

GAMMA-AMINOBUTYRIC ACID CONTENTS AND GLUTAMATE DECARBOXYLASE ACTIVITY
IN LOCAL THAI RICE AND TRANSGENIC RICE OVEREXPRESSING *OsCam1-1* GENE



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ปริมาณแกมมาเอมีโนบิวทริกแอซิดและแอกติวิตีของกลูตามิกดีคาร์บอกซิเลสในข้าว *Oryza sativa*
L. พื้นเมืองไทยและในข้าวทรานสเจนิกที่มีการแสดงออกของยีนคัลมอดูลิน *OsCam1-1*



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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โพธิธรรม์ ครรชิตานุรักษ์ : ปริมาณแกมมาเอมิโนบิวทริกแอซิดและแอกติวิตีของกลูตามิกดีคาร์บอกซิเลสในข้าว *Oryza sativa* L. พันธุ์เมืองไทยและในข้าวทรานส์เจนิคที่มีการแสดงออกของยีนคล่อมอดูลิน *OsCam1-1*. (GAMMA-AMINO BUTYRIC ACID CONTENTS AND GLUTAMATE DECARBOXYLASE ACTIVITY IN LOCAL THAI RICE AND TRANSGENIC RICE OVEREXPRESSION *OsCam1-1* GENE) อ.ที่ปรึกษาหลัก : รศ. ดร.ธีรพงษ์ บัวบูชา, อ.ที่ปรึกษาร่วม : ศ. ดร.ศุภจิตรา ชัชวาลย์, ผศ. ดร.มนนัทธ์ พงษ์พานิช

ในเมล็ดข้าวมีสารอาหารจำนวนมากที่เป็นประโยชน์ กากาเป็นผลิตภัณฑ์ของวิถีเมแทบอลิซึมจากกระบวนการ decarboxylation ของ L-glutamic acid (L-Glu) ซึ่งเร่งปฏิกิริยาโดย glutamate decarboxylase (GAD) ที่เป็นเอนไซม์ในไซโทซอลซึ่งควบคุมโดยโปรตีน Ca^{2+} -calmodulin (CaM) การสะสมของกากาเกี่ยวข้องกับกิจกรรมของ GAD และความเข้มข้นของสารตั้งต้น L-Glu ในการศึกษาการวิเคราะห์ปริมาณ GABA ในข้าวพันธุ์พื้นเมือง (*Oryza sativa* L.) ระหว่างการงอกที่ 0-72 ชม. พบว่าความเข้มข้นของกากาเพิ่มขึ้นอย่างรวดเร็ว และรูปแบบการเพิ่มขึ้นแตกต่างกันไปตามพันธุ์ต่าง ๆ จากการทำ GWAS โดยใช้ 2,009,083 SNPs ในประชากรข้าวพื้นเมืองจำนวน 100 พันธุ์ตามปริมาณของ GABA ที่ 48 ชั่วโมงหลังการงอกสามารถระบุ SNPs จำนวน 23 ตำแหน่งซึ่งอยู่ในหรือใกล้เคียงกับยีนจำนวน 5 ยีน โดย LOC_Os02g40320, LOC_Os02g40330 และ LOC_Os11g35580 เป็นยีนที่มี SNP ในบริเวณเข้ารหัส นอกจากนี้ยังพบ SNP ที่อยู่บริเวณ upstream ของยีน LOC_Os01g18390 และ LOC_Os04g13480 โดยจากการวิเคราะห์ด้วย STRING ของโปรตีนที่ถอดรหัสจาก LOC_Os02g40320 พบว่าเกี่ยวข้องกับโปรตีนอื่น ๆ จำนวนมาก ซึ่งรวมถึงไนเตรดรีดักเตส 1 (NIA1) และซูโครสซินเทส 6 (SUS6) และพบว่าโปรตีนที่มีโดเมนตัวรับตัวควบคุมการตอบสนองซึ่งถอดรหัสโดย LOC_Os04g13480 และทำงานในวิถีการส่งสัญญาณ His-to-Asp phosphorelay มีปฏิสัมพันธ์กับโปรตีนหลายชนิดใน two-component system ซึ่งตอบสนองต่อไซโตไคนิน โดยก่อนหน้านี้นี้มีรายงานแสดงให้เห็นแล้วว่าไซโตไคนินอาจมีหน้าที่เกี่ยวข้องกับการสะสม GABA

เพื่อตรวจสอบบทบาทของคล่อมอดูลินในการสะสมกากา ปริมาณของกากาในข้าวพันธุ์ข้าวดอกมะลิ 105 ทรานส์เจนิคที่มียีน *OsCam1-1* แสดงออกเกินปกติ (L1, L2 และ L7) ได้รับการตรวจสอบเปรียบเทียบกับข้าวพันธุ์ข้าวดอกมะลิ 105 wild type (WT) ซึ่งมีความทนทานต่อเกลือต่างกัน ปริมาณกากาในข้าวทรานส์เจนิคที่มียีน *OsCam1-1* แสดงออกเกินปกติเพิ่มขึ้นใน 24 ชั่วโมงแรกของการงอกขณะที่ WT ไม่เพิ่ม ขณะที่ภายใต้ความเครียดจากเกลือ (100 mM NaCl) ระดับของกากาเพิ่มขึ้นเล็กน้อยและไม่แตกต่างกันในแต่ละสายพันธุ์ หลังจาก 4 วันที่มีการงอกที่มองเห็นได้ภายใต้สภาวะปกติและสภาวะความเครียดจากเกลือพบว่าปริมาณกากาในข้าวทั้งหมดเพิ่มขึ้นอย่างรวดเร็ว โดยปริมาณกากาภายใต้สภาวะความเครียดจากเกลือยังคงเพิ่มขึ้นอย่างต่อเนื่องในวันที่ 6 และ 8 ในข้าวทรานส์เจนิคที่มียีน *OsCam1-1* แสดงออกเกินปกติทำให้มีระดับที่สูงกว่า WT หลังจากผ่านไป 10 และ 12 วัน ระดับกากาในสภาวะปกติก็เพิ่มขึ้นอย่างรวดเร็วในขณะที่ระดับกากาภายใต้ความเครียดจากเกลือเมื่อต้นกล้าได้รับผลกระทบรุนแรงลดลงอย่างมีนัยสำคัญ นอกจากนี้พบว่าแอกติวิตีของ GAD ในข้าวทรานส์เจนิคและ WT เพิ่มขึ้นในสัปดาห์แรกของการงอก และแอกติวิตีในข้าวแปลงพันธุ์นั้นสูงกว่า WT ในวันที่ 12 หลังการงอก ผลลัพธ์เหล่านี้ร่วมกันบ่งชี้ว่าระดับกากาที่สูงขึ้นในข้าวทรานส์เจนิคที่มียีน *OsCam1-1* แสดงออกเกินปกติอาจเป็นผลมาจากแอกติวิตี GAD ที่เหนี่ยวนำทำให้ข้าวตัดแปรพันธุ์กรรมมีความสามารถในการทนต่อเกลือได้ดีขึ้น

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Potitorn Kanchitanurak : GAMMA-AMINOBUTYRIC ACID CONTENTS AND GLUTAMATE DECARBOXYLASE ACTIVITY IN LOCAL THAI RICE AND TRANSGENIC RICE OVEREXPRESSING *OsCam1-1* GENE. Advisor: Assoc. Prof. TEERAPONG BUABOOCHA, Ph.D. Co-advisor: Prof. SUPACHITRA CHADCHAWAN, Ph.D., Asst. Prof. MONNAT PONGPANICH, Ph.D.

In rice grain, many considerable nutrients are shown to have potential functional food benefits. GABA is a metabolic product that is primarily produced by the decarboxylation of *L*-glutamic acid (*L*-Glu), catalyzed by glutamate decarboxylase (GAD), which is a cytosolic enzyme regulated by Ca^{2+} -calmodulin (CaM) complex. The accumulation of GABA is related to the activity of GAD and substrate concentration of *L*-Glu. In this study, analysis of GABA contents in local Thai rice (*Oryza sativa* L.) during 0-72 h of germination revealed that GABA concentrations rapidly increased and the pattern of increment varied among different rice varieties. Genome-wide association mapping using 2,009,083 SNPs from a population of 100 varieties based on the GABA concentrations 48 hours after germination was carried out. As a result, 23 SNPs associated to five genes were identified. LOC Os02g40320, LOC Os02g40330, and LOC Os11g35580 were three genes having significant SNPs in their coding regions. The additional genes with upstream significant SNPs were LOC_Os01g18390 and LOC_Os04g13480. The protein encoded by LOC_Os02g40320 was related to numerous other proteins by STRING analysis, including nitrate reductase 1 (NIA1) and sucrose synthase 6. (SUS6). The response regulator receiver domain protein, which is encoded by LOC_Os4g13480 and functions in the His-to-Asp phosphorelay signal transduction pathway, was discovered to interact with a number of proteins in the two-component system responding to cytokinin, which has previously been shown to have a functional relationship with GABA.

To investigate the role of calmodulin in GABA accumulation, GABA contents in the three transgenic 'Khoa Dawk Mali 105' (KDML105) rice overexpressing *OsCaM1-1* gene (L1, L2, and L7) were examined compared with the wild-type 'KDML105' (WT), which are differing in salt tolerance. Under normal condition, GABA contents in the transgenic rice overexpressing *OsCaM1-1* calmodulin gene increased in the first 24 hr of germination while that of WT did not. Under salt stress (100 mM NaCl), their levels slightly increased and were not different among the rice lines. After 4 days with visible sprouting under both normal and salt stress conditions, GABA contents in all rice lines rapidly increased. GABA contents under salt stress condition continued to increase and reached higher levels at day 6 and 8 in all transgenic overexpressing lines when compared with that of WT. After 10 and 12 days, GABA levels under normal condition then rapidly increased whereas those under salt stress when the seedlings were severely affected significantly decreased. In addition, GAD activity in all transgenic rice lines and WT appeared to increase in the first week of germination and those of the transgenic rice were higher than WT at day 12 after germination. Together these results indicated that higher GABA content in the transgenic rice overexpressing *OsCaM1-1* gene may result from the induced GAD activity, which in turn confers the salt tolerance ability to the transgenic rice.

Field of Study: Biotechnology

Academic Year: 2022

Student's Signature

Advisor's Signature

Co-advisor's Signature

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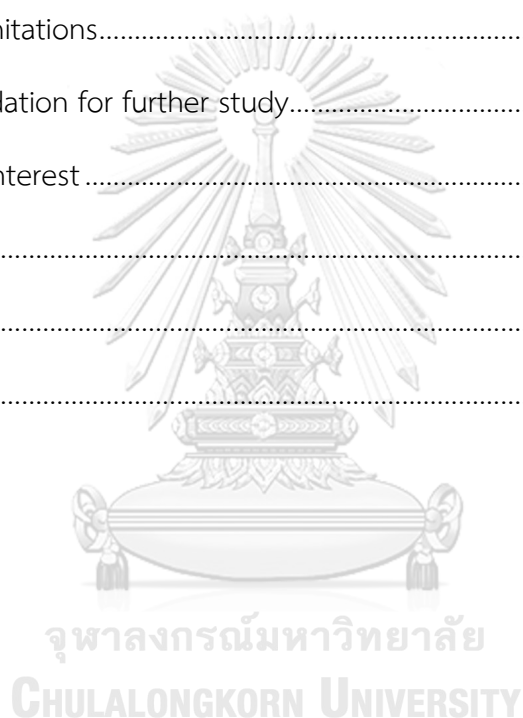
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CHAPTER I

INTRODUCTION

Examination of γ -aminobutyric acid (GABA) content in local Thai rice and identification of genes involved by GWAS can lead to the knowledge of GABA accumulation in local Thai rice varieties, which are valuable genetic resources. GABA content in plants is associated with the activity of glutamate decarboxylase (GAD), which is regulated by Ca^{2+} -calmodulin (CaM) complex, resulting in increased accumulation of GABA. Therefore, GABA content and activity of glutamate decarboxylase during germination were studied in various local Thai rice (*Oryza sativa* L.) varieties and the transgenic rice overexpressing a calmodulin: *OsCam1-1* gene, where two research articles are parts of the dissemination of this thesis.

Rice is the major staple food of world population, especially in Asia. In a small grain of rice, many considerable nutrients are shown to have potentially functional food benefits (Luangmalawat *et al.*, 2008). The majority of the nutrients found in the white polished grain is carbohydrate while proteins, vitamins and minerals are present in rice bran. These nutrients include gamma oryzanol, tocopherol, tocotrienol, and GABA. An accumulation of nutrients also originates from digestion of grain proteins, which results in amino acids, and peptides.

