4 FOLATE METABOLIC PROFILING OF TRANSGENIC LINES, LACKING AND OVEREXPRESSING FOLATE-RELATED PROTEINS

Abstract

Metabolic profiling using LC-MS/MS was employed to investigate the correlation between inactivation and/or overexpression of folate-related proteins with the concentration of folate metabolites. Transgenic rice lines either lacking or overexpressing folylpolyglutamate synthetase (*FPGS)* genes (*Os03g02030* and *Os10g35940*), and expressing folate binding proteins (cFBP and cGNMT) were evaluated using the optimised LC-MS/MS method. A decrease in both mono- and polyglutamated folates was observed in FPGS knockout lines while an increase (2.5 to 8.8-fold total folate) was observed in penta- and hexaglutamates levels in rice lines overexpressing FPGS, cFBP and cGNMT genes. This new information will aid designing the strategies to increase folate levels in rice grains of popular cultivars for enhanced supply of the vitamin to the consumers.

4.1 Introduction

4.1.1 The role of folate polyglutamylation in plants

Most enzymes prefer the polyglutamylated folate forms in the cell (McGuire and Coward, 1984) and the chain length varies from organism to organism, tissues to tissues (Sirotnak *et al*., 1963; Shane, 1982; Zheng *et al*.,1992; Cossins, 2000) and at different stages of development (Rebeille *et al*., 2006). While penta- and hexaglutamates are the major forms found in mammalian cells, tetra- and pentaglutamates and di- and heptaglutamates are the major forms in pea leaves and in tomato leaves, respectively (Imeson *et al*., 1990). Folylpolyglutamate synthetase (FPGS, EC 6.3.2.17), is the enzyme responsible for glutamylation of folate monoglutamate to create polyglutamylated folate forms in various plant species (Chan *et al*. 1986; Ravanel *et al*. 2001). The polyglutamyl tail in folate polyglutamate is hypothesised to increase the anionic nature of the conjugated folates (Hoffbrand *et al*., 1973) thus aiding in their organellar retention (Appling, 1991). Glutamate residues are sequentially conjugated with monoglutamate folate via γ-carboxyl peptide linkages (Suh *et al*., 2001) as shown in Figure 4.1. According to Ravanel *et al*. (2001), FPGS is encoded by three different isoforms in the mitochondria, cytosol and chloroplast. Tetrahydropolyglutamates are suggested to be the preferred substrates for enzyme activities and turnover rates (Besson *et al*.,

1993; Kirk *et al*., 1994; Cossins and Chen, 1997; Scott *et al*., 2000) necessary to meet the high folate demand during plant development.

These three distinct isoforms in the three subcellular compartments were identified in Arabidopsis (Scott *et al*., 2000) but comparative genomics study in rice revealed only two *FPGS* genes namely *FPGS* Os03g02030 and *FPGS* Os10g35940.

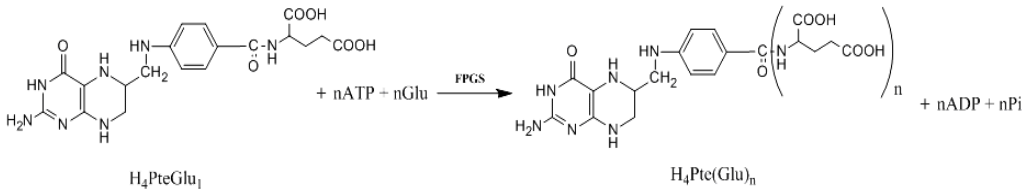

Figure 4.1 Polyglutamylation reaction of monoglutamate by the FPGS enzyme

In mammals, the effect of FPGS impairment is well documented. It has shown to adversely affect cell growth and development as a result of defect in one-carbon metabolism. However, to date, very few studies reported the physiological effect from the impairment of FPGS enzyme activity in plants although Gallardo *et al*. (2002); Heineke *et al*. (2001); Wingler *et al*. (1997); Collakova *et al*. (2008), observed physiological defect due to the impairment of folate-dependent enzymes and metabolites involve in one-carbon metabolism in plant systems. The functional importance of polyglutamylated folate to plant development was recently examined in *Arabidopsis* mutants by Mehrshahi, 2008 (PhD thesis, University of Nottingham) where the knock out mutants in all three *FPGS* genes exhibited defective proliferation rate, dwarfism and significant reduction of methionine level. In his study, a double

knock out *Arabidopsis* mitochondria and plastidic *FPGS* genes caused embryo lethality, while the lack of mitochondria and cytoplasmic *FPGS* resulted in seedling lethality and in the reduction of polyglutamylated folate pool in all three compartments.

4.1.2 Folate binding proteins

Folate binding proteins (FBP) play an important role in the assimilation, distribution and retention of folate in mammalian cells (Henderson, 1990). Cow's milk folate binding protein (cFBP) is known to mediate delivery of 5-CH_3-H_4PteGlu to the intestinal cells during folate absorption (Verwei *et al*., 2004) while rat's liver glycine N-methyltransferase (GNMT) plays a role in methylation process to catalyze the methylation of glycine using S-adenosylmethionine (SAM) to form sarcocine and S-adenosylhomocysteine (SAH) (Blumenstein and Williams, 1960). This function however, can be inhibited by 5-CH_3-H_4PteGlu$_5$ by binding to GNMT (Yeo and Wagner, 1992) but its binding affinity to other folate derivatives is still unknown.

As screening of rice varieties using the microbiological assay showed a wide range of total folate concentration and LC-MS/MS revealed the various folate species present in rice (discussed in the previous chapter) particularly the most preferred polyglutamated folate form for one-carbon metabolism in plants, the study on the T-DNA knockout lines (i.e. T-DNA insertion lines FST

number A16772 which disrupts the *FPGS Os03g02030* gene in rice *Oryza sativa* cv. Dongjin background and FST number D02773 which disrupts *FPGS* Os10g35940 gene in rice *Oryza sativa* cv. Hwayoung background) and overexpression of these two FPGS genes and expression of cow's milk folate binding protein (cFBP) and rat liver's glycine N-methyltransferase (cGNMT) enzyme was made in Nipponbare *japonica* rice variety. Rice materials generated from this work were profiled using the LC-MS/MS method established in the previous study and described fully in the last chapter. The results of this profiling are reported in this chapter. Knowledge of the folate content and forms in these rice materials is very useful for selecting the best strategy to enhance folate in the grain in a future work.

4.2 Aims and Objectives

This chapter intends to determine the function of FPGS genes in rice by characterising the effect of knocking out and over-expressing the FPGS (*FPGS Os03g02030* and *FPGS Os10g35940*) genes on the mono- and polyglutamylated folate levels in rice. This chapter also investigates the effect of expressing cow's milk folate binding protein (cFBP) and rat liver's glycine N-methyltransferase (GNMT) in the rice endosperm on this tissues folate species and concentrations.

