โครงการ การศึกษาการลดการดูดซึมและขนส่งโลหะแคดเมียม ที่มีผลมาจากการแข่งขันการดูดซึมและขนส่งแร่ธาตุ Ca, Mg, Mn ผ่านโปรตีนเลือกผ่านของต้นข้าว

โดย

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เลขที่สัญญา TRG6180007

รายงานวิจัยฉบับสมบูรณ์

โครงการ การศึกษาการลดการดูดซึมและขนส่งโลหะแคดเมียม ที่มีผลมาจากการแข่งขันการดูดซึมและขนส่งแร่ธาตุ Ca, Mg, Mn ผ่านโปรตีนเลือกผ่านของต้นข้าว

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สถาบันพัฒนาและฝึกอบรมโรงงานต้นแบบ

มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี

บทสรุปย่อสำหรับผู้บริหาร (Executive Summary)

Cadmium (Cd) accumulation in rice has been serious issue in many countries around the world. In Thailand, this problem effect highly on economic around 7,800-14,000 tons per a year. Previous study, application of soil amendment and soil endophytic bacteria presented ability to reduce Cd in rice significantly. The result suggested that competition between Cd and other divalent cation (Ca, Mg and Mn) uptake and translocation in rice might be the major mechanism to reduce Cd concentration in rice. This experiment, we aims to study the effect of rice nutrient-divalent cation including Mn, Ca and Mg on reduction of Cd uptake and accumulation in rice plant. Competition of Ca, Mg, Mn and Cd uptake and translocation in rice plant will be focused. First part of this experiment, supplement Ca into hydroponic condition can effectively inhibit Cd uptake and translocation while supplement Mg into hydroponic condition cannot significantly reduce Cd concentration in rice plant. Therefore, second part of this experiment focused on the effect of Ca on reduction of Cd uptake and translocation.

Calcium signaling has an essential role in regulating plant responses to various abiotic stresses. Application of Ca in various forms (Ca acetate and $CaCl_2$) and concentration to reduce Cd concentration in rice and study the possible mechanism by which Ca acts to control the Cd concentration in rice were the aims of this part of experiment. The results showed that supplementation of Cd-contaminated soil with Ca acetate reduced the Cd concentration in rice after exposure for 7 days in both hydroponic and soil. The possible involvement of the auto-inhibited Ca^{2+}-ATPase (ACA) gene acts to control the primary signal of the Cd stress response. The messages from ACA3 and ACA13 tended to up-regulate low-affinity cation transporter (OsLCT1) and down-regulate Cd uptake and the Cd translocation transporter, including natural resistance-associated macrophage protein 5 (Nramp5) and Zn/Cd-transporting ATPase 2 (HMA2), which resulted in reducing the Cd concertation in rice. After cultivation for 120 days, the application of Ca acetate into Cd-contaminated soil inhibited Cd uptake in rice. Increasing the Ca acetate concentration in the soil lowered the Cd concentration in rice shoots and grains. Moreover, Ca acetate maintained the rice productivity and quality while both aspects decreased under Cd stress.

The result suggested that Ca acetate have an ability to apply as a composition for effective fertilizer to reduce Cd concentration in rice plant and grain.

บทคัดย่อ

รหัสโครงการ: TRG6180007

ชื่อโครงการ: การศึกษาการลดการดูดซึมและขนส่งโลหะแคดเมียม ที่มีผลมาจากการแข่งขันการดูดซึมและขนส่งแร่ธาตุ Ca, Mg, Mn ผ่านโปรตีนเลือกผ่านของต้นข้าว

ชื่อนักวิจัย: ชัยรัตน์ ตรีทรัพย์สุนทร สถาบันพัฒนาและฝึกอบรมโรงงานต้นแบบ มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี

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ระยะเวลา: 1 ปี

แคดเมียมปนเปื้อนในข้าวเป็นปัญหาสำคัญที่ส่งผลกระทบต่อเศรษฐกิจการส่งออกข้าวไทย การประยุกต์ใช้แคลเซียมเป็นสารปรับปรุงดินมีส่วนช่วยลดการสะสมของแคดเมียในต้นข้าวและเมล็ดข้าวได้ งานวิจัยนี้มุ่งศึกษาประสิทธิภาพและกลไกการลดการดูดซึมและขนส่งโลหะแคดเมียมของต้นข้าวโดยการเติมแคลเซียมลงในอาหารเหลวและดินปนเปื้อนจากสถานที่จริง ผลการทดลองพบว่าการเติมแคลเซียมอะซิเทรตลงในดินปนเปื้อนโลหะแคดเมียมมีประสิทธิภาพสูงมากในการลดการดูดซึมสะสมโลหะแคดเมียมในต้นข้าวภายหลักการรับสัมผัสแคดเมียมเป็นเวลา 7 วัน โดยกลไกที่แคลเซียมใช้ในการควบคุมการดูดซึมสะสมแคดเมียมในต้นข้าวเป็นกลไกที่ควบคุมผ่านการเพิ่มการแสดงออกของยีนส์ในกลุ่ม auto-inhibited Ca^{2+}-ATPase (ACA) โดยเฉพาะอย่างยิ่ง *ACA3* และ *ACA13* โดยการเพิ่มขึ้นของ ACA จะมีผลต่อเนื่องในการลดการแสดงออกของโปรตีนดูดซึมโลหะแคดเมียม ได้แก่ natural resistance-associated macrophage protein 5 (*Nramp5*) และ Zn/Cd-transporting ATPase 2 (*HMA2*) การลดการแสดงออกของยีนส์ *Nramp5* และ *HMA2* มีผลให้ต้นข้าวลดการดูดซึมสะสมแคดเมียมได้อย่างมีประสิทธิภาพ นอกจากนี้การเติมแคลเซียมอะซิเทรตในดินยังมีส่วนช่วยเพิ่มคุณภาพและปริมาณผลผลิตข้าวที่ปลูกในดินปนเปื้อนแคดเมียมได้อย่างมีประสิทธิภาพอีกด้วย

คำสำคัญ: แคดเมียมม แคลเซียม, ข้าว, โปรตีนเลือกผ่าน, ไพรมาริ่ซิกแนลทรายซ์ดักชั่น

Abstract

Project Code: TRG6180007

Project Title: Competition of Ca, Mg, Mn on Reduction of Cd Uptake and Translocation in Rice Plant though Transporter

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Time: 1 Year

Cadmium (Cd) accumulation in rice has been serious issue. Supplement Ca into hydroponic condition can effectively inhibit Cd uptake and translocation. This study aimed to apply Ca in various forms (Ca acetate and CaCl2) and concentration to reduce Cd concentration in rice and propose the possible mechanism by which Ca acts to control the Cd concentration in rice. The results showed that supplementation of Cd-contaminated soil with Ca acetate reduced the Cd concentration in rice after exposure for 7 days in both hydroponic and soil. The possible involvement of the auto-inhibited Ca2+-ATPase (ACA) gene acts to control the primary signal of the Cd stress response. The messages from ACA3 and ACA13 tended to up-regulate low-affinity cation transporter (OsLCT1) and down-regulate Cd uptake and the Cd translocation transporter, including natural resistance-associated macrophage protein 5 (Nramp5) and Zn/Cd-transporting ATPase 2 (HMA2), which resulted in reducing the Cd concertation in rice. After cultivation for 120 days, the application of Ca acetate into Cd-contaminated soil inhibited Cd uptake in rice. Increasing the Ca acetate concentration in the soil lowered the Cd concentration in rice shoots and grains. Moreover, Ca acetate maintained the rice productivity and quality while both aspects decreased under Cd stress.

Keywords: Cadmium, Calcium-acetate, Cd transporter, Oryza sativa, Primary signal transduction.

Acknowledgements

Investigator would like to thank Thailand Research Fund (TRF) for the financial support under the Thailand Research Fund Grant for New Researcher (TRG6180007) and Assoc. Prof. Dr. Paitip Thiravetyan, mentor, who directed the work. In addition, she give very effective suggestion for data analysis and improve the quality of manuscript. The authors would like to express my gratitude to Prof. Jian Feng Ma, Professor of Plant Nutrition, Institute of Plant Science and Resources, Okayama University for providing Dr. Chairat with the opportunity to visit his laboratory and learn the hydroponic technique of growing rice.

Content

Chapter 1
Introduction

Indica rice grains (*Oryza sativa*) contaminated with cadmium (Cd) has become a serious issue in many countries, such as China, Thailand, Bangladesh, and Sri Lanka (Williams *et al*. 2009; Sriprachote *et al.* 2012; Meharg *et al*. 2013) because this rice species can uptake and translocate high concentrations of Cd (Meharg *et al*., 2013). In Thailand, this problem affects around 7,800–14,000 tons of rice per year (Simmons *et al.*, 2005; Phaenark *et al.*, 2009). Several research studies have presented efficient methods to lower the Cd concentration in plant. For example, Adhikari *et al.* (2018) supplement sulfate to reduce Cd accumulation and phytotoxicity in maize. Application of fluoride to Cd-contaminated soil can inhibit Cd uptake by radish plants (Chen *et al.* 2017). In addition, Huang *et al.* (2017a) applied selenium to reduce Cd phytotoxicity and accumulation in rice and increase rice productivity. Qin *et al.* (2018) reported that increasing of wheat root growth by selenium is a mechanism to reduce Cd concentration and phytotoxicity in plant. Wu *et al.* (2018) presented Cd uptake inhibition by ammonium nutrition. In this case, ammonium nutrition can down-regulate rice Cd transporters. Sebastian and Prasad (2016) applied ferrous sulphate to reduce Cd accumulation in rice. Previous study, the application of biochar and microorganisms also significantly decreased the Cd concentration in rice plants and grains and all treatments that presented a low Cd concentration also had a significantly high calcium (Ca) level (Suksabye *et al.* 2015; Treesubsuntorn *et al.* 2018). Besides, some investigations demonstrated the Cd uptake could be limited by the concentration of Ca in the environment (Hayakawa *et al.* 2011; López-Climent *et al.* 2013; Eller and Brix, 2016).

