Effects of calmodulin overexpression on gamma-aminobutyric acid (GABA) levels and glutamate decarboxylase activity in rice seedlings

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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. Two of the three transgenic overexpression lines also exhibited statistically significantly higher GAD activity while the other line had a trend of having higher activity compared to the WT. These results suggest that transgenic rice plants overexpressing *OsCam1-1* had enhanced GAD activity facilitated by calmodulin overexpression, resulting in higher GABA content.

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

INTRODUCTION

Soil salinity is a major constraint on rice production worldwide [1]. 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 [2]. 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 [3, 4]. 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 [5]. GABA provides a source of carbon skeletons and energy for biosynthetic pathways through the GABA shunt, and it participates in signaling pathways [6]. Under salt stress, plants have adapted defense mechanisms provided by GABA to resist the damaging consequences of salt stress-induced ROS production [7, 8].

In plants, GABA is primarily produced by the decarboxylation of L-glutamic acid (L-Glu), which is catalyzed by glutamate decarboxylase (GAD) [9]. The accumulation of GABA is related to the activity of GAD and concentration of L-Glu [10]. Rice plants overexpressing *OsGAD* accumulate GABA at high concentrations under various pH conditions [11]. 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 [12].

GAD, which catalyzes the irreversible decarboxylation of L-Glu to produce GABA, is a cytosolic enzyme regulated by the Ca^{2+}-calmodulin (CaM) complex [13]. 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 [14]. 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.

MATERIALS AND METHODS

Sample preparation

Three lines of KDML 105 rice (*Oryza sativa* L.) seeds overexpressing *OsCam1-1* gene previously produced [15] 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 [16]. 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 °C. For each germination condition, the samples were analyzed in five replicates.

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 [17]. 2,3,5 Triphenyl tetrazolium chloride (TTC) was dissolved in deionized water to make 100% (w/v) stock solution and stored at 4 °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.

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 4,415g at 4 °C for 20 min. The samples were filtrated through a 0.2 µm cellulose acetate membrane, collected, and transferred to a new 500-µl microcentrifuge tube. Finally, the GABA content was measured using the GABase method [18, 19].

Preparation of crude proteins

A modified procedure described by Khwan-chai et al [20] was used to extract proteins for measuring GAD activity. Rice seedlings (10 grains) were ground in liquid nitrogen and extracted with 0.5 ml of 50 mM phosphate buffer (pH 5.8) containing 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 4,415g for 20 min at 4 °C using a refrigerated high-speed centrifuge. The protein content of the crude extract was determined using Bradford reagent [21].

GAD activity assay

The modified procedure described by Johnson et al [22] was used to determine GAD activity. The GAD activity assay reaction (200 µl) consisted of 50 mM sodium phosphate (pH 5.8), 30 mM L-glutamate, 20 µM pyridoxal-5-phosphate (PLP), and sufficient protein to produce a reaction rate such that the 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 incubation in a heat block at 100 °C. The samples were filtrated through a 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 [18, 19]. One unit of GAD activity was defined as the amount of enzyme that produced 1 µmol GABA per minute. GAD activity units per 100 mg of protein were calculated.

Statistical analysis

To evaluate the differences among the treatments, 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.

RESULTS AND DISCUSSION

GABA content during rice grain germination and seedling growth

During soaking (0–24 h)

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 (Fig. 1). They were approximately 6–8 mg per 100 g of rice grain. However, the GABA content in the transgenic rice increased after 6–24 h of soaking in water, whereas those in the WT only slightly increased, resulting in significantly higher GABA contents in the transgenic rice within the first 24 h of soaking (Fig. 1a). At 24 h, GABA contents in all three transgenic rice lines were two-fold higher than that in the WT. Their GABA contents reached 13–16 mg/100 g FW (approximately two-fold higher than that of non-germinated grains).

