CHAPTER 4

DISCUSSION

4.1 Physiological appearance of rice during germination

To follow seedling development, length of shoot and root, weight of germinated grain and total seedling of the seven cultivars were measured as shown in Figure 3.1 (a-d). Shoot and root weights of most cultivars increased along with germination time, while grain weight decreased.

Germinated grain weight increased in the first 3 days of germination, indicating that rice grain took up the water for preparation of germination process and leads to the breaking of the seed coat (Mayer and Mayber, 1982). Thereafter, germinated grain acts as an autotroph by using carbohydrate in seed for supplying seedling growth (Smith and Dilday, 2003

As a results, grain weight began to decrease after 3 days until 30 days of germination (Figure 3.1a). Another evidence possibly showed that, starch was digested by the activated amylase (Mayer and Mayber, 1982). While, Helel *et al*, (1996) suggested the mobilization of storage starch to the other growing part during the germination period.

Although, the longer germination period gave higher GABA contents in germinated grains (Figure 3.1). This weight decreased is not acceptable for the consumers who preferably consume germinated rice as a functional food and also would like to obtain calories and texture feeling from germinated rice. This result suggested that the best period of germination is the first 5 days of early stage.

The root and leaf lengths showed a parallel increase with the GABA content, as germination time lengthened was in all seven cultivars.

The results provide the information useful for preparation of rice leave and root by appropriate germination time as shown in Figure 3.1b-c and also suggested that the change developed during seedling development.

However, at the first and second germination days, rice seed presented only coleoptile (shoot). The coleorhiza (root) first emerged from the seed followed by the prophyll at the third day as shown in Figure 3.2. The first leaf protrusion from the germinated seed occurred at fourth germination days. In this study, we start to measure the height of leave at the beginning of the fourth day as shown in Figure 3.1b.

Interestingly, this research found that during rice seedling development of all cultivars, there were only one first leaf through 30 germination days. In contrast, Counce *et al,* (2000) suggested that the full emergence of the fourth true leaves should be appeared at the end of seedling stage within 30 days of seedling emergence. However, the first leaves showed extended straight shape together with the developmental change of seedling. For possibly explanation, the seedling in this growth study was done on the moisture paper where the nutrient supply was not enough as in soil or enrich medium for longer germination time. Therefore, this study allow only first leaf to develop during 30 days as shown in Figure 3.2.

Considering the coleorhiza, it is the second tissue emerging from the seed coat in this study at the second day, as shown in Figure 3.2. This phenomenon is corresponding to Counce *et al,* (2000) who suggested that, under the water submerged rice seeds condition, the coleoptile usually emerges first and coleorhizae is suppressed.

Moreover, the agglomeration of root at the bottom of plastic boxes at the longer germination time was observed in this study. It is a critical to measure the length of root. In further study, the design of the container to allow root elongation should be examined. The work of Kikui *et al*, (2005) suggested to make a device for observation and measurement of root growth from germination stage to seedling stage in hydroponic culture (Figure 4.1).

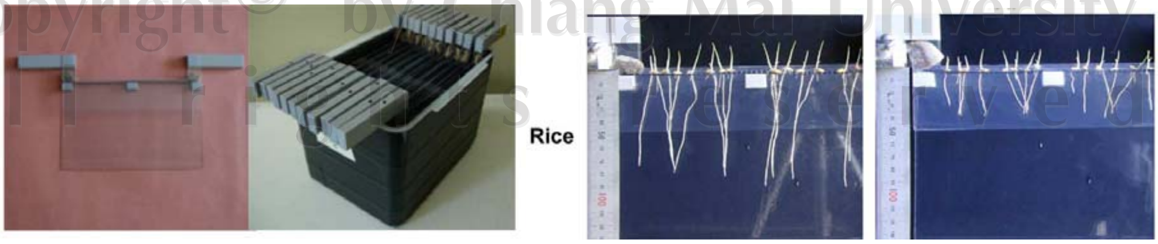

Figure 4.1 The device was made for observation and quantitative measurement of root growth during germination stage (Kikui *et al*, 2005).

