CHAPTER 3

RESULTS

3.1 Physiological appearance

Rice germinated grain weights were investigated during germination. It was found that grain weight increased starting from the non-germinated rice (control) through the first 3 germination days in all cultivars. After 3 germination days, grain weight drastically reduced until 30 days, as shown in Figure 3.2a. Raw seed weights were first found to be 0.0302, 0.0290, 0.0271, 0.0292, 0.0239, 0.0257 and 0.0285 g and decreased to 0.0231, 0.0199, 0.0186, 0.0218, 0.0198, 0.0196 and 0.0198 for SPT1, SP1, PT1, PL2, KDML105, CN1 and RD6 cultivars, respectively in 30 days. The average percentage of grain weight loss varied in the range of 22-31.11% during 3-30 germination days comparing with the initial raw seed.

The length of young leaves was measured after the first true leaf emerges from the seed during the 4 days of germination as shown in Figure 3.1. The length measurement was done from the culms base to the tip of the longest leaf for investigation the growth rate of rice seedling. Counce *et al,* (2000) suggested that seedling height usually represented the growth rate. It was found that, leaf length rapidly increased at the early stage of germination days, especially from 3 to 5 days in most cultivars (Figure 3.2b). From 4 to 30 germination days, the leaf length increased from 1.64 to 11.5, 1.32 to 17.4, 1.6 to 15.38, 1.3 to 13.62, 1.1 to 12.6, 0.8 to 11.6 and 11.1 to 11.8 for SPT1, SP1, PT1, PL2, KDML105, CN1 and RD6 cultivars, respectively. The leaves length of all the cultivars were not different, except in PT1 and SP1 that exhibited longer leaves than those in the other cultivars as shown in Figure 3.2b.

The extension of root during germination was also investigated. The root length was increased at the early stage of germination, but slowly increased during longer germination time (Figure 3.2c). Developed coleorhiza (root) emerged from the germinated grains at the second germination day (Figure 3.1). The average root lengths were 2.94

2.96, 3.14, 2.26, 2.25, 2.3, 3 cm before extended to 8.7, 11, 7.7, 7.9, 7.8, 9.24, 8.32 and 8.1 cm for SPT1, SP1, PT1, PL2, KDML105, CN1 and RD6 cultivars, respectively. The highest root length presented in PT1 cultivars, especially at 5-10 germination days as shown in Figure 3.2c.

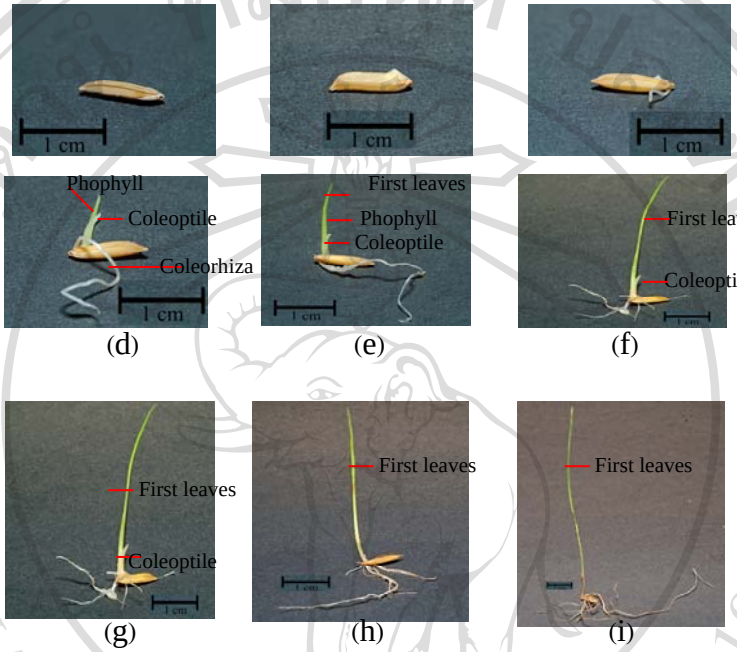

Figure 3.1 Development of represented rice of KDML 105 cultivars during germination (**a**) non-germinated grains and germinated seedling during germination at (**b**) 1 days, (**c**) 2 days, (**d**) 3 days, (**e**) 4 days, (**f**) 6 days**,** (**g**) 10 days, (**h**) 20 days and (**i**) 30 days.

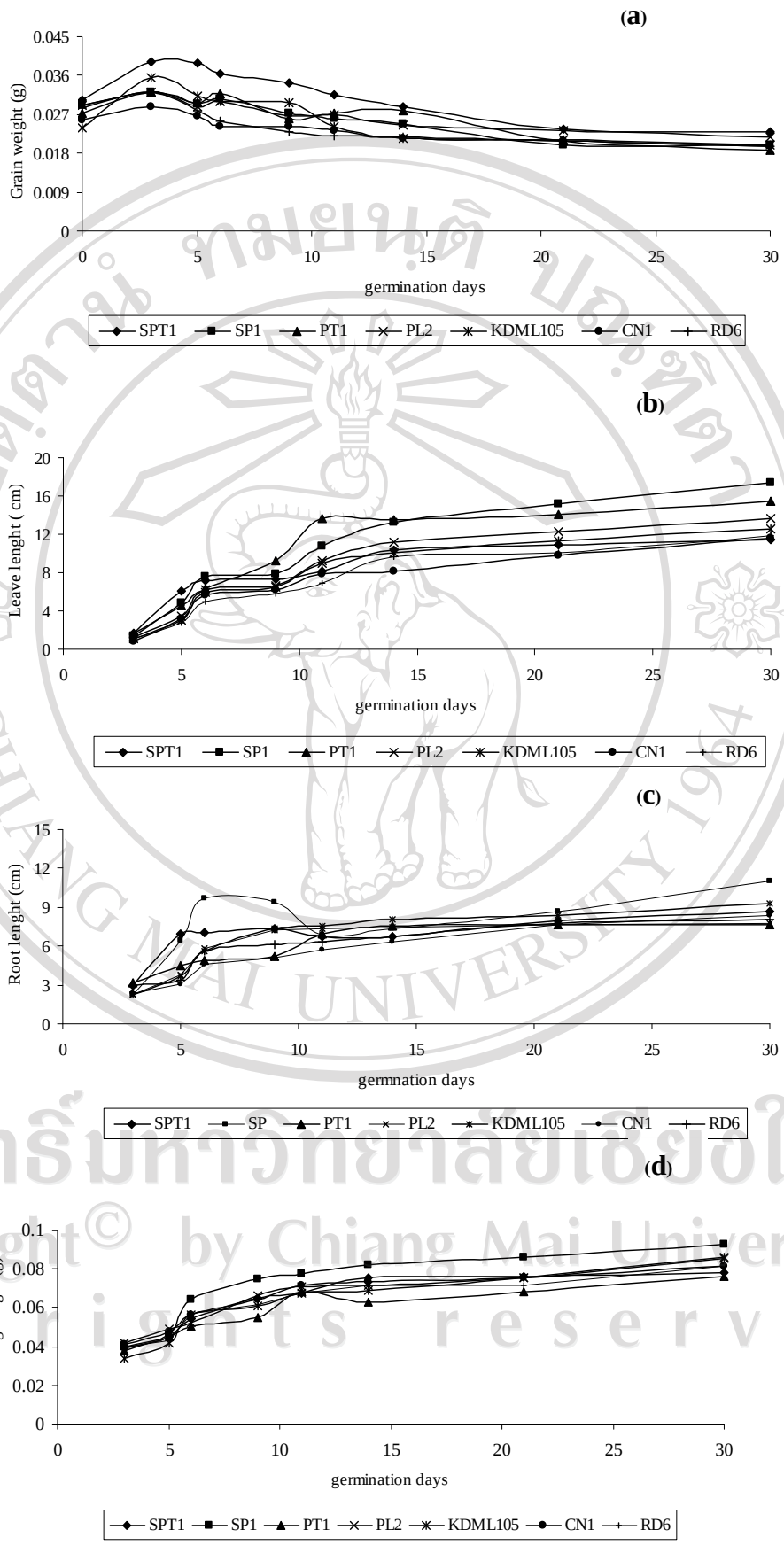

Figure 3.2 Seedling developments under aerobic and light conditions at 30°C. Change in grain weight (**a**) the length of young leaves (**b**) and roots lengths (**c**) and weight of the whole grains seedling (**d**) in seven rice cultivars.