Previous studies have similarly reported the effect of soaking as a pretreatment for germination on GABA accumulation. Soaking brown rice in water for 24 h resulted in a similar range of GABA content in the present study [23]. Soaking rice grains results in higher levels of GABA and its intermediates than before germination and upregulation of the GAD enzyme [24]. The GAD activity responsible for GABA biosynthesis was not found in rice grains before germination. However, GAD protein was detected using western blotting by Jannoey et al [25], suggesting that upon water absorption during soaking, GAD enzyme was activated. This likely resulted in increased GABA production, especially in transgenic rice overexpressing calmodulin, an activator of the GAD enzyme.
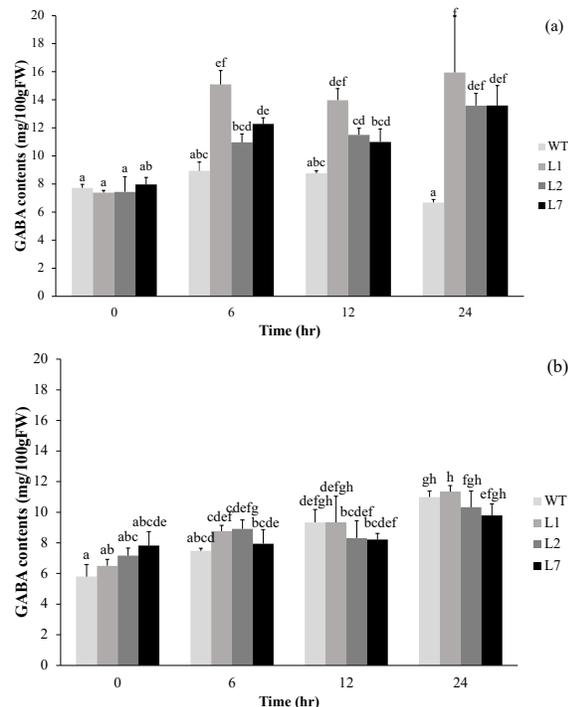
Fig. 1 GABA contents in grains of transgenic rice overexpressing *OsCam1-1* (L1, L2, and L7) compared with that in grains of WT during soaking. (a) Normal condition; (b) salt stress (100 mM NaCl). Data shown are means ± SD of five replicated experiments. Different letters on bars indicate significant difference at $p < 0.05$.

The results suggest that *OsCam1-1* overexpression led to higher GABA production – 6–24 h after the grains were soaked in water to trigger the germination process. Using cDNA expression library screening, OsCaM1 has been reported to interact with a GAD protein (LOC_Os08g51080) [26]. The overexpressed OsCaM1 likely activated GAD proteins, which in turn resulted in the higher GABA content observed in transgenic rice. *OsCam1-1* overexpressing rice lines have been shown to be more tolerant to salt stress than the WT at the early seedling stage [15], which may be partially due to 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 [27].

Salt stress can affect seed germination, resulting in biochemical and physiological changes [28]. The effect of soaking in NaCl solution on the accumulation of GABA in transgenic rice overexpressing *OsCam1-1* compared with the WT was investigated (Fig. 1b). Overall, the amount of GABA in all the rice lines increased slightly including the WT. Under salt stress, the WT exhibited an increase in GABA content, which was not observed when soaked in water. The increase

in GABA content under salt stress has been observed in other plant species including wheat and barley [12], which possibly contributes to the responses of these plants to salt stress (discussed below). However, at all time points, the transgenic rice did not exhibit different GABA contents from the WT, as the GABA content 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 content observed under normal conditions in the transgenic rice overexpressing the *OsCaM1-1* gene (Fig. 1a) would better prepare them to adapt to salt stress than the WT. The higher degree of salt tolerance of transgenic rice that overexpressed *OsCam1-1* previously reported [15], may be partly explained by the increased GABA accumulation during this early stage of germination.