Ca is a ubiquitous regulator of the signaling network in plants under abiotic and biotic stresses, whereas different signals trigger unique Ca signatures (Cho *et al.* 2012). As a secondary messenger, Ca can induce plant adaptation (Song *et al.* 2008), stabilize the plant cell

wall, and maintain cell membrane integrity (Hirschi 2004). The molecule plays a vital role in altering plant growth under a multitude of stimuli, such as light, salt, low temperature, and alkali (McCue and Hanson 1990; Sanders *et al.* 1999; Wood *et al.* 2000; Shao *et al.* 2008; Shao *et al.* 2009). One of the most important stress response mechanisms triggered in response to changes in the intracellular Ca content is the up-regulation of auto-inhibited Ca^{2+}-ATPases (*ACAs*). These *ACAs* act to maintain Ca homeostasis in eukaryotic cells, indicating that disturbance of the Ca homeostasis in plant cells might be the primary response to stress (Mahajan and Tuteja 2005; Huda *et al.* 2013a). For example, up-regulation of *ACAs* in *Arabidopsis*, *Lycopersicon*, and *Physcomitrella* under salinity stress have been reported (Chung *et al.* 2000; Geisler *et al.* 2000; Qudeimata *et al.* 2008). For cold stress at 2 °C, the activity and stability of the *ACAs* were key to inducing the cold resistance of winter wheat (Liu *et al.* 2002). *ACAs* were also expressed in response to abscisic acid, to promote *Arabidopsis* growth exposed to drought stress (Cerana *et al.* 2006). The production of reactive oxygen species (ROS), which are normally produced under Cd stress and cause phytotoxicity in plants (Wang *et al.* 2004; Heyno *et al.* 2008), can be strongly suppressed by *ACAs* (Beffagna *et al.* 2005). Previous authors have well described the role of *ACAs* in up-regulating ROS detoxification enzymes in plants and yeast but not in Cd uptake and translocation in plant. The experiment in 2014, transgenic tobacco with *ACA6* seem to reduce Cd concentration in plant by reduction of Cd translocation (Shukla *et al*. 2014). In this case, *ACA6* might play the role to regulate Cd transporter, result to decrease Cd concentration in plant. Therefore, this study, we aims to study the application of Ca in various forms (Ca acetate and $CaCl_2$) and concentration to reduce Cd concentration and improve rice productivity and quality. In addition, we also presented the pattern of candidate *ACAs* and Cd transporters gene including Cd uptake from roots to shoots: natural resistance-associated macrophage protein 5 (*Nramp5*), Cd transportation from intracellular to xylem and phloem: Zn/Cd-transporting ATPase 2 (*HMA2*)

and Cd translocation from roots to shoots: low-affinity cation transporter 1 (*LCT1*) expression in various Ca forms and concentration under Cd stress. The result can be used to predict the possible mechanism of Ca to reduce Cd uptake and translocation in rice via signal transduction from *ACA*.

Objectives

1. To study the effect of nutrient divalent cation including Ca, Mg and Mn on reduction of Cd uptake and translocation by rice plant.
2. To study the application of Ca in various forms (Ca acetate and $CaCl_2$) and concentration to reduce Cd concentration and improve rice productivity and quality
3. To present the pattern of candidate *ACAs* and Cd transporters gene expression in various Ca forms and concentration under Cd stress

Chapter 2
Literature review

2.1 Effect of Cadmium on rice

Cadmium is a heavy toxic metal of great environmental and occupational concern. It is obtained as a by-product of zinc mining (Phaenark et al. 2009). Cadmium is classified as a human carcinogen, as teratogen impacts the lungs, kidneys, liver, and reproductive organs and causes itai-itai disease (soft bones) (Boparai et al. 2011). There are many sources of cadmium in the environment such as metal plating, cadmium-nickel batteries, phosphate fertilizer, mining, pigments, photographs, dyes and textile operations, stabilizer, and alloy manufacturing (Boparai et al. 2011; Balkaya and Cesur 2008; Al-Anber and Matouq 2008; Gutierrez-Segura et al. 2012). In Thailand, some cadmium exposure health effects have been reported in Mae Sod District, Tak Province (Swaddiwudhipong et al. 2007). The source of cadmium contamination in Mae Sod District was zinc mining activities around that area (Simmons et al. 2005). More than 2,000 hectares in Mae Sod District, Tak Province, closed to Zn mines, was used for rice culture, and rice grain productivity from this area is around 7,800-14,000 tons/year. In addition, more than 80% of rice samples showed a higher Cd concentration than Cd standard guideline from Japan and FAO (Simmons et al., 2005; Phaenark et al., 2009). Moreover, Swaddiwudhipong et al. (2007) reported adverse health impacts from Cd contaminated rice in Mae Sod District, Tak Province. In plant, Cd accumulation can reduce biomass and induce leaf chlorosis (Ozturk et al., 2003; Chaturvedi, 2004).

Using cadmium-contaminated soil and underground water for irrigation can result in the increase of its concentration in rice plants (Simmons et al., 2005). Cadmium is taken up from cadmium-contaminated soil through roots and translocated into shoots and eventually grains of rice plants. Normally, cadmium depresses growth by affecting photosynthesis, chlorophyll fluorescence and nutrient uptake by plants. In addition, Cd can be translocated to edible parts of plants at levels that are not phyto toxic to the plant but may pose serious risk to the public health (Phaenark et al. 2009). Rice is one of the major food crops in many countries, including Thailand. This problem has a negative impact on Thailand’s economy and the life quality of the native people.

2.2. Effect of divalent cation element on Cd uptake and translocation

In order to reduce Cd contaminated in rice grain, many studied have proposed the ability of uptake and translocation competition between Cd and other divalent cation element including Manganese (Mn), Calcium (Ca) and Magnesium (Mg) (Hayakawa et al., 2011; Eller and Brix, 2015; Kudo et al., 2015). In addition, these 3 elements have been proposed the important role for plant growth.

Competitiveness of divalent cation uptake and translocation in rice have been rarely reported. For example, calcium may play important roles on rice plant Cd uptake and explained that higher calcium contents reduced the cadmium accumulation in rice grains (Suksabye et al., 2015; Treesubsuntorn et al., 2017) because cadmium might compete with calcium to be up taken through iron channels in root cells and guard plant cells (Tian et al. 2011). The magnesium contents in rice grains grown in Cd contaminated soil with the addition of biochars and microorganisms increased concomitantly with the decrease in cadmium concentration in rice grains. Magnesium alleviated the cadmium accumulation in rice plants (Nazar et al, 2012; Chou et al. 2011). Kashem and Kawal (2007) reported that the addition of magnesium enhanced the growth of plants suffering from cadmium toxicity and resulted in a reduced cadmium concentration in plants. Cadmium being a divalent cation may compete with magnesium in their transport across membranes. They are taken up by plants via cation transport systems normally involved in the uptake of essential elements (Nazar et al. 2012). However, there are no research precisely confirmed the competitiveness between Cd and other divalent cation in rice uptake and translocation. This project with physical research and transporter gene expression level would confirm the uptake and translocation competitiveness of Cd and other divalent cation, which the result can be benefit for soil amendment development for real site application target to limit Cd concertation in rice grains.

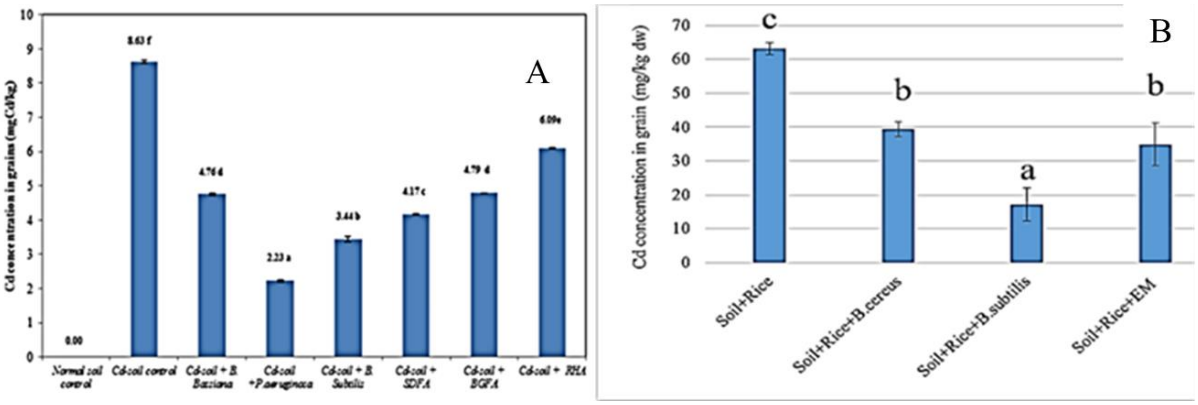

Fig 2.1. Cd uptake and translocation in rice: Cd concentration in rice grain, grown in Cd contaminated soil with and without soil amendment and microorganisms (A) and with and without microorganism inoculation (B).

Soil supplemented with plant nutrient including Ca, Mg and Mn have strong ability to reduce Cd and improve rice productivity. Nowadays, although some studies showed the reduction of Cd accumulation in plant by Mn, Ca and Mg (Eller and Brix, 2015; Kudo et al., 2015; Ma et al., 2015), in rice, the evident to show Cd uptake and translocation inhibiting by Mn, Ca and Mg have not been clearly investigated. This experiment aims to firstly present the effect of rice nutrient-divalent cation including Mn, Ca and Mg on reduction of Cd uptake and accumulation in rice plant. In addition, up regulation of nutrient-divalent cation transporter gene under various Cd concentration, which can be applied to predict the possible Cd transporter in rice plant, will be screened. The knowledge from this research can be developed for effective commercial fertilizer with suitable mineral concentration to reduce Cd accumulation in rice, benefit for native Mae Sod District, Tak Province farmer. The fertilizer can be commercialized while the investigating knowledge from this experiment can be used to develop Cd tolerance rice species, which low Cd uptake and translocation, by breeding technology.