3.2 GABA and amino acid contents determination

3.2.1. Standard calibration curve for GABA content determination

The standard curve of GABA was prepared from derivative formation of GABA using HN. The yellow color of GABA-HN derivatives was detected at 330 nm by LC-MS. Separated GABA peaks at different concentrations were detected at 5.786 min retention time (RT) (Appendix D). The standard calibration curve of GABA content was plotted between GABA concentration and peak area as shown in (Figure 3.3)

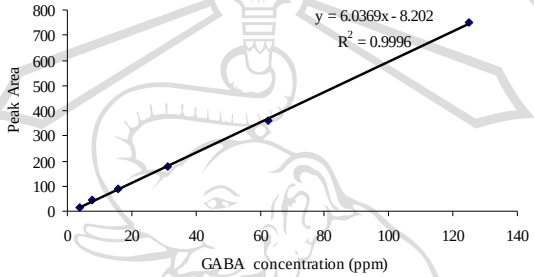

Figure 3.3 Standard calibration curve of GABA

3.2.2 GABA content

The GABA content in each sample was calculated as described in Appendix C. The concentrations of GABA were obtained from the standard curve of GABA. Crude extract from germinated rice was mixed with HN same as the standard GABA as described in previous topic before analysis by LC-MS. GABA content of each seedling part (grains and young leaves) was reported in Figure 3.4. Germinated rice showed increasing GABA content in grains comparing to the control of most cultivars (Figure 3.4a). The HPLC chromatogram shown in Figure 3.5 presented GABA peak (number 3). The results provide the data supported the presence of GABA in crude extract of germinated rice.

GABA concentrations in germinated rice grains were found to range between 0.19-1.25 mg/g in PL2; 0.30-2.01 mg/g in CN1; 0.51-2.45 mg/g in KDML 105; 0.34-1.74 mg/g in SP1; 0.38-1.38 in SPT1; 0.22-1.47 in RD6 and 0.39-1.59 mg/g in PT1 rice cultivars during germination (Figure 3.4a), (Table G.1, appendix G). Figure 3.4b presented the GABA content in young leaves. GABA contents were 0.42-1.16, 0.67-2.21, 0.56-3.91, 0.63-3.16, 1.02-2.50, 0.81-2.12 and 0.61-2.85 in SPT1, RD6, SP1, PL2, KDML105, PT1 and CN1, respectively.

The highest GABA content was found to be at 3.91 mg/g in young leaves of 15 germination days while the lowest was found to be 0.19 mg/g in non germinated grains.

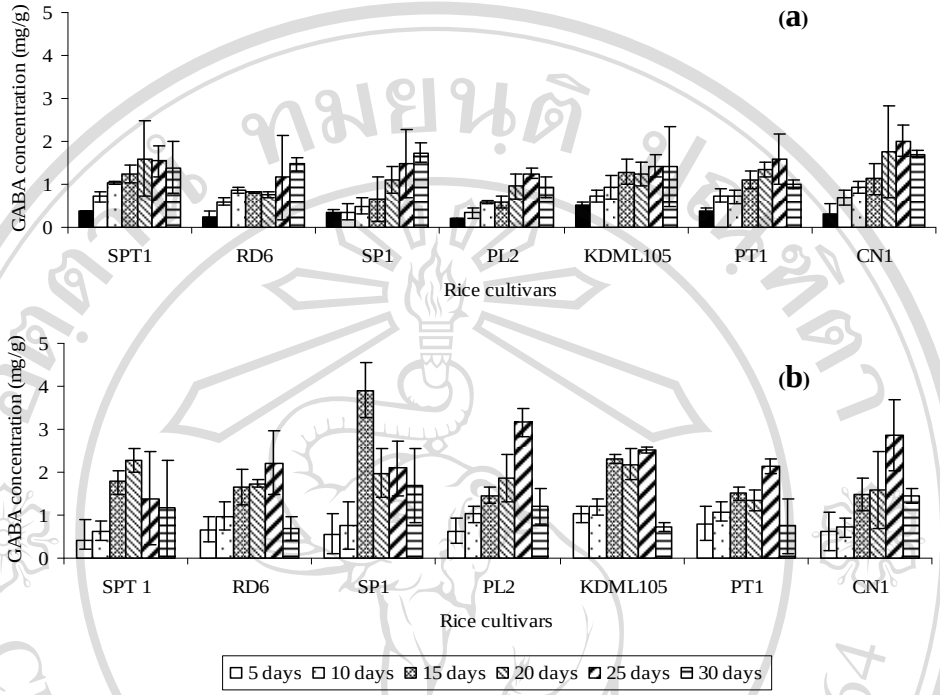

Figure 3.4 GABA contents of germinated rice grains and young leaves. (**a**) Germinated rice grains comparing to non germinated rice grain (control) (**b**) young leaves. Each bar represents the average of three determinations with error bars showing the standard deviation from the mean value.

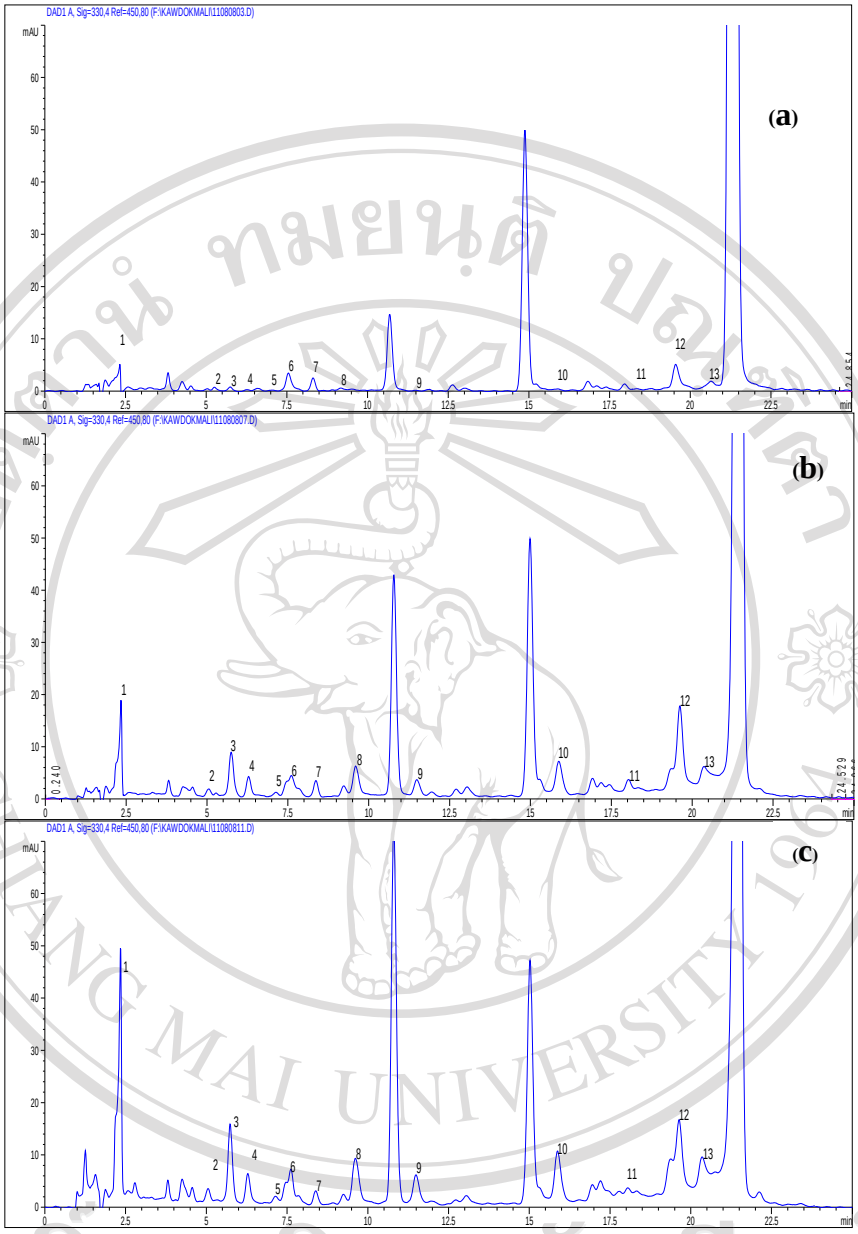

Figure 3.5 HPLC chromatograms of rice extracts after derivatization showing GABA and amino acids derivative peaks; peaks number (1) arginine, (2) asparagine, (3) GABA, (4) serine, (5) glycine, (6) threonine, (7) glutamic acid, (8) tyrosine, (9) alanine, (10) valine, (11) tryptophan, (12) isoleucine and (13) leucine in (**a**) non germinated rice grains (control) (**b**) germinated rice grain and (**c**) young leaves. The conditions were elution under the mobile phase A (0.1 % formic acid) and mobile phase B (acetonitrile) with the phase ratio of A; 35-40, 40-55, 55-35 at 0-5 min, 5-10 min and 10-20 min, respectively. Separation of crude extract was carried out using a C18 reversed phase column with UV detection 330 nm for 25 min.