4.3 Materials and Methods

4.3.1 Rice Materials

All rice materials analysed in this study were grown in the greenhouse at the Plant and Crop Sciences Division of the University of Nottingham. The established condition was fully described in the previous chapter. The grains of Dongjin and T-DNA knock out plants for *FPGS Os03g02030* gene (obtained from French Rice Functional Genomics Centre, Montpelier, France but was originally procured from Postech, Korea) and overexpressed FPGS genes (*Os03g02030* and *Os10g35940*), and expressed cFBP and cGNMT rice lines were grown for more seed samples for analyses. Germination was made on a petri dish where seeds were soaked with distilled water and kept in growth room at 26-28°C. Germinated seeds were transferred in pots containing 1:1 ratio of Levington M3 and John Innes no.3 composts (composition described in Table 3.2 in Chapter 3) and grown in the greenhouse with a 12h light cycle at 28-30°C during the day and 21°C during the night.

Folates extraction from rice leaf and grain samples was fully described in the previous chapter and summarised here as follows:

Three replicates of rice grain (0.5 g) and of leaf samples (0.2g) were homogenized using Retsch® MM301 ball mill equipment and mixed with 1 mL ice-cold 95% methanol/phosphate extraction buffer (75 mM KH_2PO_4, 0.4 M ascorbic acid, 0.8% 2-mercaptoethanol, pH 6.0) and 25 µL internal standard

mixture consisting of 0.1mg/mL of each of methotrexate (MTX), tri-MTX and hexa-MTX (1:1:1 v/v) for 15 min. α-Amylase (20 µL of a 0.5 mg/mL solution) was added only to the rice grain extract. The sample extracts were centrifuged (15 000 x g, 10 min, 4°C), and the supernatants were filtered through a 0.45 µm Whatman Vectaspin microfilter (15 000 x g, 3 min, 4°C). They were evaporated to dryness under nitrogen gas and re-suspended in 200 µL of extraction buffer (75 mM KH_2PO_4, 52 mM ascorbic acid, 0.1% 2-mercaptoethanol, pH 6.0). Samples were kept at -80°C until used or maintained at 4°C in the HPLC autosampler for not more than 12 hours before LC-MS/MS analysis.

4.3.2 LC-MS/MS measurement of folates

The LC-MS/MS method was based on a previously described method (Garratt *et al*., 2005). HPLC analysis was performed using Shimadzu VP series HPLC system (Milton Keynes, UK) using a Luna C18 (2) 100Å analytical column (150 x 2.0 mm, 5 µm particle size) and a compatible C18 guard column (Phenomenex,Macclesfield, UK). Mobile phase A consisted of methanol/water (5:95, *v/v*) with 5 mM dimethylhexylamine (DMHA, pH 8.1 and mobile phase B was 5 mM DMHA in methanol. A linear gradient from 22% B to 80% B over 20.5 min was followed by a 5 min isocratic hold at 80% B and re-equilibration for 12.5 min at 22% B. The flow rate was 200 µL/min and the injection volume was 20 µL. The column was maintained at 35^{o}C throughout the run.

A hybrid triple quadrupole ion trap mass spectrometer (4000 QTRAP) from Applied Biosystems (Foster City, CA, USA) was run using negative polarity. The TurboIonSpray source conditions were optimized for optimal ionization of folates as follow: gas 1 and 2 at 20 and curtain gas at 40. The ion spray voltage was set at 4 kV and the turbo probe was heated at 500^{o}C. Declustering potential and collision energies for each folate standard were optimized for compound dependent parameters using the quantitative optimization wizard of the Analyst software (version 1.4.2).

Quantification of individual folate used three internal standards enumerated in Chapter 2 and extracted calibration standards for all the folate species and confirmation of folate structural identity was confirmed by comparison with an in-house folate spectral library.

4.4 Statistical Analysis

Significance of differences for the means of folate analytes, total folate concentration and percent distribution of folate derivatives in different samples and treatments were analysed by one-way ANOVA using Dunnett's post test performed using GraphPad Prism version 4.02 for Windows, GraphPad software, San Diego, California USA, www.graphpad.com.

4.5 Results and Discussion

4.5.1 Folates in FPGS Os03g02030 knock out line and wild type rice

There is a demand for folate during rice grain filling as shown by the transcript abundance of folate genes as mRNA abundance represents *de novo* folate synthesis machinery being detected in every stage of rice grain development sampled (Anukul, 2009). Folate requirements seem to be particularly high after germination during the vegetative growth stage, until anthesis (flowering). Observations made by Anukul (PhD thesis, University of Nottingham, 2009) showed that the knock out rice line was reaching the heading stage one week later compared to wild type and was also delayed every subsequent step, reaching the mature stage approximately two weeks later than the Dongjin wild type plant. The demand for folate drops during seed filling stage where most of starch accumulation happens.

Despite the seed development phenotype of the knock out plants, the mature seeds looked no different in appearance and in the ability to germinate (Figure 4.2) compared to the wild type. The germination rate of the *fpgs*03g mutant however, was just about 95% compared to the germination rate of the Dongjin wild type. The seed yield per panicle/plant (~60 seeds) of the *fpgs*03g was approximately the same with the wild type.