2.2.1. Effect of Ca on Cd uptake and translocation

Calcium is an essential macronutrient for plants and has a major physiological role in plants. These are functions such as cell wall stabilization, membrane stabilization, and transduction of signals coming from the environment (Cho et al. 2012). If calcium is deficient, it results in plant membrane injury and lower antioxidant capacity (Schmitz-Eiberger et al.

2002; Cho et al. 2012). In 1998, the low-affinity cation transporter (LCT1) from Arabidopsis thaliana-expressing yeast cells showed decreasing of Cd uptake when concentration of Mg and Ca was increased (Clemens et al., 1998). Ca can inhibit Cd translocation in *Gamblea innovans*, result was presented in low concentration of Cd in stem and leaves when Ca was in the system comparing with without addition of Ca into the system (Hayakawa et al., 2011) (Fig 1.). In 2015, *Sesbania sesban* and *Brassica juncea* was grown under difference concentration of Ca and with and without Cd. The result found that high concentration of Ca can reduce Cd content in both shoot and root (Eller and Brix, 2015).

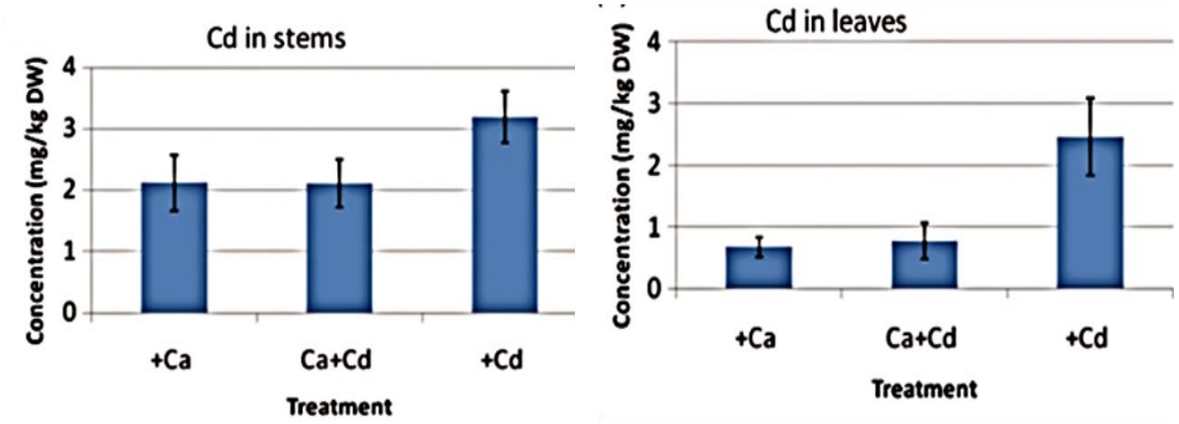

Fig 2.2. Reduction of Cd in stems and leaves of *Gamblea innovans* by Ca (Hayakawa et al., 2011).

2.2.2. Effect of Mg on Cd uptake and translocation

Magnesium is an indispensable mineral for plant growth and has major physiological and molecular roles in plants such as being a component of chlorophyll molecule, a cofactor for many enzymatic process associated with phosphorylation, dephosphorylation, the hydrolysis of various compounds, and a bridging element for the aggregation of ribosome subunits necessary for protein synthesis (Chou et al. 2011). The experiment in barley, Mg can effectively reduce Cd uptake resulting in plant healthy and high biomass although it was grown under Cd contaminated condition (Fig.2.) (Kudo et al., 2015).

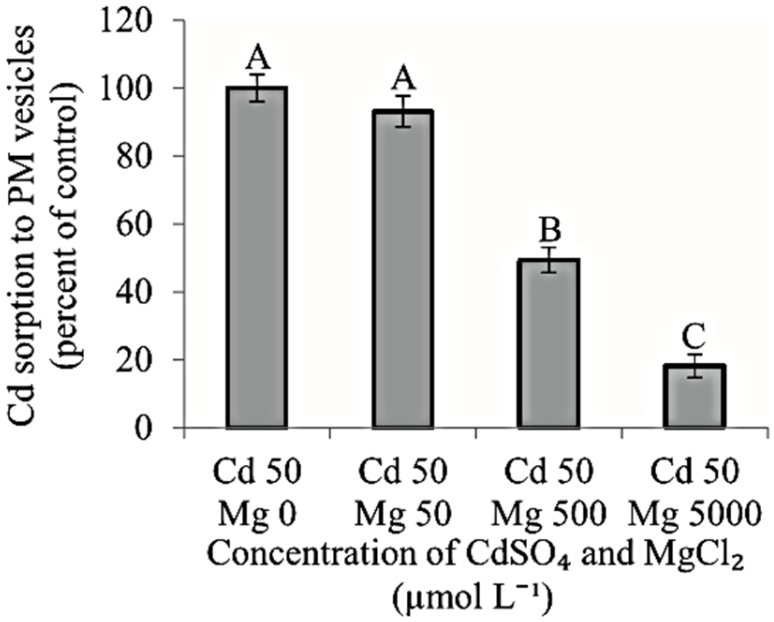

Fig 2.3. Magnesium inhibits cadmium translocation from roots to shoots, rather than the uptake from roots, in barley (Kudo et al., 2015).

2.2.3. Effect of Mn on Cd uptake and translocation

Manganese (Mn) being an essential plant nutrient involves in enzymatic activities, act as a co-factor of many enzymes (Millaleo et al., 2010; Grundmeier and Dau, 2012) and required for many metabolic pathways (Shalygo et al., 2000). Mn deficient soils negatively affects plant growth, and quality attributes (Narwal et al., 2012). In addition, Mn fertilization improves crop yield and quality characters by improving plant nutritional status and photosynthetic efficiency (Lidon et al., 2004; Mousavi et al., 2007). Dynamic roles of Mn in plant morpho-physiological and agronomic characters have been well-established, however, effects of Mn on rice aroma formation in terms of 2-AP contents and enzymes involved in its biosynthesis has not been reporter yet. Mn reduce Cd in rice and be taken up thought similar transporter (Sasaki et al., 2015), which might probably reduce Cd in by Mn.

Chapter 3
Materials and Methods

3.1. Growing rice under hydroponic conditions

The variety of rice used in this experiment was Pathum Thani 1, which breed from BKNA6-18-3-2 and PTT85061-86-3-2-1 species. Healthy rice seed was chosen and immerged in deionized water under dark conditions at 30^{o}C for two days. After small roots appeared, the seedling was transferred to a net, which floated on a 0.5 mM $CaCl_2$ solution under dark conditions at 30^{o}C for another six days. The 0.5 mM $CaCl_2$ solution was changed every two days. Then, the seedling was grown continuously in a 0.5 mM $CaCl_2$ solution under light conditions in a greenhouse for two days. After that, the net with the seedling was transferred to a ½ Kimura B medium that contained the following macronutrients: $(NH_4)_2SO_4$ (0.18 mM), $MgSO_4 \cdot 7H_2O$ (0.27 mM), KNO_3 (0.09 mM), $CaNO_3.4H_2O$ (0.18 mM) and KH_2PO_4 (0.09 mM), the following micronutrients: Na_2EDTA-Fe(II) (20 μM), $MnCl_2.4H_2O$ (9 μM), H_3BO_3 (46 μM), $Na_2MoO_4.4H_2O$ (9 μM), $ZnSO_4.7H_2O$ (0.7 μM) and $CuSO_4.5H_2O$ (0.3 μM) and had a pH of 5.6 that was adjusted by 1 M NaOH. The ½ Kimura B medium was changed every two days. The seedling was grown continuously until it was 30 days old. During this process, the seedling presents small roots and leaves, which are strong enough to expose to Cd. Rice that was 30 days old was transferred to a hydroponic pot and stand on the pot lid by sponges. These hydroponic pots contained ½ Kimura B medium as the main rice nutrient. A ½ Kimura B medium without Cd-contamination was used as the control and a ½ Kimura B medium that contained 50 μM $CdSO_4$ and ½ Kimura B mediums containing 50 μM $CdSO_4$ that were supplemented with 45 mM $CaCl_2$ or 45 mM Ca-acetate were used as the experimental treatments. All experimental condition, pH was adjusted to 5.6 by 1 M NaOH. The solution was changed every two days. The rice was grown under these conditions in a greenhouse at

35°C with normal light conditions for seven days. The shoots and roots of the rice were sampled after the experiment. For rice roots, the plant was immerged in cool (4°C) 5 mM $CaCl_2$ for 1 min to clean the root surface and slow down the metabolism of the rice plant. This process was repeated twice. The roots were then dried with tissue paper and cleaned twice using 4°C, deionized water for one minute each time. The roots were again dried and carefully separated from the shoots. After the roots were separated from the shoots, the rice base was cut out around 1 mm to ensure that the sample was a shoot zone. Root and shoot (node 1) samples weighing 100 mg were used to measure gene expression. In addition, the concentration of Ca and Cd in the roots and shoots was also measured.

3.2. *ACAs* gene expression level

We used the NCBI database to select *ACAs* for *Oryza sativa* and design primers. We searched the keywords "Ca-transporting ATPase and *Oryza sativa*" in the database. Ten *ACAs* of *Oryza sativa* were chosen for this study. DNA sequences for each of the *ACAs* (FASTA file) were used to design the specific primers using the free online NCBI program. Specific *ACA* genes of the *Oryza sativa* primers are shown in Table 1.