In plant, GABA is a metabolic product that is primarily produced by the decarboxylation of *L*-glutamic acid (*L*-Glu), catalysed by GAD (Bouche & Fromm, 2004). Though many studies have been done to improve GABA production in rice. In the past, substantial research has been conducted on GABA accumulation in japonica and indica germinated brown rice related to GABA shunt. In previous studies, soaking at 30 °C and germination at 35 °C during 36 h resulted in the highest GABA in distilled soaking water with pH 7 (Zhang *et al.*, 2014). Besides, GABA content in 'Khao Dawk Mali 105' rice (Tipkanon, 2014) is affected by many factors. Four factors, which were pH, CaCl₂ concentration, soaking temperature, and soaking time, were tested using the eight-run Plackett-Burman design. The results indicated that GABA content in the rice germ soaked at 40°C for 8 h was highest (307.1 mg/100 g of rice germ, dry basis) (Tipkanon, 2014). Moreover, the accumulation of GABA in the rice germ by protease was investigated. After the proteolytic hydrolysis of germ protein by trypsin, the amount of GABA reached about 2.26 g per 100 g of germ. This demonstrates that the GABA yield could be significantly increased (Zhang *et al.*, 2006).

1.1 Introduction to the research problem and its significance

1.1.1 GABA in plants

In the plant, GABA is produced by the decarboxylation of *L*-glutamic acid (*L*-Glu), catalyzed by glutamate decarboxylase (GAD) (Bouche & Fromm, 2004). Its

accumulation is related to L-Glu concentration and GAD activity (Zhang et al., 2014). GAD is a cytosolic enzyme regulated by the Ca^{2+} - calmodulin (CaM) complex, which catalyses the irreversible decarboxylation of glutamate to produce GABA. Then, GABA is converted into succinic semialdehyde by GABA transaminases using either α -ketoglutarate or pyruvate as amino acid acceptors in mitochondria. This organic substance can enter tricarboxylic acid cycle after the conversion to succinate by succinic semialdehyde dehydrogenase (Zhang et al., 2016). Under stress, GAD activity undergoes changes of catalytic properties mediated by calcium/calmodulin in rapid responses. Previous study found that rice plants overexpressing *OsGAD* gene were able to accumulate GABA at high concentration under short-term salinity (Shimajiri et al., 2013).

1.1.2 Research significance

Forward and reverse genetics have been performed to identify loci/genes such as genetic mapping of quantitative trait loci (QTLs) using cross population (Wu et al., 2005). Nowadays, functional genomics and molecular breeding studies are based on the high-throughput sequencing information, including the genome-wide association study (GWAS) (Guo et al., 2014). A number of traits in rice, including numerous agronomic characteristics and biosynthesis of metabolites have been analysed using GWAS (Huang et al., 2012; Yano et al., 2016). Thus, to fill the knowledge gap between genotypes and phenotypes of GABA synthesis in rice seeds, one of the objectives of this research is to investigate GABA contents and GAD

activity in local Thai rice varieties. These data on different rice accessions will be clustered based on the physiological responses and used to perform GWAS to identify regions/genes responsible for the trait. Furthermore, to investigate the role GABA synthesis on improved tolerance under salt stress in the transgenic rice overexpressing *OsCam1-1* gene, GABA contents and GAD activity of the overexpressing rice and the wild-type 'KDML105', which are different in salt tolerance will be compared.

1.2 Objectives

1.2.1 To Investigate GABA content in local Thai rice and perform a genome-wide study of genetic variants in different varieties to see if any variants are associated with the trait

1.2.2 To investigate GABA accumulation and GAD activity associated with the improved tolerance under salt stress in the transgenic rice overexpressing *OsCam1-1* gene compared with the wild-type 'KDML105'

1.3 Research scope

1.3.1 GABA contents during germination were examined in 100 local Thai rice varieties and GWAS on the genome-wide set of genetic variants in this population was carried out based on the GABA contents.

1.3.2 GABA contents and GAD activity under normal and stress conditions were examined in the transgenic rice lines overexpressing *OsCam1-1* gene compared with the wild-type 'KDML105'.

1.4 Beneficial outcome from research

Information on GABA levels during germination in different Thai landrace rice varieties and the involvement of calmodulin on its accumulation in the transgenic rice overexpressing *OsCam1-1* gene would be obtained, which can be applied for selection of local Thai rice varieties and production of GABA for commercial use.



CHAPTER II

MANUSCRIPTS

2.1 Manuscript I

Genome-wide association mapping of GABA contents during germination in local Thai rice (*Oryza sativa* L.) population.

(Preparing to publish on Scientific Reports (Q1))

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2.1.1 Abstract

Gamma-aminobutyric acid (GABA) and other beneficial components of germinated rice make it an invaluable functional food. We surveyed 100 local Thai rice accessions to determine the GABA levels accumulated during germination. GABA contents in the grains ranged from 5.02–10.82 $\mu\text{g}/\text{grain}$ while those at 48 h after germination ranged from 6.72–17.69 $\mu\text{g}/\text{grain}$. The increase in GABA content varied among the varieties ranged from 0.49–11.81 $\mu\text{g}/\text{grain}$ with the average at 3.78 $\mu\text{g}/\text{grain}$. Based on these parameters, 2,009,083 SNPs identified from this population were used for GWA mapping, and 23 SNPs associated with five genes on chromosomes 1, 2, 4, and 11 were identified. They were highly expressed in the endosperm especially LOC_Os02g40330 and LOC_Os4g13480. STRING analysis revealed that the protein encoded by LOC_Os02g40330 was associated with proteins involved in carbon and nitrogen metabolism and metabolic processes, including nitrate reductase and sucrose synthase. A response regulator receiver domain containing protein encoded by LOC_Os4g13480 interacted with proteins involved in the two-component system responding to cytokinin, which was previously shown having a functional connection with GABA. The knowledge on the varieties with high GABA content and the genes involved in GABA accumulation will greatly benefit future improvement of GABA production.

2.1.2 Introduction

Rice is a great source of energy, is abundant in carbohydrates, and acts as a fuel for the body. It is a central component of many cuisines including those in Thailand, India, China, and Southeast Asia. Therefore, it is unsurprising that there are growing concerns about how rice production can cope with the increasing global population (Charoenthaikij et al., 2009). There are many types of rice, which can be categorized by shape (long seed or short seed) or color (white or brown). Brown rice (BR), which is unmilled or partially milled, contains more nutrients than plain white rice. It has a high content of bioactive compounds and is rarely consumed as a staple food due to its dark color and hard texture (Wu et al., 2013). BR has a higher content of non-starch nutrients, such as proteins, minerals, fatty acids, dietary fiber, and phenolic compounds, than polished rice. These nutrients are concentrated in the bran layer (Yan et al., 2020). Germination is a technique used to improve the texture of cooked BR. It also causes numerous changes in the composition and chemical structure of the bioactive components. Consumption of germinated BR, which contains bioactive compounds such as gamma-aminobutyric acid (GABA) has increased in many Asian countries due to improvements in eating quality and health-promoting function (Cho & Lim., 2016). GABA biosynthesis must be optimized to produce food products with enriched GABA content (Diana et al., 2014). According to reported data, BR, barley, and corn have higher concentrations of GABA than other grains (Ramos-Ruiz et al., 2018). GABA-containing products have gained attention,

giving rise to many GABA-rich foods such as sprouted BR and fermented milk products (Ou et al., 2009; Park & Oh, 2007).

GABA is found in both unicellular and multicellular organisms and is involved in many aspects of the plant life cycle (Michaeli & Fromm, 2015). It is a non-protein amino acid, an essential component of the free amino acid pool in living organisms (Ramos-Ruiz et al., 2019), and is a signaling molecule that acts as an inhibitory neurotransmitter (Bown & Shelp, 2016). In plants, GABA is mainly involved in growth and development through the GABA shunt, which bypasses the TCA cycle. GABA is synthesized from glutamate by irreversible decarboxylation, and catalyzed by glutamate decarboxylase (GAD) in the cytosol. GABA is transferred to the mitochondria and then subjected to succinic semialdehyde (SSA) conversion by GABA transaminase (GABA-T) (Li et al., 2021). GABA accumulates rapidly in plant tissues such as during seed soaking in water (Saikusa et al., 1994; Varayanond et al., 2005), or in response to environmental stress (Roberts, 2007) including heat stress (Nayyar et al., 2014), and cold stress (Yoon et al., 2017). Increasing GABA concentrations enhance plant stress resistance by reducing the production of reactive oxygen species (ROS), promoting photosynthesis, and controlling stomatal opening under drought stress (Li et al., 2021).

Forward and reverse genetics have been used to identify loci/genes, such as the genetic mapping of quantitative trait loci (QTLs) using cross populations (Wu et

al., 2005). Currently, functional genomic and molecular breeding studies are based on high-throughput sequencing information, including genome-wide association studies (GWAS) (Guo et al., 2014). Several traits in rice, including numerous agronomic characteristics and biosynthesis of metabolites, have been analyzed using GWAS (Huang et al., 2012; Yano et al., 2016). Thus, to fill the knowledge gap between genotypes and phenotypes of regulation of GABA biosynthesis in rice seeds, the objective of this study was to identify genetic variants that potentially contribute to GABA biosynthesis in local Thai rice varieties by GWAS. These data on different rice accessions were clustered based on GABA content during germination and used to perform GWAS to identify regions/genes responsible for GABA accumulation.

2.1.3 Methods

2.1.3.1 Chemicals and plant materials

The chemicals used in this study, including γ -aminobutyric acid (GABA) (purity $\geq 99\%$), nicotinamide adenine dinucleotide phosphate (NADP⁺), 5-sulfosalicylic acid dihydrate (C₇H₆O₆S·2H₂O), and *Pseudomonas fluorescens* GABase (0.97 units/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA) while disodium 2-Oxoglutarate (C₅H₄Na₂O₅), DL-dithiothreitol, and sodium sulfate anhydrous (Na₂SO₄) were obtained from TCI (Tokyo, Japan), Vivantis (Selangor Darul Ehsan, Malaysia), and KEMAUS (Cherrybrook, Australia), respectively. A total of 100 local Thai rice varieties were obtained from the Pathum Thani Rice Research Center, Pathum Thani Province,

Thailand. The 100 different rice cultivars used in this study were harvested during cultivation in December 2020.

2.1.3.2 Sample preparation

Ten rice varieties Leuang Tah Aiam, Ta Nod, Tawng Mah Eng, Khao Tod Lawng, Leb Nok, Khao' Glam, Mali Daeng, Mali Dam, Gam Nai Prom, and Daeng Hawm Gulahb were selected for examination of GABA contents at varying time points. Germination method, according to Kamjijam et al. (2020), was conducted in a controlled growth chamber at 30 °C with 16/8 h light/dark photoperiod for 0, 24, 48, and 72 h using 10 grains at each time point. Deionized water was replaced every 24 h during incubation. At each time point, samples were harvested in liquid nitrogen and stored at -80 °C. The experiments were conducted in five replicates. For GWAS, one hundred local Thai rice varieties (Supplementary Table S1) were germinated for 48 h in the same conditions and samples were prepared as described above.

2.1.3.3 Extraction of GABA

Samples were ground in liquid nitrogen, and the frozen powdered samples were extracted with 0.3% (w/v) sulfosalicylic acid, and vortexed for 20 s. They were then sonicated for 20 min, and centrifuged at 4,500 rpm for 20 min at 4 °C. The samples were then filtrated using a 0.2- μ m cellulose acetate membrane and the supernatant was transferred to a new tube (500 μ L). GABA content was measured using the GABase method (Nonaka et al., 2017; Kittibunchakul et al., 2017).

2.1.3.4 Statistical analysis

GABA content is expressed as mean±standard deviation. Analysis of Variance (ANOVA) was used to determine the differences among the mean values. When significant differences were detected, the mean values were compared using Tukey's HSD. Statistical analysis was performed at a significance level ($P < 0.05$).

2.1.3.5 Genome-wide association mapping

One hundred local Thai rice varieties' short reads from the Illumina Genome Analyzer were aligned against the reference genome using Burrow-Wheeler Aligner (BWA version 0.7.17) (Li & Durbin, 2009). The genomic analysis toolbox (GATK version 4.1.6.0) was used to identify variations (McKenna et al., 2010). PLINK 1.07 was used to eliminate SNPs with a minor allele frequency of less than 5% (Purcell et al., 2007) or when more than 10% of the varieties lacked the genetic information. The ungenotyped markers produced were imputed using Beagle 5.0. (Browning et al. 2018). Based on the phenotypic and SNP data, genome-wide association (GWA) mapping was performed using GEMMA software (Zhou., 2014). A Manhattan schematic was produced using the R 'qqman' package, with the chromosome position on the X-axis versus the $-\log(p\text{-values})$ of all SNPs. A quantile-quantile (Q-Q) diagram of the observed p-values in relation to the expected p-values was also created. SNP marker's p-value must be less than 0.05 to be considered as significant after adjusting for multiple hypothesis testing.