3.2.3 Standard calibration curve of amino acids

Amino acid standard curve was prepared from amino acids derivatized-HN solution. The yellow color of amino acids-HN was detected under the wavelength at 330 nm by LC-MS simultaneous with GABA. Standard calibration curve for amino acid contents were separately plotted between the amino acid concentration and peak area as shown in Figure 3.6.

3.2.4 Amino acid content

Amino acid content was determined at the same time during GABA analysis as described in Chapter 2. The standard calibration curve of amino acids contents as shown in Figure 3.6 and the chromatograms of rice samples containing derivatized amino acids are in Figure 3.5. Most of the peaks presented in the chromatogram are of amino acids, arginine, asparagine, serine, glycine, threonine, glutamic acid, tyrosine, alanine, valine, tryptophan, isoleucine and leucine. (Figure 3.5b-c).

The dominant amino acids in this study are arginine, asparagine, isoleucine and leucine (Figure 3.7), (Table G.2, appendix G), while sulphur containing amino acids (methionine, cystein), aromatic amino acid (phenylanine) and proline were not detected in rice grains and young leaves.

Amino acid contents in germinated rice grains range between 2.75-10.25, 1.92-11.94, 0.58-4.00, 0.89-5.55, 0.34-2.09, 0.6-9.04, 0.32-13.24, 0.35-4.53, 0.19-2.97, 0.31-2.90, 0.77-11.20 and 0.96-7.80 mg/g for arginine, asparagine, serine, glycine, threonine, glutamic acid, alanine, tyrosine, valine, tryptophan, isoleucine and leucine, respectively.

In young leaves, amino acid contents increased during germination as well as in germinated rice grains. Amino acid content in young leaves showed higher amount than in germinated rice grains within the same germination time. Amino acid contents in young leavess were found to be 3.03-16.51, 1.92-71.25, 0.78-5.29, 0.34-6.31, 0.25-2.92, 0.92-7.88, 0.51-16.84, 0.35-5.3, 0.34-7.17, 0.54-8.26, 0.86-19.30 and 0.96-15.82 mg/g for arginine, asparagine, serine, glycine, threonine, glutamic acid, tyrosine, alanine, valine, tryptophan, isoleucine and leucine, respectively.

Asparagine showed the highest content in germinated grain of 11.74 mg/g. In young leaves, it was found that the highest found at 65.351 mg/g for asparagine. Valine showed the lowest amount of 0.19 and 0.34 mg/g in germinated rice grains and young leaves, respectively.

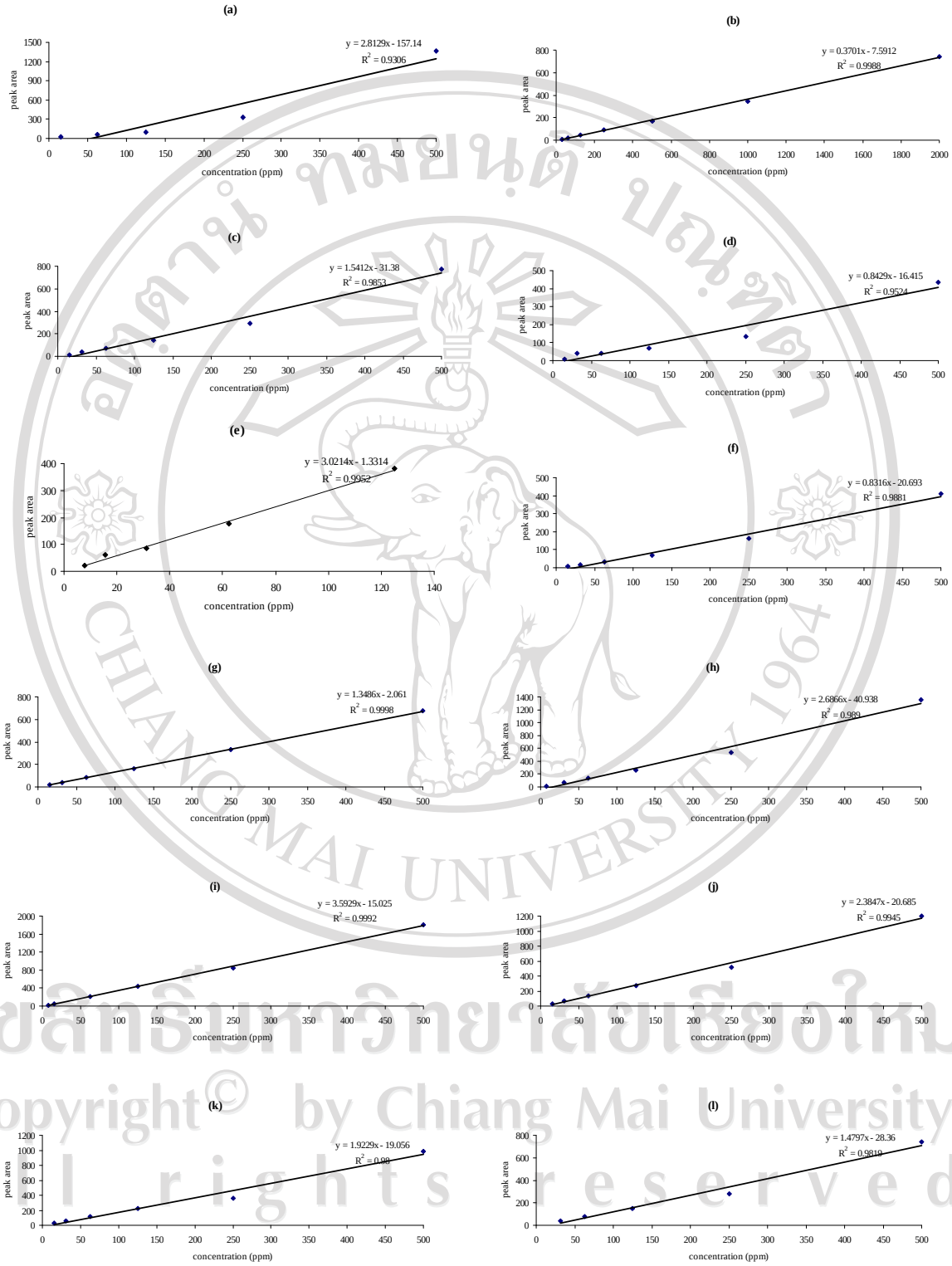

Figure 3.6 Standard calibration curve of amino acids (**a**) arginine, (**b**) asparagine, (**c**) serine, (**d**) glycine, (**e**) threonine, (**f**) glutamic acid, (**g**) alanine, (**h**) tyrosine, (**i**) valine, (**j**) tryptophan, (**k**) isoleucine and (**l**) leucine.