Table 3.1. Specific *ACAs* gene of *Oryza sativa* primers in this study

Gene description	Gene symbol	Locus tag	Forward (5'->3')	Reverse (5'->3')
ACA2	LOC4331984	OSNPB_030203700	TCTGCTTGGCATTCAGGGAG	AGCACTTTCAAATTACCTGGCA
ACA3	LOC4326507	OSNPB_010939100	CCCTCAAGCTGACAGCCTAC	ACATTGGTCGGCATTCTTTGC
ACA13	LOC4348639	OSNPB_100418100	CTACGGCAAGATGGTGGTCA	TCTTCATGGAGAAGGCCAGC
ACA8	LOC9267724	OSNPB_080517200	AAACCTGTCTTCCCCCAAAGG	TTACCTGACATTCCCACAGCC
ACA8	LOC4328480	OSNPB_020176700	CGAGACCGTGACTTGTGTGA	TTATTTAGCCCAGCCCAGCC
ACA8	LOC4336912	OSNPB_040605500	AGATCCAGCAGCTTATGGTGAC	GCAATTGGGTTTTCCGGGTG
ACA6	LOC4339199	OSNPB_050495600	GCCAGGTATGTGAACTACAAGTC	TGTGAAATGCGCACGGAAAA
ACA5	LOC4349735	OSNPB_110140400	AGGTCTTTCCGAAGTCTGCC	GCCAGTTGAGCGGAACAGTA
ACA4	LOC4351449	OSNPB_120136900	GCTCTCATGTTTGGTGGGGA	GCAGACTTCGGAAAGACCTCA
ACA1	LOC4352664	OSNPB_120586600	TGCATTAGCTACCGAGCCAC	AGGGCAACTTTGACAACACC
18S rRNA			TGTCCAAGATTCCCCACTGC	GTTGGTCGGGTAAAGGCTGA

Plant shoots and roots weighing a total of 100 mg from each treatment were pulverized using liquid nitrogen. The RNA from the plant samples was extracted using Plant Total RNA Purification mini kits (Favorgen Biotech Corp., Taiwan). Our protocol followed the manufacturer's instructions. DNase I (New England Biolabs, UK) was used to purify the RNA. The UV 260 nm/280 nm absorption ratios for all of the RNA samples were between 1.8 and 2.1. Then, all RNA samples were reverse-transcripted to cDNA using iScript™ Reverse Transcription Supermix for RT-PCR (Bio-rad Laboratories Inc., USA). The cDNA concentration was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific). We added a qPCR reaction mixture containing 10 µL KAPA SYBR FAST qPCR Master Mix (2) Universal (KAPA BIOSYSTEMS, USA), 0.4 µL of each primer (10 mM), 1 µL of cDNA (100 ng) and 8.2 µL of milli-Q water (PCR grade) for a total volume of 20 µL to each sample. The gene expression level was determined by qPCR that used a CFX96 Optical Real-Time Detection system (Bio-rad Laboratories Inc., USA). The qPCR was performed as follows: an

initial denaturation step at 95ºC for 1 min, 44 cycles of denaturation at 95ºC for 5 s, annealing and extension steps at 60ºC for 30 s, followed by one cycle of 95ºC for 1 min, 55ºC for 30 s, and 95ºC for 30 s. The relative expression of the genes was calculated using the $2^{-\Delta\Delta Cq}$ method. The average of the cycle threshold values of 18S rRNA was used as an internal reference gene for normalization (Table 1). The presented values with their standard deviations (SD) were based on triplicate samples.

3.3. Rice Grown in Cd-contaminated Soil

Rice seeds were immersed in deionized water under dark conditions at 30 °C for 2 days. After the small roots appeared, the seedlings were transferred to Cd-free paddy soil (pH 6.5-7, cation exchange capacity of 23.4 cmol mg/kg, and 0.64% organic matter) and grown continuously for 28 days. At 30 days old, rice plants were transferred to soil contaminated with an initial 82.3 ± 5.28 mg total Cd/kg. For Cd-contaminated soil preparation, Cd-contaminated zinc silicate residues (obtained from Mae Sod District, Tak Province, Thailand) was taken from surface layer (0–20 cm in depth). Therefore, main form of Cd in this residues is Cd silicate, which normally, can be solubilized in form of Cd ion. Cd-contaminated zinc silicate residues were dried and passed through a 2-mm sieve. Cd-contaminated soil (82.3 ± 5.28 mg total Cd/kg) was prepared by mixing between Cd-contaminated zinc silicate residues and paddy soil (Sao et al. 2006).

Each pot contained 1 L of water and 2 kg of Cd-contaminated soil (pH was around 6.5-7, cation exchange capacity of 23–25 cmol mg/kg, and 0.64–0.70% organic matter). For Ca treatment, Ca-acetate solution 1 L with concentration 11.25 mM, 22.5 mM and 45 mM was used to replace water. Cd-contaminated soil without Ca supply served as the positive control and rice grown in normal paddy soil without Cd was used as the negative control. Supplement of Ca acetate into Cd-contaminated soil can normally decrease soil pH, which decreasing of

pH can increase Cd water solubility (Temminghoff et al., 1995). Therefore, NaOH was used to adjusted pH in this experiment in the range of 6.5-7 in order to control the effect of pH in this experiment.

For short-term soil Cd exposure (7 days), all conditions were done in triplicate, with 5 rice plants per pot. Shoots and roots of rice were sampled after the treatment. For rice roots, the plant was immersed in cool 5 mM $CaCl_2$ (4 °C) for 1 min, to clean the root surface and slow the rice plant metabolism. This process was repeated twice. After drying with tissue papers, the roots were cleaned again in 4 °C deionized water for 1 min. This process was repeated twice before the shoots were carefully separated. Once the roots were separated, around 1 mm of the rice base was cut out, to confirm the sample was a shoot zone. The 100 mg roots and shoots (node 1) samples were measured for gene expression. The Ca and Cd concentrations in roots and shoots were also determined.

For 120 days Cd exposure, all conditions were done in triplicate, with 5 rice plants per pot. Rice plants were grown for 120 days until the grains were fully developed, and the rice grains then analyzed for productivity and Cd concentration. Fertilizers (N-P-K=16-20-0) were added to pot experiments at 0.5 g/pot, once the plants reached 60 days old (one time). After Cd exposure for 60 days, chlorophyll fluorescence (Fv/Fm) and leaf chlorophyll (SPAD values) were recorded by using a chlorophyll fluorescence meter (model FMS2, Hansatech Instruments, UK) and SPAD 502DL Plus Chlorophyll Meter with Data Logger, respectively, according to the instrument instructions.

3.4. Cd and Ca Concentration Analysis

Shoots and roots samples were oven-dried at 70 °C for 2 days. The shoots and roots dry weights were recorded and the samples digested with 2 mL of 61% HNO_3 per digestion tube, at 100 °C for 8 h, 120 °C for 2 h, and 140 °C for 1–2 h. The digested solutions were diluted

with deionized water to a final volume of 20 mL and the element content analyzed by using inductively coupled plasma–optical emission spectroscopy (ICP–OES; Shimadzu, ICPE-9000, Japan) with 0.3 mg/L limit of detection.

3.5. *Nramp5, HMA2, LCT1* Gene Expression

The *OsNramp5* (Cd and Mn root uptake transporter), *OsHMA2* (P-type heavy metal ATPase; delivery of Cd to developing tissues), and *OsLCT1* (Cd transporter from roots to shoots expressed in rice node) Cd transporter genes of *O. sativa* were selected for assessment of their expression levels. The primers for assaying *OsNramp5* and *OsHMA2* were chosen based on Sasaki *et al.* (2012) and Takahashi *et al.* (2012), respectively, while those for *OsLCT1* were designed by the NCBI program. qPCR was conducted as described above for *ACA3* and *ACA13*, using the primers shown in Table 2. The mean cycle threshold values of 18S rRNA, used as an internal reference gene for normalization, were similar under different conditions. Primer sets for *OsNramp5*, *OsHMA2*, *OsLCT1*, and *18S rRNA* was presented in table 2.

Table 3.2. Primer sets for *OsNramp5*, *OsHMA2*, *OsLCT1*, and *18S rRNA*

Gene description	Forward (5'–3')	Reverse (5'–3')
OsNramp5	CAGCAGCAGTAAGAGCAAGATG	GTGCTCAGGAAGTACATGTTGAT
OsHMA2	GCAGATCAAGTCACCCCATGG	GCCATCACCAACCATCAGCGT
OsLCT1	TGTTCTTATCGACCTCGGCG	CAGTATGGTCATGGCCGCTT
18S rRNA	TGTCCAAGATTCCCCACTGC	GTTGGTCGGGTAAAGGCTGA

3.6. Rice Productivity and Rice Quality Analysis

After 120 days, completely yellowed rice grains were harvested. The total grain dry weight in each pot was recorded. All rice grains in each pot were oven-dried at 60 °C for 2

days. The dry weight of rice grains was measured to four decimal places. Moreover, the Cd and Ca concentrations in rice grains were quantified by ICP–OES, as described above.

3.7. Data Analysis

All data analysis was undertaken using SPSS version 21, at a 95% confidence level. A descriptive statistic mode was applied to calculate the mean and standard deviation. The mean of each treatment was compared by using one-way ANOVA, and Fisher's LSD method was used to classify the groups of data. All parts of the experiment were studied in triplicate, with more than 3 samples per repetition.

Chapter 4
Results and discussion

4.1. Effect of $CaCl_2$ and Ca-acetate on Cd reduction in rice plants growing in Cd-contaminated hydroponic conditions

Application of $CaCl_2$ and Ca-acetate to a system can significantly reduce Cd concentration in every part of rice (Fig 4.1). After exposure for 7 days, Cd concentration in the roots of rice growing under 50 µM Cd was two times higher than that growing under 50 µM Cd with 45 mM Ca-acetate. The same result was also found in the shoots, while Cd concentrations in the shoots of rice growing under 50 µM Cd was three times higher than of those growing under 50 µM Cd with 45 mM Ca-acetate. For Ca concentration, the results showed that rice can take up Ca-acetate faster than $CaCl_2$ at the same concentration. Ca concentration in the roots of rice growing in 50 µM Cd with 45 mM Ca-acetate was higher than that growing in 50 µM Cd with 45 mM $CaCl_2$. The same result was also found in the shoots (Fig 4.1). Borchert (1986) presented the finding that Ca-acetate can induce Ca uptake in plants compared to other forms of Ca. These results confirmed that Ca-acetate plays an important role in reducing Cd concentration in rice. In addition, Hayakawa et al. (2011) showed that the application of $CaCl_2$ in a hydroponic system can reduce the Cd concentration of stem and leaves of *Gamblea innovans* (Hayakawa et al., 2011). Increasing the Ca concentration in a hydroponic system can reduce Cd accumulation in the roots and shoots of *Sesbania sesban* and *Brassica juncea* (Eller and Brix, 2016). This result might be explained by the fact that Ca, which is an important primary signal transduction element (Cho et al., 2012), can induce Cd stress response genes inside rice plants and cause plants to tolerate Cd stress conditions. In addition, low concentrations of Ca in the plant cells can also cause plant membrane injury and a low

antioxidant capacity (Schmitz-Eiberger et al., 2002). A Ca supplement for rice that is growing in Cd-contaminated soil could be important for future fertilizer development.

