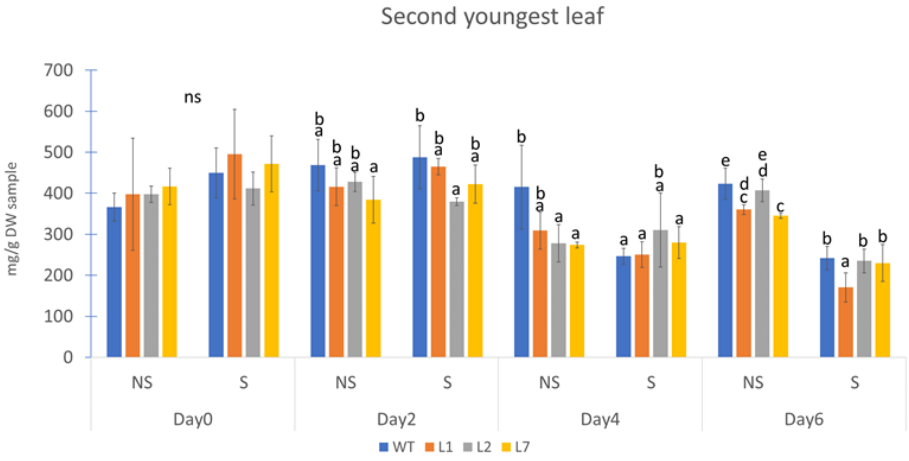


**Figure 32** Unsaturated fatty acid content in the youngest fully expanded leaf (A) and the second youngest leaf (B) of the three lines of transgenic rice overexpressing *OsCam1-1* (L1, L2, L7) comparing with the wild type (WT) at days 0, 2, 4, and 6 after exposure to 115 mM NaCl salt stress treatment from five independent biological replicates using Randomized Complete Block Design (RCBD) with Duncan multiple comparison test ($p<0.05$), the error bars represent SD and different letters indicate statistically significant difference between rice lines under stress and non-stress conditions.

# CHAPTER IV

# DISCUSSIONS

In our previous report, transcriptome profiling of the transgenic 'Khao Dawk Mali 105' rice overexpressing *OsCam1–1* and wild type rice showed 1,434 salt responsive genes under salt stress. The overexpression of *OsCam1–1* extensively affected the expression of genes implicated in several major cellular processes including signaling and stress responses, hormone-mediate regulation, secondary metabolism, transcription, lipid metabolism, glycolysis, TCA cycle, glyoxylate cycle, photosynthesis, and carbohydrate metabolism [130]. Here, the phenotypes of the transgenic rice overexpressing *OsCam1–1* grown under salt stress have been investigated compared to wild type. The result revealed that the transgenic rice overexpressing *OsCam1–1* had higher shoot fresh weight and dry weight than wild type after salt stress at day 4 and day 6 (Figure 16 and 17). The results correspond to previous studies including the transgenic rice overexpressing *OsCam1–1* grown in hydroponic mWP nutrient solution treatment [129], the transgenic rice overexpressing *OsMSR2*, which is a calmodulin like gene [146] , the transgenic tomato overexpressing *ShCML44* [147] and the transgenic Arabidopsis overexpressing calmodulin-like gene, *CmCML13* from melon [148], which better maintained shoot fresh weight and dry weight when compared to wild type after salt stress treatment. The root fresh weight was decreased after salt stress treatment but, in root dry weight, not as much decrease was observed. In addition, no significant difference between the transgenic rice overexpressing *OsCam1–1* and wild type was found both normal and salt stress condition (Figure 16 and 17). This may be because the dry weight of roots is much less than the dry weight of shoot and in general, roots have higher salt tolerance than shoots [149] so, it is difficult to compare between transgenic rice and wild type moreover, some research focuses on shoot weight because rice seedling shoot is further developed to vegetative stage and reproductive stage, which is important for rice yield [81]. In this study, clear difference in shoot fresh weight and dry weight between wild type and all three transgenic rice lines overexpressing *OsCam1-1* grown under salt stress suggests that *OsCaM1-1* plays an important role in salt stress tolerance.

The SPAD value is a chlorophyll meter to estimate the green index of rice leaves. The result reported that SPAD value of the wild type had decreased to a greater extent when compared

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to the transgenic rice overexpressing *OsCam1–1* at day 6 after salt stress treatment (Figure 25) because, SPAD value was significantly related with concentration of nitrogen, which is an important nutrient for plant growth, which may improve leaf dry weight [150] [151] [152] [153]. Therefore, the transgenic rice may be able to better absorb nitrogen and/or maintain its concentration than wild type. From the overall phenotype, the results suggest that the *OsCam1–1* overexpression could improve transgenic rice salt tolerance ability observed from the maintenance of the shoot fresh weight and shoot dry weight under salt stress possibly by protecting the plant from nutrient deficiency and death.

Our previously reported transcriptome analysis showed that expression of genes in the glyoxylate cycle including isocitrate lyase gene (*OsICL*) and malate synthase (*OsMS*) were highly induced by salt stress and its expression was increased by the associated effect of salt stress and overexpression of a rice calmodulin gene (*OsCam1-1*) [130]. Therefore, isocitrate lyase and malate synthase activities were examined to confirm the relationship between the possible role of glyoxylate cycle and the function of *OsCam1-1* gene under salt stress. The result of isocitrate lyase activity showed that the youngest fully expanded leaf of transgenic rice had higher isocitrate lyase activity than wild type after salt stress at day 2 and day 4 (Figure 26A) while, the second youngest leaf had higher isocitrate lyase activity than wild type both under stress and non-stress conditions at day 6 (Figure 26B). For malate synthase, the youngest fully expanded leaf of the transgenic rice had higher activity than wild type at day 2 under salt stress (Figure27A). Similarly, second youngest leaf has tendency to have higher malate synthase than wild type after salt stress treatment (Figure 27B). The result of glyoxylate cycle enzyme activity are consistent with transcriptome profiling in KDML 105 rice overexpressing *OsCam1-1* and wild type [130]. When focused on *OsICL* gene expression, the expression levels of *OsICL* in young leaf of transgenic rice increased after salt stress treatment, but in wild type, it was not affected by salt stress while the expression levels of *OsICL* in senesced leaf both of wild type and transgenic rice increased after salt stress treatment [131]. Expression levels in this previous report correlated with the isocitrate lyase activity of the youngest fully expanded leaf at day 2 and day 4 and of the second youngest leaf in day 6. From these previous results and these finding suggested that *OsICL* was induced by salt stress and related with the overexpression *OsCam1-1* gene. In the presence of *OsCaM1-1*, the youngest fully expanded leaf of the transgenic rice appeared to be

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more responsive to salinity than the wild type, causing a more rapid change in isocitrate lyase activity at 2-4 days after salt stress treatment after that, it may be difficult to see the change at day 6 because leaf was further developed to older leaf. In contrast, the second youngest leaf had not change in early days until day 4 after salt stress treatment after that, isocitrate lyase was changed both under normal and salt stress condition at day 6, which may be because it was less responsive to salinity than the youngest fully expanded leaf. The isocitrate lyase was highly increased under salt stress both in transgenic rice and wild type, which corresponds to *OsICL* gene expression level. In our previous result, malate synthase gene was highly induced by salt stress and expressed at higher levels in the transgenic rice [130]. Here, malate synthase activity was found increased in a short period of time in the second day after exposure to salinity stress after that malate synthase activity did not changed both in the youngest fully expanded leaf and the second youngest leaf These results correlated with the study on the *icl* mutant in *Arabidopsis* seedings, which reported that isocitrate lyase has an important function at other stages of the life cycle [128].

The role of isocitrate lyase and malate synthase in plant glyoxylate cycle involves the bypass of the TCA cycle and the conversion of lipid to sugar via a gluconeogenesis process; therefore, sucrose, glucose, and, fructose contents were determined. Overall, sucrose content appeared to decrease over time and after salt stress treatment in both the youngest fully expanded leaf and the second youngest leaf (Figure 28). In contrast, the glucose content appeared to increase in the second youngest leaf, however, similar to the sucrose content, upon salt stress its content was decreased especially in the second youngest leaf. Importantly, the second youngest leaf of the transgenic rice had lower sucrose and glucose contents than wild type at day 2 after salt stress treatment (Figure 28B and 29B). For fructose, its content in the wild type appeared to increase over time and increased upon salt stress in the youngest fully expanded leaf at day 2 and especially in the second youngest leaf from day 2 to day 6. However, this induction in the second youngest leaf was absent in all three transgenic rice lines after salt stress treatment (Figure 30). These results correspond to the previous studies reporting that the glucose and fructose contents of the *Aticl* mutant, which was the most salt sensitive line were highest among the five Arabidopsis wild type and *OsICL*-expressing rice lines [131]. In both studies, the results suggest that *OsICL* is regulated by *OsCam1-1* as its overexpression, which up-regulated *OsICL* expression probably results in the transgenic rice being able to utilize glucose and fructose, which

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were monosaccharides used as carbon sources for plant growth better than wild type. In part of sucrose content, normally starch is converted to sucrose to be transported to plant cells in the sink. It is possible that when the plant leaves were developed to mature leaf at day 4 and day 6, sugars were mobilized to produce younger leaf thus the lower sucrose levels observed, moreover, when the plant was exposed to the stress of salinity, the plant was more stimulated to transport the carbon source to the younger leaves for survival. Overall, the sugar levels upon salt stress in the transgenic rice overexpressing *OsCam1-1* had decreased at a higher degree than wild type since day 2 and the lower contents continued to be observed particularly for fructose in the second youngest leaf onto day 4 and day 6 of treatment.

In previous report, the granule-bound starch synthase (GSSB) was inhibited by salt stress and as a result starch content was decreased in rice leaf and another reported that KDML105 rice, which was a salt sensitive cultivar had lower starch content than Pokkali, which was a salt tolerant cultivar [45, 154]. In this study, starch contents in both of the youngest fully expanded leaf and the second youngest leaf decreased after salt stress treatment at day 2, day 4 and, and day 6 but, the youngest fully expanded leaf of the transgenic rice had only a slightly better trend in maintaining the starch content than wild type (Figure 31). this result is consistent with the previous report in a hydroponic experiment revealing that the transgenic overexpressing *OsCam1-1* of KDML105 rice exhibited significant decrease in starch levels, but to a lesser extent than the wild type [130]. In contrast with previous study, the second youngest leaf of transgenic rice had lower starch content than wild type after salt stress treatment at day 6 (Figure 31B). This report suggest that when plants were exposed to salinity stress, the ability to accumulate carbon sources in the form of starch was reduced. Especially at day 6, the second youngest leaf possibly start to senesce so, plant may have adapted by converting starch into sucrose and mobilizing this carbon source to younger parts for use under salt stress condition. This result suggested the transgenic rice may progress further into senescence, accelerating the process of carbon source mobilization from older leaves earlier than the wild type.