2.1.4 Results

2.1.4.1 GABA contents in Thai rice during germination

To survey GABA accumulation in local Thai rice grains during germination, 10 selected varieties were germinated for 0, 24, 48, and 72 h (Supplementary Figure S1), and their GABA content was examined (Figure 1). Five varieties were white rice ('Leuang Tah Aiam', 'Ta Nod', 'Tawng Mah Eng', 'Khao Tod Lawng', and 'Leb Nok') and the other five varieties ('Khao Glam', 'Mai Daeng', 'Mali Dam', 'Gam Nai Prom', and 'Daeng Hawm Gulahb') were colored rice. The results showed that GABA content increased in all 10 rice varieties, mostly from 48 h. No difference was observed between the white rice and colored rice groups. At 0 h, the GABA content in the grains of all 10 varieties was similar, ranging from 6–7 $\mu\text{g}/\text{grain}$. Except for 'Mali Dam', the GABA content in all varieties did not increase after 24 h of soaking in water. An increase in GABA content was observed from 24 to 48 h at different degrees among the rice varieties except Leb Nok and Mali Dam. From 48 to 72 h, GABA content continued to increase in most varieties and reached different levels, ranging from 9–12 $\mu\text{g}/\text{grain}$. As a result, to represent their potential for GABA accumulation, GABA contents at 48 h after soaking were used, as some varieties showed only slightly increased contents at this time or did not increase at all, while others exhibited substantial increment.

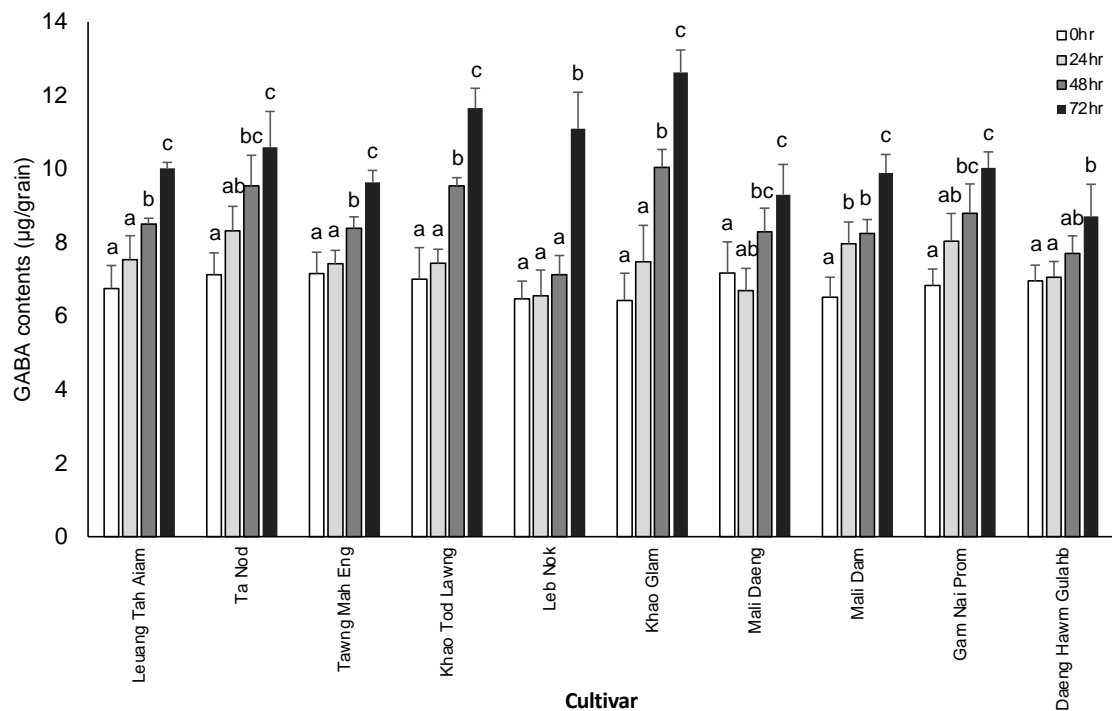


Figure 1 GABA contents in the 10 rice varieties after soaking at 30 °C for 0–72 h.

GABA content levels of each variety at 0, 24, 48 and 72 hr after germination were compared. The different letters above the bars represent the significant difference among means of the same variety by DMRT at $p < 0.05$.

2.1.4.2 Phenotypic variation of GABA content among Thai rice varieties

Figure 2 shows the GABA content in 100 local Thai rice varieties at 48 h after the grains were soaked with water (48 h) to start the process of germination compared with those at the start of water soaking (0 h). GABA contents at 0 h ranged from 5.02 to 10.82 $\mu\text{g}/\text{grain}$ while those at 48 h ranged from 6.72 to 17.69 $\mu\text{g}/\text{grain}$ (Table 1). The box plots (Figure 3a) demonstrate the distribution of the GABA contents of the 100 Thai rice varieties at 0 h and 48 h, and the increased contents

during 0–48 h of water soaking. The increase in GABA content from 0 to 48 h after soaking varied among the varieties ranged from 0.49 to 11.81 $\mu\text{g}/\text{grain}$ with the average at 3.78 $\mu\text{g}/\text{grain}$ (Table 1 and Figure 3a).

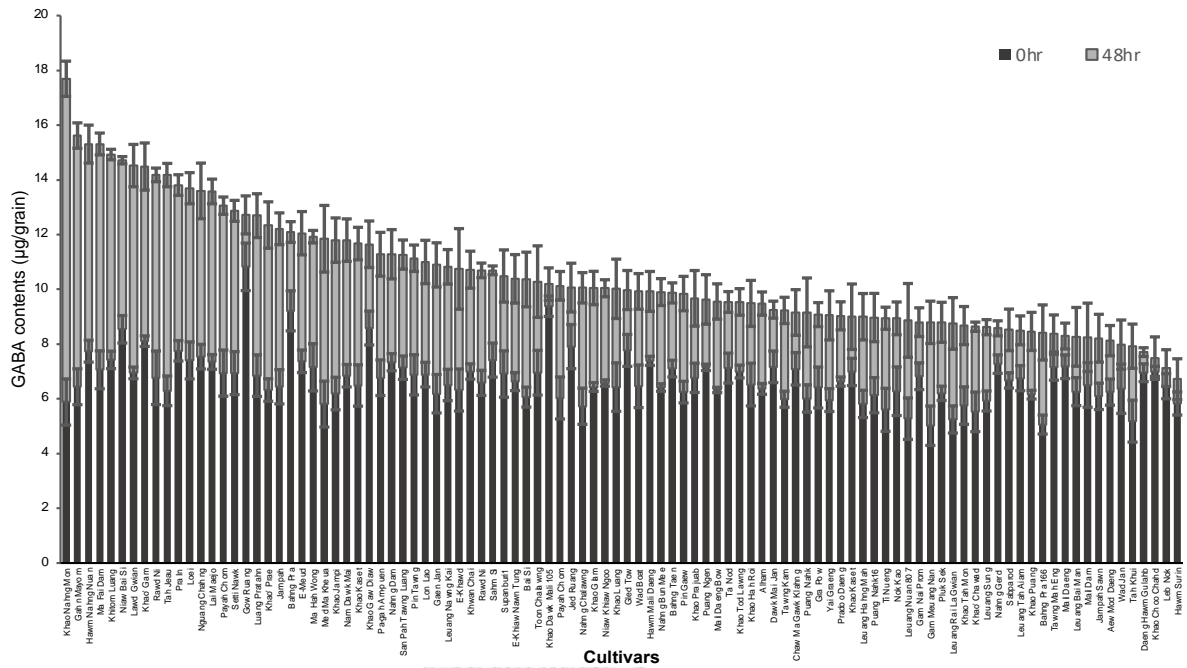


Figure 2 GABA contents in 100 local Thai rice varieties after soaking in water for 0 and 48 h.

Table 1 Mean and range of phenotypic values of GABA contents in 100 varieties for soaked in water at for 0 h and 48 h.

GABA contents	Mean	Range	CV%
0 hr ($\mu\text{g}/\text{grain}$)	6.73 ± 0.89	5.02 – 10.82	13.16
48 hr ($\mu\text{g}/\text{grain}$)	10.51 ± 2.18	6.72 – 17.69	20.78
Increased during 0-48 hr ($\mu\text{g}/\text{grain}$)	3.78 ± 2.01	0.49 – 11.81	53.28
0 hr ($\text{mg}/100 \text{ g FW}$)	24.56 ± 5.32	15.20 – 49.52	21.66
48 hr ($\text{mg}/100 \text{ g FW}$)	20.83 ± 6.00	11.77 – 41.11	28.80

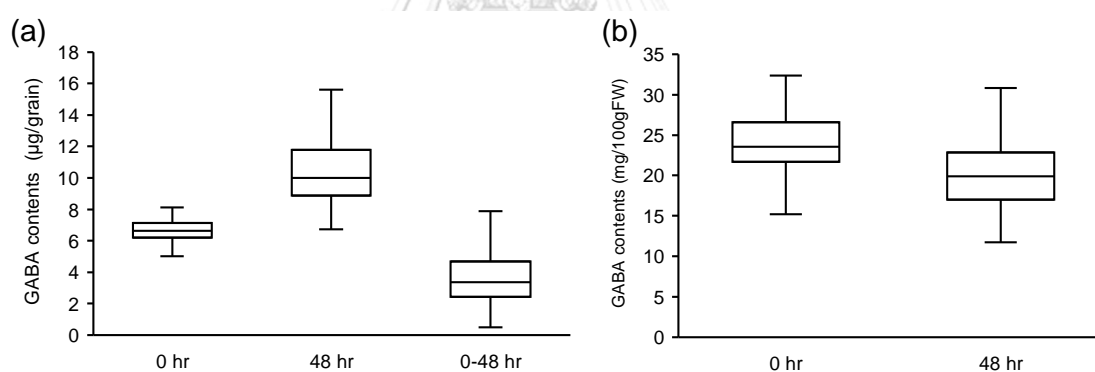


Figure 3 Box plots of GABA contents of the 100 Thai rice varieties at 0 h, at 48 h, and the increased contents during 0–48 h of soaking (a) and GABA contents at 0 h and at 48 h when calculated as $\text{mg}/100 \text{ g FW}$ (b).

The top five local Thai rice varieties that exhibited the highest GABA contents at 0 h were 'Gow Ruang' ($10.82 \mu\text{g}/\text{grain}$), 'Khao Dawk Mali 105' ($9.38 \mu\text{g}/\text{grain}$), 'Bahng Pra' ($9.22 \mu\text{g}/\text{grain}$), 'Khao Gaw Diaw' ($8.58 \mu\text{g}/\text{grain}$) and 'Niaw Bai Si' ($8.54 \mu\text{g}/\text{grain}$)

(Supplementary Table S1). The histogram shows that the largest number of rice varieties had GABA content at 0 h of approximately 6 $\mu\text{g}/\text{grain}$ (Supplementary Figure S2a). At 48 h, the top five GABA accumulators at 48 h were 'Khao Nahng Mon' (17.69 $\mu\text{g}/\text{grain}$), 'Gahn Mayom' (15.62 $\mu\text{g}/\text{grain}$), 'Ma Fai Dam' (15.31 $\mu\text{g}/\text{grain}$), 'Hawm Nahng Nuan' (15.31 $\mu\text{g}/\text{grain}$), and 'Khitom Luang' (14.92 $\mu\text{g}/\text{grain}$) (Supplementary Table S1). The largest number of rice varieties had GABA content at 48 h of approximately 9 $\mu\text{g}/\text{grain}$ (Supplementary Figure S2b) and an increment of approximately 3 $\mu\text{g}/\text{grain}$ (Supplementary Figure S2c). The top five highest increased GABA contents were from 'Khao Nahng Mon' (11.81 $\mu\text{g}/\text{grain}$), 'Gahn Mayom' (9.17 $\mu\text{g}/\text{grain}$), 'Ma Fai Dam' (8.25 $\mu\text{g}/\text{grain}$), 'Tah Jeua' (7.88 $\mu\text{g}/\text{grain}$) and 'Lawd Gwian' (7.57 $\mu\text{g}/\text{grain}$), respectively (Figure 2). The three rice varieties with the highest increased GABA contents were also the top three accumulators at 48 h.

To account for the difference in germinating grain weight, GABA content was calculated as mg/100 g fresh weight (FW; Table 1). The mean values of GABA content ranged from 15.20–49.52 mg/100 g FW at 0 h and 11.77 – 41.11 mg/100 g FW at 48 h. Figure 3b shows the distribution of GABA content per fresh weight of the 100 Thai rice varieties after 0 h and 48 h of soaking. Although GABA content increased during germination, its content per fresh weight appeared to decrease (Table 1) due to the increase in weight. It should be noted that when calculated as content per weight, the majority of rice varieties fall into a narrower range of GABA content both at 0 h (Supplementary Figure S2d) and 48 h (Supplementary Figure S2e).