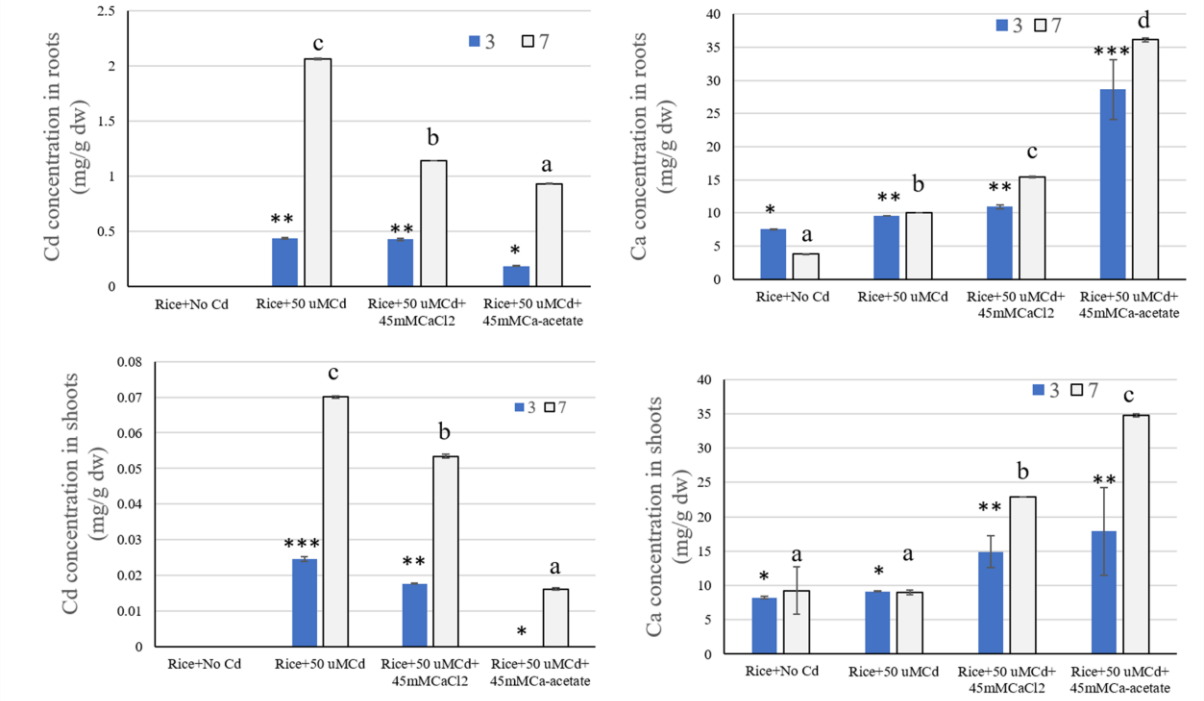

Fig. 4.1. Cd and Ca contents in roots and shoots of rice seedling growing in control (½ KimuraB without Cd) and treatment (½ KimuraB contained with 50 µM Cd, ½ KimuraB contained with 50 µM Cd and 45 mM $CaCl_2$ and ½ KimuraB contained with 50 µM Cd and 45 mM Ca-acetate) for 3 and 7 days at pH 5.6. (*, **, ***, ****) and (a, b, c, d) represented the group classification by Fisher's LSD method at 95% confident level.

4.2. *ACA* expression in rice plants growing in Cd-contaminated hydroponic conditions

ACAs, which are a group of Ca ATPase transporters, control Ca homeostasis and primary signal transduction in plants in response to various stresses (Pei et al., 2000; Kudla et al., 2010; Bose et al., 2011). Supplementing Ca to rice might increase the expression of this group of transporters and thus reduce Cd concentration in rice. Ten *ACA*s were selected from the NCBI database: OSNPB_030203700 (*ACA2*), OSNPB_010939100 (*ACA3*),

OSNPB_100418100 (*ACA13*), OSNPB_080517200 (*ACA8*), OSNPB_020176700 (*ACA8*), OSNPB_040605500 (*ACA8*), OSNPB_050495600 (*ACA6*), OSNPB_110140400 (*ACA5*), OSNPB_120136900 (*ACA4*) and OSNPB_120586600 (*ACA1*). The sequence of each transporter was analysed using the MEGA7 program to create the phylogenetic tree (Fig 4.2A). The pattern of *ACA* expression level was compared using normal rice plants without Cd exposure as a reference point of gene expression and calculated using the $2^{-\Delta\Delta Cq}$ method. The heat map showed up-regulation (green colour), down-regulation (red colour) and similar expression level (black colour). The results showed that after adding Ca-acetate to rice growing under Cd-contaminated conditions, the level of *ACA3* and *ACA13* gene expression was strongly up-regulated in both the roots and the shoots (Fig 4.2B and 4.2C).

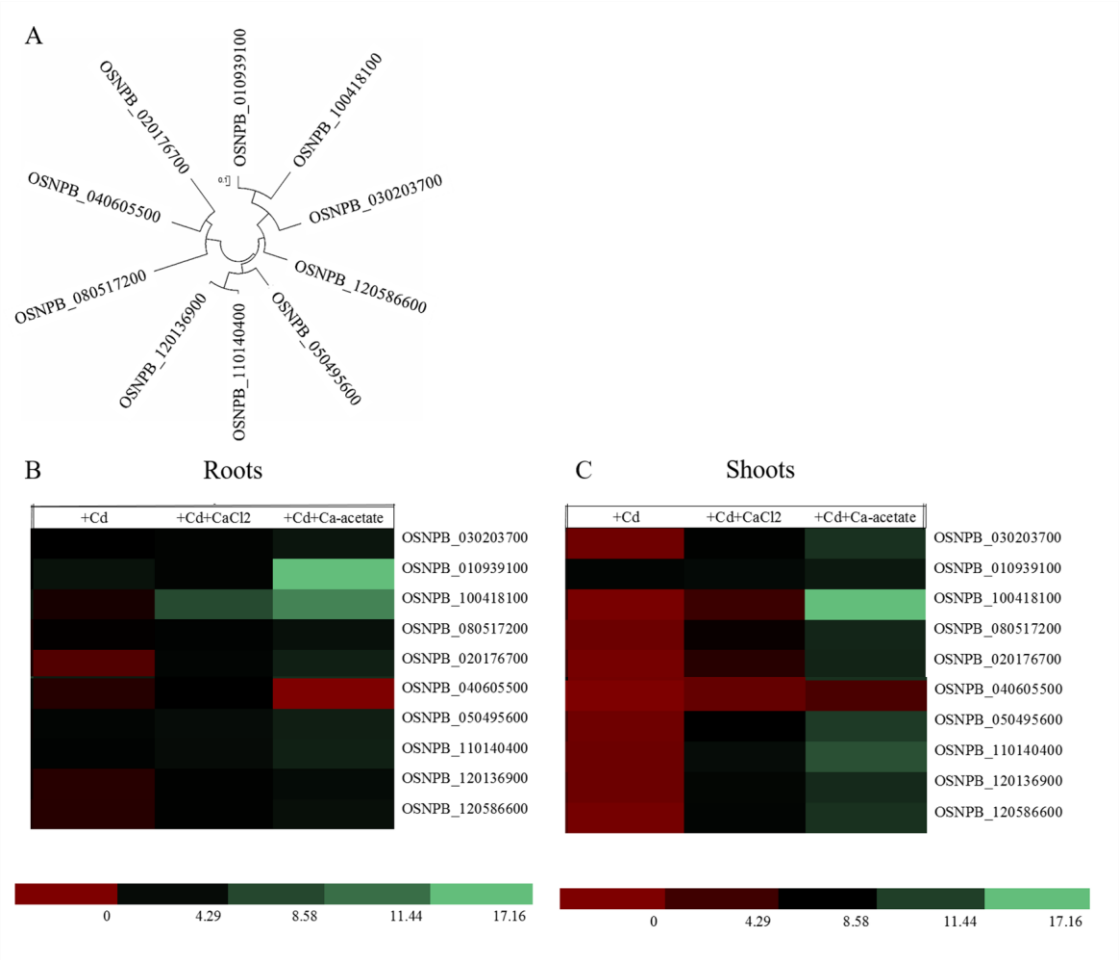

Fig. 4.2. ACA phylogenetic tree and pattern of ACAs gene relative expression in rice roots (B) and shoots (C) (30 days old) growing in treatments including ½ KimuraB contained with 50 µM Cd, ½ KimuraB contained with 50 µM Cd and 45 mM $CaCl_2$ and ½ KimuraB contained with 50 µM Cd and 45 mM Ca-acetate for 7 days at pH 5.6 while control (½ KimuraB without Cd) was used to calculate relative expression by using $2^{-\Delta\Delta Cq}$ method.

Many studies have reported the function of *ACAs* in stress responses (Geisler et al., 2000; Chung et al., 2000; Qudeimata et al., 2008; Kudla et al., 2010; Huda et al., 2013b). For example, *ACAs* can induce the adaptation of wheat growing under cool stress at 2^{o}C (Liu et al., 2002). In addition, *ACAs* (*ACA8* and *ACA9*) can induce ABA biosynthesis under drought stress in *Arabidopsis* (Cerana et al., 2006). For osmotic stress, Geisler et al. (2000) showed that *ACA4*

was induced under high NaCl and KCl concentrations and the up-regulation of *ACA4* can improve salt tolerance in *Arabidopsis*. Moreover, *ACA7* is involved in salt tolerance by regulating various functional behaviours of rice (Singh et al., 2014). In addition to abiotic stress responses, biotic stress responses can be regulated by *ACAs*. Dit Frey et al. (2012) showed that *ACA8* can increase plant immunity and protect plants from pathogenic bacteria. In this study, *ACA3* and *ACA13* might play an important role inhibiting Cd uptake and translocation in rice.