In previous report of Arabidopsis during germination, isocitrate lyase was found to play an important role in lipid conversion to sugar by using the acetyl unit from acetyl CoA, which is a product of β-oxidation, via the glyoxylate cycle and gluconeogenesis [127, 128]. In another previous study, the activity of *OsCam1–1* might affect energy metabolism, which might possibly

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be linked to lipid metabolism during salt stress [130]. Moreover Aziz and co-worker reported that polyunsaturated fatty acids, particularly linolenic acid, were the major fatty acids found during the seeding stage to vegetative stage in rice under saline soil [155]. So, unsaturated fatty acid contents were determined. This study showed that the youngest fully expanded leaf of transgenic rice had lower unsaturated fatty acid content than wild type at day 2 under salt stress (Figure 32A) while the second youngest leaf of transgenic rice had trended to exhibit lower unsaturated fatty acid content than wild type in normal condition. Furthermore, some of transgenic rice had decreased unsaturated fatty acid content when compared to wild type under salt stress (Figure 32B). These results suggested that when the youngest fully expanded leaf was stressed with salinity and it may be more responsive to salinity than the second youngest leaf so, the youngest fully expanded leaf quickly responded at day 2 after salt stress treatment. The result suggested that transgenic rice was able to breakdown lipid for use to produce sugar in gluconeogenesis process better than wild type. On the other hand, the second youngest leaf of transgenic rice had better trend to breakdown lipid than wild type under normal condition because, the second youngest leaf maybe entered to senesced leaf at day 4 and day 6 so, it had mechanism to breakdown lipid for creating sugar and mobilized to younger leaf or plant cell. From this result, it can be concluded that salinity stress affected lipid breakdown in the youngest fully expanded leaf of the transgenic rice more than the second youngest leaf.

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# CHAPTER V

# CONCLUSIONS

From this study, the glyoxylate cycle was probably influenced by the *OsCam1-1* gene function as its overexpression in the transgenic rice exhibited higher glyoxylate cycle enzyme activity than wild type, which led to improved salt tolerance. The enhanced glyoxylate cycle enzyme activities may result in the earlier conversion of fatty acids to sugars and mobilization of these carbon sources. As a result, the transgenic rice may better use and mobilize its carbon sources in order to better adapt and survive in the earlier days after salt stress treatment than wild type. Taken together, this study shows that the glyoxylate cycle, which in this study was enhanced by the overexpression *OsCam1-1* gene, probably helps maintain and balance carbon source and energy metabolism in rice under salt stress condition.

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# APPENDIX

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# APPENDIX A

# CHEMICAL SOULTION PREPARATION

**1. Yoshida's stock solutions**

| Nutrient element | Reagent (AR grade) | Preparation (g/10 liters of distilled water) | |
|---|---|---|---|
| N | $NH_4NO_3$ | 914 | |
| P | $NaH_2PO_4.2H_2O$ | 403 | |
| K | $K_2SO_4$ | 714 | |
| Ca | $CaCl_2$ | 886 | |
| Mg | $MgSO_4.7H_2O$ | 3240 | |
| Mn | $MNCl_2.4H_2O$ | 15.0 | |
| Mo | $(NH_4)_6Mo_7O_{24}.4H_2O$ | 0.74 | separately dissolve and |
| B | $H_3BO_3$ | 9.34 | mix with 500 ml of |
| Zn | $ZnSO_4.7H_2O$ | 0.35 | concentrated $H_2SO_4$ |
| Cu | $CuSO_4.7H_2O$ | 0.31 | after that make the |
| Fe | $FeCl_3.6H_2O$ | 77.0 | volume to 10 liters |
| | Citric acid (monohydrate) | 119 | with distilled water |

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

and adjust pH to 5.8 by KOH

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

| | | |
|---|---|---|
| $MgSO_4H_2O$ | 1820 g/10 L | for "Mg" solution |
| $NaH_2PO_4H_2O$ | 356 g/10 L | for "P" solution |
| $CaCl_2.2H_2O$ | 1174 g/10 L | for "Ca" solution |
| $KNO_3$ | 615 g/10 L | for "K" solution |
| $FeSO_4.4H_2O$ | 74.8 g/10 L | for "Fe" solution |
| $FeSO_4.7H_2O$ | 91.8 g/10 L | for "Fe" solution |

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**2. Extraction buffer for isocitrate lyase and malate synthase activities assay**

| | | |
|---|---|---|
| 1 M Tricine buffer pH 7.5 | 8.5 | ml |
| 1 M KCl | 500 | µl |
| 1 M $MgCl_2$ | 50 | µl |
| 500 mM EDTA | 100 | µl |
| 1 M DTT | 100 | µl |
| Sterile distilled water | up to 50 | ml |

**3. Reaction buffer for isocitrate lyase assay**

| | | |
|---|---|---|
| 1 M Tricine buffer pH 7.4 | 5.95 | µl |
| 1 M $MgCl_2$ | 175 | µl |
| 500 mM EDTA | 140 | µl |
| Phenylhydrazine | 21.1 | µl |
| Sterile distilled water | up to 35 | ml |
| 4 mM DL-isocitric acid (substrate) | 25 | µl |

**4. Reaction buffer for malate synthase assay**

| | | |
|---|---|---|
| 1 M HEPES buffer pH 7.8 | 1.125 | ml |
| 1 M $MgCl_2$ | 67.5 | µl |
| Acetyl CoA | 0.02 | g |
| DTNB | 0.0089 | g |
| Sterile distilled water | up to 11.25 | ml |
| 5 mM sodium glyoxylate (substrate) | 25 | ml |

**5. Determination of starch content**

- **50 mM acetate buffer**

  Prepare stock solution as following

  1) 0.2 M acetic acid 11.6 ml

  Dissolve in sterile distilled water and make up the volume to 1 L and store in room temperature.

  2) 0.2 sodium acetate 27.2 g

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Dissolve in sterile distilled water and make up the volume to 1 L and store in room temperature.

Prepare 50 mM acetate buffer as following

| | | |
|---|---|---|
| 1) 0.2 M acetic acid | 20 | ml |
| 2) 0.2 M sodium acetate | 30 | ml |

Adjust pH utilizing acetic acid solution and, make up the volume to 200 ml with sterile distilled water.

**6. Determination of lipid content**

- **Vanillin-phosphoric acid reagent**

Prepare 0.2 mg/ml vanillin

| | | |
|---|---|---|
| vanillin | 0.012 | g |

Prepare 17% phosphoric acid

| | | |
|---|---|---|
| 85% phosphoric acid | 12 | ml |

Dissolve in sterile distilled water and make up the volume to 60 ml and store in brown glass bottle.

**7. Determination of protein**

- **Bradford reagent**

| | | |
|---|---|---|
| Coomassie Brilliant Blue (G250) | 0.03 | g |
| 95% ethanol | 15 | ml |

The solution is stirred under dark condition until completely dissolved.

| | | |
|---|---|---|
| 85% phosphoric acid | 20 | ml |

Make up the volume to 300 ml with sterile distilled water and filter the solution using Whatman filter paper. The solution must store at 4 $^{\circ}$C in the brown glass bottle (available for several weeks).

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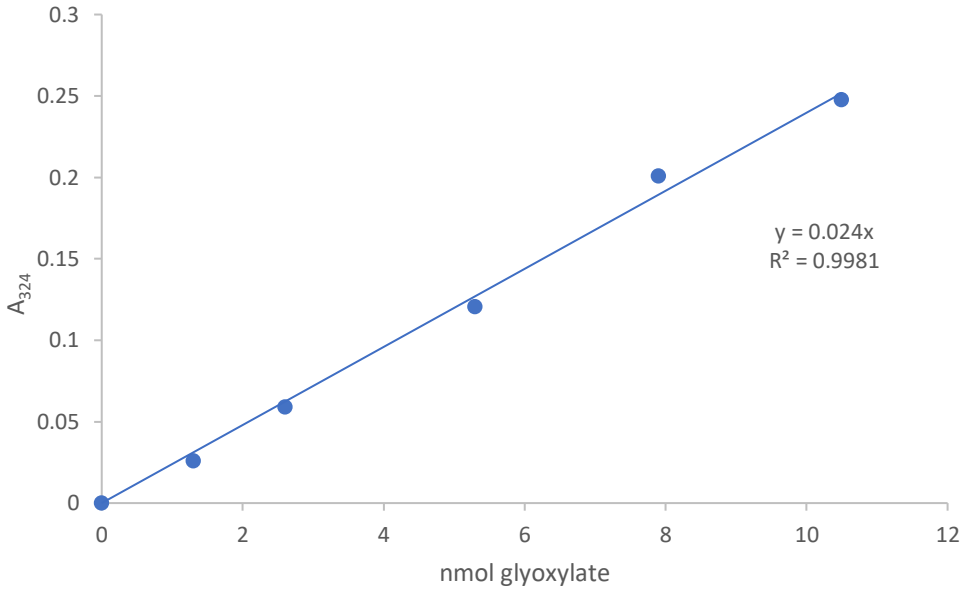
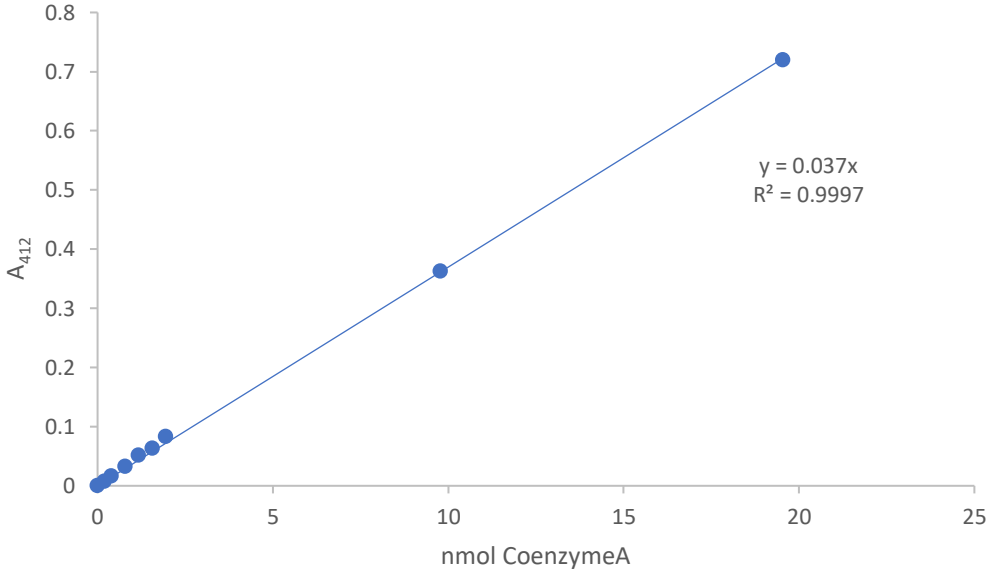
# APPENDIX B

## STANDARD CURVES

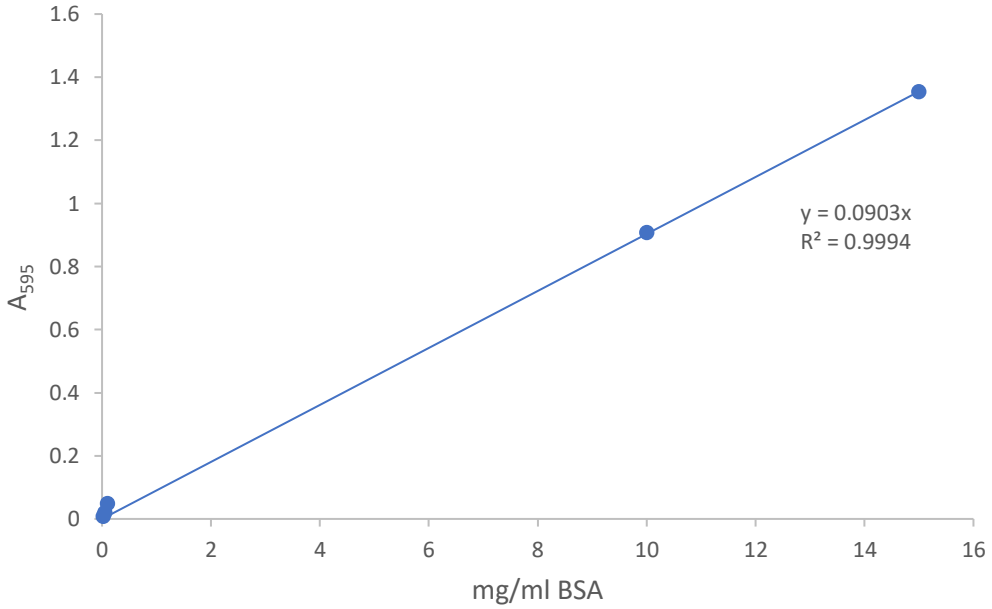
**1. Standard curve of glyoxylate content for isocitrate lyase activity assay**