Two plastic plates (145×215mm and 50×215mm) were combined together with a 10 mm space. In this space a plastic mesh was set. Then the sterilized and pre-soaked seeds were transferred on the mesh, and the device was placed on the top of a tank. Water filled til the mesh position (6 L/10 devices), then the seeds were germinated and grown on the mesh. (Kikui *et al*., 2005)

However, the extension of root can still be measured in this study and the results are similar to the report of Smith and Dilday, (2003), who reported that the normal single root grew 3-5 cm within the first 3 days after germination and typically extended to 12 cm under aerobic condition and light.

They suggested that root development contributes to plant nutrient uptake from emergence through the seven-leaves stage as described in the introduction section.

4.2 GABA contents in rice during germination

In this research, whole rice grains were subjected to germination for different days. The germination process leaded to increasing of GABA contents during germination time both in grains and young leaves, while the control contained less GABA as shown in Figure 3.4 and Figure 3.5.

This result agreed with the previous report of Oh and Choi, (2001) which showed higher GABA concentrations in rice, barley and soybeans after germination than in the control. Beside these, this result was the same as the previous reports which studied the effect of germination on GABA accumulation in brown rice and other plant species. For example, Korean and Japanese brown rice prepared by soaking in water, showed higher GABA concentrations of 13 and 3.41 fold higher than the control, respectively (Oh, 2003; Miura *et al*., 2006). Moreover, GABA is likely to accumulate upon soybean [*Glycine max* (L.) Merr] seed growing, about 11-17 fold increase as compared with the control plant (Oh and Choi, 2001). While, germinated wheat had increased GABA content 18 times greater than the control (Nagaoka, 2005).

During germination, hydrolytic enzymes were activated in grains, especially GAD enzyme which were effectively converted L-glu to GABA upon water absorption during soaking and germination (Komatsuzaki *et al*., 2007).

Another reason explained that germination process can increase GABA concentrations in many seed species, possibly because the plant germination has a metabolic change in seed and reserved materials were breakdown after hydration, which results in an accumulation of a soluble nitrogen compound such as GABA (Mayer and Mayber, 1982). A related reason is that extensive breakdown of seed-storage compounds occurs and protein and other cell components are synthesized during this process (Kuo *et al.,* 2004).

Moreover, Komatsuzaki *et al*, (2007) suggested that storage proteins were decomposed by water absorption during germination, changed into transportable amide and supplied to the growing parts of rice seedling as a nitrogen source for embryo growth during germination.

However, the GABA contents were found in control to be 0.19, 0.24, 0.34, 0.39, 0.51, 0.31 and 0.23 mg/g in PL2, CN1, SP1, PT1, KDML 105, SPT1 and RD6 respectively. The values were not different among the cultivars (p>0.05) before germination. After germination, germinated whole grain rice showed different GABA concentration levels. Saikura *et al,* (1994) suggested that the amount and pattern of GABA accumulation varied with each cultivar. This variation might be due to the different GAD activity.

The increasing of L-glu content during germination was also considered, although it has been converted to GABA. L-glu might be resulted from activating of enzymes during germination, for example, a variety of proteolytic enzymes. As a result, proteins are broken down into free amino acids including L-glu (Kigel and Galili, 2003).

Another type of enzymes involving in L-glu formation is aminotransferase. The previous report showed that L-glu was produced in seed during germination by the action of glutamine-oxyglutarate aminotransferase (Mayer and Mayber, 1982). This enzyme reaction leads to L-glu formation and subsequent transformation to higher GABA during germination by GAD enzyme (Oh and Choi, 2001). However, some evidence suggested that, L-glu was increased because it was synthesized by the glutamate synthase (GOGAT) and glutamine syntheses (GS). The GS/GOGAT system plays an important role in glutamate accumulation during germination (Aurisano *et al*., 1995).

Although the GABA concentrations in germinated whole grain rice increased during germination time, but GABA concentration began to decrease after 20 germination days. GABA concentrations in rice grains of all decreased after 20 germination days, except SP1 and KDML105 cultivars. However, after 20 germination days, unchanged GABA contents was presented in KDML105 cultivar. While, GABA contents of SP1 slightly decreased after 25 germination days. The results indicated that GABA was produced during germination and was used as nitrogen source of growth and converted to SSA by GABA-T and SSADH enzyme in mitochondria further acted as a metabolizing compound in the TCA cycle (Kono and Himeno, 2000; Huang *et al.,* 2007). Therefore, it is of interest to examine this activity and inhibit both enzymes during germination for higher GABA accumulation in further study.