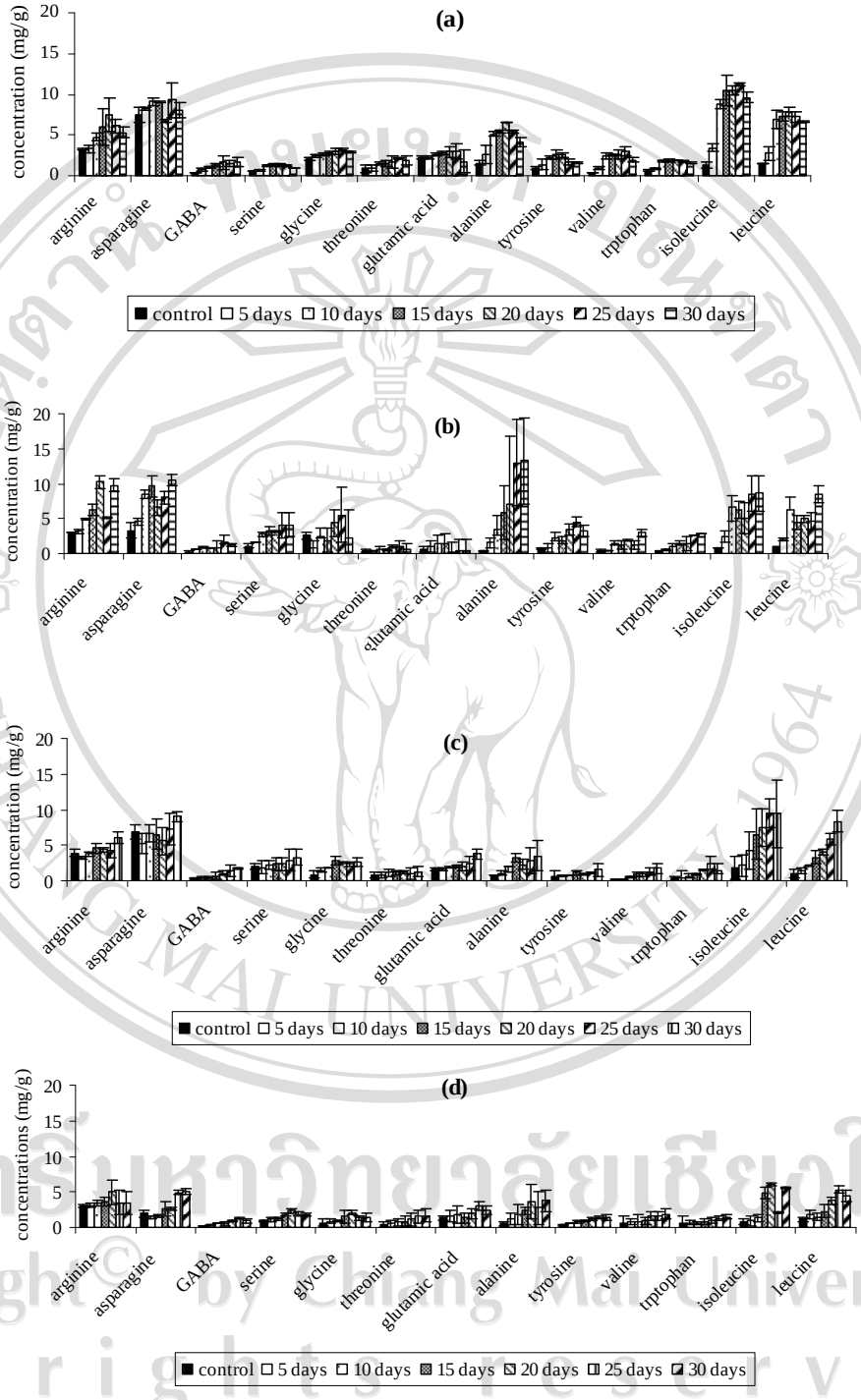

Figure 3.7 Amino acids content in germinated rice grains in different rice cultivars (**a**) Sunpatong1;SPT1 (**b**) Gorkho6;RD6, (**c**) Supan1;SP1, (**d**) Pitsanulok;PL2, (**e**) Kawdokmali105; KDML105, (**f**) Patumtanee 1;PT1, (**g**) Chainat1;CN1 at different germination time.

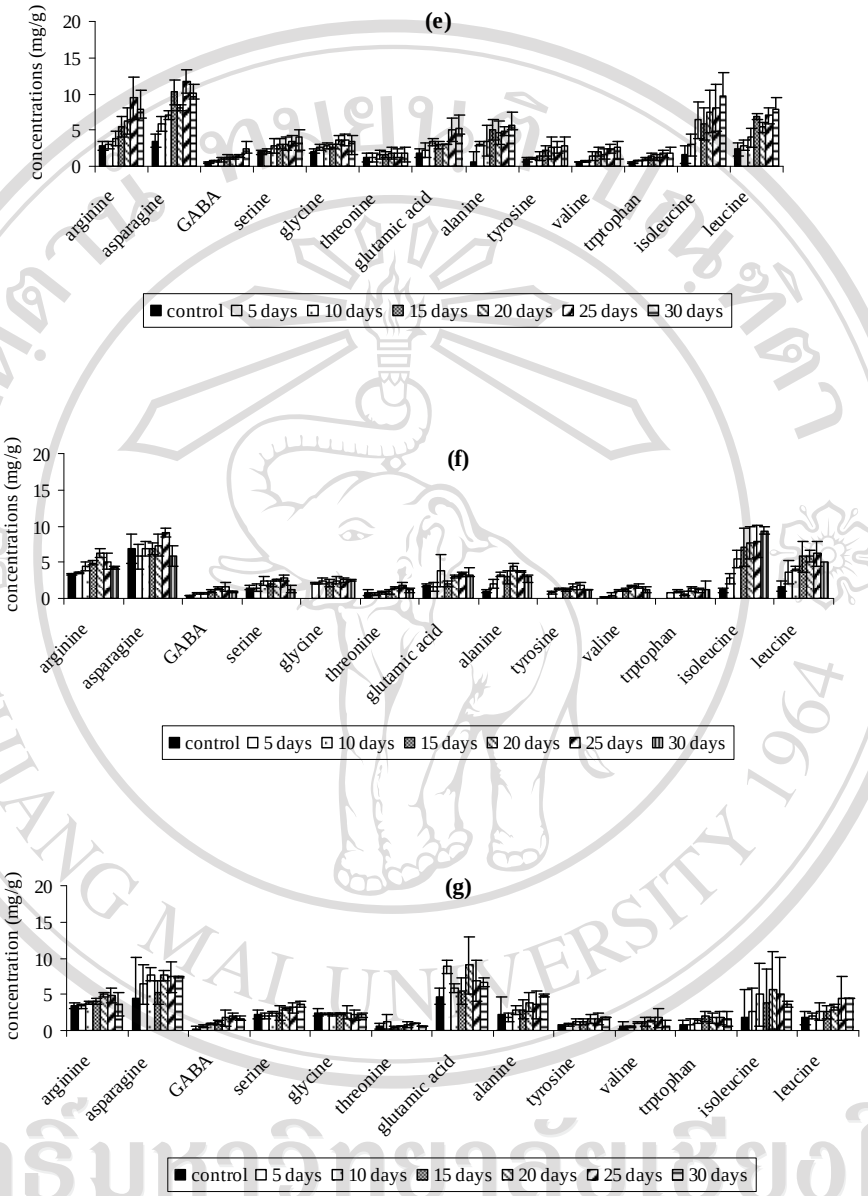

Figure 3.7 (continued)