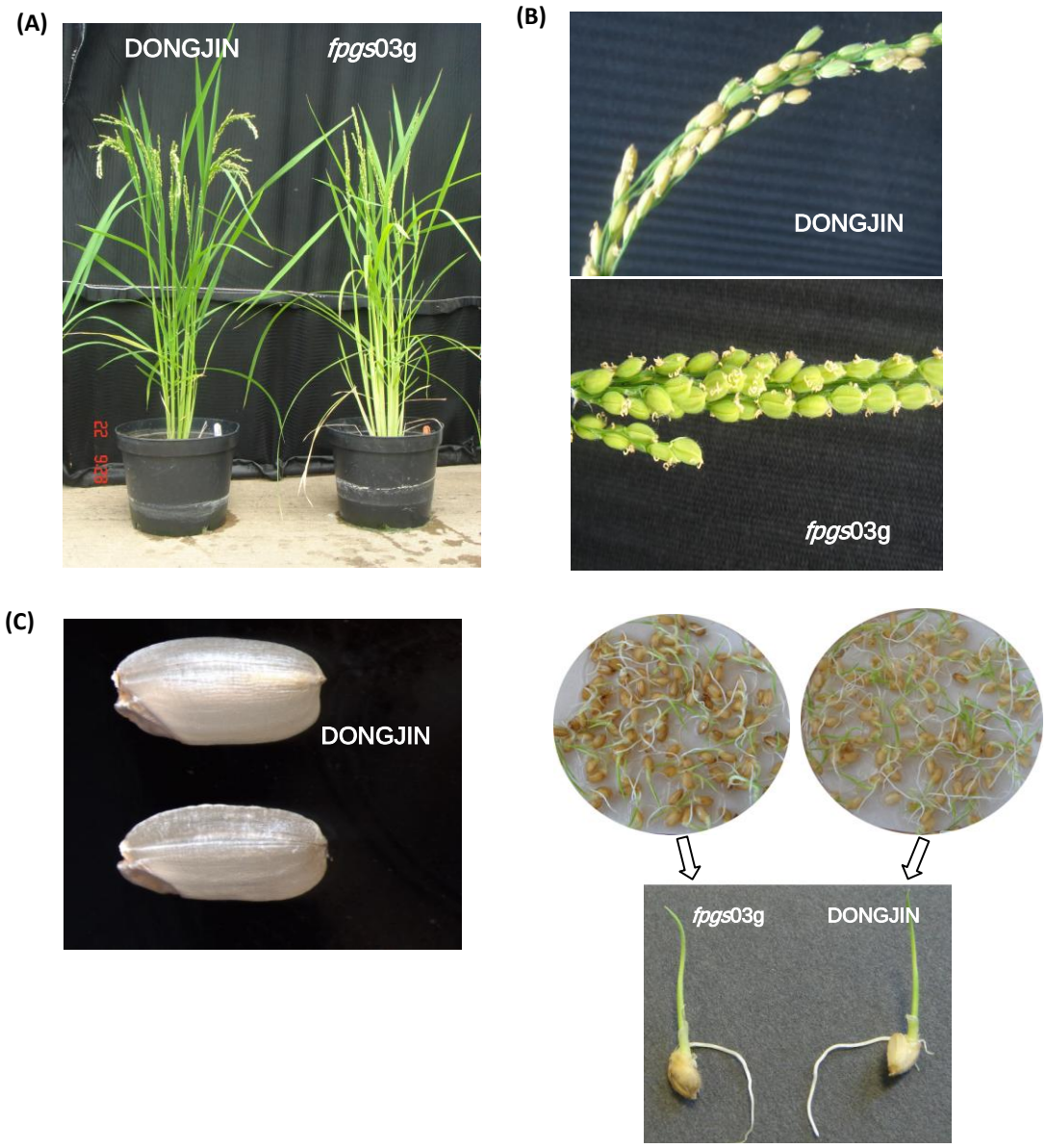

Figure 4.2. Plant phenotypes of *FPGS Os03g02030* knock out line and Dongjin wild type. **(A)** 2-month old rice plants; **(B)** 2-month old rice seeds; **(C)** hulled mature dry rice seeds; **(D)** 4 -day old germinated rice seeds (photos by Nampeung Anukul)

LC-MS/MS profiling of rice leaf and grain tissues was used to detect the qualitative and quantitative changes in the folate status of wild type versus FPGS mutants.

The results from metabolic profiling using LC-MS/MS of Dongjin wild type (WT) and FPGS knockout (FPGS KO) revealed that 5-CH_3-H_4PteGlu and other folate forms in grains are much lower than in leaves except for 5-CHO-H_4PteGlu. Nevertheless, the same five folate forms were detected in both grains and leaf tissues of WT and FPGS KO.

Interestingly, the polyglutamated folates detected using this analytical technique was higher in leaf tissues than in grain for both wild type and FPGS knockout lines. The higher folate content in leaves could be explained by the higher requirement for the vitamin during photorespiration (Roje *et al*., 2002) since folate synthesis in leaves is related to light (Jabrin et al., 2003).

Mono- and polyglutamated folates were all significantly decreased in grains of FPGS KO compared to the WT. 5-CHO-H_4PteGlu$_5$ was the most abundant form of polyglutamated folate in Dongjin and the *fpgs*03g mutant leaves. Cossins (2000) found that methyl derivatives of folate account for 45-65% of the total folate level in higher plant tissues and they are mostly in the cytosol fraction while the formyl derivatives are produced mostly in organelles with 44% of 5-CHO form dominant in mitochondria (Chen *et al*., 1997; Chan and Cossins, 2003). Overall, there was a 33% and 28% decrease in total folate concentration in grains and in leaves of the FPGS KO line compared to the WT, respectively.

The concentration of folate derivatives in rice tissues is summarised in Table 4.1. Tetra- and penta-glutamated folate forms were detected in leaf and seed samples. Approximately 40% of rice folate forms detected in leaf and 20% of folate in rice grain exist either as tetra or penta forms of the 5-CH_3- and 5/10-CHO-H_4PteGlu. A decrease in mono- and polyglutamated folate levels in the *fpgs03g* mutant rice grain was observed which shows that folate polyglutamylation is involved in retention and homeostasis of folates as shown in previous studies (DeSouza *et al*., 2000; Lin *et al*., 1993; Lin and Shane, 1994). The reduction in monoglutamated folates may be a result of folate breakdown, as polyglutamated protein-bound folates are more resistant to oxidative breakdown (Suh *et al*., 2001). Overall, there was a 33% and 28% decrease in total folate levels in grains and in leaves of the knockout line compared to the wild type, respectively. In terms of percentage distribution of the various folate forms in rice grain and in leaves, the CHO-H_4PteGlu in grains are higher than in leaves while the CHO-H_4PteGlu$_5$ folate was higher in leaf tissues than in grains. Approximately 40% and 20% of the folates are tetra- and pentaglutamated in leaves and in grains, respectively.

Table 4.1. Total folate levels and folate derivatives in leaf and seed from Dongjin wild type and *FPGS Os03g02030* mutant

Rice Variety	Folate Form (µg/100g)					
(*n = 3*)	5-CH_3 H_4PteGlu	5/10-CHO H_4PteGlu	5-CH_3 H_4PteGlu$_4$	5-CH_3 H_4PteGlu$_5$	5-CHO H_4PteGlu$_5$	Total Folate
Dongjin (L)	5.16 ± 0.72	1.62 ±0.24	0.33± 0.01	0.7± 0.40	4.36± 0.47*	12.17± 0.40*
*fpgs*03g (L)	3.88 ± 0.14	0.79± 0.18	0.67± 0.01	0.7 ± 0.01	2.65 ± 0.1*	8.69± 0.10*
Dongjin (S)	3.21± 0.70*	1.87±0.28	0.33± 0.01	0.3 ± 0.004*	0.74±0.01*	6.45± 1.00*
*fpgs*03g (S)	2.13±0.30*	1.72±0.61	0.11±0.001	0.1±0.00*	0.22±0.01*	4.28± 0.20*

