



The University of  
**Nottingham**

UNITED KINGDOM • CHINA • MALAYSIA

Abilgos Ramos, Riza (2010) Folate profiling in wild and transgenic rice. PhD thesis, University of Nottingham.

**Access from the University of Nottingham repository:**

<http://eprints.nottingham.ac.uk/12870/1/Abstract.pdf>

**Copyright and reuse:**

The Nottingham ePrints service makes this work by researchers of the University of Nottingham available open access under the following conditions.

This article is made available under the University of Nottingham End User licence and may be reused according to the conditions of the licence. For more details see:  
[http://eprints.nottingham.ac.uk/end\\_user\\_agreement.pdf](http://eprints.nottingham.ac.uk/end_user_agreement.pdf)

For more information, please contact [eprints@nottingham.ac.uk](mailto:eprints@nottingham.ac.uk)

# Folate Profiling in Rice

**Riza Abilgos Ramos, MSc**



A thesis submitted to the University of Nottingham  
for the degree of Doctor of Philosophy, September 2009.

## ABSTRACT

Folate is one of the important B vitamins needed for normal cell function, growth and development. Whilst it is naturally found in rice, the level is low in the grain and the naturally-occurring forms of the vitamin are not well studied.

Chapter 2 describes the development of LC-MS/MS based technique to profile the mono- and polyglutamated folates while Chapter 3 describes how folates in rice were initially profiled using the microbiological assay (MA). MA was used to screen 51 rice cultivars for their total folate content, and then LC-MS/MS was employed to measure naturally occurring mono- and polyglutamated forms of the vitamin in selected cultivars. This study revealed wide natural variation among rice cultivars in terms of total folate content when MA screening was made and the validated LC-MS/MS technique of simultaneous profiling of mono- and polyglutamated folates through MeOHAA/PO<sub>4</sub> extraction revealed that the naturally-occurring species in wild type rice are 5-CH<sub>3</sub>-H<sub>4</sub>PteGlu, 5/10-CHO-H<sub>4</sub>PteGlu, 5-CH<sub>3</sub>-H<sub>4</sub>PteGlu<sub>4</sub>, 5-CH<sub>3</sub>-H<sub>4</sub>PteGlu<sub>5</sub> and 5/10-CHO-PteGlu<sub>5</sub>.

Chapter 4 describes the use of the LC-MS/MS techniques to characterise folate mono- and polyglutamated forms in a variety of transgenic rice lines. There was a general decrease in polyglutamated folate forms in the folate polyglutamating enzyme (FPGS Os03g02030) knockout rice line. Conversely, there was a dramatic increase in 5-CH<sub>3</sub>-H<sub>4</sub>PteGlu<sub>4</sub>, 5/10-CHO-H<sub>4</sub>Pteglu<sub>5</sub>, 5-CH<sub>3</sub>-H<sub>4</sub>PteGlu<sub>6</sub>, and 5/10-CHO-H<sub>4</sub>Pteglu<sub>6</sub> levels, resulting in a 2.5 to 8.8-fold increase in the total folate pool in the unpolished grains of rice either overexpressing FPGS, or the folate binding proteins -cFBP and GNMT compared to wild type Nipponbare.

This study demonstrates that overexpression of the two rice FPGS genes (Os03g02030 and Os10g35940) or folate binding proteins (cFBP and GNMT) results in improved levels and overall folate profile which can be exploited in breeding programmes designed to enhance folate content in this important staple crop.