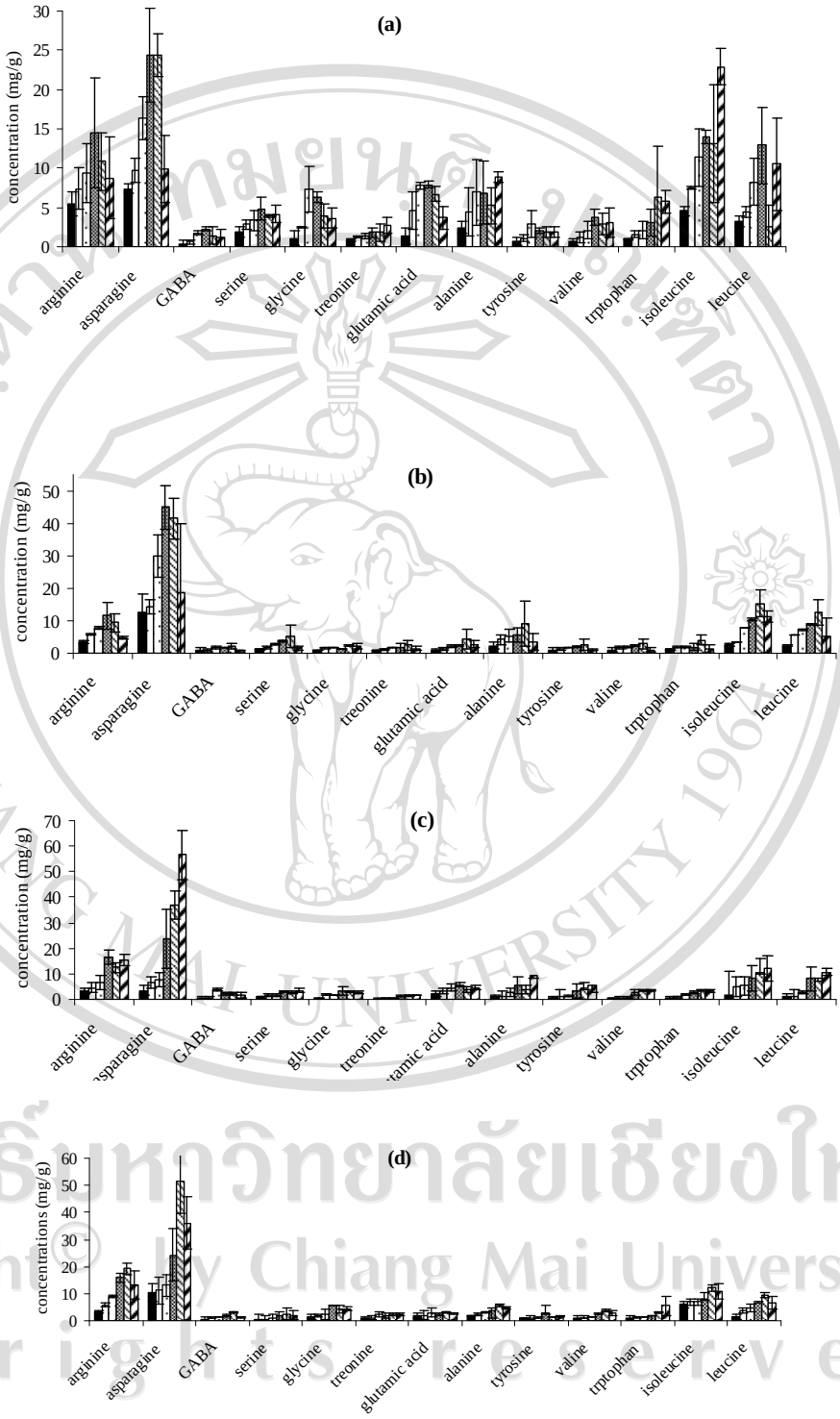

Figure 3.8 Amino acids content in young leaves of different rice cultivars (a) Sunpatong1; SPT1 (**b**) Gorkho6;RD6, (**c**) Supan1;SP1, (**d**) Pitsanulok;PL2, (**e**) Kawdokmali105; KDML105 (**f**) Patumtanee 1;PT1, (**g**) Chainat1;CN1 at different germination time.

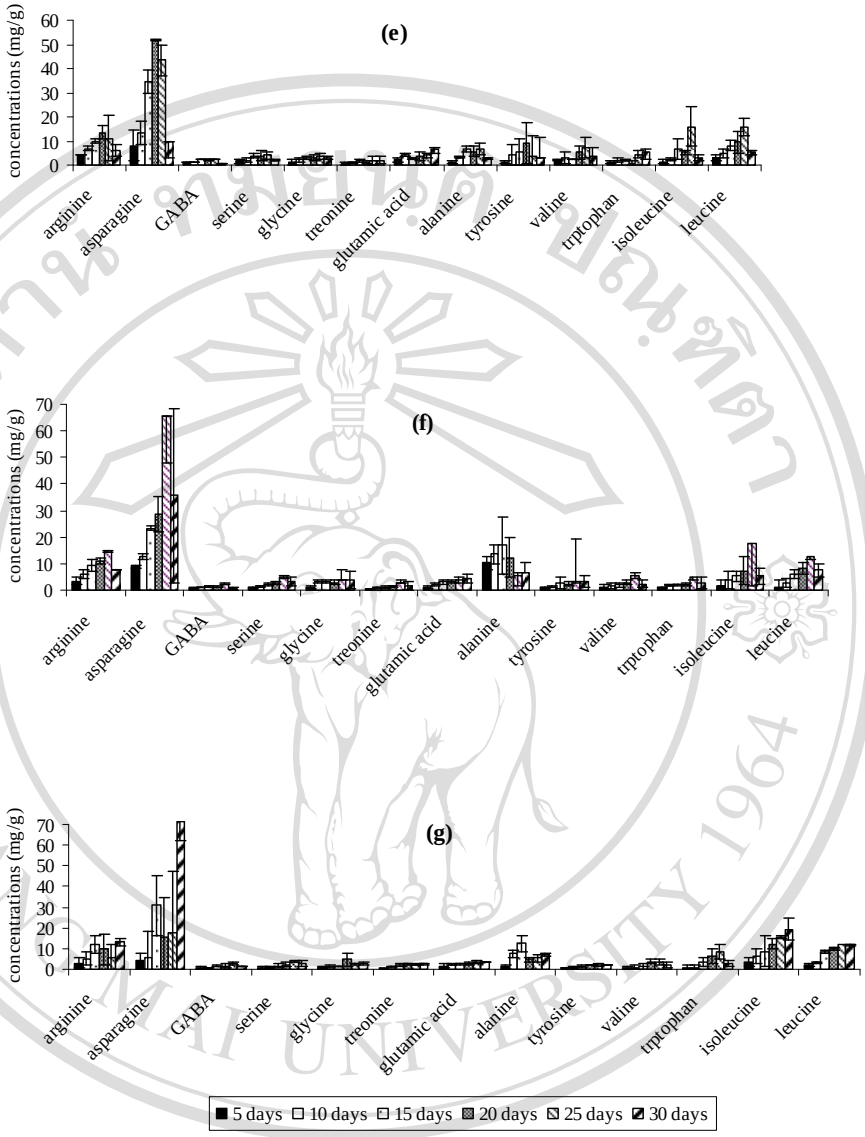

Figure 3.8 (continued)

3.2.5 Comparison of GABA and amino acid contents between rice cultivars

The GABA and amino acid contents were compared among seven rice cultivars. GABA and amino acids in germinated grain showed different content between seven rice cultivars during germination. The difference of GABA content in germinated grain can be observed at 5, 10 and 30 germination days, but the data were not significantly different when germinated at 0, 15, 20, 25 days as shown in Table F.1 (appendix F)

In view of 5 germination days in germinated gain, the results showed no difference of arginine, threonine, serine, tyrosine, isoleucine and leucine. At 10, 15, 20, 25 and 30 germination days, it was found that there was no difference for some amino acids alanine, serine at 10 days, arginine, alanine, serine at 15 days, glycine, threonine, alanine, tyrosine, isoleucine at 20 days, glutamic acid, alanine, isoleucine, leucine at 25 days and threonine, alanine, tryptophan at 30 days.

The part of young leaves showed no different content of GABA in different rice cultivars at 5, 15, 20, 25 days. There was a little difference among rice cultivars in their GABA production only at 10 and 30 germination days. The data could be divided into 3 different levels ($p \geq 0.05$) at 10 days at 0.63, 0.71, 0.75 mg/g, 0.98, 1.02, 1.07 mg/g and 1.07, 1.19 mg/g for SPT1, SP1, CN1; RD6, PL2, PT1; and PT1, KDML105, respectively. The different data level of other germination days were shown in Table F.2. Exception, valine content showed no significantly difference among the cultivars in young leaves.

While, accumulation of arginine and isoleucine differed among different rice cultivars at 10 and 30 days of germination. Arginine, glycine and tyrosine contents showed different data at 10, 15 and 30 days. Serine, threonine, alanine contents presented different value at 10 and 15 days, while at other germination days there were not much difference.

Moreover, the seven selected cultivars showed some difference for leucine and tryptophan contents at the 10, 15 and 25 germination days. Finally, the content variation of glutamic acid was found in most germination days, except at 5 and 25 days that we found a little difference.

3.3 GAD enzyme activity determination

The optimal amount of substrate, activation time and various pHs of GAD enzyme activity assay were investigated. The reaction of enzyme activity assay was then terminated and analyzed for GABA product, by LC-MS, immediately.

3.3.1 Effect of factors influencing the GAD activity

3.3.1.1 Optimal pH

Results of GAD activity from rice leaves and germinated grain at various pHs of buffer solution were shown in Figure 3.9. Rice GAD maximally catalyzed L-glu to GABA at pH 6. Higher or lower pH of their optima result in decreased activity of GAD enzyme in both young leaves and germinated grains (Figure 3.9). For example, their activities were nearly 0 from germinated grains during germination. While, GAD activity of young leaves showed nearly 0 at pH 4-5 and pH 8, and increased at interval of pH 5.8-7.

The optimum pH values of GAD in this study differed from other studies. The optimum pHs are 3.5-5 among the bacteria GAD, 5.5-6.5 among the plant GAD, and about 6.8-7.0 among the animal GAD (Matsumoto *et al*., 1986; Satyanarayan and Nair., 1985). Nevertheless, the optimum pH of the rice in this study were quite similar to other plant GAD (Inatomi and Slaughter, 1975; Satyanarayan and Nair, 1985). In addition, Saikusa *et al,* (1994) found that the GABA content in the rice germ increased remarkably after water soaking under slightly acidic conditions. However, this study found that the GAD was most inactivated in acidic pH.