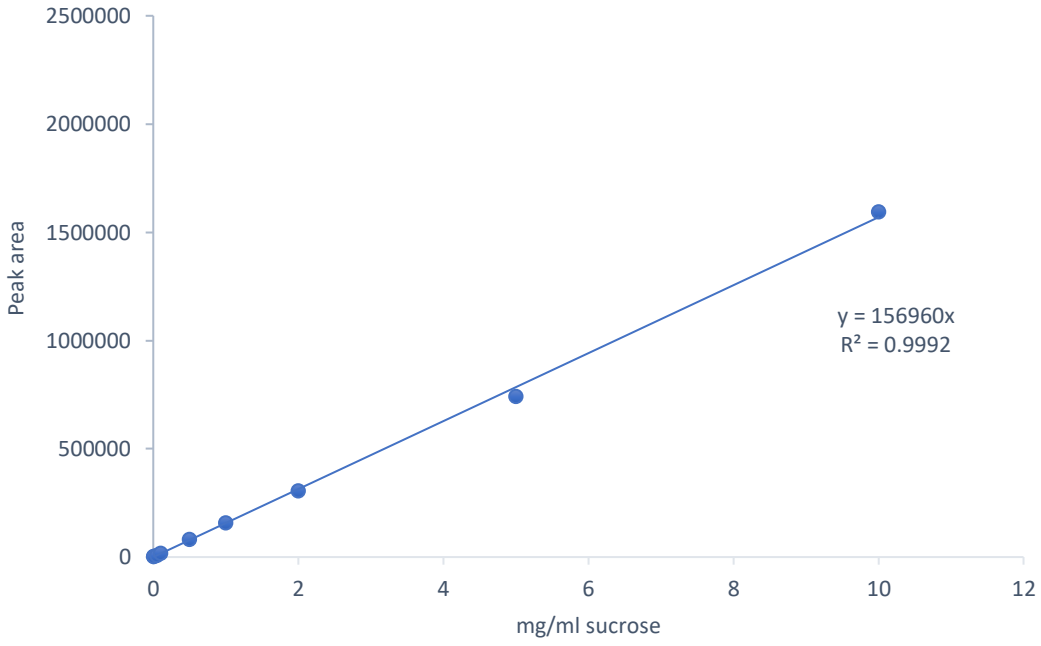

**2. Standard curve of glyoxylate content for malate synthase activity assay**



**3. Standard curve of Bradford assay**

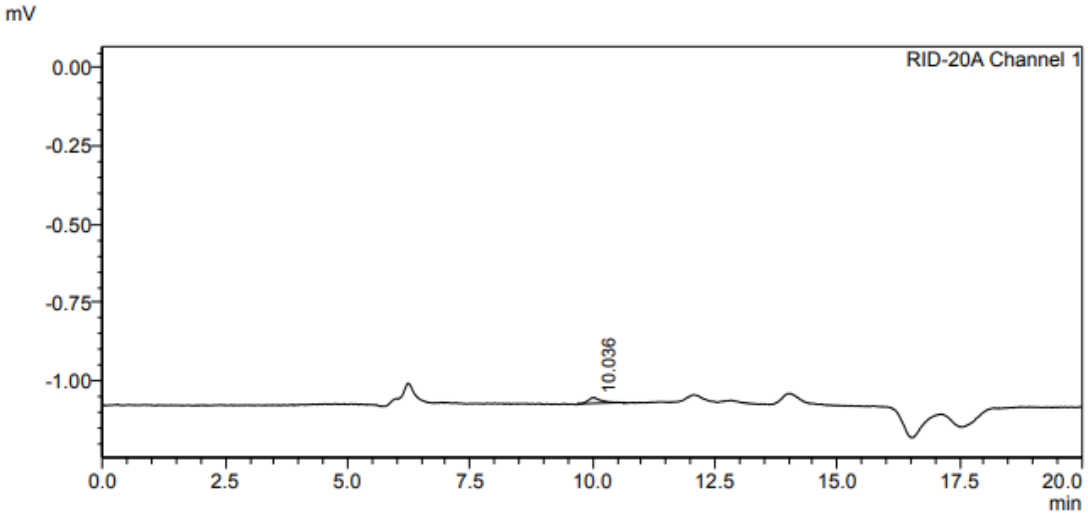



**4. Standard curve of sucrose content**

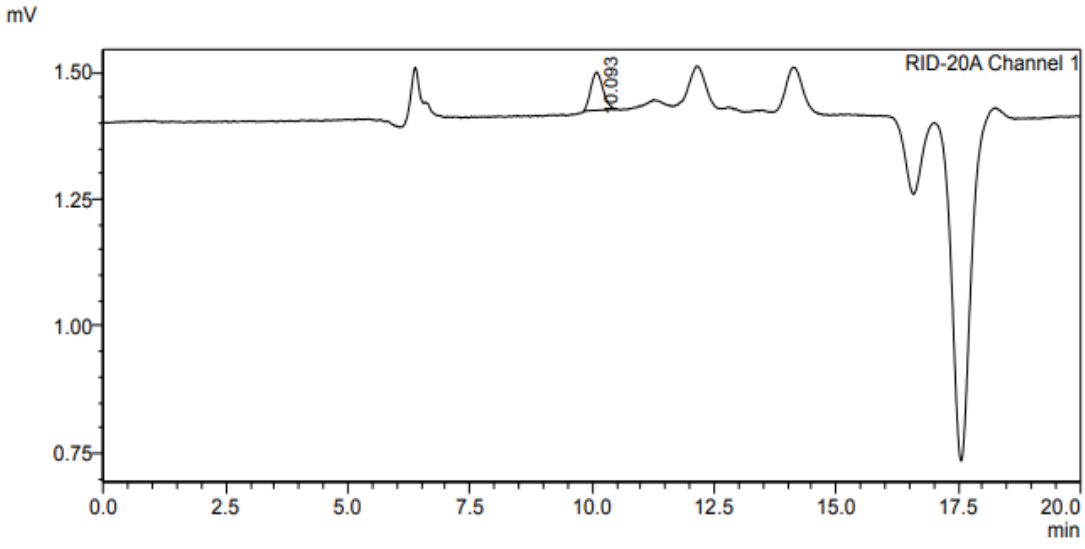


**5. Analysis report of sucrose content**

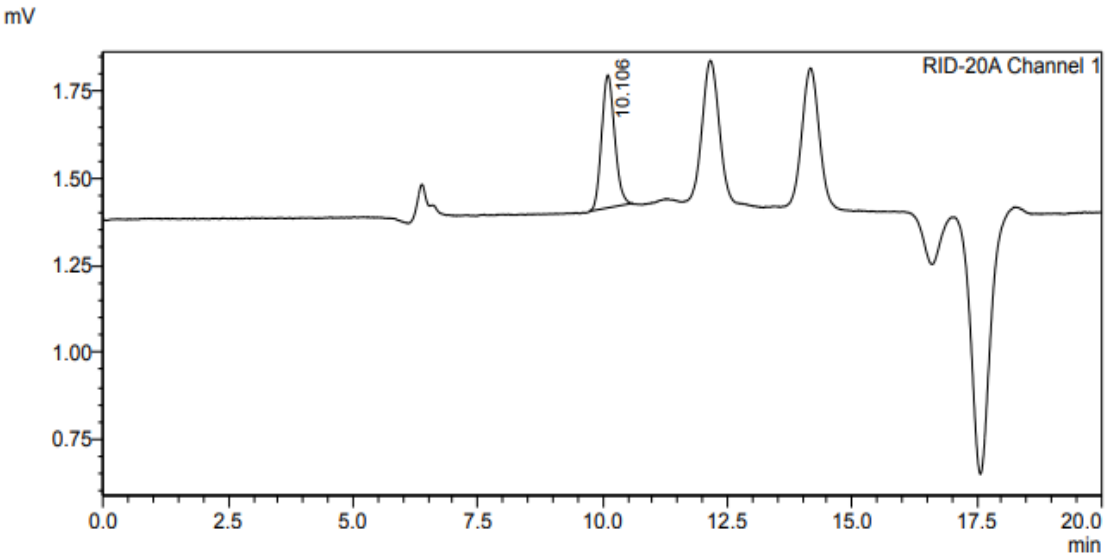
0.005 mg/ml



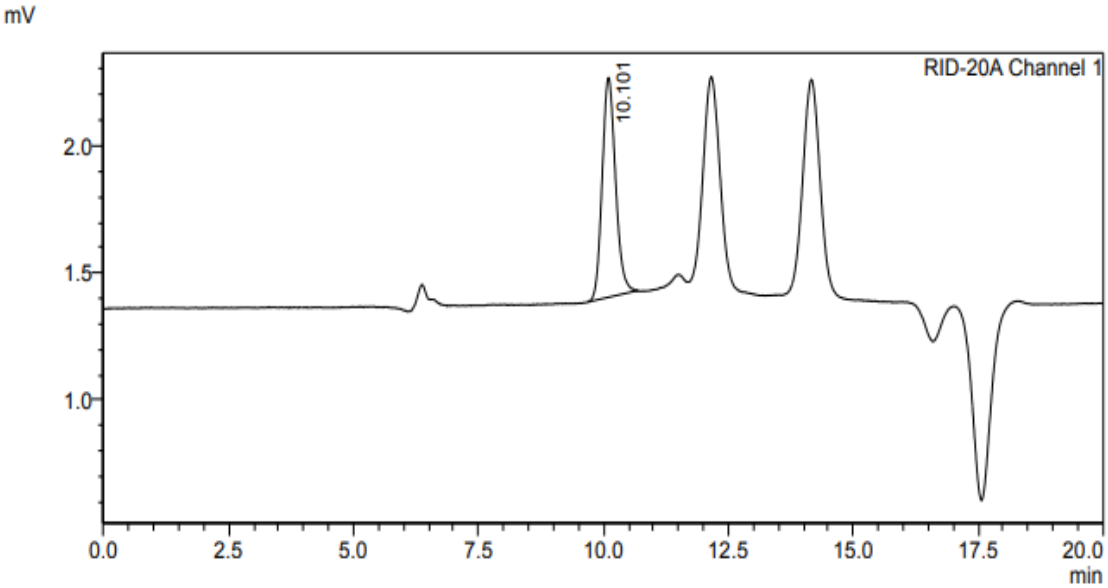

0.01 mg/ml



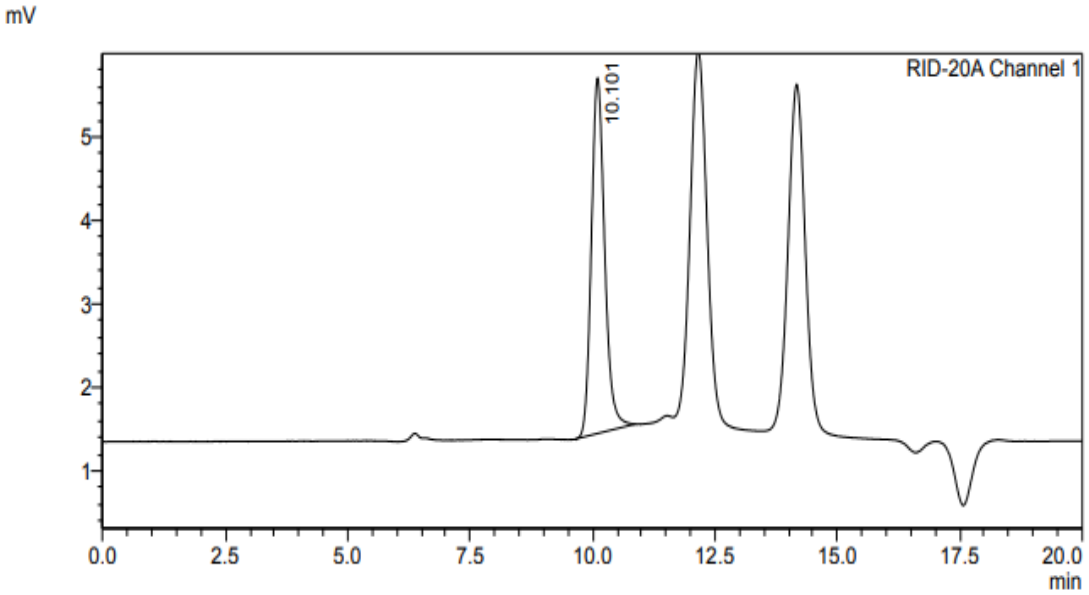
0.05 mg/ml