# TABLE OF CONTENTS

<b>ABSTRACT</b>	II
<b>TABLE OF CONTENTS</b>	III
<b>LIST OF FIGURES</b>	V
<b>LIST OF TABLES</b>	VII
<b>ABRREVIATIONS</b>	IX
<b>1 GENERAL INTRODUCTION</b>	<b>1</b>
1.1 RICE FACTS AND FIGURES	1
1.1.1 <i>Rice Production and Utilisation</i>	1
1.1.2 <i>Rice Structure, Chemistry and Composition</i>	4
1.1.3 <i>Rice Nutritional Value and Importance</i>	6
1.2 FOLATES AND FOLIC ACID	8
1.2.1 <i>Folate Chemistry, Structure and Properties</i>	8
1.2.2 <i>Folate in Plants and Foods</i>	12
1.2.3 <i>Folate in Cereals, Cereal Products and Rice</i>	15
1.2.4 <i>Absorption, Bioavailability and Nutritional Importance</i>	17
1.2.5 <i>Methods of Measurements and Analysis</i>	20
1.3 THE BIOFORTIFICATION PROSPECTS	26
1.4 AIMS AND OBJECTIVES	28
<b>2 DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR THE MEASUREMENT OF MONO- AND POLYGLUTAMATED FOLATES IN RICE</b>	<b>29</b>
ABSTRACT	29
2.1 INTRODUCTION	30
2.1.1 <i>The complexity of folates</i>	30
2.1.2 <i>Methods of folate measurement</i>	31
2.1.3 <i>Folate extraction and LC-MS/MS technique</i>	32
2.2 AIMS AND OBJECTIVES	35
2.3 MATERIALS AND METHODS	36
2.3.1 <i>Chemicals, reagents, standards and quality control</i>	36
2.3.2 <i>Method development and optimisation</i>	39
2.3.3 <i>Mono- and polyglutamated folate measurement using LC-MS/MS</i>	43
2.4 STATISTICAL ANALYSIS	50
2.5 RESULTS AND DISCUSSION	51
2.5.1 <i>Optimisation of rice folate extraction</i>	51
2.5.2 <i>Method validation</i>	53
2.5.3 <i>Application of the method to certified reference material (CRM 121) and plant (spinach) quality control (QC)</i>	61
2.6 CONCLUSION	63
<b>3 APPLICATION OF MICROBIOLOGICAL ASSAY AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY IN THE QUANTIFICATION OF RICE GRAIN FOLATES</b>	<b>64</b>
ABSTRACT	64
3.1 INTRODUCTION	65
3.1.1 <i>Measurements of food folates</i>	65
	III

3.1.2 <i>Folates in cereals and cereal products</i>	67
3.2 AIMS AND OBJECTIVES	69
3.3 MATERIALS AND METHODS	70
3.3.1 <i>Rice materials</i>	70
3.3.2 <i>Microbiological determination of total folate concentration in unpolished rice grains</i>	73
3.3.3 <i>Monoglutamate folate measurement in unpolished rice grain using HPLC-UV/FLD</i>	75
3.3.4 <i>Mono- and polyglutamated folate profiling of rice using LC-MS/MS</i>	78
3.4 STATISTICAL ANALYSIS	79
3.5 RESULTS AND DISCUSSION	80
3.5.1 <i>Total folates in rice varieties as measured by microbiological assay with dienzyme treatment</i>	80
3.5.2 <i>Determination of monoglutamate folate species in unpolished rice grains using HPLC – UV/FLD</i>	88
3.5.3 <i>LC-MS/MS measurement of folate forms in the rice grain</i>	92
3.5.4 <i>Comparison between the MA, HPLC-UV/FLD and LC-MS/MS results</i>	101
3.6 CONCLUSION	103
<b>4 FOLATE METABOLIC PROFILING OF TRANSGENIC LINES, LACKING AND OVEREXPRESSING FOLATE-RELATED PROTEINS</b>	<b>104</b>
ABSTRACT	104
4.1 INTRODUCTION	105
4.1.1 <i>The role of folate polyglutamylation in plants</i>	105
4.1.2 <i>Folate binding proteins</i>	107
4.2 AIMS AND OBJECTIVES	108
4.3 MATERIALS AND METHODS	109
4.3.1 <i>Rice Materials</i>	109
4.3.2 <i>LC-MS/MS measurement of folates</i>	110
4.4 STATISTICAL ANALYSIS	111
4.5 RESULTS AND DISCUSSION	112
4.5.1 <i>Folates in FPGS Os03g02030 knock out line and wild type rice</i>	112
4.5.2 <i>Folates in rice with overexpressed FPGS genes</i>	116
4.5.3 <i>Folate profile of rice with cFBP and cGNMT</i>	122
4.6 CONCLUSION	129
<b>5 GENERAL CONCLUSION</b>	<b>130</b>
5.1 FOLATES AND RICE	130
5.2 FOLATE ANALYSES IN RICE	131
5.3 BIOFORTIFICATION OF FOLATES IN RICE EMPLOYING GENETIC AND TRANSGENIC APPROACHES	132
5.4 FUTURE DIRECTIONS	133
<b>6 REFERENCES</b>	<b>134</b>