Hierarchical analysis was used to cluster the Thai rice varieties based on their GABA contents at 0 h, 48 h, and the increased contents during 0–48 h of water soaking. Based on GABA content, the 100 rice varieties were categorized into four clusters (Figure 4). The varieties in the first cluster (cluster I) showed a relatively low GABA content after 48 h of soaking, as they either exhibited a small increment in GABA content or had a relatively low content at the start. Most colored rice varieties were found in this cluster (in red rectangles); therefore, the colored rice did not appear to exhibit higher GABA content compared to the white rice. The varieties in cluster II exhibited a medium increase, resulting in a relatively higher GABA content than that in the first cluster. Cluster III, which contained four varieties, originally had the highest GABA content in the grain. However, it showed a relatively small increment after soaking in water for 24 h. In cluster IV, high accumulation of GABA was observed at 48 h, which resulted from the relatively higher increment during 0–48 h of soaking. There were 14 varieties in this cluster including the top three GABA accumulators at 48 h of soaking ('Khao Nahng Mon', 'Gahn Mayom', and 'Ma Fai Dam'). Initially, they had a relatively low GABA content in the grain. However, they exhibited very high increments. 'Khao Nahng Mon' was the best accumulator, with over 200% increase in GABA content.

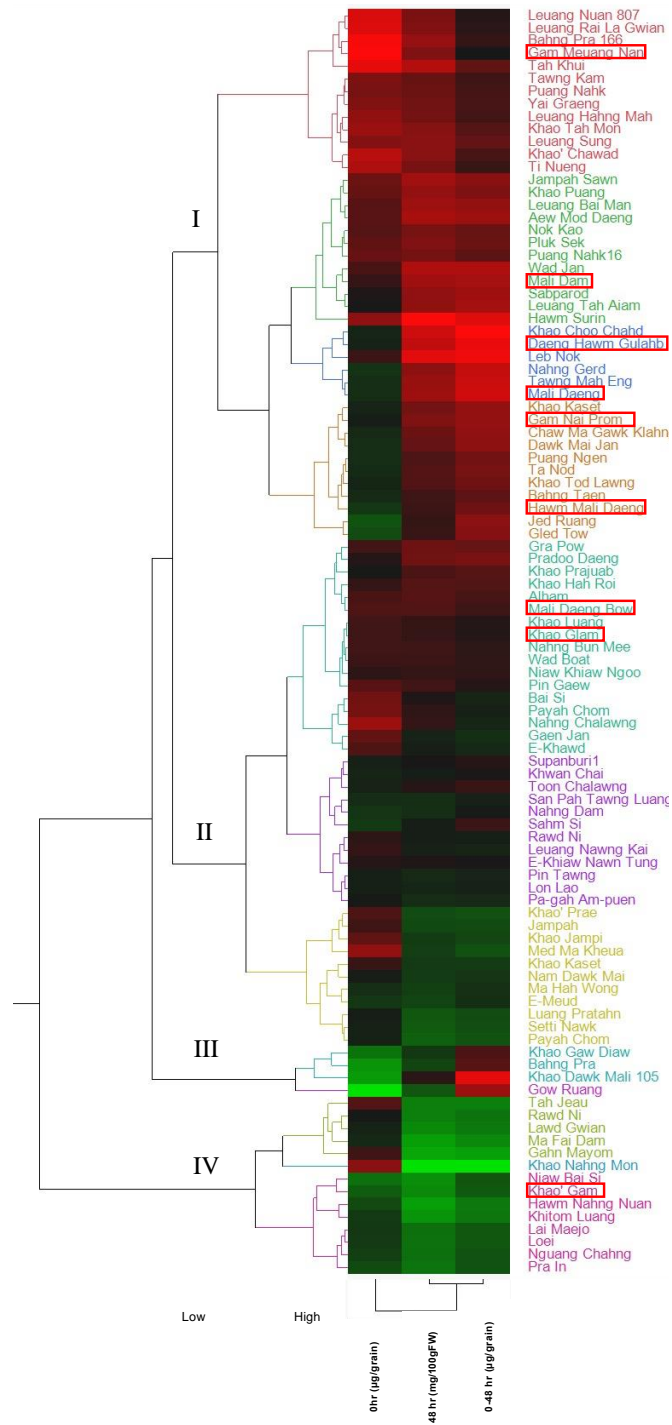


Figure 4 Hierarchical clustering analysis of 100 Thai rice varieties evaluated on contents of GABA

at 0 h, 48 h, and increased GABA contents at 0–48 h of soaking ($\mu\text{g}/\text{grain}$). Colored rice has been represented in the red box.

2.1.4.3 Genome-wide association mapping and candidate loci associated with GABA contents

We performed GWAS using 2,035,945 SNPs called from the whole-genome sequences of the 100 varieties to identify potential genes associated with GABA accumulation in the 48 hr germinating rice grains (Figure 5). A total of 23 significant SNPs were identified from GWA mapping of these parameters. One SNP peak consisting of five SNPs on chromosome 4 from GWA mapping of the 48 h GABA contents (Figure 5a), one SNP peak consisting of eight SNPs on chromosome 2, and three additional SNPs on chromosomes 5 and 11 from GWA mapping of the 48 h GABA contents ($\text{mg}/100 \text{ g FW}$; Figure 5b), and one SNP peak consisting of five SNPs on chromosome 4, and two additional significant SNPs on chromosomes 1 and 2 from the increased GABA contents during 0–48 h, were found (Figure 5c).

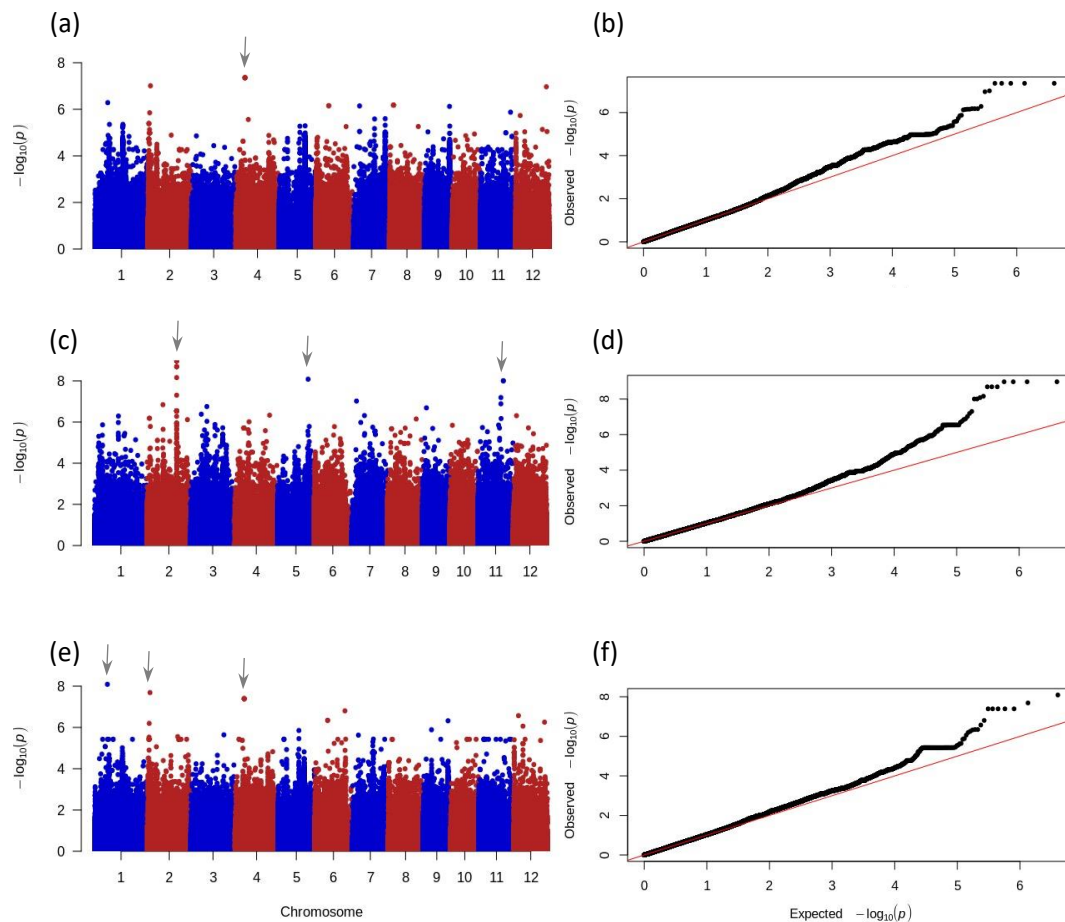


Figure 5 Manhattan and Quantile-quantile (Q-Q) plots demonstrating the GWAS.

SNP markers were used in the GWAS analysis to determine the relationship between SNPs and phenotypes based on GABA contents for 48 h ($\mu\text{g}/\text{grain}$) (a and b) 48 h ($\text{mg}/100 \text{ g FW}$) (c and d) Increasing 0–48 h (e and f). For Manhattan plots, the x-axis displays SNP sites over the whole rice genome by chromosome, and the y-axis is the negative logarithm p-value: $-\log_{10}(p)$ of each SNP. The expected $-\log_{10}(p)$ of each SNP is displayed on the x-axis of Q-Q plots, while the observed $-\log_{10}(p)$ is displayed on the y-axis.

Table 2 presents the list of all significant SNPs and their information from the GWA mapping of all three GABA content parameters. The identified SNP peak containing five SNPs on chromosome 4 associated with the 48 h GABA content was also found to be associated with the increased GABA content during 0–48 h. The SNPs associated with 48 h GABA (mg/100 g FW) on chromosomes 2 and 11 were found in the coding regions of the following loci: *LOC_Os02g40320*, *LOC_Os02g40330*, and *LOC_Os11g35580*. They encode a protein similar to argonaute MEL1, a conserved hypothetical protein, and the disease-resistance protein PRM1, respectively. Other SNPs were found in proximal regions upstream of the following genes: *LOC_Os01g18390* and *LOC_Os04g13480*, which encode a putative low-temperature and salt response protein, and a response regulator receiver domain-containing protein, respectively. Supplementary Table S1 provides a list of loci within 100 kb of each of these significant SNPs. Their annotated proteins have various functions, including metabolism, transcriptional regulation, and stress signaling. In addition, numerous retrotransposon proteins have also been identified.

Table 2 List of significant SNPs from GWA mapping of GABA contents.

Parameter	chr	Position (IRGSP-1.0)	allele1	allele0	af	<i>p</i> -value (MLM)	Locus	Annotation			
48 h GABA Content (µg/grain)	4	7519195	G	A	0.49	4.40E-08	LOC_Os04g13480 (upstream)	A response regulator receiver domain-containing protein			
	4	7519202	C	A	0.49	4.40E-08					
	4	7519210	T	C	0.49	4.40E-08					
	4	7519211	A	G	0.49	4.40E-08					
	4	7519223	A	G	0.49	4.40E-08					
48 h GABA Content (mg/100 g FW)	2	24408288	A	G	0.06	6.93E-09	LOC_Os02g40320	A protein similar to argonaute MEL1			
	2	24408374	T	C	0.05	1.06E-09					
	2	24408423	C	T	0.07	2.04E-09					
	2	24408451	C	G	0.07	2.04E-09					
	2	24408783	T	C	0.05	1.06E-09					
	2	24408966	G	A	0.05	1.06E-09					
	2	24410001	C	G	0.07	2.04E-09			LOC_Os02g40330	A conserved hypothetical protein	
	2	24410352	G	A	0.05	1.06E-09					
		5	25122470	G	A	0.16			8.27E-09	-	
		11	20861728	C	A	0.065			9.89E-09	LOC_Os11g35580	The disease- resistance protein PRM1
	11	20861729	C	A	0.065	9.89E-09					
Increased GABA Content during 0- 48 h (µg/grain)	1	10340396	G	A	0.49	8.14E-09	LOC_Os01g18390 (upstream)	A putative low- temperature and salt response protein			
	2	2184159	A	G	0.075	2.05E-08			-		
	4	7519195	G	A	0.49	4.07E-08	LOC_Os04g13480 (upstream)	A response regulator receiver domain-containing protein			
	4	7519202	C	A	0.49	4.07E-08					
	4	7519210	T	C	0.49	4.07E-08					
	4	7519211	A	G	0.49	4.07E-08					
4	7519223	A	G	0.49	4.07E-08						

Figure 6 presents the gene expression profiles in various tissues/organs obtained from the Rice Expression Profile Database (RiceXPro) (version 3.0) (<https://ricexpro.dna.affrc.go.jp/>) for candidate loci either containing or lying immediately downstream of the identified significant SNPs. All candidate genes were highly expressed in the endosperm. STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) analysis was conducted using the STRING database version 11.5 (<https://string-db.org/>) to construct a protein-protein interaction network for the two genes highly expressed in the endosperm: *LOC_Os02g40330* and *LOC_Os04g13480*. The results showed a complex protein association network in which proteins had some sort of direct or indirect interaction with other proteins. In the STRING analysis of *LOC_Os2g40330*, a candidate gene discovered by GWA mapping of GABA content at 48 h (mg/100 g FW), proteins linked to carbon and nitrogen metabolism and metabolic pathways were found in the highly interconnected networks, which included nitrate reductase 1 (NIA1) and sucrose synthase 6 (SUS6) (Figure 7a), though the interactions were from only text mining. In the STRING analysis of *LOC_04g13480*, which was discovered by GWA mapping of both GABA content at 48 h and the increased GABA contents during 0–48 h of soaking, interconnected networks of protein components in the two-component system, which are involved in cytokinin signalling, were found (Figure 7b).