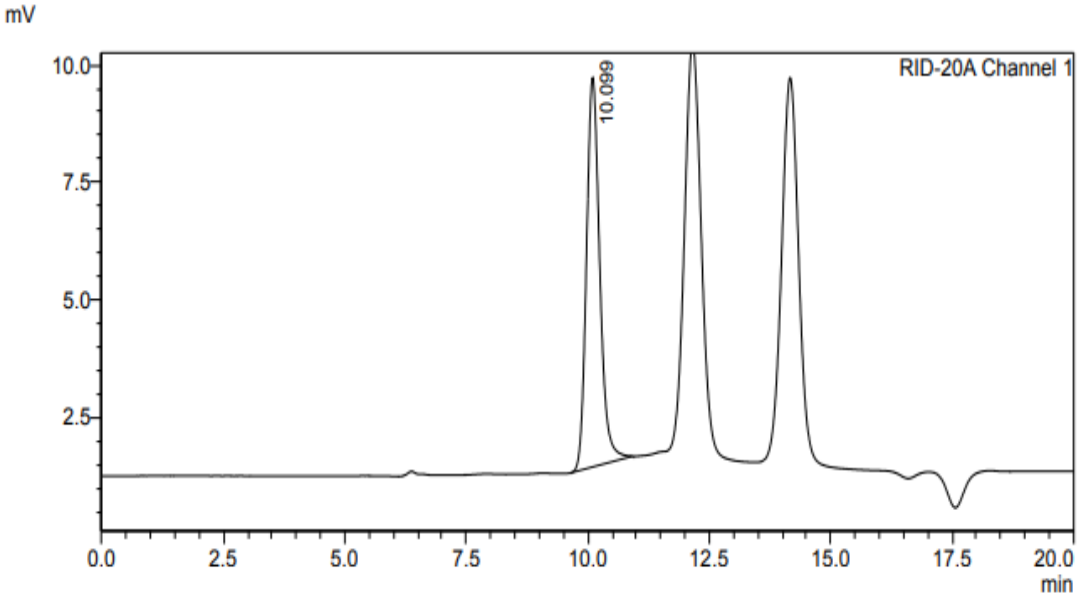

0.1 mg/ml



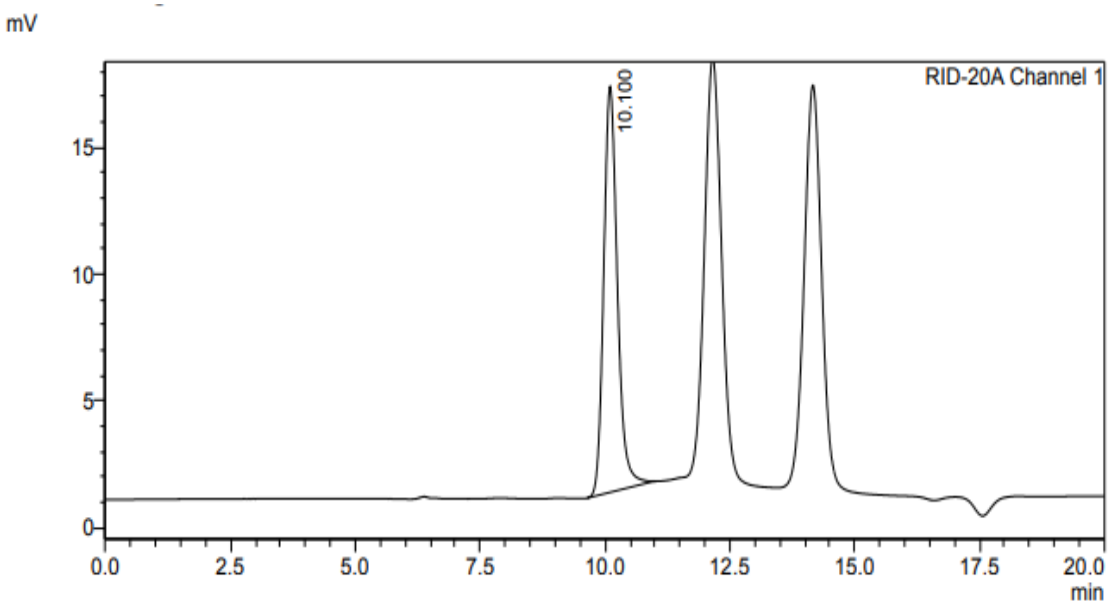
0.5 mg/ml




1 mg/ml



2 mg/ml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10
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7.5
10.0
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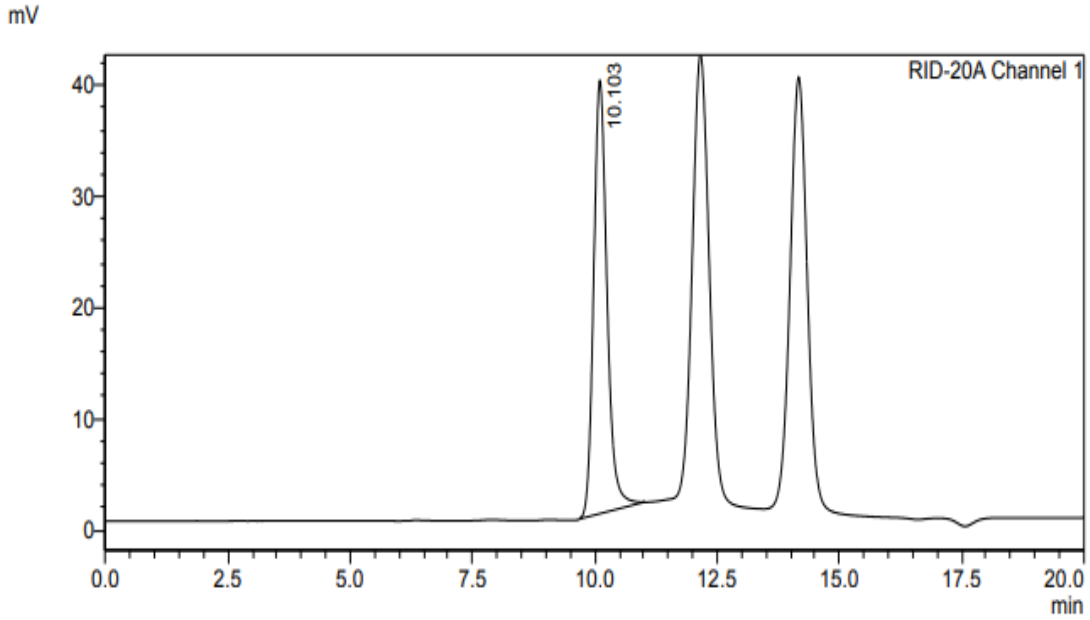
15.0
17.5
20.0
min

5 mg/ml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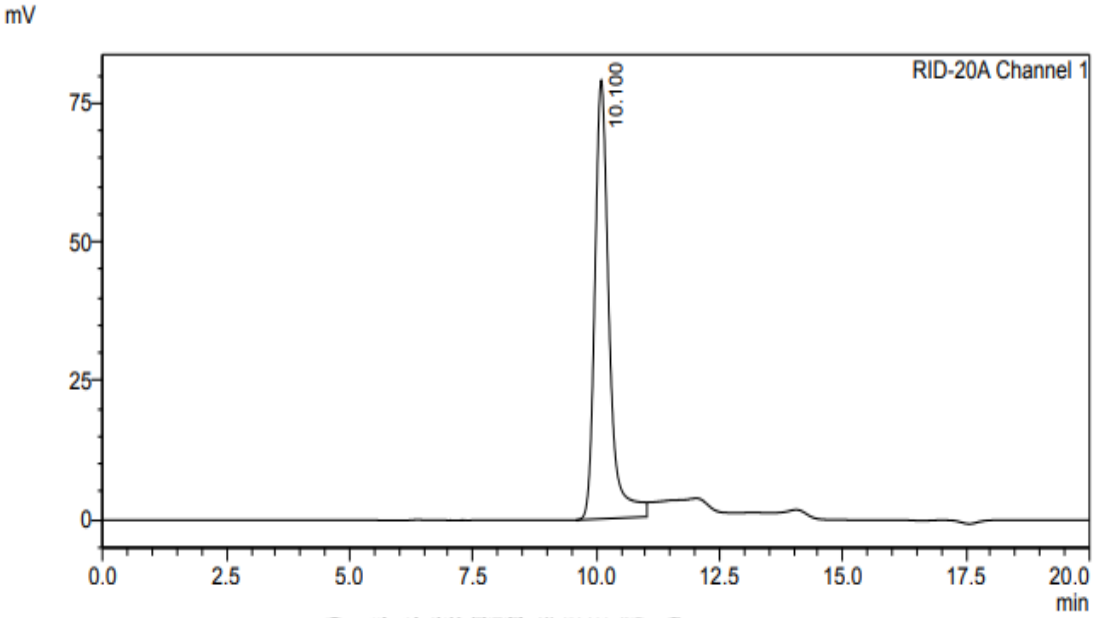
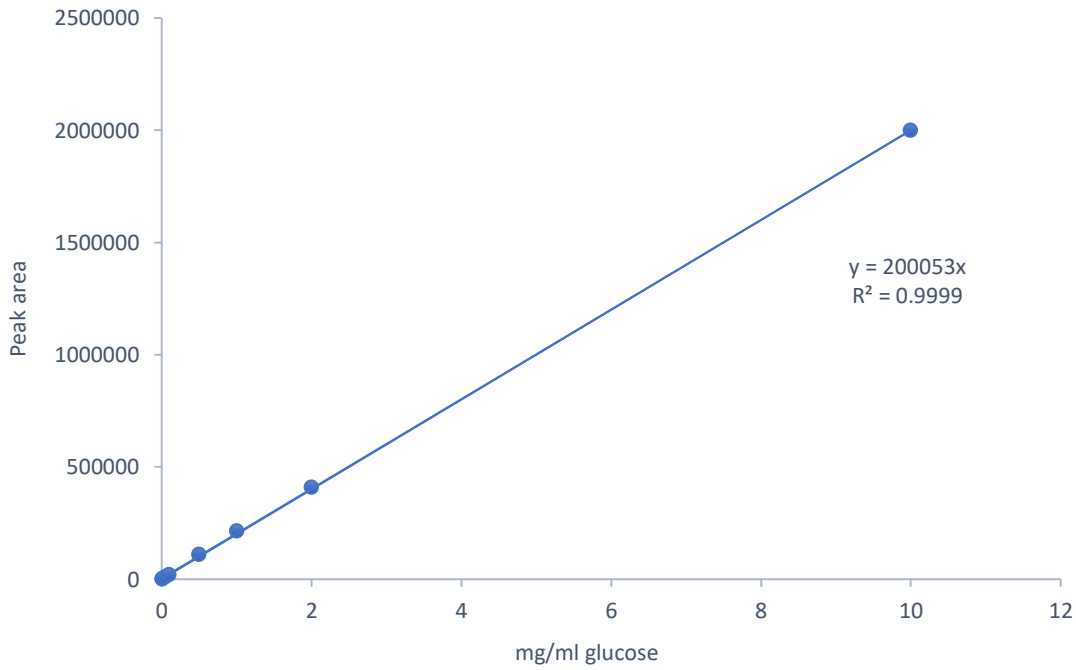
17.5
20.0
min