4.3. Cd and Ca Contents in Rice Grown in Cd-contaminated Soil with Various Ca Acetate Concentrations

Rice grown in Cd-contaminated soil had three times more Cd than rice grown in Cd-contaminated soil supplemented with Ca acetate. It demonstrated that application of 22.5 and 45 mM Ca acetate, as a soil amendment in real Cd-contaminated soil, limited Cd uptake and translocation. Ca acetate significantly reduced Cd in rice shoots after Cd exposure for 7 days (Fig. 4.3.). Interestingly, no Cd was detected in rice shoots of plants exposed to 45 mM Ca acetate. Application of Ca acetate into Cd-contaminated soil can be effective method to reduce Cd uptake in rice.

Previous study, Jopony and Young (1994) showed that a mixture of 5 mM $CaCl_2$ and $Ca(NO_3)_2$ with Cd solutions can generate both CdCl and $CdNO_3$ complexes, which can probably increase Cd water solubility and should be easily take up by plant. In form of Ca acetate in this experiment, acetate might be able to react with Cd and generate Cd acetate. However, this form of Ca acetate can be rapidly take up into the plant when compare to other form of Ca. Borchert (1986) presented that acetic acid on Ca acetate, as a permeable weak acid property and small size organic acid, can be easily taken up into plant. Therefore, intracellular Ca concentration might be the main effect to reduce Cd uptake in this experiment.

Auto-inhibited Ca^{2+}-ATPase (ACA3 and ACA13) and Cd Transporter Genes (OsHMA2, OsNramp5, and OsLCT1) Expression in Rice Plants Grown in Cd-contaminated Soil

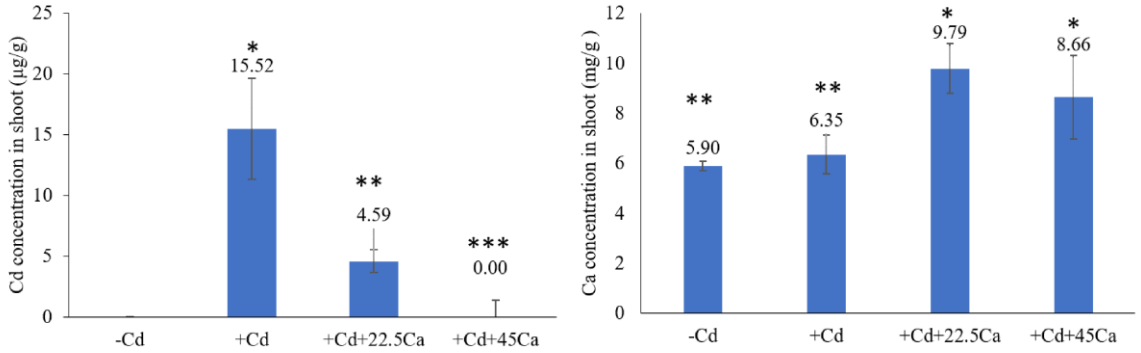

Fig 4.3. Cd and Ca contents in shoots of rice seedlings grown in control (paddy soil without Cd) and treatments (Cd-contaminated soil containing an initial 82.3 ± 5.28 mg Cd/kg and Cd-contaminated soil containing an initial 82.3 ± 5.28 mg Cd/kg and supplemented with different Ca acetate concentrations (22.5 and 45 mM)) for 7 days at soil pH 6.5–7.0. Group classification by Fisher's LSD method at 95% confidence level (*, **, ***).

Ca acetate up-regulated *ACA3* and *ACA13* by 17-fold under hydroponic conditions, suggesting these two *ACAs* contribute to controlling the Cd concentration in rice under Cd stress. Therefore, both these *ACAs* were investigated in this study. The result evidenced the expression of *ACA13* in rice roots was up-regulated after the plant was exposed to Cd and Ca acetate, especially, a high Ca acetate concentration (45 mM), which increased the *ACA13* gene 9,022.56 times compared to rice grown in paddy soil without Cd (control) (Figure 4.4C). In addition, *ACA13* expression displayed a Ca acetate dose-dependent increase. For *ACA3* expression in rice roots, the result was not significantly different in each treatment (Figure 4.4A). For rice shoots, Ca acetate exerted a dose-dependent increase on the expression levels of both *ACA3* and *ACA13* (Figure 4.4B and 4.4D), confirming that both 22.5 and 45 mM Ca acetate can increase the relative expression levels of *ACA3* and *ACA13* in rice shoots

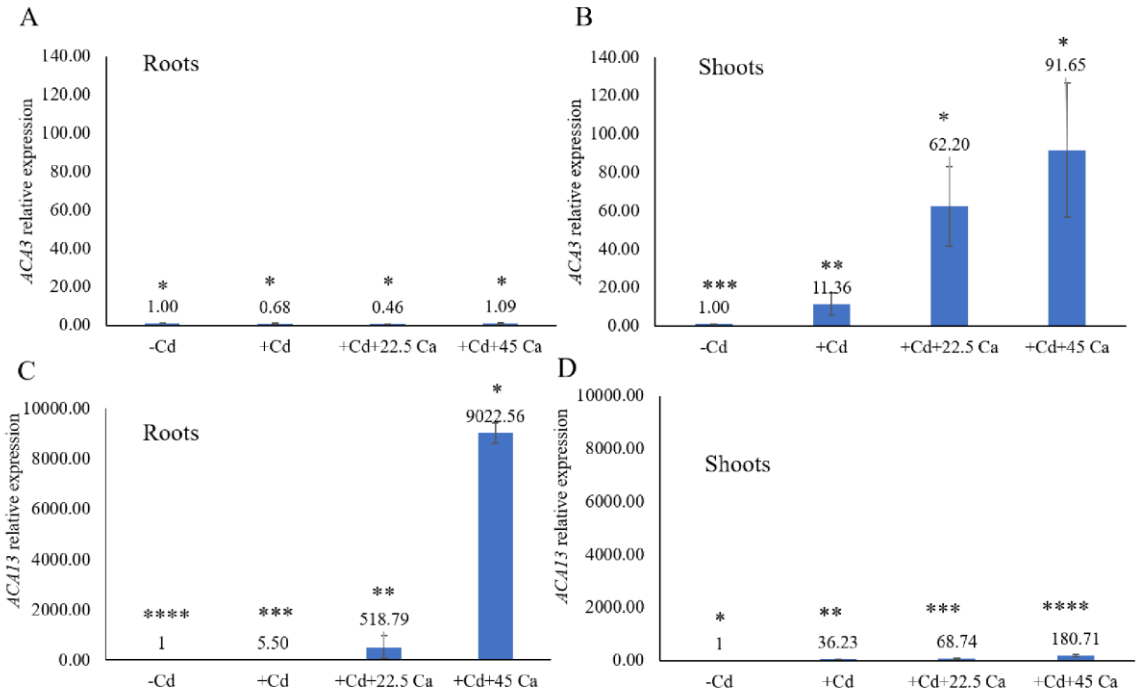

Fig 4.4. *ACA3* and *ACA13* relative expression in rice seedling roots and shoots (30 days old) grown in control (paddy soil without Cd) and treatments (Cd-contaminated soil containing an initial 82.3 ± 5.28 mg Cd/kg and Cd-contaminated soil containing an initial 82.3 ± 5.28 mg Cd/kg and supplemented with different Ca acetate concentrations (22.5 and 45 mM)) for 7 days at soil pH 6.5–7.0. Group classification by Fisher's LSD method at 95% confidence level (*, **, ***, ****).

The downstream generation of ROS (Beffagna *et al.* 2005) that occurs in response to the transient increase in cytosolic Ca^{2+} can cause phytotoxicity in plants (Wang *et al.* 2004; Heyno *et al.* 2008). In many crop plants, like rice, abiotic stresses, including Cd, lead to excessive production of ROS, causing plant cell membrane injury due to lipid peroxidation. Up-regulating of *ACAs* can effectively reduce the generation of ROS, by inducing antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, catalase, and glutathione peroxidase (Wang *et al.* 2004; Beffagna *et al.* 2005; Heyno *et al.* 2008). For example,

transgenic tobacco with *ACA6* can improve plant tolerance to Cd stress, by increasing the activity of ROS detoxification enzymes and increasing the non-enzymatic antioxidant content (glutathione and ascorbate) (Shukla *et al.* 2014). Although the role of *ACAs* in up-regulating of ROS detoxification enzymes has been well described (Shukla *et al.* 2014), few studies have presented the response of Cd transporters under the regulation of *ACAs*. In 2014, *OsACA6*-overexpressing tobacco growing under Cd-contaminated medium showed lower Cd concentration and Cd translocation in plant. This result suggested that signal transduction from *ACAs* does not only effect on ROS detoxification but can also effect to Cd uptake and translocation in plant (Shukla *et al.* 2014). Therefore, the current study focused on the role of *ACAs* in regulating Cd uptake and translocation.

Sasaki *et al.* (2012) identified low manganese (Mn) and Cd concentrations in both roots and shoots of an *OsNramp5*-knockout line of rice. Transgenic rice with *OsNramp5* can absorb more Mn and Cd than wild-type rice (Wu *et al.* 2016). Experiments confirmed that this transporter corresponded to the uptake of Cd and Mn from the environment to root cells. The expression of this gene was evident in rice roots while in the shoots, a very low *OsNramp5* expression level was detected (Ishimaru *et al.* 2012; Sasaki *et al.* 2012; Oono *et al.* 2014; Wu *et al.* 2016). *OsNramp5* expression in rice roots was highly decreased by Ca acetate when compared to rice grown without Ca acetate (Figure 4.5A). For rice shoots, the expression of *OsNramp5* was not detected (Figure 4.5B). The result implies the transporter is involved in Cd uptake from the environment to roots but is not related to Cd translocation in rice plants (Wu *et al.* 2016).