5-CH_3-H_4PteGlu = 5-methyltetrahydropteroylmonoglutamate;
5-CHO-H_4PteGlu = 5-formyltetrahydropteroylmonoglutamate;
5-CH_3-H_4PteGlu$_4$ = 5-methyltetrahydropteroyltetraglutamate;
5-CH_3-H_4PteGlu$_5$ = 5-methyltetrahydropteroylpentaglutamate;
5-CHO-H_4PteGlu$_5$ = 5-formyltetrahydropteroylpentaglutamate;
(L) represents a leaf sample and (S) represents a mature seed sample
The mean ± SD of the folate contents was presented with *n = 3*
* - samples within tissues which are significantly different (P<0.05)

4.5.2 Folates in rice with overexpressed FPGS genes

To study the effect of over-expressing *FPGS* genes (*FPGS Os03g02030* and *FPGS Os10g35940*) on folate content of rice grains, profiling of mono- and polyglutamate folate forms was conducted by LC-MS/MS. Two mono and five polyglutamated folates were detected in all the samples. Confirmation of identity between the mass spectral library for standard and sample spectra led to the positive identification of 5-CH_3-H_4PteGlu, 5-CHO-H_4PteGlu, 5-CH_3-

$H_4PteGlu_4$, 5-CH_3-$H_4PteGlu_5$, 5/10-CHO-$H_4PteGlu_5$, 5-CH_3-$H_4PteGlu_6$ (Figure 4.3) and 5/10-CHO-$H_4PteGlu_6$ (Figure 4.4). In previous investigations of other plant materials such as *Arabidopsis thaliana* wild type and transgenic lines, 5-CH_3-$H_4PteGlu$ and its polyglutamated forms are the predominant forms but mono and polyglutamated 5/10-CHO were shown to be also present (Roos and Cossins, 1971; Spronk and Cossins, 1972; Goyer *et al*.,2005). The work of De La Garza *et al*. (2007) on crossed tomato lines overproducing both PABA and pteridines showed up to 25-fold more folate in the fruits. When the group of Gillies *et al*. (2008) introduced in rice the wheat 6-hydroxymethyl-7,8-dihydropterin/7,8- dihydropteroate synthase (HPPK/DHPS) which operates at the central point of the folate biochemical pathway, they observed higher folate levels (doubling from control) in the transgenic seeds. Naqvi *et al.* (2009) also observed a two-fold increase in the amount of total folate in transgenic corn when they expressed *Escherichia coli folE* in the corn endosperm. Storozhenko *et al*., (2007) already showed a 100-fold increase in folate levels in rice grains when they expressed the *Arabidopsis thaliana gch1* and *adcs* cDNAs. So far, no report was made on the expression of *fpgs* in any cereals, particularly on rice.

In this study, there was no significant change in the level of 5-CH_3-$H_4PteGlu$ in most of the *FPGS* transgenic lines analysed when compared with Nipponbare (wild type). The observed slight decrease in 5/10-CHO-$H_4PteGlu$ levels in FPGS lines was also not significant compared to the control. The significant increase in the total folate (2.5 to 4.7-fold) of *FPGS* lines can be largely accounted for

by the increase in penta- and hexaglutamates folate forms (5-CH_3-H_4PteGlu$_5$, 5/10-CHO-H_4PteGlu$_5$, 5-CH_3-H_4PteGlu$_6$ and 5/10-CHO-H_4PteGlu$_6$). Summary of these results is shown in Table 4.2. Whilst these forms are either very low or undetectable in wild type, they can account for up to three-fold more folate than the monoglutamated total. Overexpression of either FPGS genes can therefore be used to significantly enhance folate abundance.

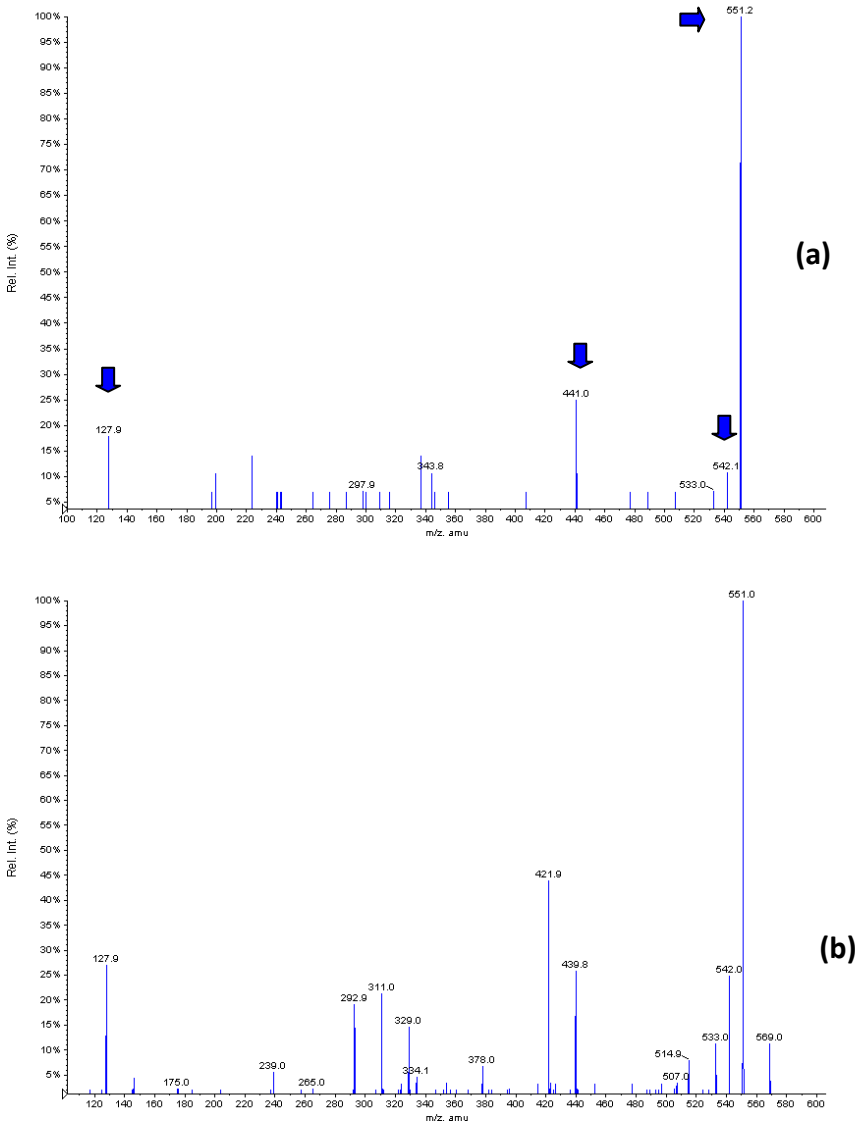

Figure 4.3. Mass spectra for the confirmation of 5-CH_3-H_4PteGlu$_6$ in grains of transgenic rice lines. Spectra (a) correspond to the sample and (b) to the respective folate standard. Arrows indicate the common ion fingerprints.