10 mg/ml

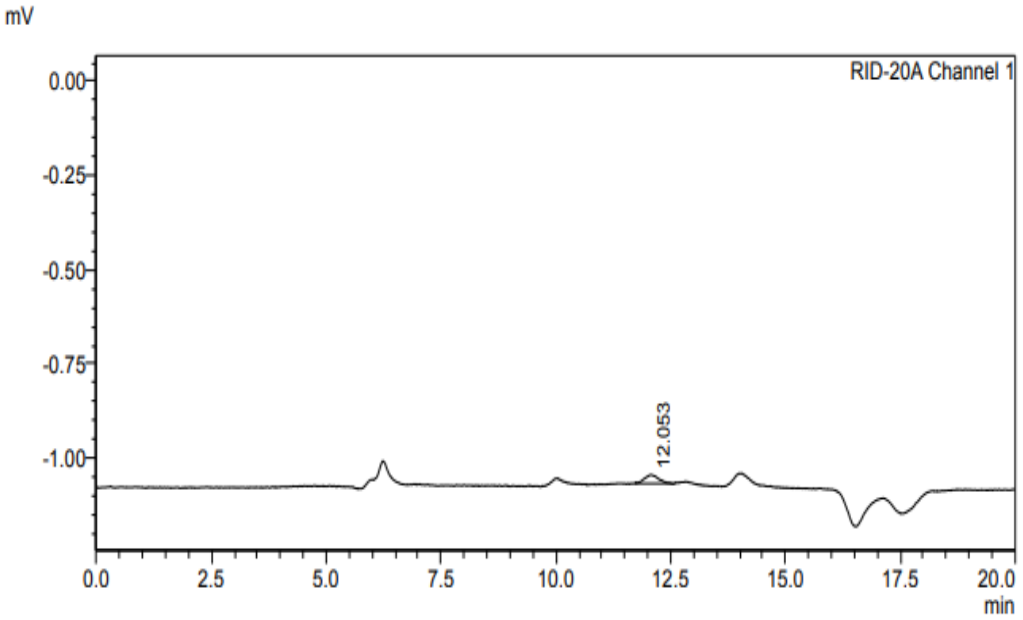



**6. Standard curve of glucose content**

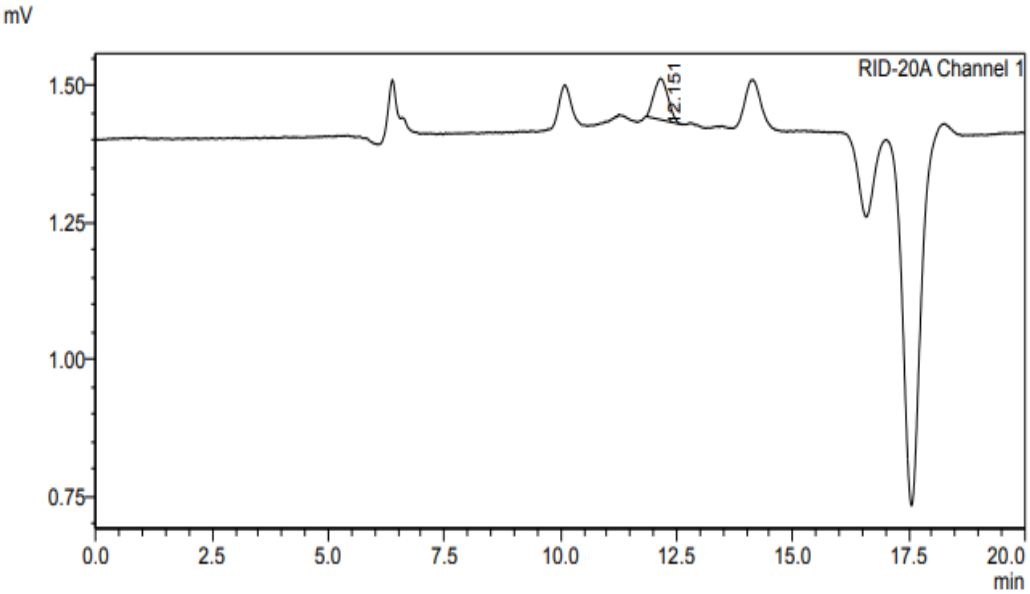


**7. Analysis report of glucose content**

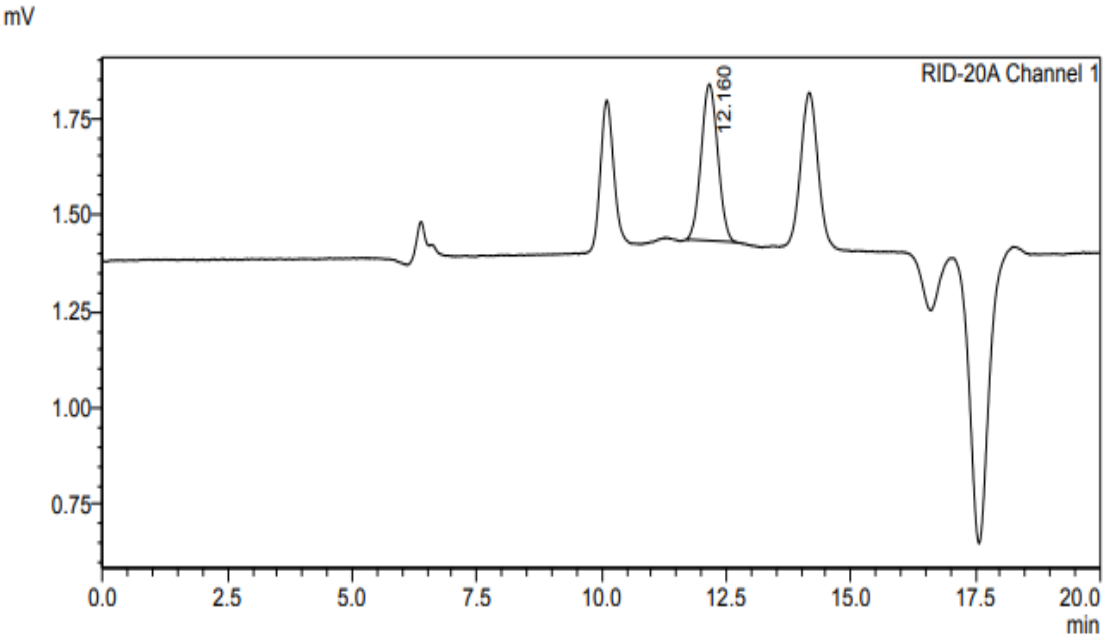
0.005 mg/ml



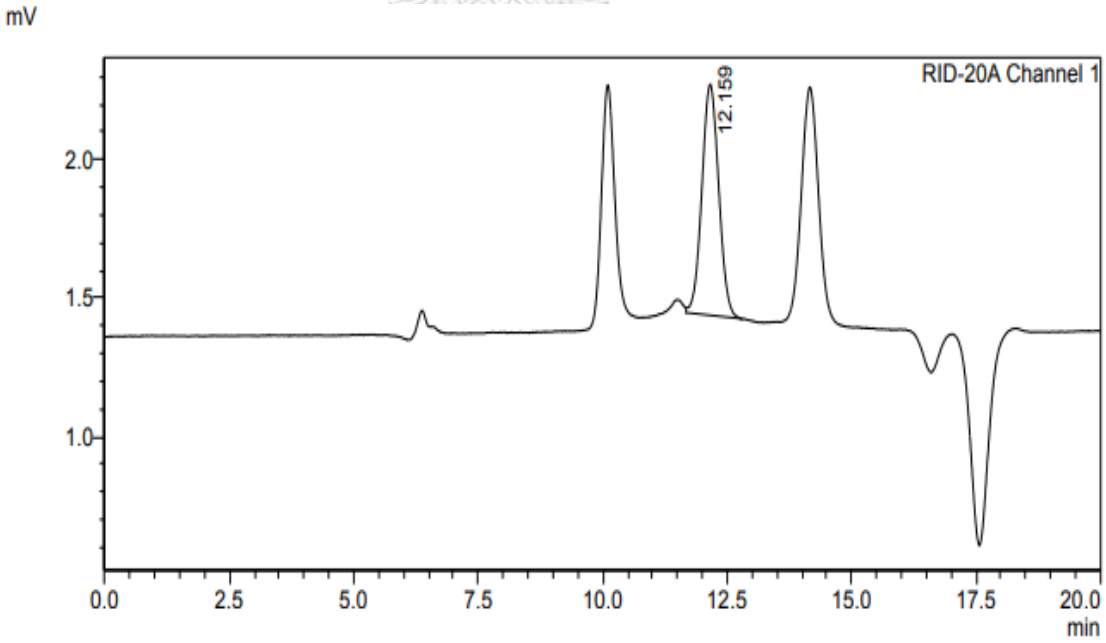

0.01 mg/ml



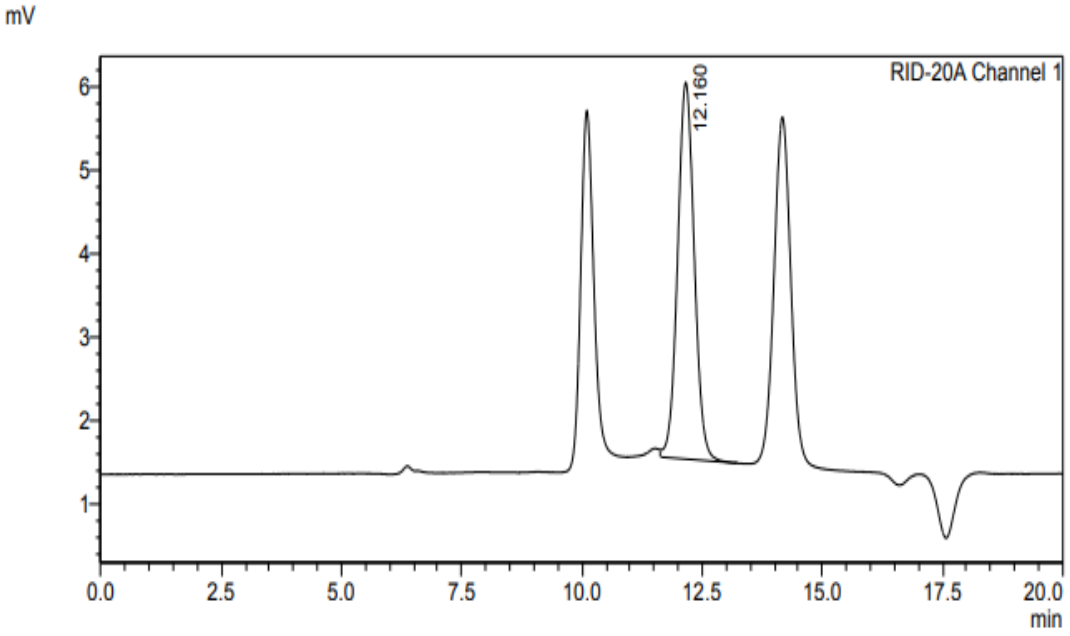