The *OsHMA2* gene is localized to the plasma membrane and transports Cd from the roots to the shoots, by pumping out the intracellular Cd in rice roots to the xylem and from the xylem to the phloem (Gao *et al.* 2016). This gene is mainly expressed in rice roots (Takahashi *et al.* 2012). Rice possesses nine *HMA* genes, which encode proteins crucial for transitional metal

homeostasis (Oono *et al.* 2014). Overexpression of *OsHMA2* enhanced the Cd defensive system in rice (Ueno *et al.* 2010). The present investigation revealed Ca acetate can control the level of *OsHMA2* expression in both roots and shoots, to close to that seen in rice grown under paddy soil without Cd (Figure 4.5C, D). A low level of *OsHMA2* expression might act to control Cd translocation and reduce Cd concentration in rice shoots (Gao *et al.* 2016).

OsLCT1 is a well-known non-specific cation transporter in rice, which is localized to the shoot node and is a key channel for Cd and Ca translocation in the rice plant. In addition, the rice node has been proposed as a key contributor to restricting Cd translocation in rice plant (Huang *et al.* 2017b). In the current research, *OsLCT1* was poorly expressed in the roots but was markedly up-regulated by Ca acetate in the shoots (Figure 4.5E, F).

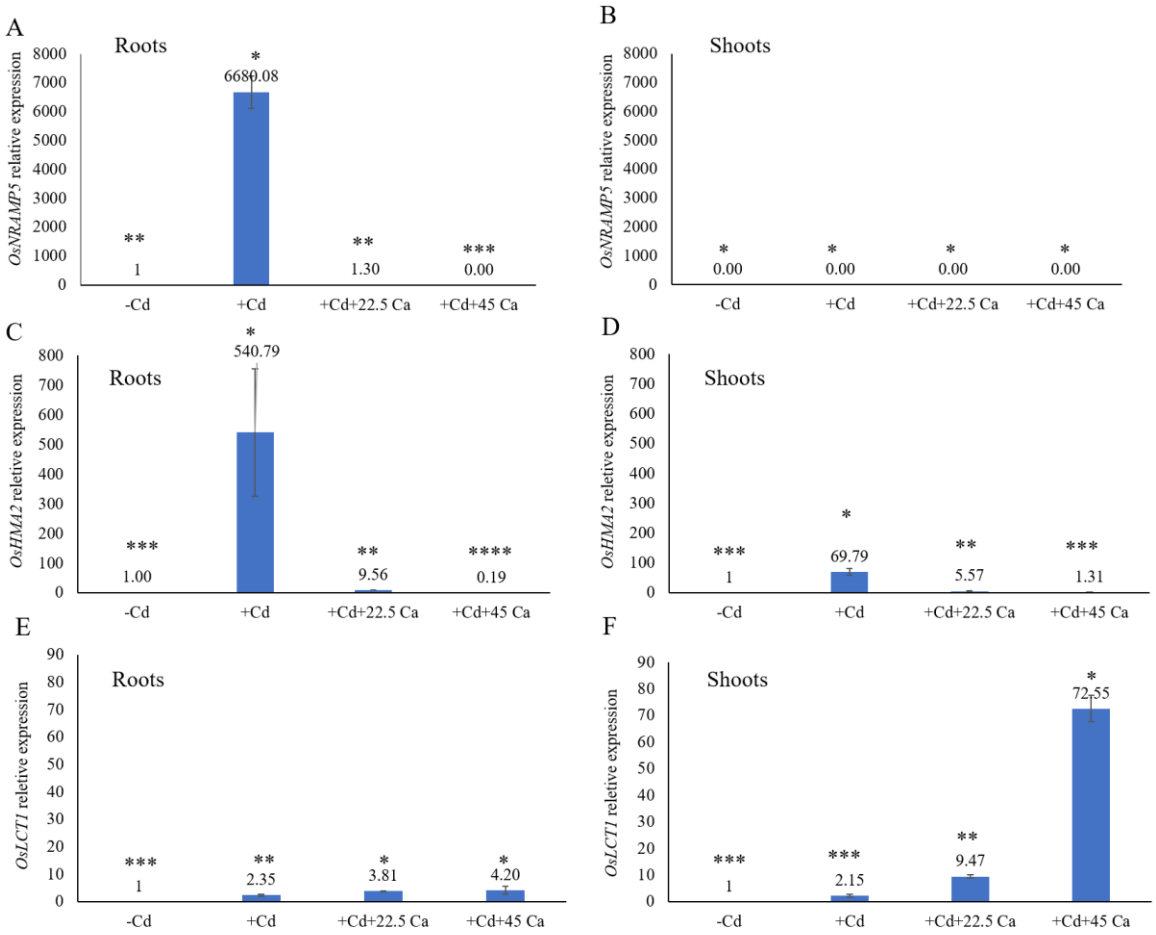

Fig 4.5. Relative expression of Cd transporter genes (*OsHMA2*, *OsNramp5*, and *OsLCT1*) in rice seedling roots and shoots (30 days old) grown in control (paddy soil without Cd) and treatments (Cd-contaminated soil containing 82.3 ± 5.28 mg Cd/kg and Cd-contaminated soil containing 82.3 ± 5.28 mg Cd/kg supplemented with different Ca acetate concentrations (22.5 and 45 mM)) for 7 days at soil pH 6.5–7.0. Group classification by Fisher's LSD method at 95% confidence level (*, **, ***, ****).

From the Cd concentrations and gene expressions, Ca acetate, in suitable amounts, showed the potential to be a soil amendment to inhibit Cd uptake and translocation in rice plants. The mechanism of Ca acetate to control Cd concentration in rice might relate to the functions of *ACA3* and *ACA13*. Up-regulation of these genes in the presence of Cd may be responsible for regulating the Cd transporters in rice seedlings. This study verified that the

addition of Ca acetate could down-regulate Cd transporters (*OsHMA2* and *OsNramp5*) at the transcription level, resulting in the lowering of Cd uptake and translocation.

Although Ca acetate up-regulated *OsLCT1* in node 1, because of the non-specific cation transporter property of this transporter, the translocation competition between Ca and Cd could also be an important mechanism to reduce Cd translocation during high Ca concentration in rice.

4.4. **Rice Productivity and Quality Analysis**

Various Ca acetate concentrations were applied to Cd-contaminated soil (82.3 ± 5.28 mg Cd /kg soil dry weight), to reduce Cd uptake by the plants and its transport to the rice grains. Ca acetate could promote rice growth under Cd-contamination as evidenced by the Fv/Fm and SPAD indicators (Figure 4.6A, B). At 60 days, Cd drastically reduced the Fv/Fm and SPAD values, whereas, in rice supplemented with Ca acetate, these two values were similar to those in the control plant without Cd stress (Figure 4.6C, D).

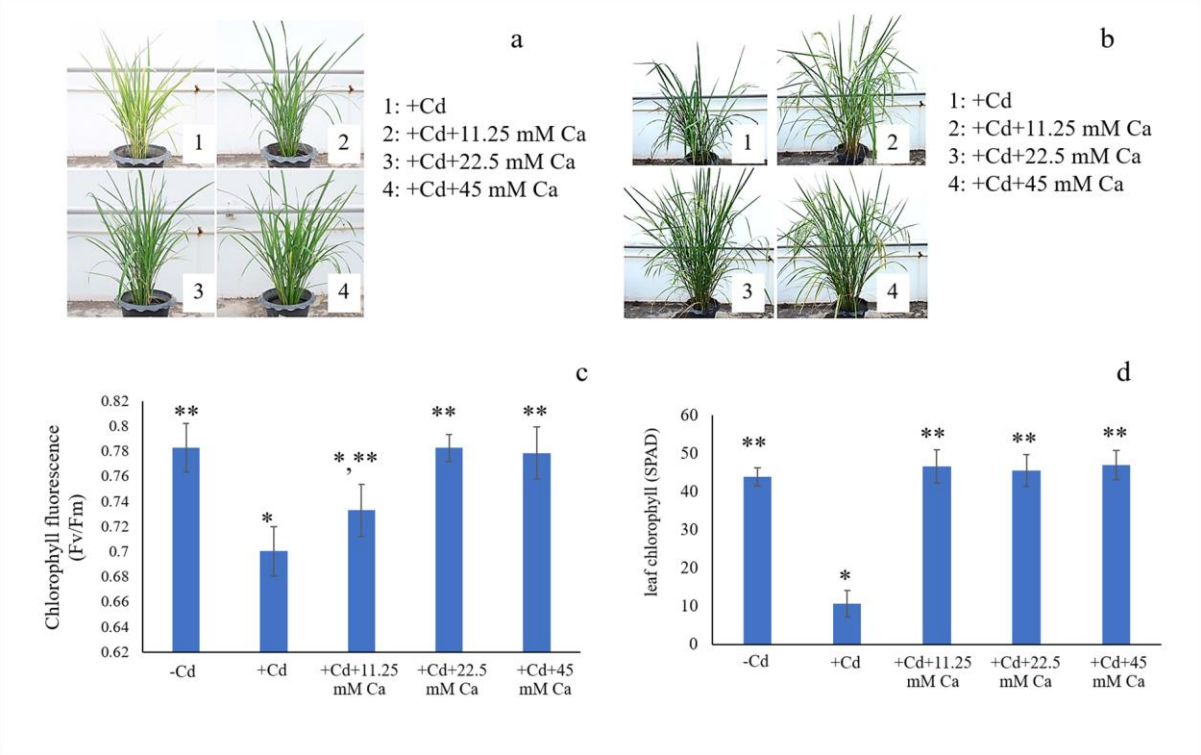

Fig 4.6. Rice growth, including appearance, at 60 (A) and 90 (B) days. Chlorophyll fluorescence (Fv/Fm) (C) and leaf chlorophyll (SPAD) (D) at 60 days under each condition. Rice was grown in control soil without Cd (-Cd) and in soil with an initial 82.3 ± 5.28 mg Cd/kg (+Cd) supplemented with Ca acetate at 11.25 mM (+Cd+11.25 mM Ca), 22.5 mM (+Cd+22.5 mM Ca), and 45 mM (+Cd+45 mM Ca). Group classification by Fisher's LSD method at 95% confidence level (*, **).