During germination and seedling growth (2–12 d)

Seedling growth was monitored under normal conditions (Fig. 2a) and NaCl treatment (Fig. 2b). The rice seeds used were tested and confirmed for their viability by TTC staining (Fig. 2). Under salt treatment, rice seeds geminated more slowly than under normal conditions. However, growth of the WT and the transgenic rice lines did not appear to be significantly different both under normal condition and salt stress. GABA content on day 2 under both normal (Fig. 3a, Table S1) and salt treatments (Fig. 3b, Table S2) was around 6–8 mg/100 g FW. From days 2 to 4, GABA content significantly increased under both normal and salt stress conditions. While it remained at similar levels under normal conditions (Fig. 3a), GABA content from day 4 to day 6 continued to increase under salt stress (Fig. 3b). Compared with the WT from day 6 to day 8, all three transgenic lines showed a trend of higher GABA accumulation under salt-stress treatment (Table S2): 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/100 g 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/100 g FW, respectively). GABA content values under salt stress were higher than those obtained from their respective plants grown under normal conditions. However, from days 8 to 10, GABA content increased rapidly under normal conditions (Fig. 3a) as the seedlings grew rapidly. In contrast, the GABA content under salt stress (Fig. 3b) decreased by 50% during the same period. After 12 days of germination under normal conditions, all transgenic lines exhibited statistically significantly higher GABA contents than WT (Fig. 3a, Table S1).

These results were consistent with those of 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 germina-
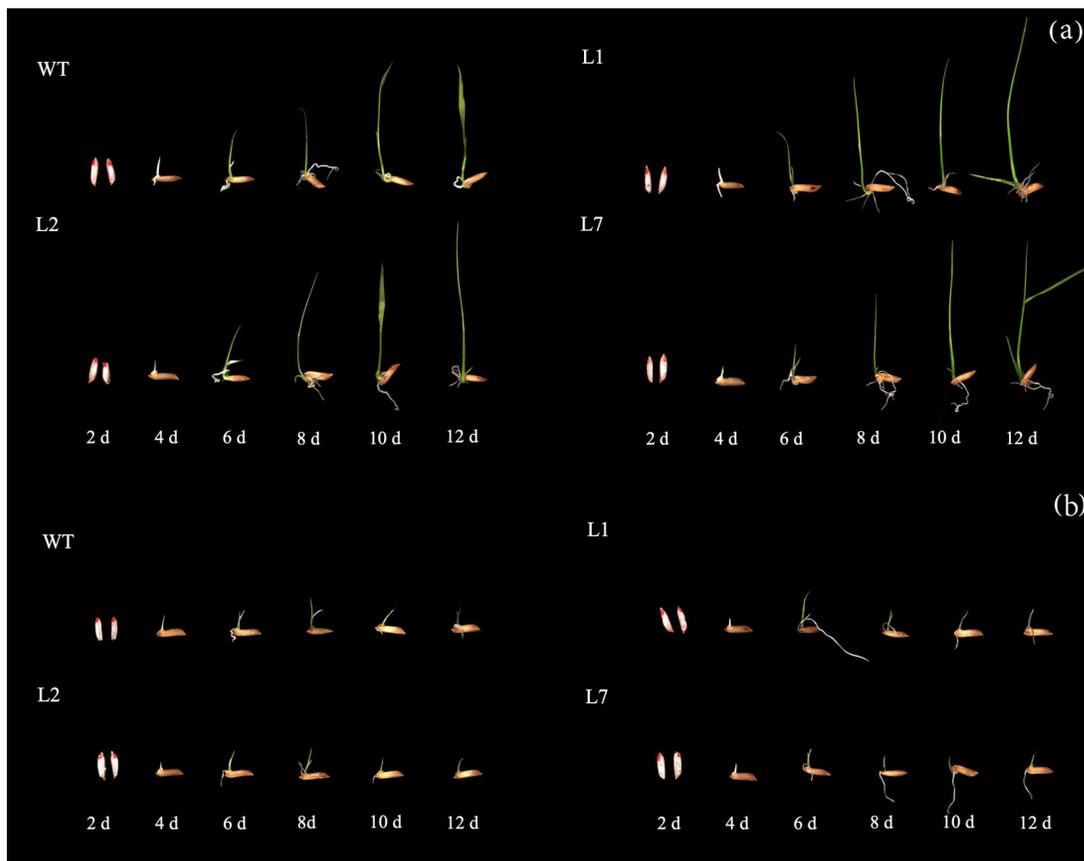
Fig. 2 Grain germination at various time points for the transgenic rice overexpressing *OsCam1-1* and WT under normal condition (a) or salt stress (b).
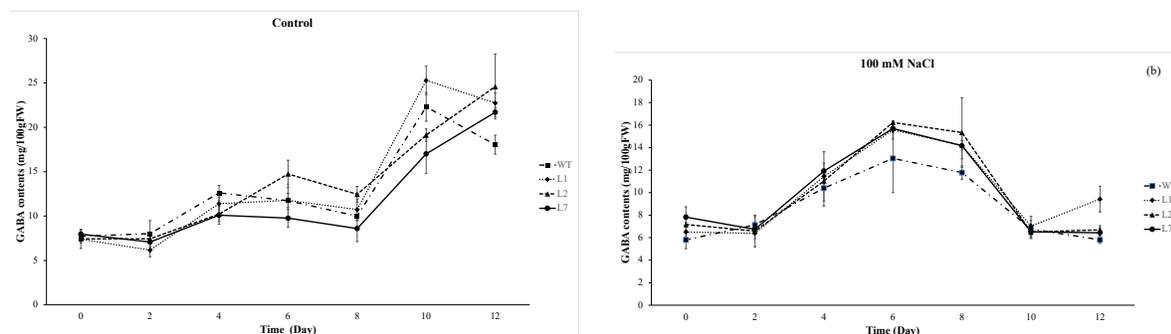
Fig. 3 GABA contents during rice grain germination under (a) normal condition (Control) and (b) salt stress (100 mM NaCl). Data shown are means ± SD of five replicated experiments.