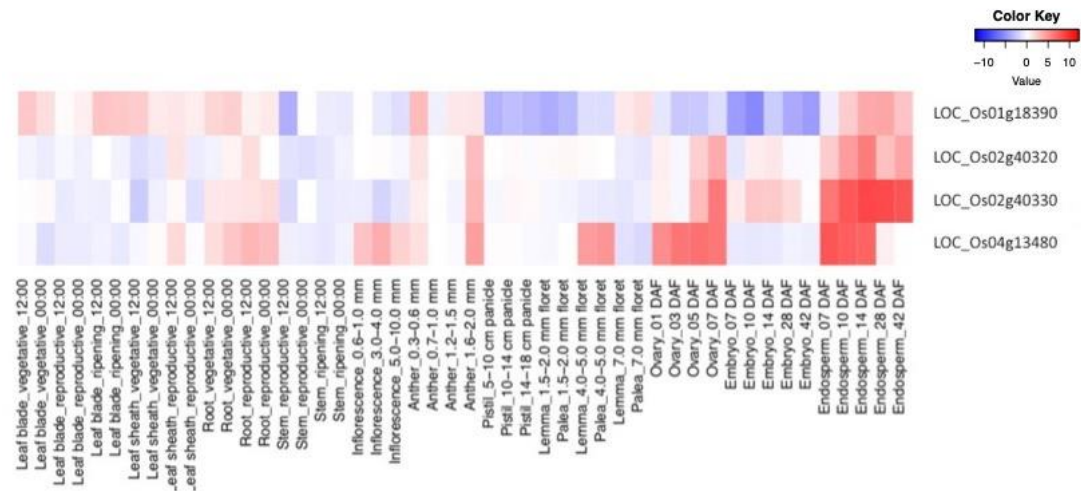


Figure 6 Heat map of expression levels in various tissues/organs throughout the entire growth of genes containing or lying immediately downstream of the significant SNPs identified from GWA mapping based on GABA contents at 48 h (μ g/grain), 48 h (mg/100 g FW), and increased GABA contents at 0–48 h.

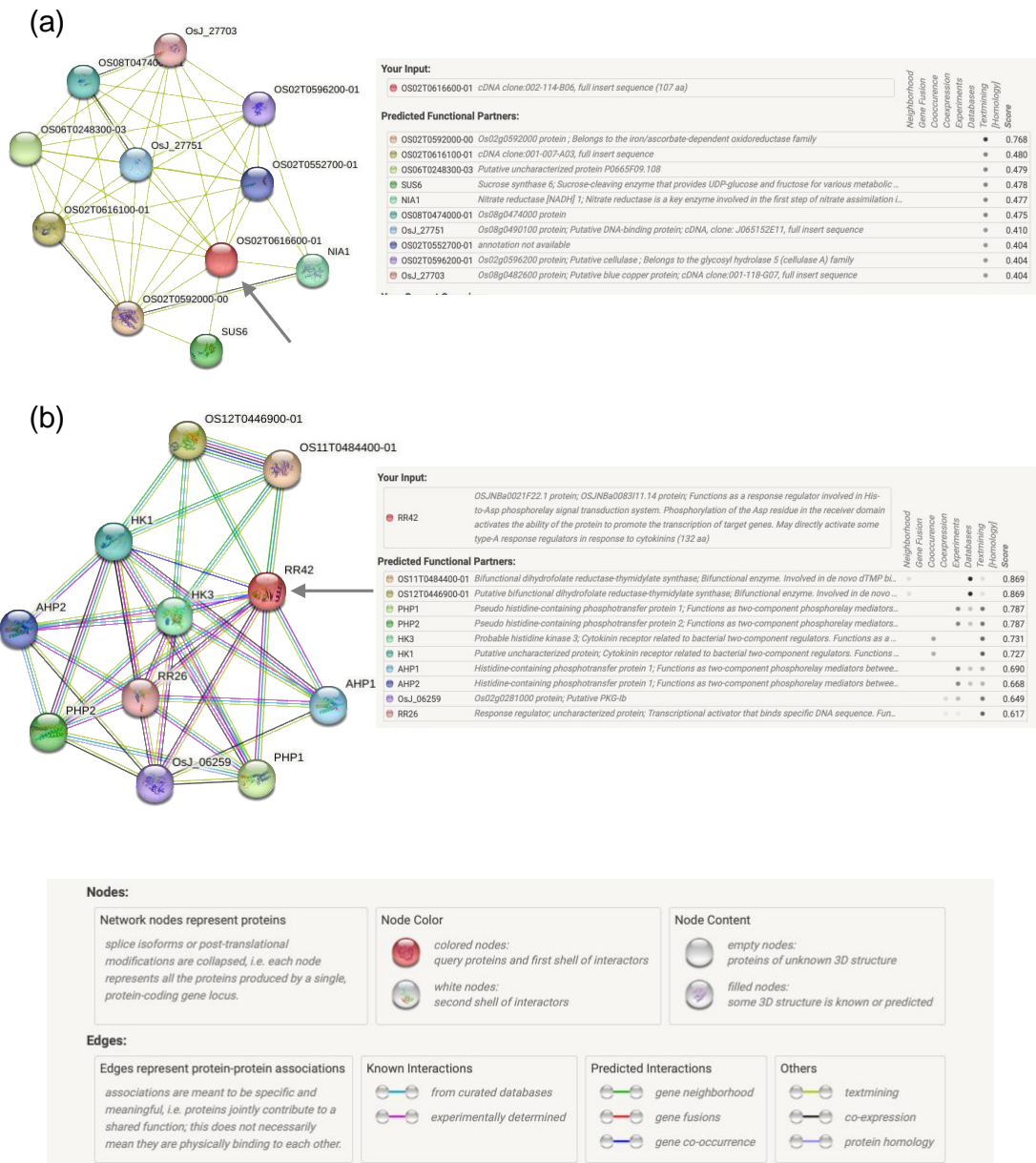


Figure 7 STRING analysis uncovering protein-protein interaction networks of LOC_Os02g40330 (a) and LOC_Os04g13480 (b) of GABA contents in Thai rice varieties. A gray arrow indicates the query protein.

2.1.5 Discussion

Varietal differences among rice cultivars are likely due to their genetic and environmental adaptations to different regions of the world. Water soaking can increase GABA content in all types of rice germ, and its accumulation varies with the rice variety and soaking time (Varanyanond et al., 2005). Because soaking can directly lower the hardness of germinated whole grains, it is a traditional pre-treatment technique and a crucial stage in the process of whole-grain germination. In the present study, imbibition likely started as grains were soaked, which further promoted metabolic processes, including those of amino acids. GABA was likely synthesized, which showed a rapid increase after 48 h of soaking in water. A previous study similarly showed that the production of GABA had the highest rates in the 'Khao Dawk Mali 105' (white rice) and 'Riceberry' cultivars (colored rice) within 48 h (26.12 and 34.28 mg/100 g) and reached their highest levels at 96 h (31.36 and 38.75 mg/100 g). 'Khao Dawk Mali 105's GABA and amino acid levels were lower than those of the germinating 'Riceberry' seed. The use of germinating rice as a functional food is therefore supported by the dramatic increase in GABA and other important amino acids that occur after germination (Kamjijam et al., 2020). The genetic basis of GABA synthesis and accumulation during germination would help to increase our understanding of its biological functions and optimize its use as a functional food.

GWAS can identify genes that affect the trait of interest in large phenotypic variations. In this study, GWAS using more than two million SNPs from 100 rice accessions uncovered 23 SNPs linked to GABA accumulation in the Thai rice population. There are no reports of GWAS on GABA content in rice; however, GWAS has been used to identify genetic variants associated with numerous phenotypes, including those occurring during germination and seedling growth. Panhabadi et al. (2022) reported that the results of GWAS identified several plausible candidate genes for features related to germination and seedling development, which will significantly advance our understanding of the genetic complexity underlying the relevant phenotypes. GWAS investigation of seedling growth characteristics, relative water content, and cell membrane stability under salt stress has identified 25 candidate genes (Kojonna et al., 2022). Using an Arabidopsis mutant line with a T-DNA insertion, OsCRN was found to have a significant impact on the ability of rice to withstand salt stress during the seedling stage. In this study, GWAS based on GABA content at 48 h of germination, either as the total amount in each grain ($\mu\text{g}/\text{grain}$) or the content per fresh weight ($\text{mg}/100 \text{ g FW}$), yielded significant SNPs; however, they were all different. In contrast, using the parameter of increased GABA content during 0–48 h of germination, five SNPs in a single peak were all the same SNPs that were associated with the 48 h GABA content ($\mu\text{g}/\text{grain}$) as both parameters were clustered closer together than with the 48 h GABA content ($\text{mg}/100 \text{ g FW}$) (Figure 4). Overall, GWAS revealed five genes associated with the 23 significant SNPs identified.

Proteins connected to one of the candidate genes highly expressed in the endosperm, LOC_Os2g40330, were found to be involved with carbon and nitrogen metabolism. One of these proteins was SUS6 (Figure 7a), which is involved in the metabolism of sucrose and affects seed germination and seedling development (Gibson, 2005). LOC_Os2g40330 (Os02g0616600) was also found to be connected with NIA1, a nitrate reductase that reduces nitrate to nitrite before entering the GS-GOGAT pathway to be incorporated into amino acids. Beginning with α -ketoglutarate, which stands for the meeting point of the carbon and nitrogen metabolic pathways, the GABA shunt, a two-step bypass of the TCA cycle, is initiated (Huergo and Dixon, 2015). Blocking α -ketoglutarate dehydrogenase activity evoked an upregulation of the GABA shunt, increasing the carbon and nitrogen transfer from α -ketoglutarate to GABA (Araujo et al., 2008). GWA mapping based on GABA content at 48 h ($\mu\text{g}/\text{grain}$) identified the protein encoded by LOC_Os04g13480 (RR42), which plays a role in the His-to-Asp phosphorelay signal transduction system as a response regulator. The capacity of the protein to enhance the transcription of target genes is activated by phosphorylation of the Asp residue in the receiver domain in response to cytokinins. Several proteins in the two-component system, including AHP1, AHP2, PHP1, PHP2, and RR26, were associated with RR42. Authentic histidine phosphotransfer proteins (AHPs), type-B response regulators (RRs), and histidine kinase receptors comprise the phosphorelay that constitutes the cytokinin signaling pathway. Type-A RRs, which are cytokinin primary response genes, and pseudo histidine phosphotransfer proteins

(PHPs), which lack the His residue necessary for phosphorelay, have both been identified as negative regulators of cytokinin signaling (Vaughan-Hirsch et al., 2021). Cytokinin has a functional connection with GABA (Podlešáková et al, 2018) as evidenced by a previous report on barley expressing cytokine dehydrogenase 1 (Pospíšilová et al., 2016).

GWA mapping of complex quantitative traits is facilitated by high-quality genotyping data from high-throughput sequencing, in conjunction with reliable statistical analysis. We performed GWAS of GABA accumulation based on high-density SNPs in Thai rice. The 23 significant SNPs were found to be associated with five loci on chromosomes 1, 2, 4, and 11. Their annotation was consistent with their putative involvement in the GABA content. These SNPs significantly assisted in identifying the likely underlying genetics of GABA accumulation. Future improvements in the effectiveness of GABA production will benefit from knowledge of the varieties with high GABA content and the genes involved in GABA accumulation.

2.2 Manuscript II

Calmodulin overexpression increases gamma-aminobutyric acid (GABA) levels and glutamate decarboxylase activity in rice during germination

(Preparing to publish on Journal of Science Asia (Q2))

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2.2.1 Abstract

The transgenic ‘Khao Dawk Mali 105’ (KDML105) rice (*Oryza sativa* L.) lines overexpressing *OsCam1-1* calmodulin gene are more tolerant to salt stress and drought than the wild type (WT) plants. Here, we examined the production of gamma-aminobutyric acid (GABA) and glutamate decarboxylase (GAD) during germination in such plants. During the first 24 h of soaking in water under normal conditions, the GABA content increased to a higher degree in all transgenic rice lines than in the WT. During seed germination (from day 2 to day 4) under both normal

and salt stress conditions, the GABA content in all rice lines rapidly increased. GABA content under salt stress conditions then continued to increase and reached higher levels in all transgenic overexpression lines than in WT after 6-8 days of germination. The transgenic overexpression lines also exhibited induced GAD activity during germination compared to the WT. These results indicate that transgenic rice plants overexpressing *OsCam1-1* had enhanced GAD activity facilitated by calmodulin overexpression, resulting in higher GABA content that conferred salt tolerance.