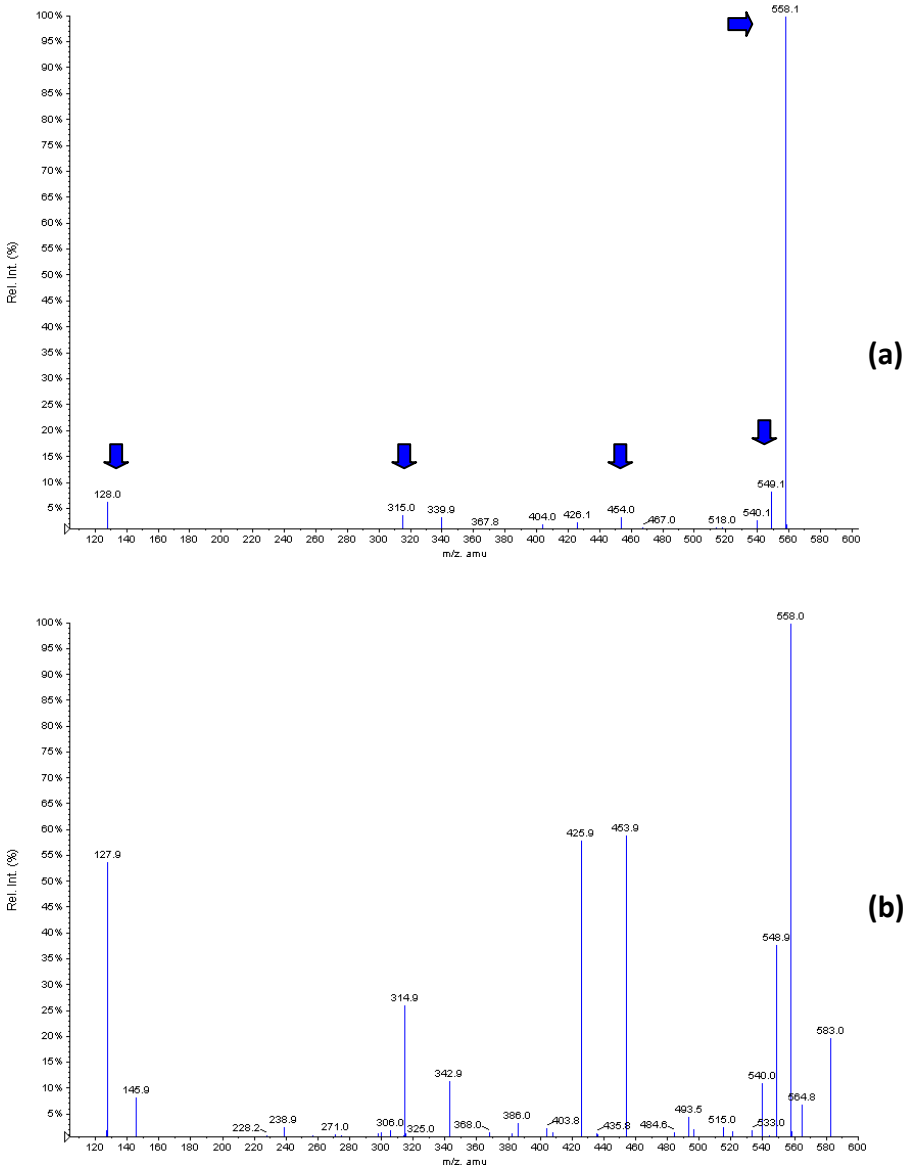

Figure 4.4. Mass spectra for the confirmation of 5/10-CHO-H_4PteGlu$_6$ in grains of transgenic rice lines. Spectra (a) correspond to the sample and (b) to the respective folate standard. Arrows indicate the common ion fingerprints.

Table 4.2. Folate forms and total concentration in unpolished rice grains with over-expressed *FPGS* genes, cFBP and GNMT (mean ± SD; *n=3*) compared to the WT (Nipponbare). Statistical differences are indicated with asterisks (*p<0.01*; **, *p<0.05*; *)

Rice Sample	Folate Form (µg/100g)							
	5-CH_3-H_4PteGlu	5/10-CHO-H_4PteGlu	5-CH_3-H_4PteGlu$_4$	5-CH_3-H_4PteGlu$_5$	5/10-CHO-H_4PteGlu$_5$	5-CH_3-H_4PteGlu$_6$	5/10-CHO-H_4PteGlu$_6$	Total Folate
3g*FPGS* 11-1	13.21 ± 3.1	0.30 ± 0.1*	0.48 ± 0.05	0.85 ± 0.1*	0.63 ± 0.1**	14.15 ± 4.1**	4.81 ± 0.5**	34.43 ± 2.3**
2 3g*FPGS*3-1	21.81 ± 2.3	0.36 ± 0.04*	0.50 ± 0.1	1.10 ± 0.1*	0.38 ± 0.04*	34.43 ± 3.5**	5.16 ± 0.6**	63.74 ± 1.2**
3g*FPGS*17-3	13.91 ± 1.3	0.14 ± 0.01*	0.50 ± 0.03	0.82 ± 0.1*	0.38 ± 0.1*	32.23 ± 1.2**	3.13 ± 0.2**	51.11 ± 0.5**
3g*FPGS*5-2	18.41 ± 2.5	0.15 ± 0.03*	0.49 ± 0.1	0.81 ± 0.0*	0.30 ± 0.02*	24.11 ± 7.0**	2.09 ± 0.1**	46.36 ± 1.4**
10g*FPGS*1-3	13.31 ± 0.7	0.28 ± 0.0*	0.56 ± 0.05	0.97 ± 0.2*	0.23 ± 0.03	31.31 ± 4.5**	1.54 ± 0.3**	48.20 ± 0.8 **
10g*FPGS*3-1	11.71 ± 0.9	0.78 ± 0.1*	0.53 ± 0.1	1.13 ± 0.4*	0.31 ± 0.0	30.27 ± 3.9**	3.07 ± 0.2**	48.25 ± 0.8**
2 10g*FPGS*16-2	8.11 ± 3.0	0.19 ± 0.0*	0.59 ± 0.1	1.04 ± 0.2*	0.39 ±0.1*	43.19 ± 12.3**	2.83 ± 0.9**	56.34 ± 2.4**
2cFBP20-1	23.01 ± 6.0**	0.69 ± 0.04*	0.99 ± 0.1	2.38 ± 0.3**	-	21.94 ± 4.3**	6.72 ± 0.5**	55.73 ± 1.9**
cFBP5-1	50.11 ± 5.4**	0.74 ± 0.01*	0.64 ± 0.2	1.22 ± 0.2*	0.28 ± 0.2	26.19 ± 4.7**	3.98 ± 1.5**	83.16 ± 3.1**
cFBP4-1	38.81 ± 3.5**	0.88 ± 0.03*	0.67 ± 0.3	1.37 ± 1.1*	0.65 ± 0.4**	25.79 ± 1.0**	5.60 ± 1.9**	73.77 ± 1.2**
cFBP15-1	13.21 ± 5.6	0.69 ± 0.04*	1.70 ± 0.6*	1.80 ± 0.1**	0.17 ± 0.01	29.24 ± 8.3**	5.19 ± 2.9**	52.00 ± 2.5**
2GNMT25-2	51.56 ± 6.1**	5.43 ± 1.9**	4.24 ± 0.9*	0.97 ± 0.1*	-	30.19 ± 12.9**	4.86 ± 0.8**	97.25 ± 3.3**
GNMT15-3	62.61 ± 11.3**	8.38 ± 2.8**	0.71 ± 0.5	1.01 ± 0.4*	0.67 ± 0.2**	-	4.99 ± 0.7**	78.37 ± 2.7**
GNMT18-3	40.16 ± 4.9**	5.18 ± 0.8**	0.81 ± 0.03	0.92 ± 0.1*	2.12 ± 1.6**	34.35 ± 17.1**	-	83.54 ± 4.1**
GNMT4-2	86.61 ± 7.2**	5.43 ± 2.4**	2.03 ± 0.9*	2.30 ± 0.9**	-	22.73 ± 7.6**	4.69 ± 0.4**	119.10 ± 3.3**
Nipponbare	11.09 ± 0.5	1.69 ± 0.04	0.71 ± 0.5	-	-	-	-	13.49 ± 0.4