In addition, grain length and width could also be improved by Ca acetate (Figure 4.7A, B). When the rice was grown in Cd-contaminated soil, the productivity was highly reduced, probably due to Cd phytotoxicity (Wahid and Khaliq 2015; Okem *et al.* 2016; Ronzan *et al.* 2018). However, the supplementation of Ca acetate to Cd-contaminated soil slowed down the decrease in rice productivity. When 45 mM Ca acetate was added to Cd-contaminated soil, the grain weight per pot was not significantly decreased compared to rice grown under paddy soil

without Cd (Figure 4.7C). For the grain weight per 100 rice seeds, the result was also not significantly different with rice grown under paddy soil without Cd (Figure 4.7D).

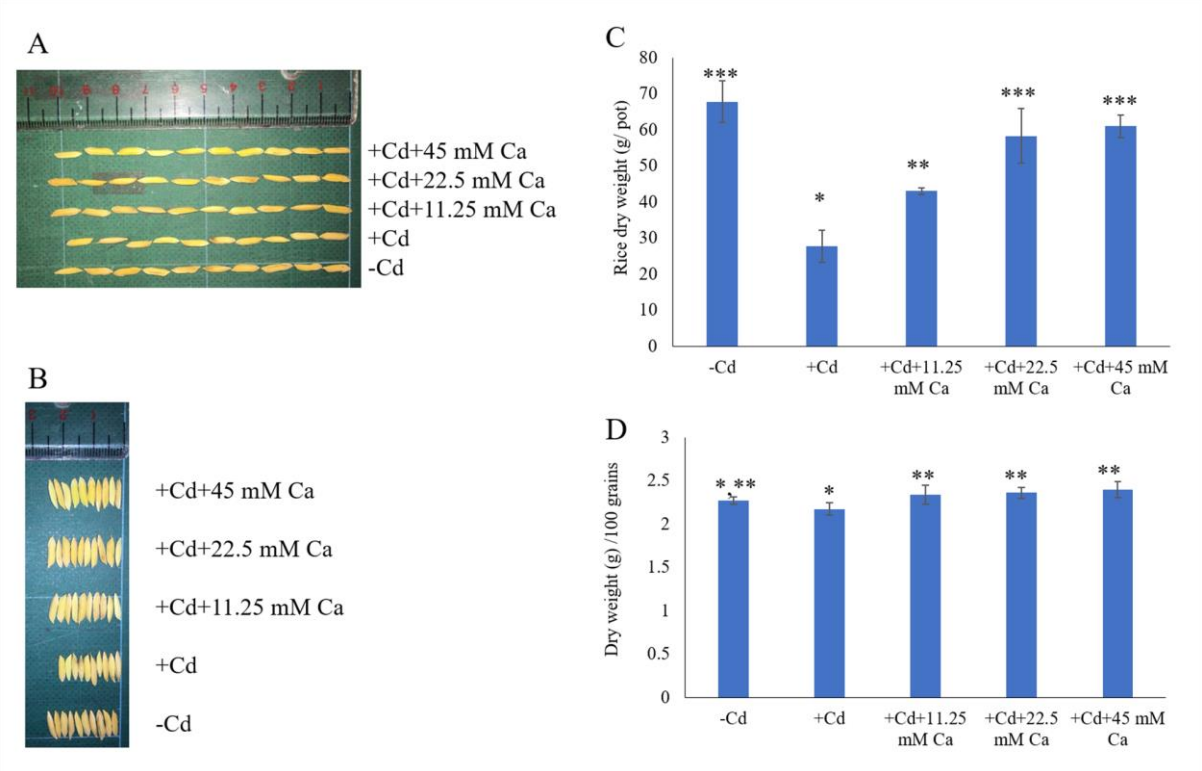

Fig 4.7. Rice productivity, including length (A) and width (B) of rice grains at 120 days. Weight of rice grain per pot (C) and dry weight of 100 rice grains (D) under each condition. Rice was grown in control soil without Cd (-Cd) and in soil with an initial 82.3 ± 5.28 mg Cd/kg (+Cd) supplemented with Ca acetate at 11.25 mM (+Cd+11.25 mM Ca), 22.5 mM (+Cd+22.5 mM Ca), and 45 mM (+Cd+45 mM Ca). Group classification by Fisher's LSD method at 95% confidence level (*, **, ***).

For Ca and Cd concentration in shoots and grains, the addition of Ca acetate effectively reduced Cd concentration in rice shoots (first leaf) and grains (Figure 4.8). Ca acetate lowered the Cd concentration in the plant, thereby decreasing Cd phytotoxicity and maintaining a high

rice grain productivity, close to that recorded in the rice grown under paddy soil (without Cd). Although the increase in Ca of rice shoots associated with the added Ca acetate was not apparent, the application of Ca acetate can help support Ca homeostasis in rice (Figure 8) (Aslam *et al.* 2017). This statement might explain why increasing the Ca supplementation in the soil does not increase the Ca concentration in rice shoots and grains, in a long-term application. However, increasing the Ca acetate amount might induce the expression of *ACAs*, especially *ACA3* and *ACA13*, to improve rice development under Cd stress. Up-regulation of these two *ACAs* can regulate Cd transporters, resulting in inhibition of Cd uptake and translocation and lowering the Cd content in rice plants.

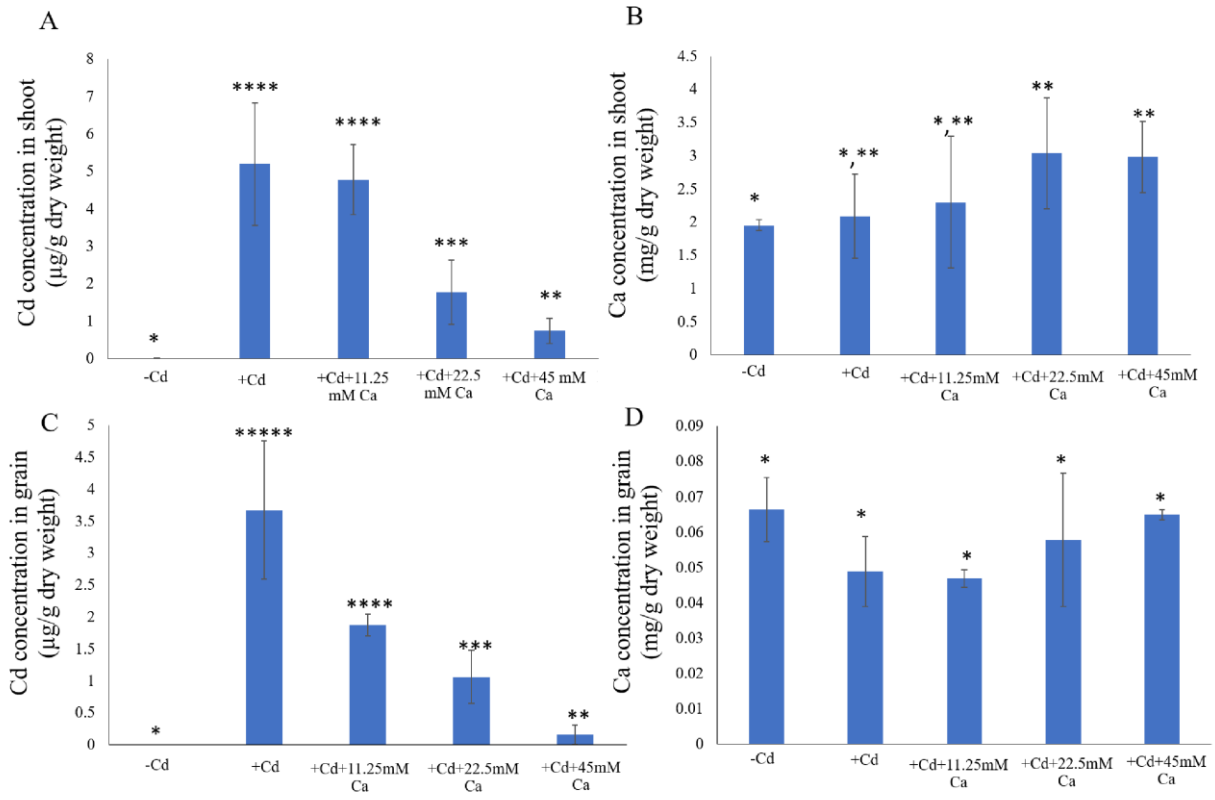

Fig 4.8. Cd and Ca concentration in rice shoots and grains at 120 days growth. Rice was grown in control soil without Cd (-Cd) and in soil with an initial 82.3 ± 5.28 mg Cd/kg (+Cd) and supplemented with Ca acetate at 11.25 mM (+Cd+11.25 mM Ca), 22.5 mM (+Cd+22.5 mM

Ca), and 45 mM (+Cd+45 mM Ca). Group classification by Fisher's LSD method at 95% confidence level (*, **, ***, ****).

The presented results confirmed that Ca acetate can effectively inhibit Cd uptake and translocation, and this compound can be applied to rice cultivated in Cd-contaminated paddy fields.

Chapter 4
Conclusions

Supplementation of Cd-contaminated soil with Ca acetate reduced the Cd concentration in rice plant. The high Ca concentration in the plant cells induced the expressions of the *ACA3* and *ACA13* genes, which function to drive primary signal transduction under Cd stress. Increasing the Ca acetate concentration inhibited the Cd transporters (*OsNRAMP5* and *OsHMA2*) at the transcription level, resulting in decreased Cd uptake and translocation. In addition, Ca and Cd showed the possible translocation competition in rice plants from the roots to shoots, via *OsLCT1*. Therefore, the application of Ca acetate to reduce Cd concentration in rice plants grown under Cd-contaminated soil has the potential to be used in real fields. In the pot experiment, Ca acetate reduced the Cd concentration effectively and protected the plant from Cd phytotoxicity. Ca acetate also seemed to increase the quality of rice and its productivity. For further study, the reaction between Ca acetate and Cd and effect of Ca acetate on Cd solubility can be interesting topic to explain the effect of Ca acetate outside plant that might probably involve the reduction of Cd uptake into rice. In addition, the application of agricultural waste containing high Ca acetate contents needs to be developed for real rice field application.

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Appendix