In addition, the GABA content in this research was compared with other data, such as Japanese rice prepared by soaking in water for 96 hours at 30^{o}C, contained GABA at 1.49 mg/g (Komatsuzaki *et al.,* 2007) and GABA contents of 0.207 mg/g for Korean rice (Oh, 2003). But when Japanese rice was soaked in water and gas treatment during germination, 2.49 mg/g of GABA were reported (Ohtsubo *et al.,* 2005). It can be seen that after soaking with gaseous treatment, the content of GABA was higher than that by the conventional soaking method. It is possible that, rice treated by gas during germination can reduce microorganisms and prevent fermentation during rice soaking. Fermentation process leads to decrease amount of GABA during germination (Komatsuzaki *et al*., 2007).

While, the highest of GABA content of our germinated rice was 3.16 mg/g, which was higher than the previous reports because the different methods (germination and long soaking time, temperature and variety of rice cultivars) used for treatment during germination which had the effect on GABA contents.

Another reason is different cultivars showed the varying of embryo weights. There was a report that GABA in the brown rice with giant embryo drastically increased after germination when soaked in water at 30^{o}C for 24 hours (Matsuo *et al*., 1997). This result related with the previous report in which *O. sativa* mutant with giant embryo was enhanced in GABA concentration and several japonica rice (*O. sativa* var *japonica*.) mutants with giant embryo were found to be nutritionally enhanced (Zhao and Jiang, 2002).

Beside these, the GABA accumulation in young leaves was investigated (Figure 3.4b). Young leaves are considered as waste from GABA-enriched rice production since they are removed from grains before milling. Young rice leaves showed the higher GABA concentrations than in rice grains in all cultivars during germination time. The highest GABA contents of leaves were 2.25, 2.53, 1.02, 1.33, 1.42, 1.32, 1.46- folds of those in grains of SP1, PL2, KDML105, PT1, CN1, SPT1, and RD6 respectively. The difference in the level of GABA in each seedling part was seen in previous report. The GABA concentrations were detected depending on the stage of tissue development with the highest level found in the apical regions such as root and leaves (Nagaoka, 2005).

However, it was reported that, a loss of nitrogenous compounds such as GABA in seed was observed together with its increase in growing part and pumule of seedling (Kigel and Galili, 2003).

4.3 Amino acid contents in rice during germination

During the germination of rice grain, amino acids were also produced in this study. The polar amino acids (arginine, asparagine, serine, glycine, threonine, glutamic acid, tyrosine), non-polar amino acids (alanine, valine, tryptophan, isoleucine and leucine) were eluted under the extraction condition in this study and showed increased level after germination as shown in Figure. 3.7, but aromatic and sulfur amino acids were not found.

In most legume (Kuo *et al.,* 2004) and Korean rice cultivar (Lee *et al.,* 2007), the sulfur amino acid was not detected during germination which was similar to that found in this study. Asparagine and Isolecine were found in very high level the same as that found in this study, but their content lowest in Japanese germinated rice (Maeda *et al.*, 2007) and lpum cultivars of Korean rice (Lee *et al.,* 2007). While, germinated brown rice of Haiminori Japanese cultivar, almost amino acid content increased about 1.07-7.11 fold, except aspartic acid, serine, asparagine and glutamic acid which decreased.

These results support the fact that the amount and pattern of amino acid accumulation varied with each cultivar and region.

In this study, the alanine presented at the highest increase, 8.38-fold at 30 germination days. While, arginine, asparagine, GABA, serine, glycine, threonine, glutamic acid, alanine, tyrosine, valine, trptophan, isoleucine and leucine, amino acid showed the highest content in germinated rice grain at 3.42, 3.34, 4.81, 2.01, 1.73, 1.53, 2.89, 2.92, 4.43, 3.25, 6.19 and 3.33-fold when compared to the control, respectively.

These results indicated that germination process lead to the increasing of amino acids in germinated rice. Previous report suggested that amino acids stored in raw rice seed as storage protein are breakdown by protease after water absorption during germination, changed into amino acid transportable amide, supplied to the growing parts of the rice seedling (Lea *et al.,* 1990).