KEYWORDS: gamma-aminobutyric acid, GABA, GAD, *OsCam1-1*, salt stress

2.2.2 Introduction

Soil salinity is a major constraint on rice production worldwide (Reddy et al., 2017). Globally, 20% of cultivated land and 33% of irrigated land are damaged and degraded by salinity, negatively affecting agricultural production and food security for the general population (Machado et al., 2017). Salinity causes a two-phase response in plant growth: a fast osmotic phase that hinders the development of young leaves, and a longer ionic phase that accelerates the aging of mature leaves. Plant adaptations to salinity include osmotic stress tolerance, Na^+ or Cl^- exclusion, and tissue tolerance to accumulated Na^+ or Cl^- ions (Munns & Tester., 2008). Gamma-aminobutyric acid (GABA) accumulation is induced by salinity and several other environmental stressors, including oxygen deprivation, cold shock, heat shock,

drought, and mechanical damage (Kinnersley et al., 2000). GABA provides a source of carbon skeletons and energy for biosynthetic pathways through the GABA shunt, and it participates in signaling pathways (Ramos-Ruiz et al., 2019). Under salt stress, plants have adapted defense mechanisms provided by GABA to resist the damaging consequences of salt stress-induced ROS production (Alqarawi et al., 2016; Sheteiwy et al., 2019).

In plants, GABA is primarily produced by the decarboxylation of L-glutamic acid (L-Glu), which is catalyzed by glutamate decarboxylase (GAD) (Bouché et al., 2004). The accumulation of GABA is related to the activity of GAD and concentration of L-Glu (Zhang et al., 2014). Rice plants overexpressing OsGAD accumulate GABA at high concentrations under various pH conditions (Akama & Takaiwa, 2007). In germinating wheat and barley seeds, there is a positive correlation between the abundance of GABA shunt metabolites and salt concentration. To balance carbon and nitrogen metabolism and osmolyte production in wheat and barley seeds germinating under salt stress, the increased expression level of GAD under salinity conditions supports the need for increased activity of the GAD-mediated conversion of L-Glu to GABA during seed germination (AL-Quraan et al., 2019).

GAD, which catalyzes the irreversible decarboxylation of L-Glu to produce GABA, is a cytosolic enzyme regulated by the Ca^{2+} -calmodulin (CaM) complex (Snedden et al., 1995). All eukaryotes use CaM as the primary calcium sensor. In

response to calcium signals, CaM binds to calcium and controls the activity of several effector proteins. In rice, *OsCam2* and *OsCam3* encode proteins with only two amino acid changes that share 98.7% identity with *OsCam1*; *OsCam1-1*, *OsCam1-2*, and *OsCam1-3* encode identical *OsCam1* proteins (Boonburapong & Buaboocha., 2007). To examine the role of CaM1-regulated GAD under salt stress in rice, we investigated GABA accumulation and GAD activity associated with improved salt tolerance in transgenic rice overexpressing *OsCam1-1* and compared them to those in wild-type 'KDML105' rice.

2.2.3 Materials and Methods

2.2.3.1 Sample preparation

Three lines of KDML 105 rice (*Oryza sativa* L.) seeds overexpressing *OsCam1-1* gene previously produced (Saeng-ngam et al., 2012) and the wild-type 'KDML105' rice, which differed in salt tolerance ability were used. The rice grains were washed once with deionized water, spread on sterile Petri dishes, and then soaked with deionized water or 100 mM NaCl (Solangi et al., 2016). The grains germinated in a controlled growth chamber at 30 °C with 16/8 h light/dark photoperiod for 0, 6, 12, 24, 48, 96, 144, 192, 240, and 288 h. The treatment solutions were replaced every 24 h during the incubation. At each time point, germinating seeds were collected, quick-

frozen in liquid nitrogen, and stored at $-80\text{ }^{\circ}\text{C}$. For each germination condition, the samples were analyzed in five replicates.

2.2.3.2 TTC staining

The rice grains were washed once with deionized water. A horizontal and symmetrical incision was made in all seeds. The methods used were modified from those described by Zhao et al. (2010). 2,3,5 Triphenyl tetrazolium chloride (TTC) was dissolved in deionized water to make 100% (w/v) stock solution and stored at $4\text{ }^{\circ}\text{C}$, which was diluted to 10% with deionized water before use. The samples were soaked in 10% TTC for 1 h and then washed three times with deionized water.

2.2.3.3 Extraction of GABA

Ten grains were ground in liquid nitrogen and were extracted with 0.3% (w/v) sulfosalicylic acid by vortexing for 20 s. Then, samples were sonicated for 20 min and centrifuged at 4,500 rpm at $4\text{ }^{\circ}\text{C}$ for 20 min. The samples were filtrated through a $0.2\text{ }\mu\text{m}$ cellulose acetate membrane, collected, and transferred to a new $500\text{-}\mu\text{L}$ microcentrifuge tube. Finally, the GABA content was measured using the GABase method (Nonaka et al., 2017; Kittibunchakul et al., 2017).

2.2.3.4 Preparation of crude proteins

The modified procedure described by Khwanchai et al (2014) was used to extract proteins for measuring GAD activity. Rice seedlings (10 grains) were ground in liquid nitrogen and were extracted with 0.5 mL phosphate buffer (pH 5.8) 50 mM, 0.2

mM pyridoxal phosphate (PLP), 2 mM 2-mercaptoethanol, 2 mM CaCl_2 and 1 mM PMSF in an ice bath by vortexing for 20 s. The homogenate was centrifuged at 4,500 rpm for 20 min at 4 °C using a refrigerated high-speed centrifuge. The supernatant containing the crude enzyme was used for assay of the GAD activity.

2.2.3.5 GAD activity assay

The modified procedure described by Johnson et al (1997) was used to determine the GAD activity. The GAD activity assay reaction consisted of 50 mM sodium phosphate, pH 5.8, 30 mM L-glutamate, 20 μM pyridoxal-5-phosphate (PLP), and enough protein to produce a reaction rate such that velocity was linear and proportional to the amount of protein added. This solution was incubated at 40°C for 1 h, and the reaction was then stopped by incubating in a heat block at 100°C. After that, samples were filtrated by 0.2- μm cellulose acetate membrane and collected. GAD activity was measured according to a previously described method for GABA analysis by directly measuring GABA production using the GABase method (Nonaka et al., 2017; Kittibunchakul et al., 2017). GAD activity units per 100 mg of the enzyme were calculated.

2.2.3.6 Statistical analysis

To evaluate the differences among the treatments, the analysis of variance (ANOVA) followed by Tukey's HSD multiple range test was performed using SPSS software version 28. Results with $p < 0.05$ indicated that the treatments were significantly different.

2.2.4 Results and Discussion

2.2.4.1 GABA content during rice grain germination and seedling growth

2.2.4.1.1 During soaking (0 – 24 hr)

The GABA contents in the non-germinated grains of the transgenic rice lines overexpressing *OsCam1-1* gene (L1, L2, and L7) and the wild-type 'KDML105' (WT) were not significantly different (Figure 8). They were approximately 6-8 mg in 100 g of rice grain. However, the GABA contents in the transgenic rice increased after 6-24 h of soaking in water, whereas those in the WT only slightly increased, resulting in statistically significantly higher GABA contents in the transgenic rice within the first 24 hours of soaking (Figure 8A). At 24 h, the GABA contents in all three transgenic rice lines were two-fold higher than that in the WT. Their GABA contents reached 13-16 mg/100g FW (approximately two-fold higher than that of non-germinated grains).

Previous studies have similarly reported the effect of soaking as a pre-treatment for germination on GABA accumulation. Soaking brown rice in water for 24 h resulted in similar range of GABA content to the present study (Komatsuzaki et al., 2007). Soaking rice grains resulted in higher levels of GABA and its intermediates than before germination and the upregulation of GAD enzyme (Zhao et al., 2017). The GAD activity responsible for GABA biosynthesis was not found in the rice grains before germination. However GAD protein was detected by western blot analysis by Jannoey

et al. (2010) suggesting that upon water absorption during soaking, GAD enzyme was activated. This likely resulted in the increased GABA production, especially in the transgenic rice, which overexpressed calmodulin, an activator of the GAD enzyme.

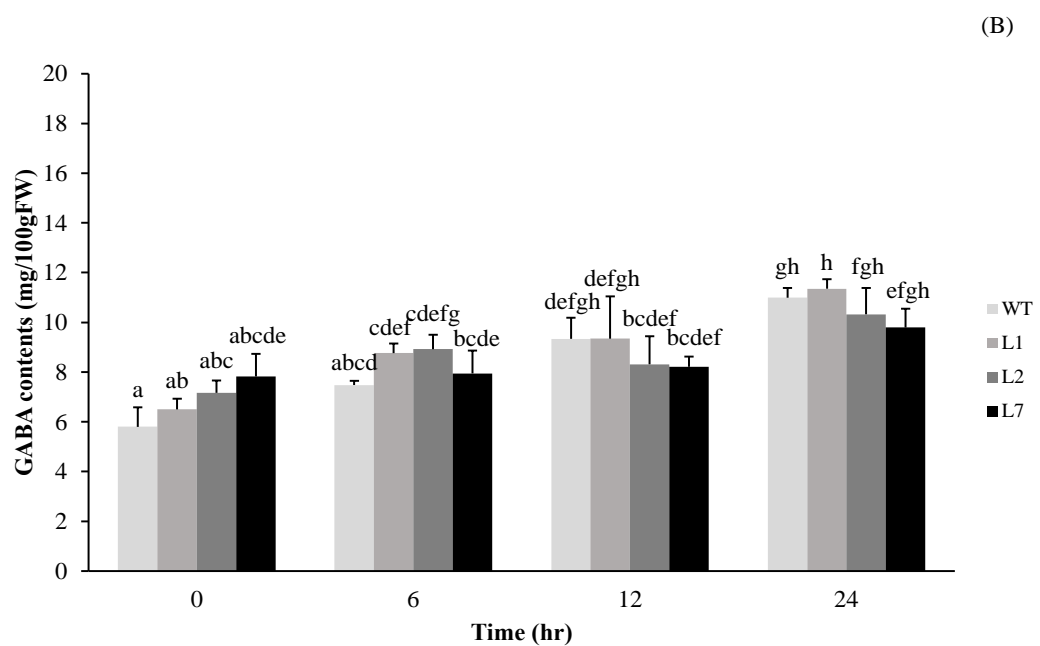
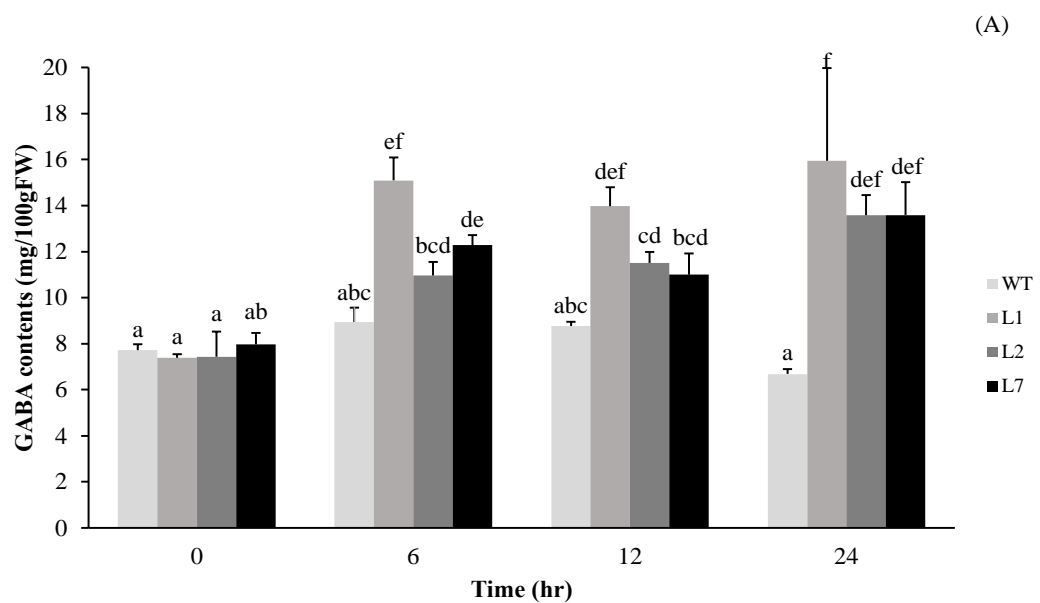


Figure 8 GABA contents in rice grains of the transgenic rice overexpressing *OsCaM1-1* (L1, L2, and L7) compared with WT during soaking. (A) normal condition; (B) salt stress (100 mM NaCl). Data shown are means \pm SD of five replicated experiments. Different letters on bars indicate significant difference at $p < 0.05$.