- not detected; 2 at the beginning of each sample indicates that each gene was transformed (rice callus) in duplicate.

4.5.3 Folate profile of rice with cFBP and cGNMT

It has been suggested that overexpression of folate binding proteins may stabilise folate content (Bekaert *et al*., 2008) after Hutchison *et al*. (2000) and Jones and Nixon (2002) showed an improvement in folate stability when bound to mammalian FBP. In this connection, cow's milk folate binding protein (cFBP) and rat liver's glycine N-methyltransferase (GNMT), were expressed in rice endosperm and to determine whether the expression of these two genes will increase total and selected folate forms in transgenic grains.

It was observed that the cGNMT and cFBP expressing rice lines did not yield many seeds compared to the over-expressed FPGS lines. All of the cGNMT and cFBP lines had less than 20 seeds per panicle or less than 50 seeds per plant respectively, compared to FPGS lines which had over a hundred seeds per plant. Hence, there appears to be a negative effect of FBP and GNMT transgene expression. This may reflect that folate bound to FBP and/or GNMT is no longer available for physiological processes, thereby impacting metabolic pathways dependent on these cofactors.

Nevertheless, results from LC-MS/MS profiling of the transgenic and non-transgenic rice (Nipponbare) revealed a significant increase ($P<0.05$) in both polyglutamated and total folate forms compared to Nipponbare (Table 4.2). There was a significantly higher 5-CH_3-H_4PteGlu levels in cFBP and GNMT lines compared to the WT while the increase in 5/10-CHO-H_4PteGlu levels was only significant for GNMT (Figures 4.4a and 4.4b). This resulted in much higher total folate enhancement in GNMT (5.8 to 8.8-fold) compared to cFBP (3.8 to 6.2-fold) expressing lines. The

increase in polyglutamyl folates in cFBP and GNMT lines were very similar to the values obtained from most of the FPGS lines (Figures 4.6a to 4.6e). In contrast to FPGS lines, FBP and GNMT expression resulted in a significant increase in both mono- and polyglutamated folate forms. This may reflect the impact of these folate binding proteins on folate homeostasis, where FBP and GNMT proteins actively sequestering mono- and polyglutamated folate forms, thereby inducing feedback mechanism(s) to elevate further synthesis. Such a feedback mechanism appears to exist in rice based on an analysis of FPGS KO lines (Anukul, PhD thesis, University of Nottingham, 2009).

Previous efforts which include expressing *gch1* from bacteria in plants (*A. thaliana*) resulted in 8.5-fold more total folates in leaves than the control (*Hossain et al*., 2004) but only two-fold in tomato fruits (De La Garza *et al*., 2004). *GCH1* activity only affects the cytosolic (pterin) branch of folate molecule (Naqvi *et al*., 2009). Another study expressing *Gallus gallus* gene (chicken *gch*1) in lettuce showed 2.1 to 8.5-fold increase in folate content (Nunes *et al*., 2009). This recent finding correlates well with the results obtained in this study.

It is not surprising that the transgenic lines with cFBP and cGNMT showed a significantly high 5-CH_3-H_4PteGlu$_6$ (Figure 4.6d) and 5/10-CHO-H_4PteGlu$_6$ (Figure 4.6e) levels detected in the unpolished grain samples as previous studies of folylpolyglutamate distributions in animal and bacterial species and in broccoli and tomato tissues showed hexa- and heptaglutamate derivatives (Cossins, 1984; Imeson *et al*. 1990).