Beside these, hydrolytic enzymes are activated and they decompose starch, nonstarch polysaccharides and proteins, which leads to the increase of oligosaccharides, and amino acids in germinated cereal grains; barley (Rimsten *et al*., 2003), wheat (Yang *et al,* 2001), oat (Mikola *et al.,* 2001) and rice (Manna *et al.,* 1995).

In young leaves, amino acid contents increased during germination, but their contents were higher than those in germinated rice grain at the same germination days. The value of amino acid content in young leave showed the highest increase between 1.31-fold to 11.20-fold of serine and isoleucine, respectively.

It might be due to the mobility of various compounds of seed to leave during germination. It was reported that, after the breakdown of materials in seed, the transport of materials from seed to another can occur. Reggina *et al*, (2000) suggested that the accumulation of amino acid is known to derive from the assimilation of the storage protein in seed followed by translocation of amino acid. For example, from the endosperm (seed) to the embryo or from the cotyledon to growing parts and lastly the synthesis of new materials from the breakdown product are formed (Mayer and Mayber, 1982).

Moreover some evidence suggested that hormone in leaves had a little direct effect on the activity of protease activity. The hormone can activate amino acid peptidase activity for breakdown storage proteins. As a results higher amino acid contents were found in leaves than the other parts of seedling. (Figure 3.7b)

Considering the increased rate of 12 amino acids with germination time (Figure 3.7), amino acid contents rapidly increased at the early stage of germination (5-10 days) when comparing with the control. After 15-30 days, the amino acid concentrations were slowly increased, only few exceptions were seen in isoleucine, leucine, arginine and asparagine. These amino acids showed continuous increase in both grain and leave, as the germination time increases.

4.4 Comparison of GABA and amino acid contents among rice cultivars

GABA and amino acids content were compared among varieties of rice cultivars. It was found that, germinated rice presented different amounts of amino acid and GABA at different germination days from 5 to 30 days.

Different GABA and amino acid content found implied that the rice cultivars had different physical properties and different enzymes for GABA and amino acid production. Although, the rice in this study are in same the species and growing area.

However, significant difference of GABA and amino acids content with long time germination were not important in rice grain, because grain weight loss and characteristic were not acceptable. The acceptable physiological appearance of germinated rice grain presented before 5 germination days.

During the first 5 days of germination, the consumer can obtain the GABA as well as amino acids content in all cultivars. It may be because 5 germination days is the early stage of germination, the metabolic change may not differ among the cultivars which corresponding to the length of leaves and root as shown in Figure 3.1. There were not difference in the length at the early germination days. For these reasons, the GABA and amino acid which produced from in this stage were not different.

Young leave also showed the difference ($p \leq 0.05$) of most of amino acids and GABA contents for the varieties of rice cultivars (Table 3.2). For example, at 10, 15, 20, 25 and 30 germination days, we observed the difference of GABA, arginine, asparagine, serine, glycine, threonine, L-glu acid, alanine, tyrosine, trptophan, isoleucine, valine and leucine. While, during 0 and 5 germination days there was not much variation of GABA and amino acid contents.

This results can be suggested that the consumer will obtain the different amounts of GABA and amino acids in leaves if the variety of cultivars were germinated at 10, 15, 20,25 and 30 germination days. Therefore, the germination time should be considered if GABA and amino acid are to be produced in leaves of different cultivars.

4.5 GAD assay by LC-MS and Western blotting

The results of GAD activity in developing rice seedling of selected KDML105 and SPT1 cultivars were shown in Figure 3.12. Both GAD activities and protein both in young leave and germinated grains during germination, but at different levels (Figure 3.13b). GAD activity was very low in the germinated rice grains, while 3.30 fold higher was found in young leaves.

When GAD protein expression patterns in young leave and grain were compared using western blotting technique. The expression of GAD protein of 70.8 Kd in young leave (lane 4,7) and germinated grains (lane 3,6) was higher than the control (lane 2,5) (Akama and Takaiwa, 2007) as shown in Figure 3.13b.

Interestingly, the GAD activity of the control was not found in the control Figure 3.12a, by LC-MS technique but its activity examined after germination. While, GAD protein in control was detected (lane 2,5) when using western blotting technique. Komatsuzaki *et al*., (2007) suggested that upon water absorption during soaking and germination, GAD enzyme be activated.