0.05 mg/ml



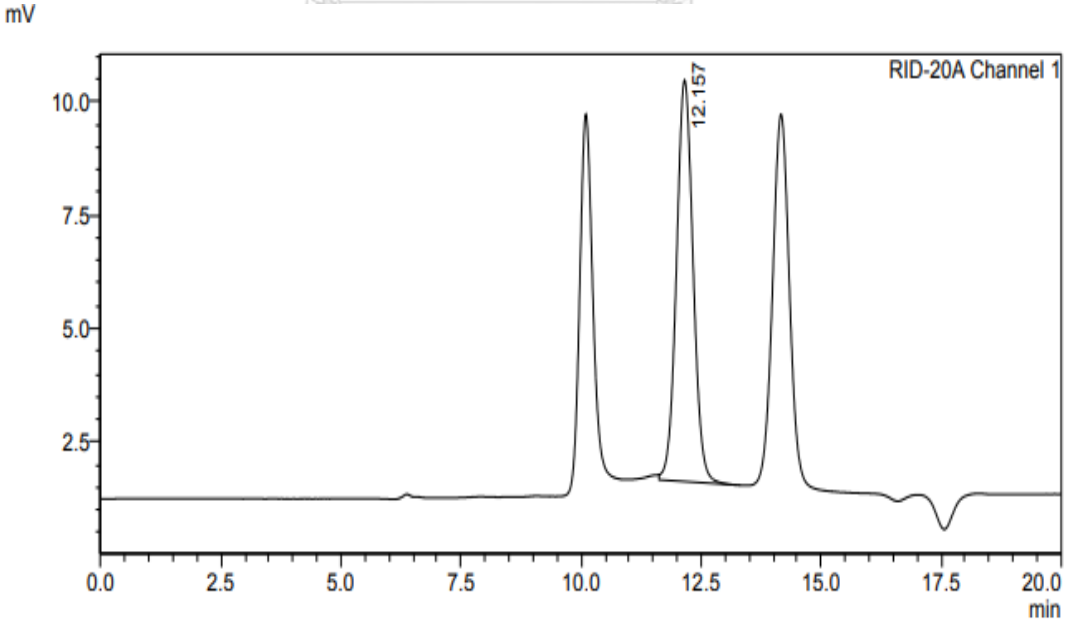

0.1 mg/ml



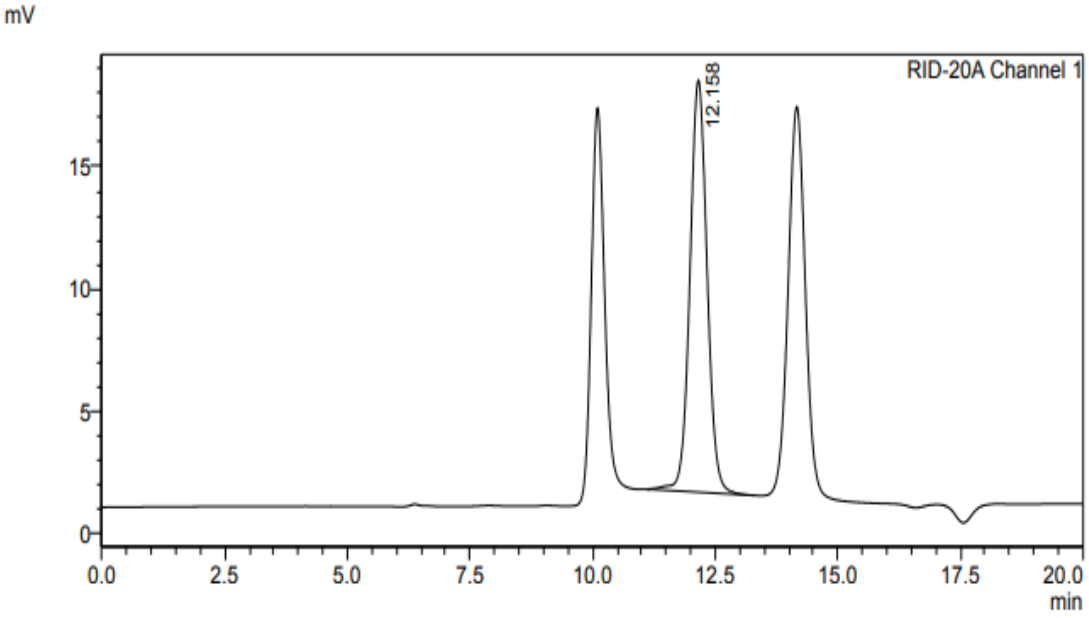
0.5 mg/ml



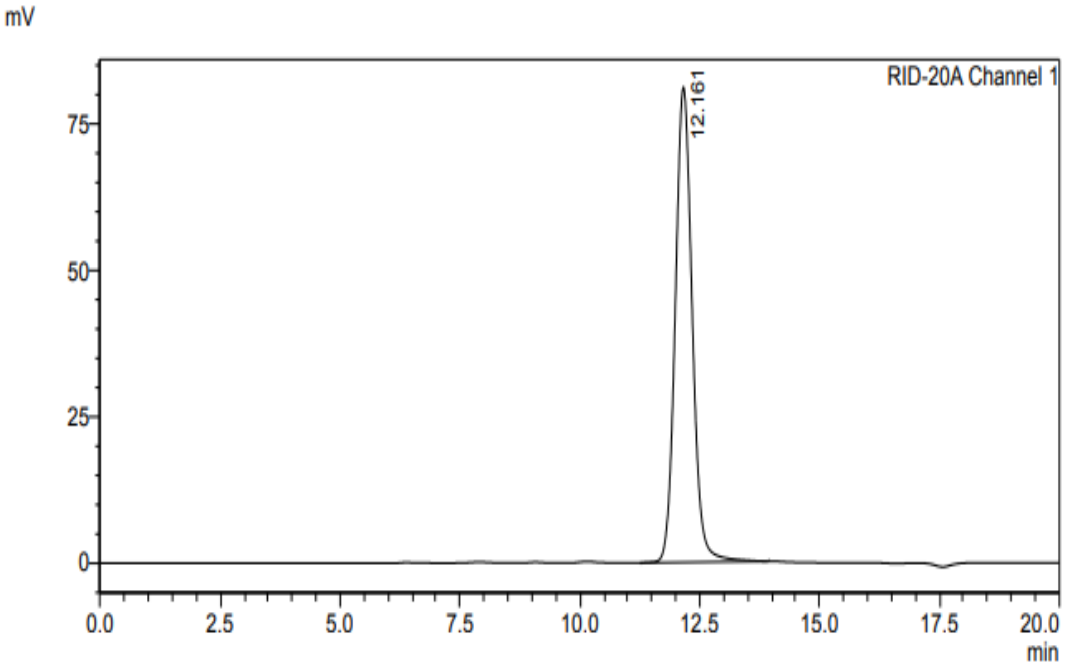

1 mg/ml



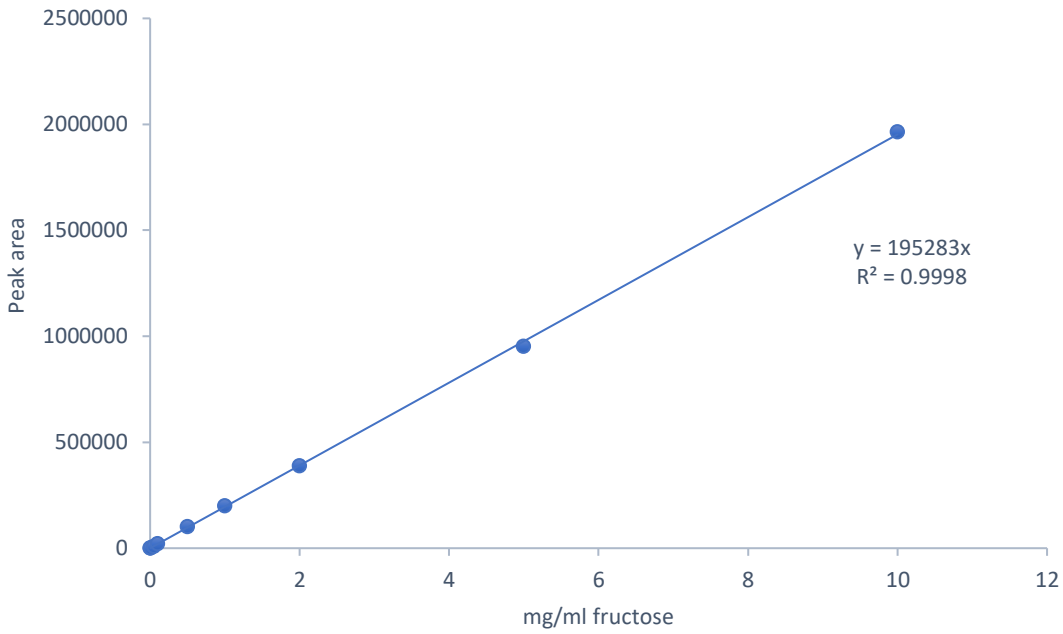
2 mg/ml




10 mg/ml

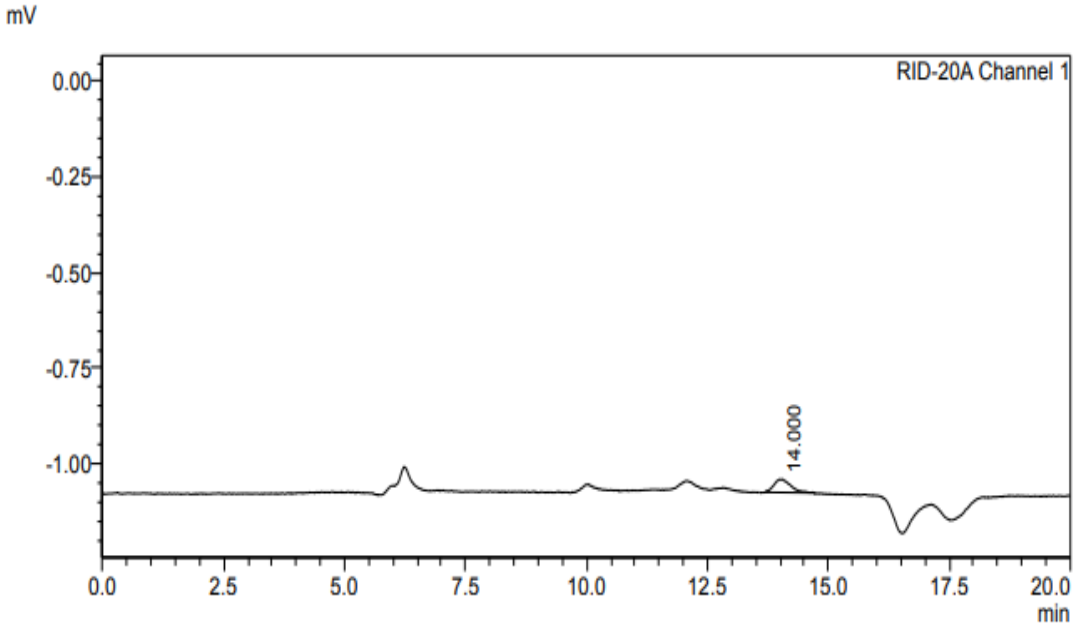