The results suggest that the *OsCaM1-1* overexpression led to the higher GABA production during 6-24 h after the grains were soaked in water to trigger the process. Using cDNA expression library screen, *OsCaM1* has been reported to interact with a GAD protein (LOC_Os08g51080) (Yuenyong et al., 2018). The overexpressed *OsCaM1* likely activated GAD proteins, which in turn resulted in the higher GABA contents observed in the transgenic rice. *OsCaM1-1* overexpressing rice lines have been shown to be more tolerant to salt stress than WT at the early seedling stage (Saeng-ngam et al., 2012), which may be partially resulted from the enhanced level of GABA. Stimulated GABA biosynthesis in germinated brown rice is correlated with the activity of partially purified GAD stimulated by the addition of calmodulin in the presence of calcium (Oh, S. H., 2003).

Salt stress can affect seed germination, resulting in biochemical and physiological changes (Ibrahim E.A., 2016). The effect of soaking in NaCl solution on the accumulation GABA content in the transgenic rice overexpressing *OsCaM1-1* gene compared with WT was investigated (Fig. 8B). Overall, the amounts of GABA in all rice

lines increased slightly. However, at all time-points, the transgenic rice did not exhibit different GABA contents from the WT, as the GABA contents in the WT also increased to similar levels when salt stress was imposed during soaking. However, if salt stress occurs later during germination, the higher GABA contents observed under normal condition in the transgenic rice overexpressing *OsCaM1-1* gene (Figure 8A) would better prepare them to adapt to salt stress than the WT. The higher degree of salt tolerant ability of the transgenic rice that overexpressed *OsCam1-1* previously reported (Saeng-ngam et al., 2012) may be partly explained by the increased GABA accumulation during this early stage of germination.

2.2.4.1.2 During germination and seedling growth (2 - 12 d)

GABA contents during germination and seedling growth were monitored under normal condition (Figure 9A) or NaCl treatment (Figure 9B). Under salt treatment, rice seeds germinate more slowly than under normal conditions. GABA contents on day 2 under both normal (Figure 10A) and salt treatments (Figure 10B) was around 6-8 mg/100g FW. From day 2 to day 4, GABA content significantly increased under both normal and salt stress conditions. While it remained at similar levels under normal conditions (Figure 10A), GABA content from day 4 to day 6 continued to increase under salt stress (Figure 10B). Compared with the WT from day 6 to day 8, higher accumulation of GABA content in all three transgenic lines under salt-stress treatment was observed: on day 6 from the highest to the lowest GABA concentrations: L2, L7, L1, and WT (16.239, 15.689, 15.564, and 13.041 mg /100g FW,

respectively) and day 8 from the highest to the lowest: L2, L1, L7, and WT (15.338, 14.204, 14.163, and 11.785 mg/100g FW, respectively). GABA content under salt stress were higher than those obtained from their respective plants grown under normal conditions. However, from day 8 to day 10, GABA content increased rapidly under normal conditions (Fig. 10A) as the seedlings grew rapidly. In contrast, the GABA content under salt stress (Fig. 10B) decreased by 50% during the same period.

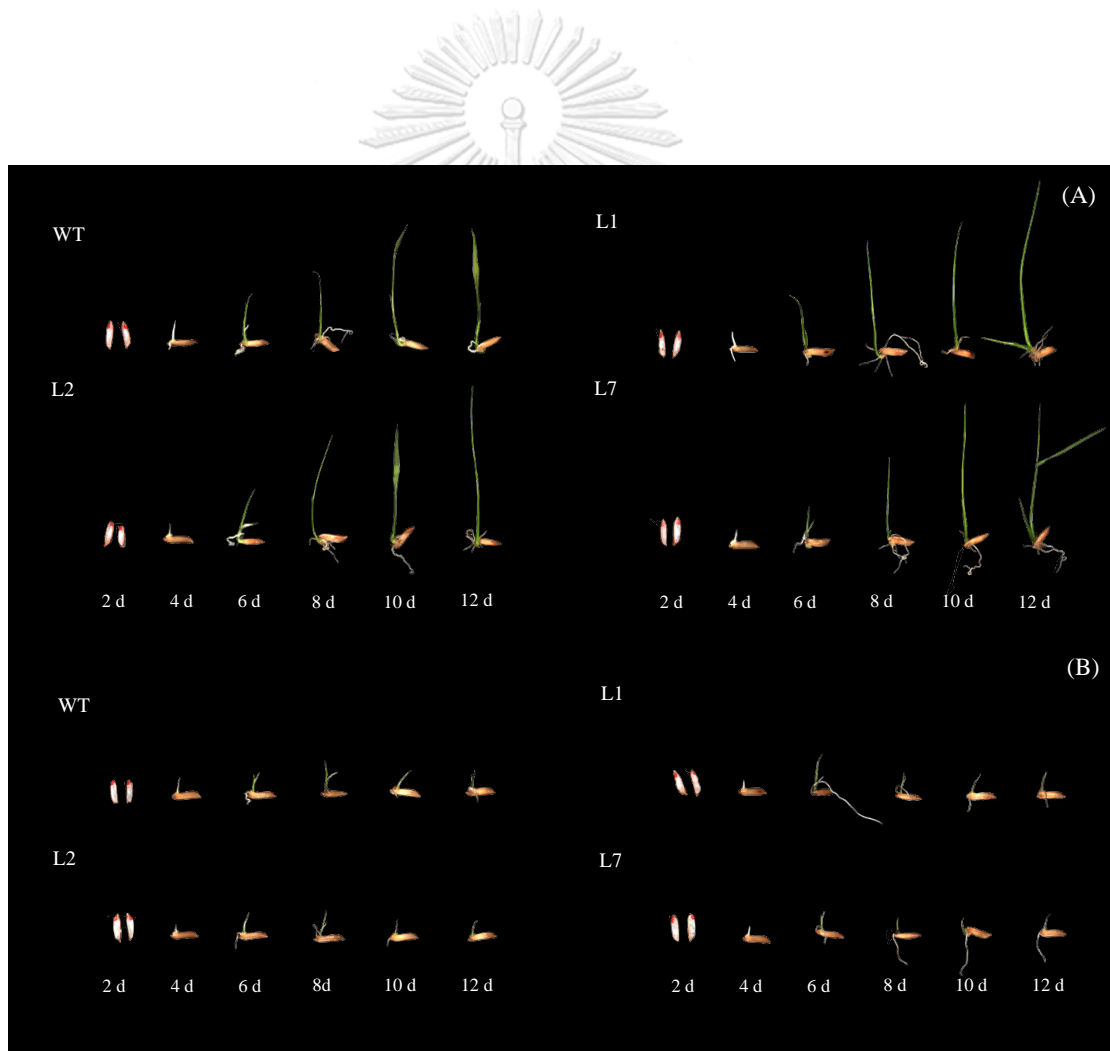


Figure 9 Rice grain germination at various time points of the transgenic rice overexpressing *OsCaM1-1* and WT under normal (A) or salt stress (B).



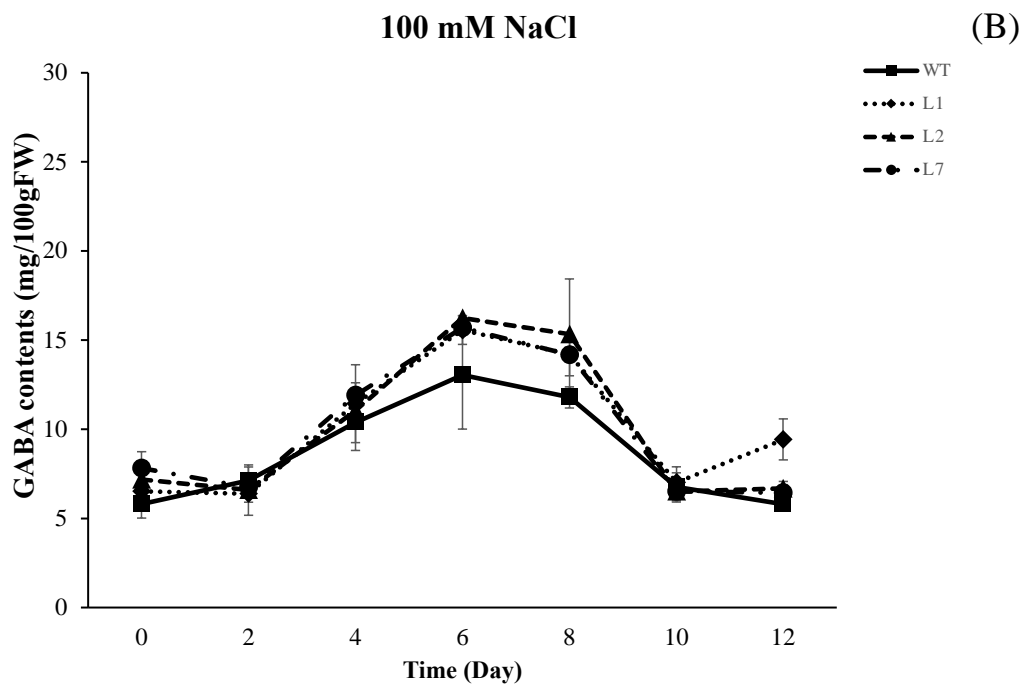
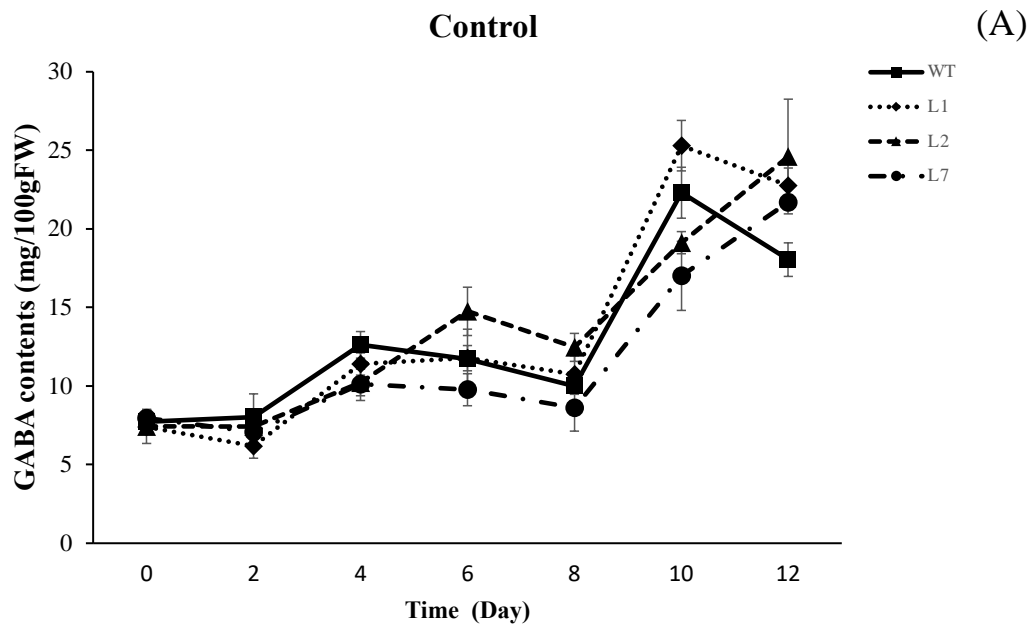


Figure 10 GABA contents during rice grain germination under (A) normal condition (Control) and (B) salt stress (100 mM NaCl). Data shown are means \pm SD of five replicated experiments.

These results were consistent to the previous investigations that focused on the effect of germination on GABA production. In soybean, GABA content was highly increased during the first 7 d of germination (Hyun et al., 2013). This effect may be caused by the strong endogenous protease and peptidase activity during the soaking phase, which leads to protein hydrolysis and increases in glutamic acid concentration, which enhances the accumulation of GABA (Li et al., 2022). Further, the GABA content in germinated grains is enhanced by salt stress. When compared to untreated wheat and barley that had not received NaCl stress treatment, the GABA concentration dramatically increased during five days of germination (AL-Quraan., 2019). In millet, the 48-h old germinating seeds treated with NaCl stress exhibited a rapid increase in GABA content, which tended to become flat with the treatment time longer than 48 h (Bai et al., 2013). In another study on white clover, endogenous GABA content decreased after 7 days of germination under salt stress, similar to what was observed in this study. In addition, soaking with exogenous GABA restored the salt-induced decrease in endogenous GABA content and alleviated

other salt stress damage during seed germination (Cheng et al., 2018). These results suggest that GABA accumulation likely contributes to the salt tolerance of plants during germination.