The results in Figure 3.13a and Figure 3.13b supported the data of GABA content (Figure 3.4), which were described above. This suggested that young leave is a better source of GAD enzyme than grains. It is possible to use GAD enzyme in other applications, such as GABA production.

4.6 Change in protein profile of rice after germination and non-germination

The SDS-PAGE results (Figure 3.14) showed the total protein patterns in germinated rice grain (lane 3,6), young leave (lane 4,7) and the control (lane 2,5). Protein profiles of germinated grain (lane 3,6), were different from the control (lane 2,5). It showed strong decreased intensity of 51.86 and 30.26 kd bands (lane 3,6) when compared with the control (lane 2,5).

Beside these, protein profile in young leave of two cultivars was also shown in lane 4 and 7. The protein profile differed from those of grains at various positions. Young leaves presented dominant bands at 95.7, 91.1 and 55.82 kd, while these proteins disappeared in grain.

When considering the electrophoresis analysis of rice protein throughout the germination time period of grain and leaves, the results in Figure 3.15 showed that when the germination time increased, the decrease in intensity of protein band was observed. These results showed inversed relationship with the amino acid level.

This may be explained that, a decrease of these band intensity (Figure 3.14) indicates that protein in germinated rice grain was hydrolyzed and which is reflected in the production of higher amino acid as shown in Figure 3.7 and Figure 3.8. The results can be the evidence supporting that proteins were broken down during germination. However, the increased intensity of protein bands in germinated grains compared to the control at 110.8, 67.9 and 64.7 kd, representing newly synthesized proteins occurred during germination.

However, strongly diminished protein at 51.86 kd will be of interest to study further on protein name, category, as well as its properties. Proteome analysis is used as a tool in this study.

4.7 Proteomic analysis of non germinated rice grains and its change during germination

Protein patterns of GABA-enriched rice after germination were also investigated using 2-D electrophoresis technique. After CBB-R 250 staining, proteins were distributed in the range of mass weight of 113-21.4 kd using IPG strip with the range of pH 3-10.

After matching of protein profile of germinated rice contain higher GABA content with the control using Image Master 2D Platinum software. It was found that, the proteome pattern were very similar. However, some protein spots could not detected in the gel of germinated rice, but they existed in the control. While, some proteins were present in germinated rice gel, but absent in the control gel.

Most of differentially down-regulated protein was found in cultivars, SPT1 and KDML105. The 24 and 19 down-regulated spots were found in KDML105 and SPT1, respectively.

However, 3 and 2 down-regulated spots of KDML105 and SPT1 that showed the approximated mass weights of 51.96 kd were interesting. Because their MW were closed to the disappeared protein in SDS-PAGE (Figure. 3.14-3.15). We chose 3 protein spots of KDML105 cultivar and 2 spots of SPT1 cultivar to be further analyzed by MALDI-TOF MS, LC-MS/MS and NCBI database search. The decreased proteins were annotated to be Os03g0793700; globulin2 and hypothetical protein OsI_13867). All of them revealed a nutrition reservoir activity, which were classified in storage protein categories.

Storage protein is one of the three major reserves (protein, starch and lipid) in the plant seeds. Along with those proteins being important for seed filling and maturation, the storage proteins were synthesized during the process of maturation and degraded as the energy and nitrogen resources for seed germination and the subsequent seedling growth (Yang, et al, 2007). Rice globulins were rich in glycine, and glutamic acid or glutamine (Komatsu and Hirano, 1992).

This study found globulin 2 to be down-regulated as in previous study. This phenomenon was also observed in Arabidopsis seed germination, rice seed (Wang *et al.*, 2008; Yang *et al,* (2007) in which many down regulated protein were defined as globulin.

In conclusion, it suggested that the major storage proteins were first broken into small fragments, which were then degraded gradually during seed germination.

In this study, the changing pattern of protein spots reflected this degradation behavior. We also found that the amino acid increase, while the disappeared Globulin and hypothetical protein OsI_13867 occurred. These results implied that energy reserve might be mobilized during germination (Yang *et al.,* 2007; Khan and Komatsu (2004).