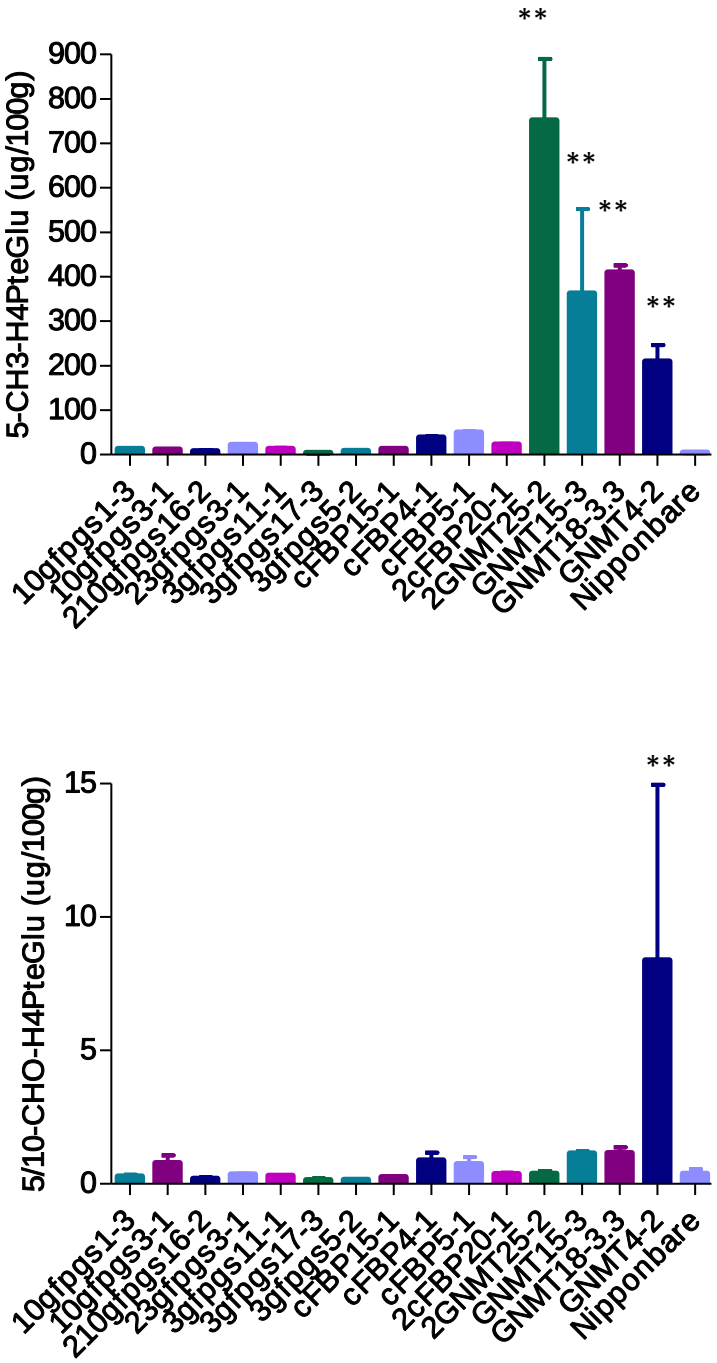

Figure 4.5a and 4.5b. Distribution of monoglutamyl (5-CH_3-H_4Pteglu and 5/10-CHO-H_4PteGlu) forms in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT. Each value represents the mean of three biological replicates ± SD. Statistical differences are indicated with asterisks (*p<0.01*; **, *p<0.05*; *).

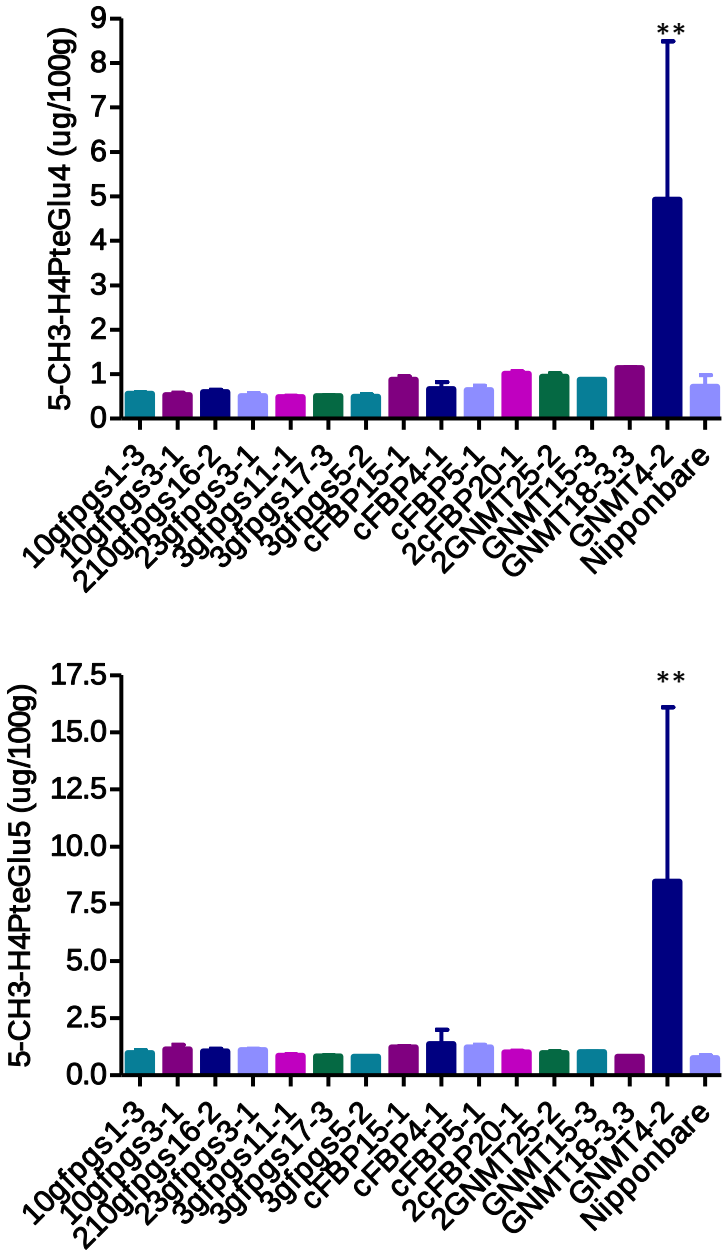

Figure 4.6a and 4.6b. Distribution of polyglutamyl forms (5-CH_3-H_4PteGlu$_4$ and 5-CH_3-H_4PteGlu$_5$) in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT. Each value represents the mean of three biological replicates ± SD. Statistical differences are indicated with asterisks (*p<0.01*; **, *p<0.05*; *).