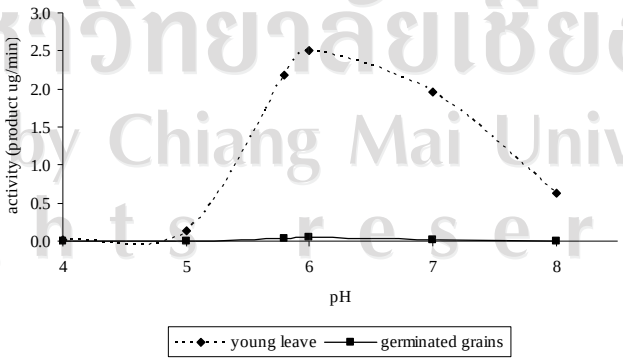

Figure 3.9 Optimal pH at 40°C of GAD enzyme from germinated grains and young leaves.

3.3.1.2 Optimal enzyme

Optimal amount of GAD enzyme from young leaves and germinated grains of rice were also investigated. The GAD enzyme were allowed to catalyzed L-glu at various concentration before measuring their activities in buffer pH 6, 40^{o}C at constant substrate concentration. Their activities were increased when raising the amount of enzyme from 200-1000 µl of both young leaves and germinated grain as shown in Figure 3.10.

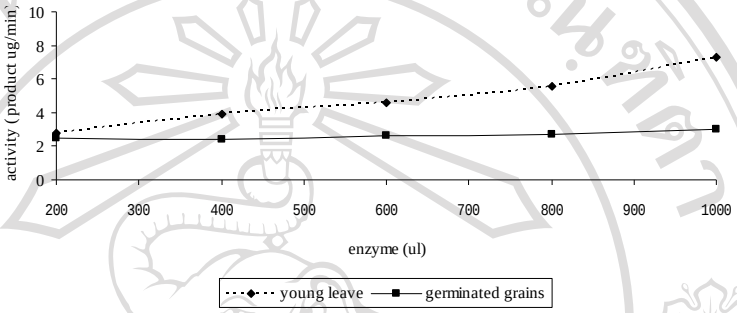

Figure 3.10 The effect of amount of GAD enzyme on their activity of young leaves and germinated rice grain

3.3.1.3 Optimal substrate and incubation time

The varying amount of substrate for GAD enzyme catalysis at different incubation times were studied. When amount of substrate increased from 200 µl (1.26 mg/ml) -400 µl (2.52 mg/ml), the activity of GAD of young leaves slightly increased as shown in Figure 3.11b. The GAD activity slightly decreased at the amount of substrate higher than 2.52 mg/ml However, the GAD activity at different substrate concentration was quite similar results.

In germinated grains (Figure 3.11a), the trends of GAD activity were the same as the young leaves, but lower activities.

The effect of incubation time was also investigated. Both types of young leaves showed the highest activity at 60 min of incubation time. While, 20, 30, 40 and 60 min of incubation time presented a lower enzyme activity. Moreover, the incubation time between 10-60 min seems to have no significant effect on GAD activity in germinated grains.

Therefore, the optimum conditions of crude GAD enzyme in this study were 10 min incubation time with 400 µl (2.52 mg/ml) substrate at 40^{o}C. This conditions will be used for GAD activity monitoring during rice germination in separate part of young leaves and grains.

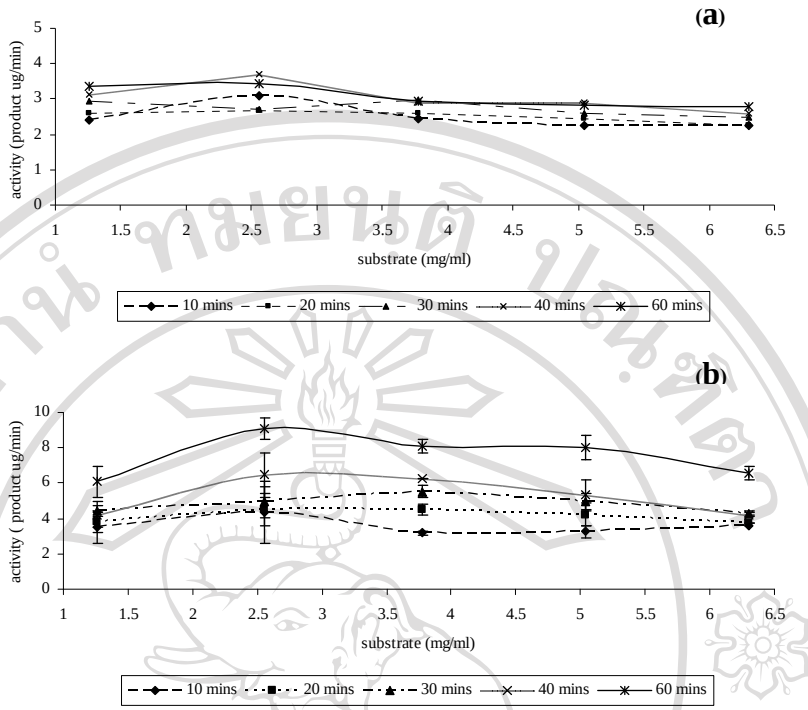

Figure 3. 11 The effect of amount of substrate and incubation time on GAD activity in (**a**) germinated grain (**b**) young leaves of rice.

3.3.2 GAD activity determination in germinated rice grain and young leaves

Only KDML105 and SPT1 cultivars, representative cultivars, were used for monitoring activity during germination in this section. The enzyme activity was investigated using two techniques in this study, LC-MS and the western blotting.

3.3.2.1 LC-MS technique

The GAD enzyme activity was quantitatively determined by LC-MS technique. Crude GAD enzyme was incubated with the substrate at appropriate condition. The GAD activities of young leaves were higher than those of germinated grain in most germination time of both cultivars.

The results in Figure 3.12 presented the GAD activity of both cultivars which were markedly increased during 5-10 days, but sharply decreased after 10 days.

The highest GAD activity that catalyzed substrate to GABA found in young leaves was 6.86 unit of SPT1 cultivars, whereas the lowest activity, almost zero, was found in grain at the later germination days.

The GAD activities of both cultivars were compared. The SPT1 cultivar contained higher GAD activity in leaves, but in germinated grain, there was no difference in GAD activity.

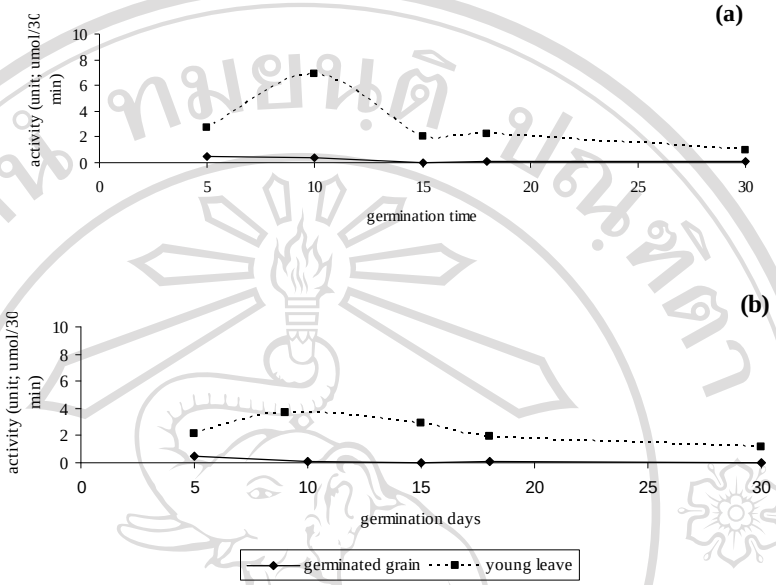

Figure 3.12 GAD activity of (a) SPT1 cultivars (b) KDML105 cultivars at different germination time of growing part (germinated grains and young leaves) of rice seedling.

3.3.2.2 Western blotting technique

Western blotting was used to qualitatively determine GAD protein in germinated rice grain and young leaves of SPT1 and KDML105 cultivars. The results were compared with the control.