**8. Standard curve of fructose content**

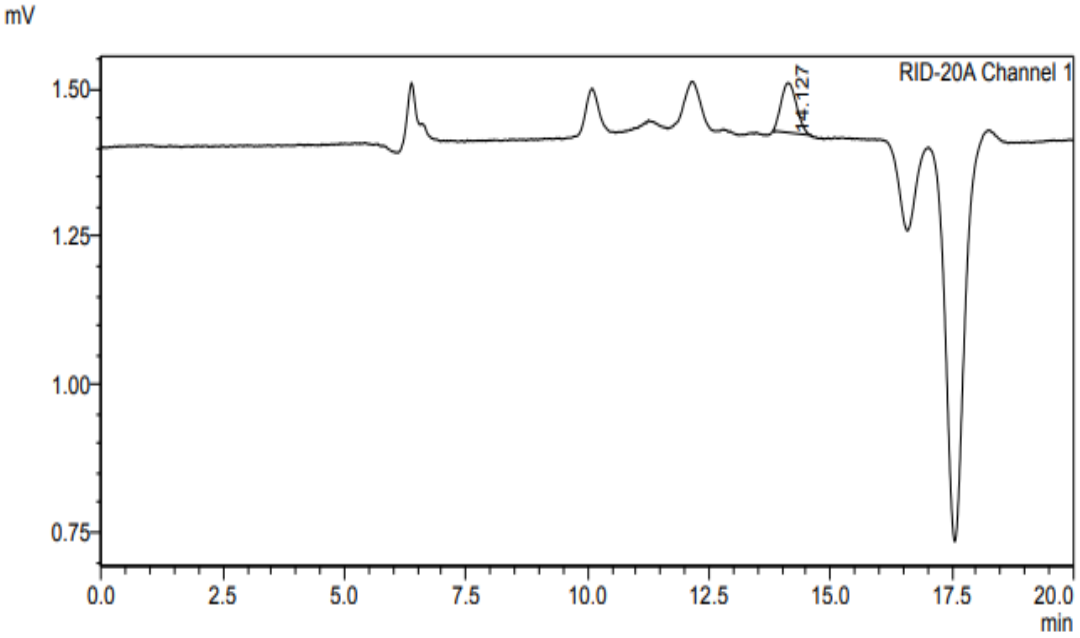



**9. Analysis report of fructose content**

0.005 mg/ml



0.01 mg/ml

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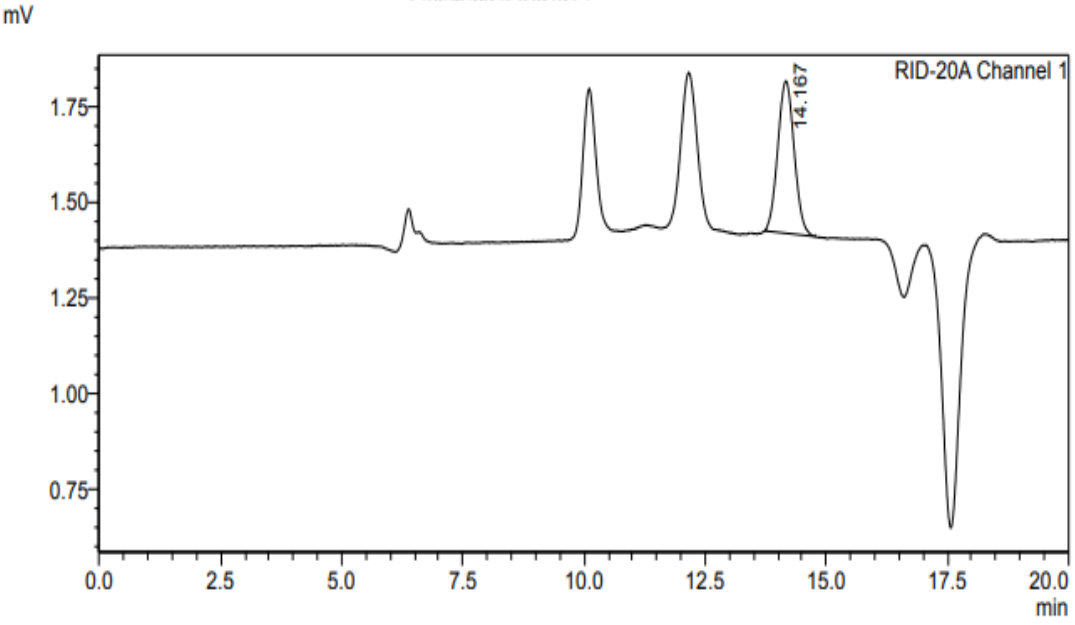
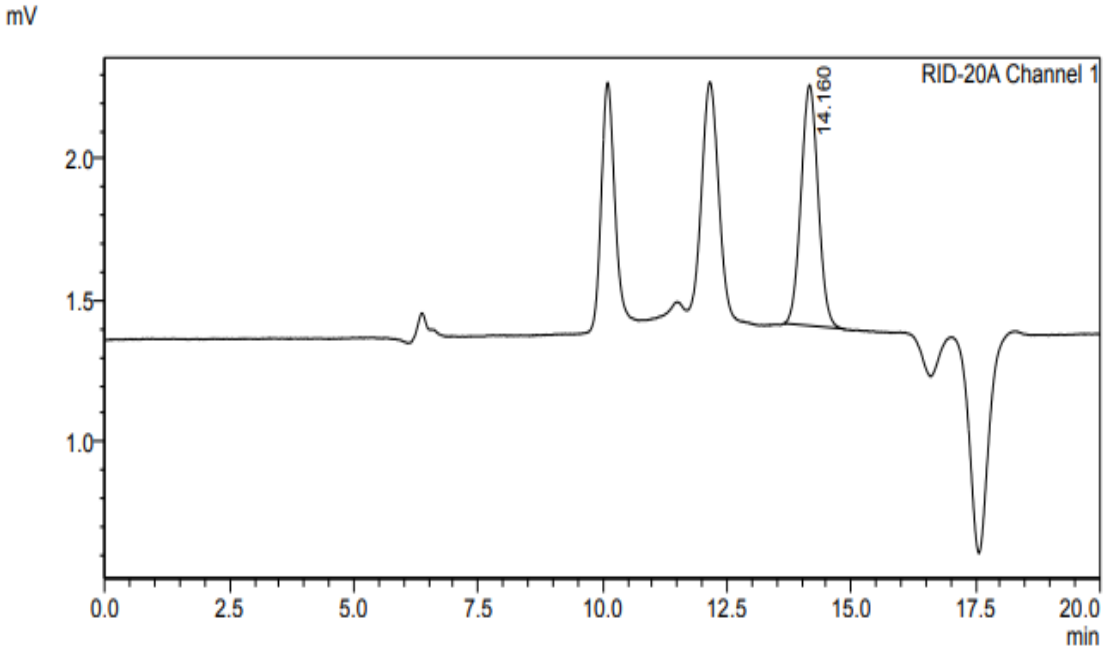
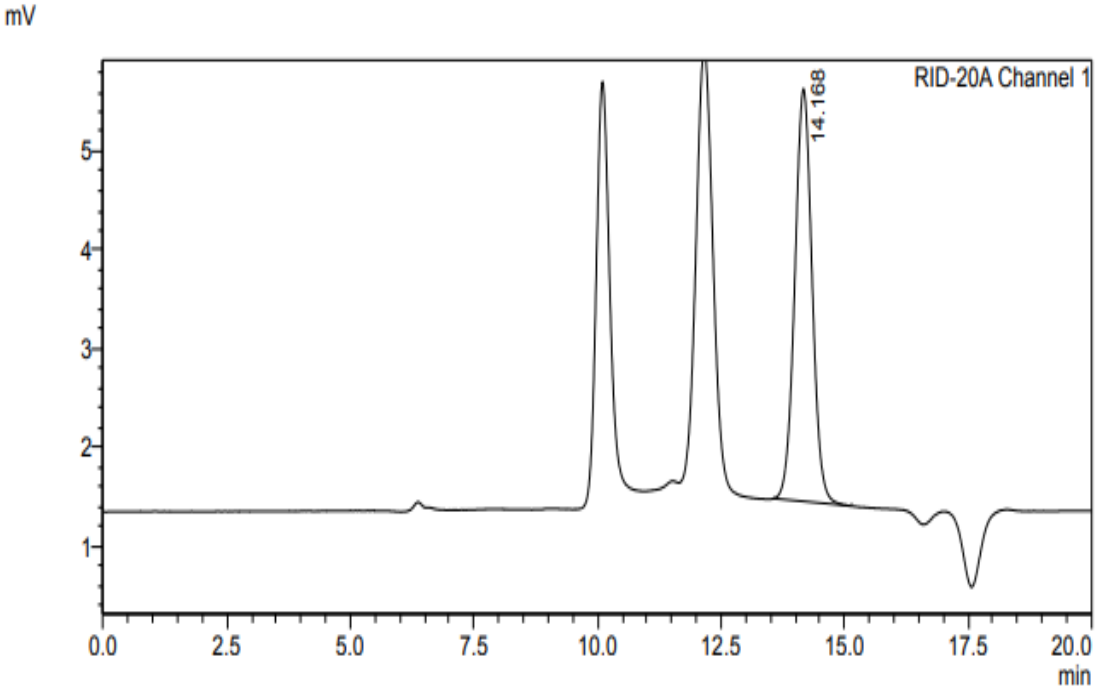