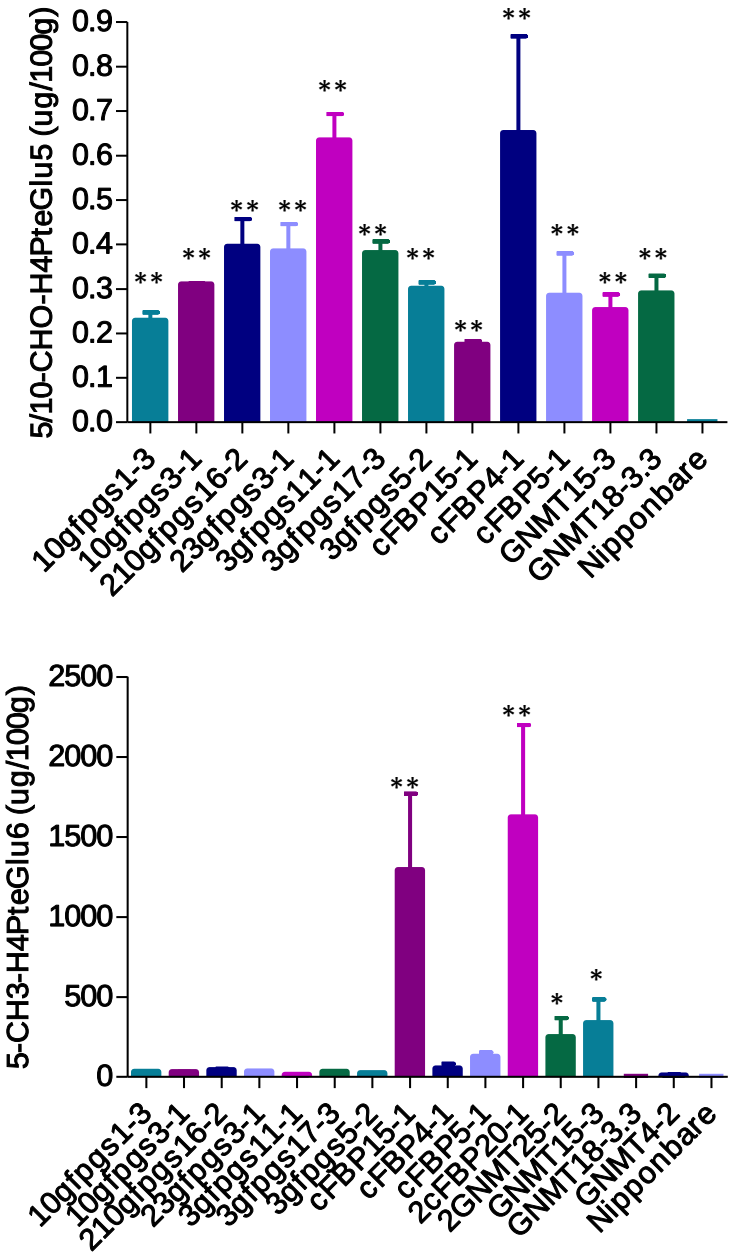

Figure 4.6c and 4.6d. Distribution of polyglutamyl forms (5/10-CHO-H_4PteGlu$_5$ and 5-CH_3-H_4PteGlu$_6$) in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT. Each value represents the mean of three biological replicates ± SD. Statistical differences are indicated with asterisks (*p<0.01*; **, *p<0.05*; *).

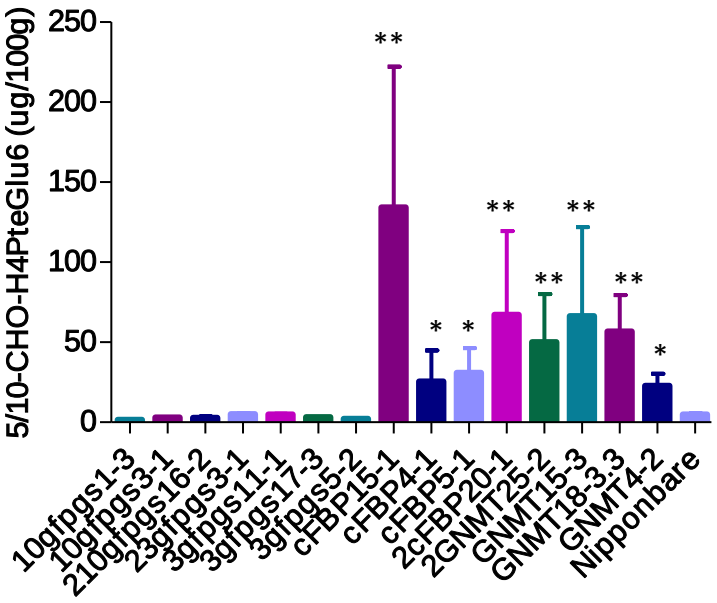

Figure 4.6e. Distribution of 5/10-CHO-H_4PteGlu$_6$ in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT. Each value represents the mean of three biological replicates ± SD. Statistical differences are indicated with asterisks (*p<0.01*; **, *p<0.05*; *).

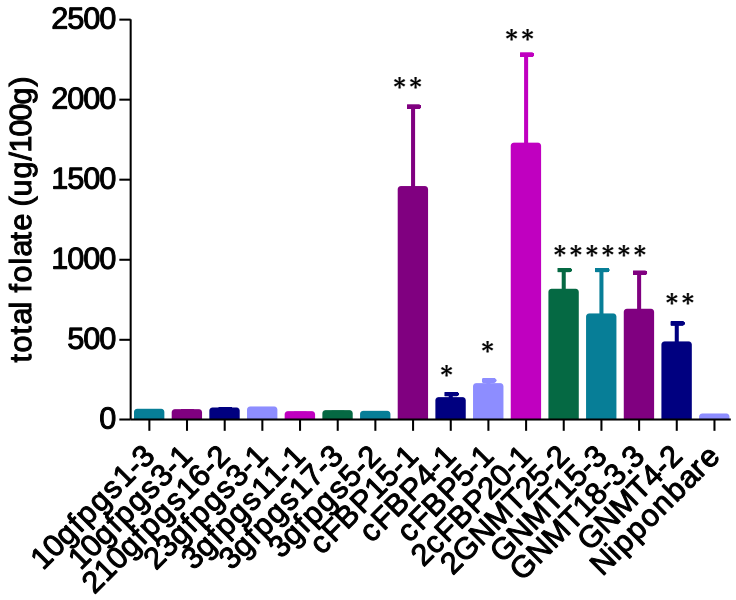

Figure 4.7. Total folate in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT. Each value represents the mean of three biological replicates ± SD. Statistical differences are indicated with asterisks (*p<0.01*; **, *p<0.05*; *).

4.6 Conclusion

LC-MS/MS based metabolite profiling of rice transgenic lines revealed that the abundance of mono- and polyglutamated folate forms were significantly decreased in seeds of FPGS knock out line, while dramatic increases in 5-CH_3-H_4PteGlu$_4$, 5/10-CHO-H_4Pteglu$_5$, 5-CH_3-H_4PteGlu$_6$, 5/10-CHO-H_4Pteglu$_6$ was observed in lines ectopically expressing FPGS, cFBP and GNMT genes in rice endosperm. There was a 2.5 to 8.8-fold overall increase in the total folate pool in the transgenic rice lines when compared to the Nipponbare. Interestingly, while FPGS expression only resulted in increased levels of polyglutamated folates, FBP and GNMT lines exhibited large increases in both mono- and polyglutamated forms. Although the highest increases in folate abundance was observed in the GNMT and cFBP rice lines, FPGS lines produced many more seeds. These initial results are very promising and will form the basis for further studies of the role of the *FPGS* genes and FBPs in enhancing folate accumulation and polyglutamylation in rice grains. This will aid the engineering of increased folate in rice grain and the development of a folate biofortified staple foodstuff.