Protein samples were separated on 10%SDS-PAGE gel (Figure 3.13a) and then transferred them to Hybond-P PVDF membrane (Figure 3.13b). The presence of GAD was detected by anti-GAD monoclonal antibody and horseradish peroxidase-labelled as a second antibody conjugate. The emission of light from peroxidase enzyme that subsequently specific with GAD protein under a chemiluminescence detector were found as single dark band on the membrane (Figure 3.13b). The molecular weight of the GAD protein that recognized by the antibody was approximately ~70.8 kD in this study (Figure 3.13b).

The control (lane 2,5) of both cultivars showed the lower intensity of GAD protein than germinated rice grain (lane 3,6) and young leaves (lane 4,7) as shown in Figure

3.12b. These data clearly suggest that RiceGAD encodes for a GAD protein, as it showed the higher expression during germination time.

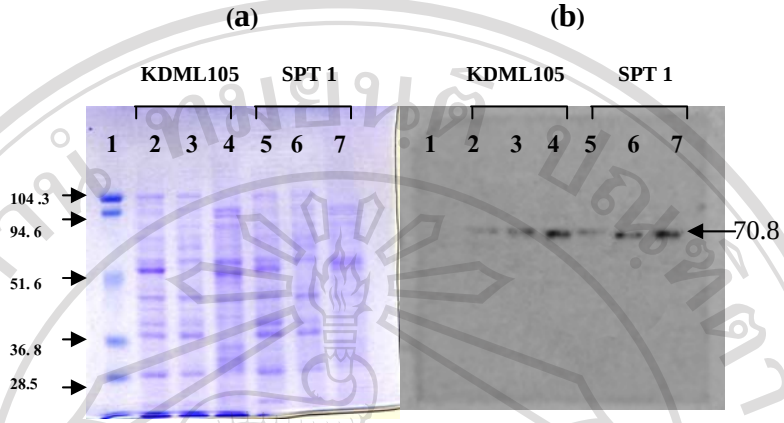

Figure 3.13 Western blot analyses of RiceGAD-encoded protein expression. Lane (1), protein marker; Lane (2,5) protein extracted from non-germinated rice (control); Lane (3,6) from germinated rice grains and Lane (4,7) from young leaves. **(a)** Coomassie blue-stained SDS-PAGE gel, **(b)** Hybond-P PVDF membranes, detected with an anti-GAD monoclonal antibody. The arrows indicate the positions of the RicGAD protein expressed in germinated grain and young leaves.

3.4 Investigation of protein profile of GABA enriched-rice using SDS-PAGE

Change of protein profile during GABA production of germinated rice was investigated. Because GABA pathway and related compounds in GABA shunt also related to nitrogen metabolism, some proteins may have function in GABA enriched-rice. Therefore, SDS-PAGE was used in this study and proteome techniques were employed for protein analysis.

3.4.1 Change in total protein between germinated rice grain, non-germinated grains and young leaves

SDS-PAGE is a well-recognized method for preliminary investigation of protein from germinated rice. According to difference in their charge and size, ability of the protein for moving forward to cathode pole is varied.

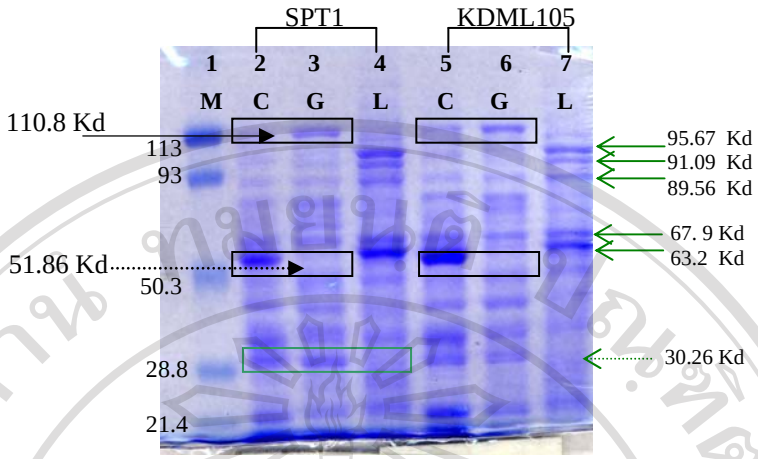

Fig 3.14 SDS-PAGE protein profile of germinated grains and young leaves extract; young leaves (lane 4,7), germinated rice grains (lane 3,6), compared to non-germinated rice grains; control (lane 2,5), and Protein marker (lane1). Protein extract (30 ug) were separated and loaded onto 10% SDS-PAGE followed CBB R-250 stained;
⇠ decreased in leaves; ← increased in leaves; ⇢ decreased in germinated grain; → increased in germinated grain.

Protein profile of GABA enrich-rice after germination process was investigated. Crude protein extract of the control, germinated grains and young leaves were introduced onto 10% SDS-PAGE. The results of germinated rice grain showed decreased and increased protein bands relative to control non-germinated grain of both cultivars (Figure 3.14) (lanes 3,6). While, young leaves (lanes 4,7) presented protein profile differently from germinated grains.

Considering the results of germinated grains of both cultivars (lane 3,6) as shown in Figure 3.14, the protein profile was different from the control (lane 2,5). It especially showed a strong decrease in the intensity of the protein band at 51.86 kd when compared with the control. However, the increased intensity of the protein band can be seen at 110.8 of lane 3 and 6 in germinated rice, representing newly synthesized proteins during germination.

The protein profile in young leaves in lane 4 and 7, differed from that of grain at various positions. Young leaves presented dominant bands at 95.67, 91.09, 89.56, 67.9, 63.2 kd, while these proteins disappeared in the grains of germinated grain and the control.

Beside these, protein band at 30.26 kd was absent in young leaves, but present in the grain.

3.4.2 Change in total protein at different germination time

Results of protein profile at different germination days were shown in Figure 3.15. Protein were gradually decreased in germinated grains and young leaves.

SDS-PAGE profiles of proteins become less stained at the last germination stage, (Figure 3.15). At 20-30 germination days, most of the protein bands were fainted in germinated grains. The major 37.06 and 51.92 kd bands decreased in intensity and so did the other MW proteins after 20 germination days.

However, in rice leaves, the most noticeable observation in the protein profile was a strongly stained band of approximately 51.92 kd. This band could be seen in extracts until 30 germination days. While, proteins with low molecular weights were not observed.

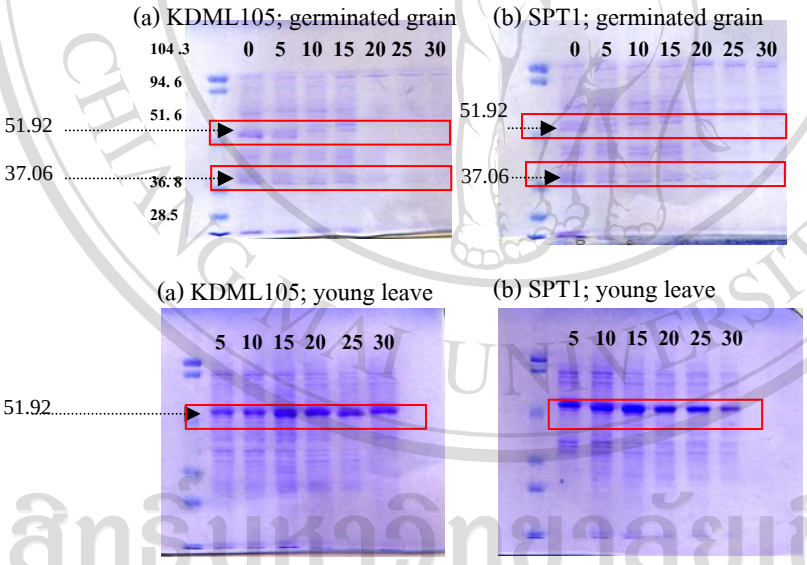

Figure 3.15 Protein profile of germinated rice grains and young leaves of (**a**) KDML 105 cultivars (**b**) and SPT1 cultivars at different germination days; lane 1; 0 days (control), lane 2; 5 days, lane 3 ; 10 days, lane 4; 15 days, lane 5; 20 days, lane 6; 25 days, lane 7; 30 days.

3.5 Proteomic analysis

The strongly diminished or appeared protein will be of interest to study further on protein name, category, as well as its characterization. Proteome analysis will be helpful for the identification of the interested protein in this study.

Therefore, this section conducted a proteomic analysis of GABA-enriched rice. Proteins were extracted from germinated grains of KDML105 and SPT1 cultivar with extraction buffer and precipitated in 100% cooled acetone. 470 µg of protein of control and germinated grains were separated according to PI value using 18 cm of NL 3-10 immobilline drystrip gel and subsequently transferred onto 10% SDS-PAGE in the second dimension.