In our study, higher GABA accumulation in all transgenic lines than in the wild type, especially under salt stress conditions, was likely due to overexpression of the calmodulin gene. Kaewneramit et al. (2019) reported that *OsCam1-1* gene overexpression possibly decreased salt-induced oxidative damage in the transgenic plants by promoting the activities of antioxidant enzymes. The GABA shunt pathway and the accumulation of GABA metabolites in *Arabidopsis* seedlings (*cam 5-4* and *cam 6-1* mutants) had been reported to contribute to the antioxidant machinery associated with ROS and the acquisition of tolerance in response to the induced oxidative stress (Al-Quraan et al., 2011). The GABA shunt can be activated under salt treatment, with the increased contents of GABA shunt metabolites such as GABA, Ala and Glu in the germinating seeds when treated with salt stress; example case include wheat and barley cultivars (Che-Othman et al., 2020). Hence, this is alternative route to the respiratory machinery.

2.2.4.2 GAD activity during rice grain germination

The formation of GABA from L-Glu (via decarboxylation) in grains during germination is catalyzed by GAD (Komatsuzaki et al 2007). GAD is well-known CaM-interacting protein (CIP) (Baum et al., 1996) and a GAD gene (LOC_Os03g51080) has

been identified to encode a CIP in rice by a cDNA expression library screening (Yuenyong et al., 2018). We examined GAD activity in the transgenic rice lines overexpressing *OsCam1-1* compared to WT under normal or salt stress conditions. Under salt stress or in the early stage of germination (0 - 2 d), GAD activity measurement in the grain did not give reliable results; therefore, we were only able to obtain data from germinating seeds and seedlings under control conditions on day 4. Table 3 showed that the activity on day 8 in L1 and L2 transgenic rice tended to be higher than that of the WT. Overall, the levels of GAD activity correlated well with the GABA content under normal conditions (Fig. 10A). GAD activity on day 10 was at the highest level, and those in the transgenic rice lines were slightly decreased on day 12, but remained at a relatively higher level than that of the WT. On day 12, GAD activity in all transgenic rice were higher than that in the WT, which corresponded to the level of GABA (Fig. 10A).

The GAD enzyme is associated with an active complex composed of CaM and Ca²⁺, which promotes GABA production (Bouche & Fromm, 2004). In this study, the GAD enzyme in the rice grains became active, as reported by Jannoey et al. (2010), and its activity increased during germination. Several previous reports have shown that the expression of these genes is induced during germination and under stress (Hyun et al., 2013; Akçay et al., 2012). Calcium/calmodulin-regulated *GAD1* has been reported to play an important role in GABA synthesis in plants under normal and heat stress conditions. Disruption of the *GAD1* gene prevented GABA accumulation in

the roots (Bouché et al., 2004). Therefore, it is likely that the enhanced accumulation of GABA in the *OsCam1-1* overexpressing rice lines under salt stress resulted from activation of GAD enzyme by OsCaM1.

Table 3 GAD activity in germinating transgenic rice overexpressing *OsCaM1-1* grown under normal condition.

Line	GAD activity (Unit/100mg Protein)				
	4 d	6 d	8 d	10 d	12 d
WT	2.070±2.60 ^{ns}	7.389±2.12 ^{ns}	6.288±4.62 ^{ns}	10.781±3.12 ^{ns}	4.380±3.01 ^a
L1	3.035±0.70 ^{ns}	6.695±5.14 ^{ns}	7.144±7.55 ^{ns}	10.390±6.47 ^{ns}	8.190±3.08 ^b
L2	2.706±3.27 ^{ns}	7.419±3.49 ^{ns}	13.633±5.57 ^{ns}	10.407±1.69 ^{ns}	9.050±2.15 ^b
L7	0.788±1.01 ^{ns}	3.075±2.41 ^{ns}	5.878±3.70 ^{ns}	7.575±0.85 ^{ns}	6.388±0.76 ^{ab}

Data shown are means ±SD of five replicated experiments. Different letters over means indicate significant difference at $p < 0.05$, while ns indicates not significant difference among means at the same timing.

CHAPTER III

CONCLUSIONS

3.1 Genome-wide association mapping of GABA contents during germination in Thai landrace rice (*Oryza sativa* L.) populations

GWA mapping of complex quantitative traits is made possible by high-quality genotyping data from high-throughput sequencing in conjunction with reliable statistical analysis. Based on high-density SNPs in Thai rice, we performed a GWAS for GABA accumulation. The 23 significant SNPs were found associated with loci on chromosomes 1, 2, 4, 5, and 11. Their annotation is consistent with their putative involvement in GABA contents. These SNPs significantly assist in identifying the likely underlying genetics of GABA accumulation. Future improvements in the effectiveness of GABA production will benefit from knowledge on the varieties with high GABA accumulation.

3.2 Calmodulin overexpression leads to increased gamma-aminobutyric acid (GABA) contents and glutamate decarboxylase activity in rice during germination

The findings of this study demonstrated that germination time and salt stress greatly affect the amounts of GABA in rice. In all *OsCam1-1* overexpressing transgenic rice lines, there was a higher accumulation of GABA content than in WT both during soaking and germination. The GAD activity in rice during germination was correlated

with the accumulation of GABA content and higher and more prolonged GAD activity was found in the transgenic rice. Therefore, we concluded that *OsCam1-1* gene could play an important role in regulating the activity of GAD enzyme and the levels of GABA in rice during germination, which in turn affecting its tolerance to salt stress.

3.3 Research limitations

If the amount of the sample is limited (<0.2 mg), we cannot use the method described in this study to measure the GAD activity.

3.4 Recommendation for further study

The GABA content increase under soaking condition. Based on the studies of optimal germination conditions of brown rice, production for germinated rice have been developed in the food industry. Nowadays, the production of rice has been enhanced with gamma-aminobutyric acid a component of this grain-based nutrition. The general caliber and substance of other functional components, however, are as important for the purpose of product evaluation. Along with the nutritional balance, the cost of the augmentation operation should also be taken into account.

3.5 Conflict of Interest

The authors declare that they have no conflict of interest.

APPENDIX



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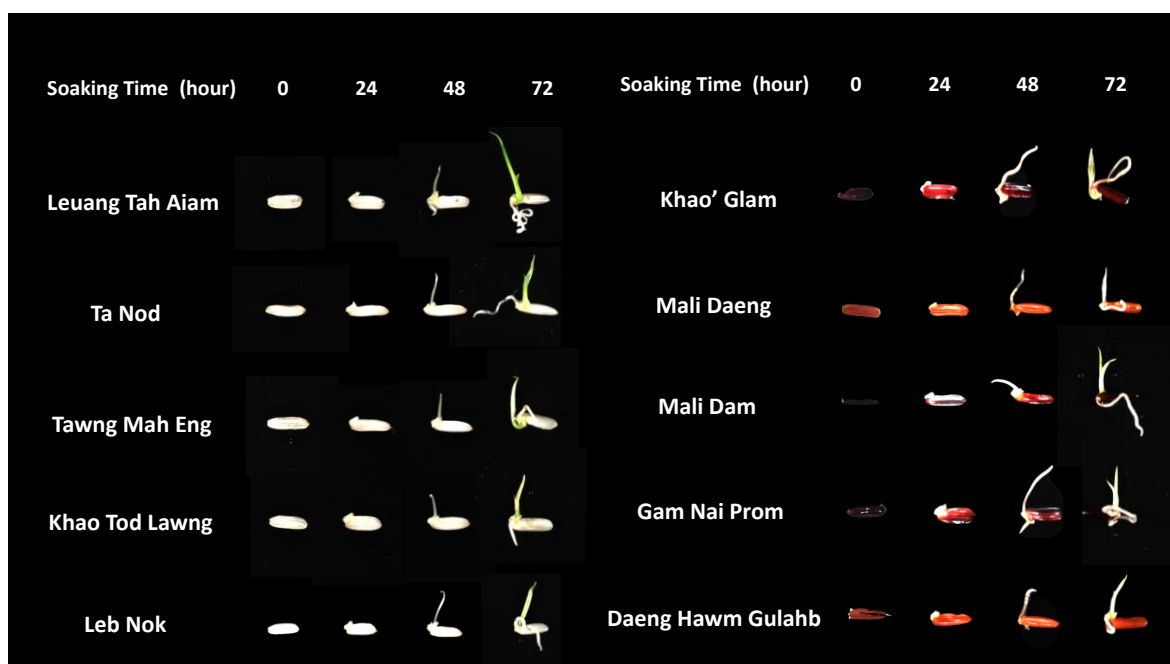


Figure S1 Germination at various time points of the 10 rice cultivars when soaked in water for 0-72 h at 35°C.



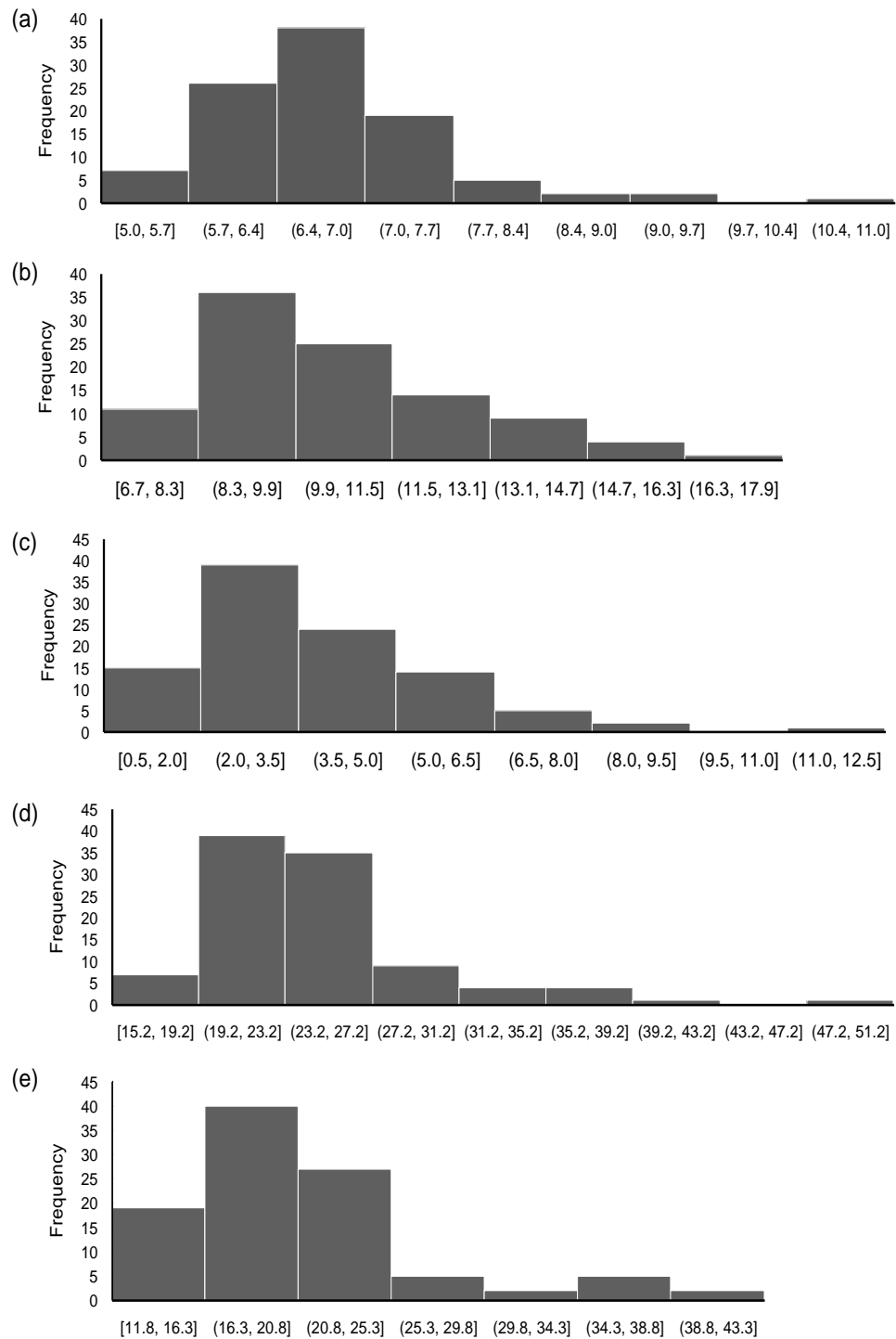


Figure S2 Histograms of GABA content values in rice grains during germination. Distribution of GABA contents at 0 h (a), at 48 h (b), increased GABA contents during 0-48 h (c), GABA contents per FW at 0 h (e), and at 48 h (f).

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