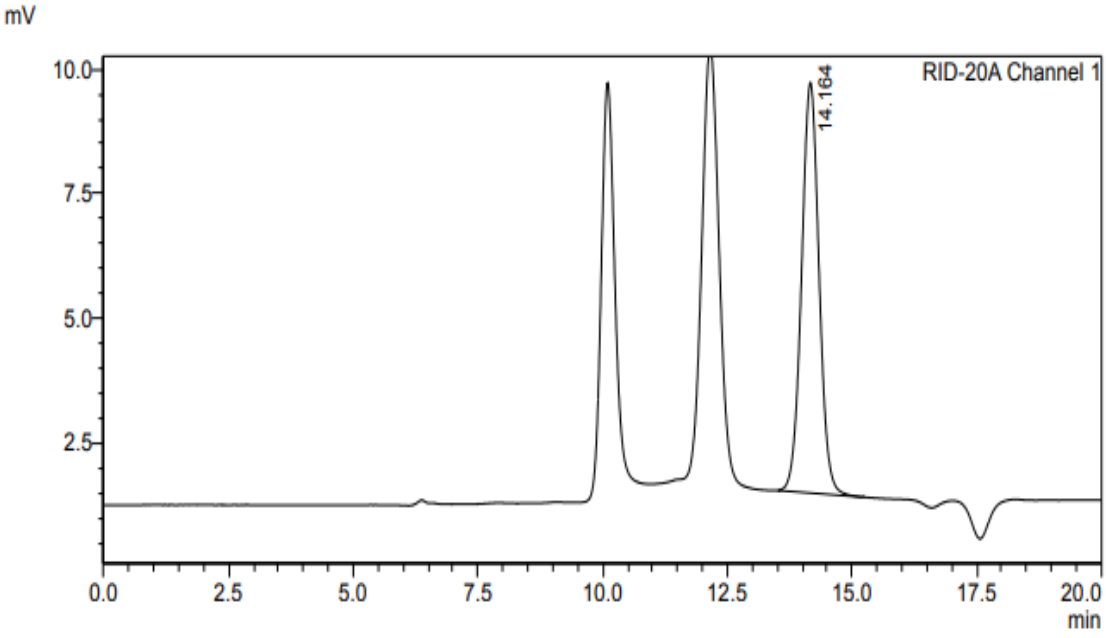
0.1 mg/ml




0.5 mg/ml



1 mg/ml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2.5
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7.5
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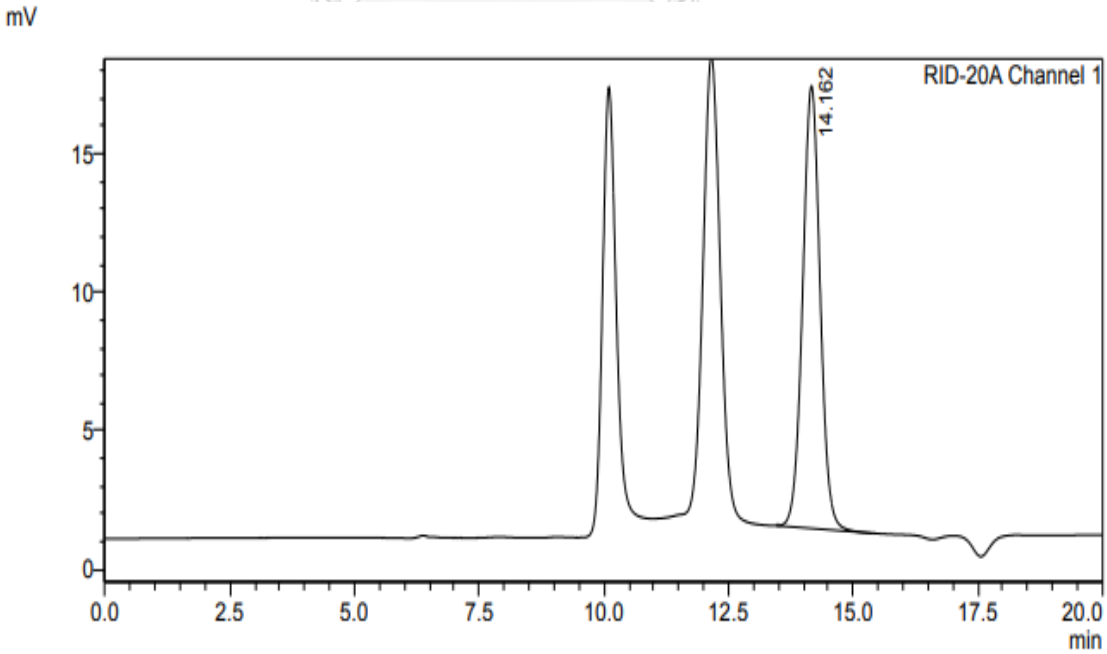
15.0
17.5
20.0
min

2 mg/ml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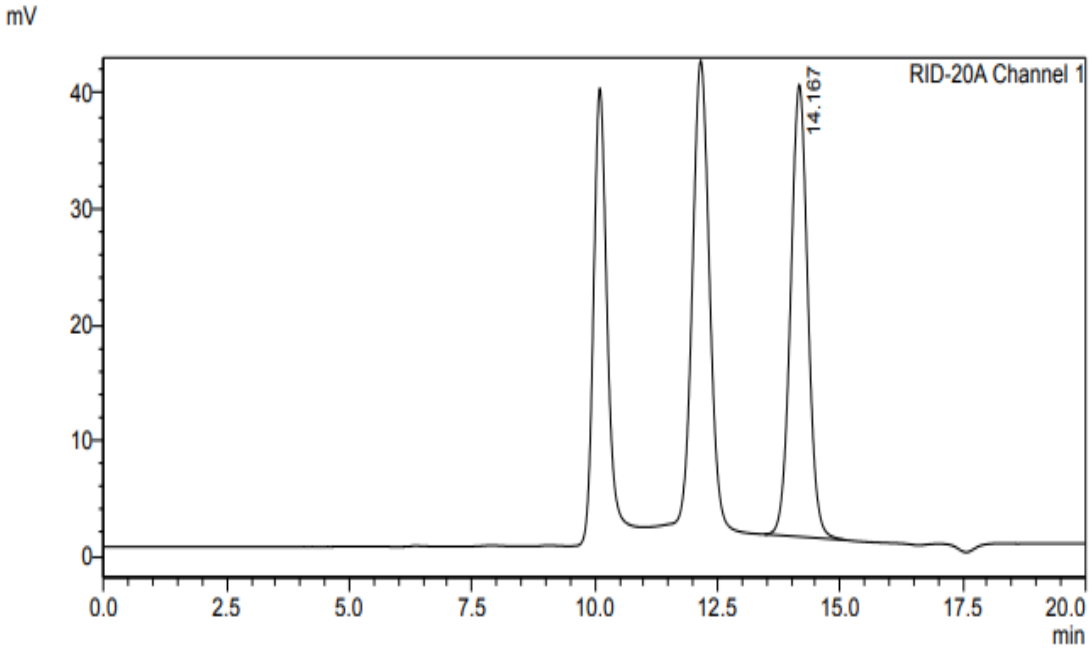
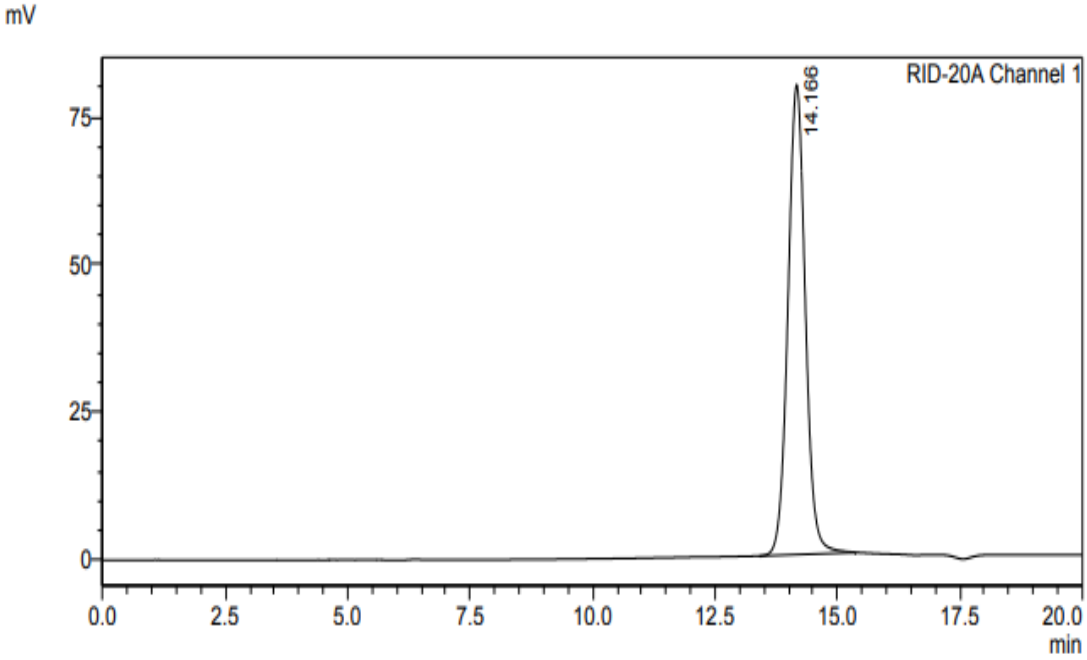
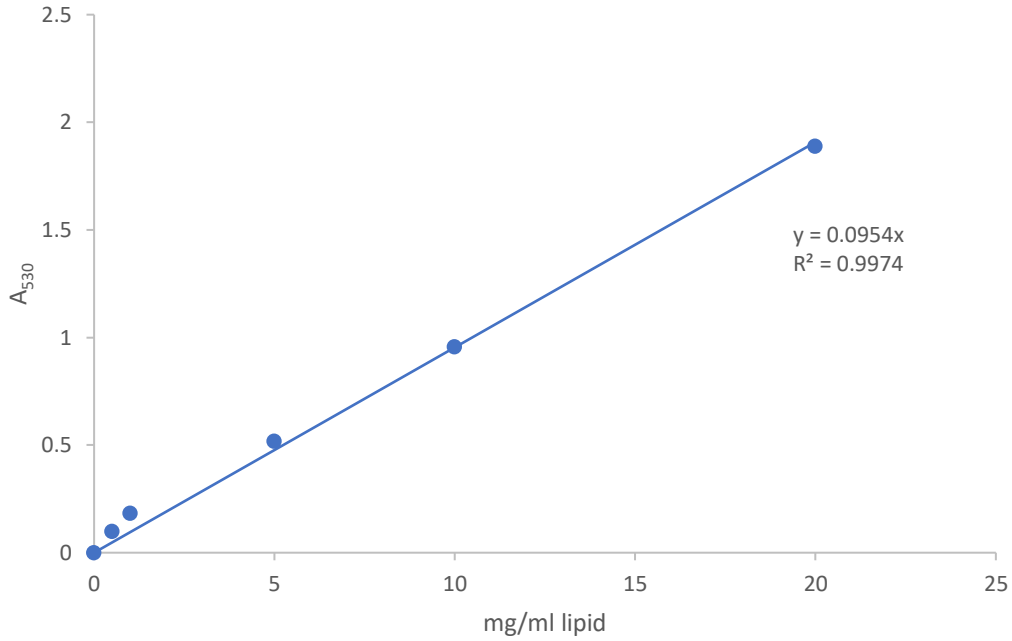
5 mg/ml




10 mg/ml



**10. Standard curve of lipid content**

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# VITA

| | |
|---|---|
| **NAME** | Miss Tanaporn Ausaha |
| **DATE OF BIRTH** | 9 May 1994 |
| **PLACE OF BIRTH** | Lopburi, Thailand |
| **INSTITUTIONS ATTENDED** | Bachelor degree of Science from Department of Biotechnology, Faculty of Science, Thammasat University in 2016. |
| **HOME ADDRESS** | 124/98 Moo 1, Soi Lom Choi<br>Tambol Khao Sam Yot<br>Amphur Mueang Lopburi<br>Lopburi province<br>15000 |
| **PUBLICATION** | Ausaha, T. Yuenyong, W. Buaboocha T. 2019. Effect of soil salinity stress on starch and sugar contents in 'KDML 105' rice (Oryza sativa L.) overexpressing OsCam1-1. Proceedings of the National Genetics Conference. 20-21 June 2019, Pattaya, pp. 207-213. |

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