The 2-D gels were visualized by CBB R-250 and were digitalized with an Epson ES-2200 scanner. Digitalized images were analyzed by Image master 2D version 5.

In KDML105 cultivars, total of 69 and 72 spots were found in germinated rice grain containing GABA and the control, respectively. 5 up-regulated protein spots (higher spot intensity of germinated grain than the control) and 24 down-regulated protein spots, of which intensity in the control were higher than germinated grain, were found. 3 of down-regulated protein spots showed strongly decreased protein expression between both conditions as shown in Fig 3.15 a.

SPT1 cultivar (Fig 3.15 b), total 126 spots in control and 94 spots in germinated rice grain were detected. The differential protein spot number was 30, were found, 19 down-regulated spots, 8 up-regulated spots and 3 spot missing (found in control and absent in germinated grain). 2 spots of down regulated ones, showed strong decreased. The identified interesting proteins of both cultivars were shown in Table 3.3.

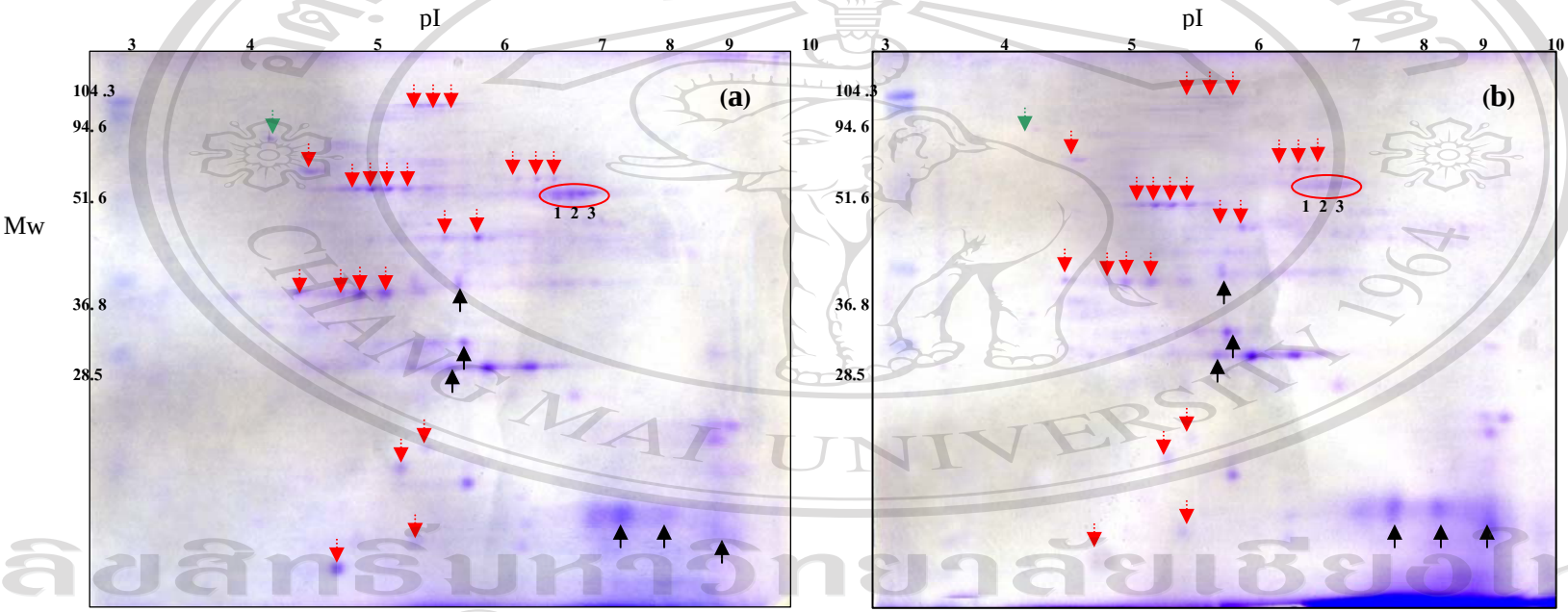

Figure 3.16 2-D gel image of coomassie brilliant blue-R 250 stained proteins profile of KDML 105 cultivar; control (**a**) and germinated rice grains (**b**) Differential expressed proteins spots are marked with arrow; ▼ = down- regulated; ▲ = up-regulated and ▼ = missing (only found in reference gel)

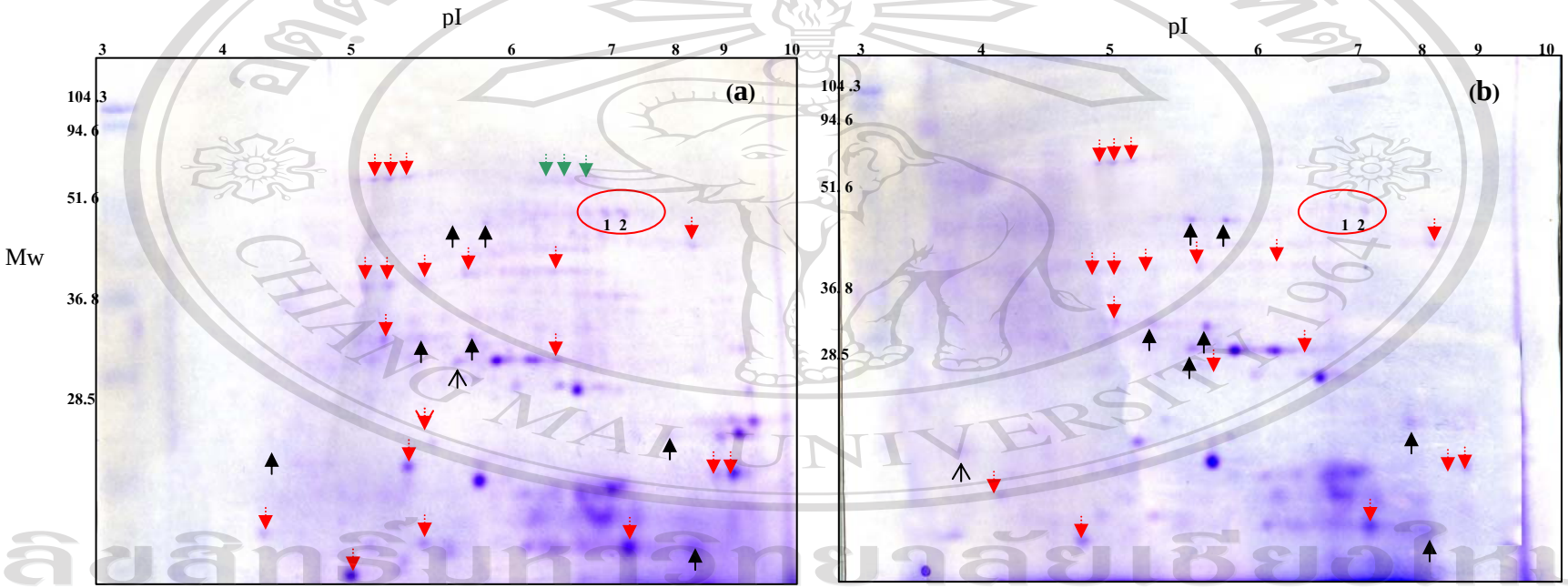

Figure 3.17 2-D gel image of coomassie brilliant blue-R 250 stained proteins profile of SPT1cultiva; control (**a**) and germinated rice grains (**b**) of Differential expressed proteins spots are marked with arrow; ▼ = down- regulated; ▲ = up-regulated and ▼ = missing (only found in reference gel).

Table 3.3 Summary of interested protein decreased expression in GABA-enriched rice of germinated grain during germination

Spot number/ rice cultivars	Protein name	Hypothetical		Experimental		Functions
		PI	MW	PI	MW	
KDML 105	Os03g0793700	6.98	52094	6.63	50074	Storage protein
1	Globulin 2					
2	hypothetical Protein OsI_13867	6.99	52028	6.43	49906	storage of nutritious substrates.
3	Hypothetical protein OsI_13867	6.99	52028	6.80	50187	storage of nutritious substrates.
SPT1	Putative globulin	6.82	51084	6.47	51047	nutrient reservoir
1	(oryza sativa (japonica cultivars))					
2	Putative globulin (oryza sativa (japonica cultivars))	6.82	51084	6.30	51823	nutrient reservoir

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