Table 1 GAD activity in germinating grains of transgenic rice overexpressing *OsCam1-1* grown under normal condition.

Line	GAD activity (Unit/100 mg 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.

tion [29]. 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 [30]. 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 the five days of germination [12]. In millet, the 48-h old germinating seeds treated with NaCl stress exhibited a rapid increase in GABA content, which tended to become flat when the treatment time was longer than 48 h [31]. 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 [32]. 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 [33] reported that *OsCam1-1* overexpression possibly decreases salt-induced oxidative damage in transgenic plants by promoting the activities of antioxidant enzymes. The GABA shunt pathway and accumulation of GABA metabolites in *Arabidopsis* seedlings (*cam 5-4* and *cam 6-1* mutants) have been reported to contribute to the antioxidant machinery associated with ROS and the acquisition of tolerance in response to induced oxidative stress [34]. The GABA shunt can be activated under salt treatment, with increased contents of GABA shunt metabolites such as GABA, Ala, and Glu, in germinating seeds when treated with salt stress; example cases include wheat and barley cultivars [35]. Hence, this is an alternative route to the respiratory machinery.

GAD activity during rice grain germination

The formation of GABA from L-Glu (via decarboxylation) in grains during germination is catalyzed by GAD [22]. GAD is a well-known CaM-interacting protein (CIP) [36], and a GAD gene (LOC_Os03g51080) has been identified to encode CIP in rice by cDNA expression library screening [26]. We examined GAD activity in 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 1 shows 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. 3a). 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 was higher than that in the WT, which corresponded to the level of GABA (Fig. 3a, Table 1).

The GAD enzyme is associated with an active complex composed of CaM and Ca^{2+}, which promotes GABA production [9]. In this study, the GAD enzyme in the rice grains became active, as reported by Jannoey et al [25], and its activity increased during germination. Several previous reports have shown that the expression of these genes is induced during germination and under stress [29, 37]. 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 [38]. Therefore, it is likely that the enhanced accumulation of GABA in the *OsCam1-1* overexpressing rice lines under salt stress resulted from activation of the GAD enzyme by OsCaM1.

In conclusion, the findings of this study demonstrated that germination time and salt stress greatly affect the amount of GABA in rice. Overall, the *OsCam1-1* overexpressing transgenic rice lines tend to exhibit a higher accumulation of GABA than in WT. GAD activity in rice during germination was correlated with the accumulation of GABA, and higher and more prolonged GAD activity was found in transgenic rice. Therefore, we conclude that *OsCam1-1* may regulate the activity of the GAD enzyme and the levels of GABA in rice during germination, which in turn affects its tolerance to salt stress.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874. 2023.065.

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Appendix A. Supplementary data

Table S1 GABA content in germinating grains of transgenic rice overexpressing *OsCaM1-1* grown under normal condition.

Line	GABA Content (mg/100 g FW)						
	0 d	2 d	4 d	6 d	8 d	10 d	12 d
WT	7.72 ± 0.75^{abc}	8.02 ± 1.48^{abcd}	12.62 ± 0.84^{fg}	11.73 ± 1.88^{efg}	10.01 ± 0.54^{bcdef}	22.30 ± 1.62^{jk}	18.04 ± 1.07^{hi}
L1	7.39 ± 0.16^{abc}	6.18 ± 0.77^{a}	11.40 ± 0.70^{defg}	11.77 ± 0.80^{efg}	10.74 ± 1.18^{cdef}	25.29 ± 1.61^{k}	22.75 ± 1.13^{k}
L2	7.44 ± 1.09^{abc}	7.44 ± 0.34^{abc}	10.22 ± 0.84^{bcdef}	14.75 ± 1.54^{gh}	12.46 ± 0.89^{fg}	19.12 ± 0.70^{ij}	24.60 ± 3.65^{k}
L7	7.97 ± 0.50^{abcd}	7.07 ± 1.09^{ab}	10.11 ± 1.03^{bcdef}	9.77 ± 1.02^{abcdef}	8.60 ± 1.47^{abcde}	17.01 ± 2.20^{hi}	21.70 ± 0.48^{jk}

Data shown are means $\pm$ SD of five replicated experiments. Different letters over means indicate significant difference at $p < 0.05$.

Table S2 GABA content in germinating grains of transgenic rice overexpressing *OsCaM1-1* grown under salt-stress condition.

Line	GABA Content (mg/100 g FW)						
	0 d	2 d	4 d	6 d	8 d	10 d	12 d
WT	5.80 ± 0.79^{a}	7.13 ± 0.76^{ab}	10.40 ± 1.59^{cde}	13.04 ± 3.03^{efg}	11.79 ± 0.59^{def}	6.78 ± 0.78^{ab}	5.81 ± 0.32^{a}
L1	6.50 ± 0.43^{ab}	6.38 ± 0.47^{a}	11.44 ± 2.19^{def}	15.56 ± 0.81^{gh}	14.20 ± 0.41^{fgh}	7.03 ± 0.87^{ab}	9.43 ± 1.15^{bcd}
L2	7.17 ± 0.50^{ab}	6.59 ± 1.41^{ab}	11.04 ± 0.58^{de}	16.24 ± 0.14^{h}	15.34 ± 3.10^{gh}	6.51 ± 6.51^{ab}	6.69 ± 0.20^{ab}
L7	7.82 ± 0.91^{abc}	6.74 ± 0.38^{ab}	11.91 ± 0.70^{def}	15.70 ± 0.37^{gh}	14.16 ± 1.16^{fgh}	6.51 ± 0.32^{ab}	6.44 ± 0.63^{ab}

Data shown are means $\pm$ SD of five replicated experiments. Different letters over means indicate significant difference at $p < 0.05$.