## LIST OF FIGURES

Figure 1.1	Farm-grown Rice ( <i>Oryza sativa</i> L)	1
Figure 1.2	Greenhouse grown IR72 at the University of Nottingham	4
Figure 1.3	Longitudinal section of the rice grain	5
Figure 1.4	Chemical structure of folates	10
Figure 1.5	Molecular structure of folate (monoglutamates)	11
Figure 1.8	Folate interconversion at different conditions	12
Figure 1.6	Folate biosynthetic pathway in plant, compiled using net based resources (TAIR-Arabidopsis thaliana Biochemical Pathways; BRENDA Enzyme database and KEGG Pathway database), and corrected/updated using available literature.	14
Figure 1.7	Absorption and activation of folic acid in mammals	18
Figure 2.1	Representative total ion chromatogram of 15 folate standards	59
Figure 3.1	Total folate content ( $\mu\text{g}/100\text{g}$ ) in 51 rice cultivars	86
Figure 3.2	Different levels of total folate ( $\mu\text{g}/100\text{g}$ ) in various rice cultivars screened using the microbiological assay	87
Figure 3.3	Changes in total folate content of unpolished IR72 grain stored in ambient (21-28°C) and cold condition (-28°C)	87
Figure 3.4	Folate forms detected in unpolished Moroberekan, IR72 and IR64 rice grains using HPLC-UV/FLD	90
Figure 3.5	LC-MS/MS chromatogram of folates detected in unpolished rice grain	94
Figure 3.6	Folate profile of rice varieties with low, medium, and high total folate concentration (a). Polyglutamate folates distribution in the same set of rice (b)	95
Figure 3.7	Chromatograms of 5-CH <sub>3</sub> -H <sub>4</sub> PteGlu (a) and of 5-CHO-H <sub>4</sub> PteGlu (b) in rice grain and their corresponding EPI spectra (c, d)	96
Figure 3.8	Chromatogram (a) and EPI spectra of 5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>4</sub> in rice grain (b) and verified with the library spectra (c)	100
Figure 3.9	Chromatogram (a) and EPI spectra of 5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>5</sub> in rice grain (b) and verified with the library spectra(c)	101
Figure 3.10	Chromatogram (a) and EPI spectra of 5-CHO-H <sub>4</sub> PteGlu <sub>5</sub> in rice grain (b) and	

	verified with library spectra (c)	102
Figure 3.11	Percent distribution of different folate species in rice	98
Figure 4.1	Polyglutamylation reaction of monoglutamate by the FPGS enzyme	106
Figure 4.2	Plant phenotypes of FPGS Os03g02030 knock out line and Dongjin wild type	113
Figure 4.3	Mass spectra for the confirmation of 5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>6</sub> in grains of transgenic rice lines	118
Figure 4.4	Mass spectra for the confirmation of 5/10-CHO-H <sub>4</sub> PteGlu <sub>6</sub> in grains of transgenic rice lines	119
Figure 4.5a and 4.5b	Distribution of monoglutamyl (5-CH <sub>3</sub> -H <sub>4</sub> Pteglu and 5/10-CHO-H <sub>4</sub> PteGlu) forms in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT	124
Figure 4.6a and 4.6b	Distribution of polyglutamyl forms (5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>4</sub> and 5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>5</sub> ) in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT	125
Figure 4.6c and 4.6d	Distribution of polyglutamyl forms (5/10-CHO-H <sub>4</sub> PteGlu <sub>5</sub> and 5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>6</sub> ) in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT	126
Figure 4.6e	Distribution of 5/10-CHO-H <sub>4</sub> PteGlu <sub>6</sub> in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT	127
Figure 4.7	Total folate in unpolished rice grains of WT and in lines with expressed FPGS, cFBP and cGNMT	128

## LIST OF TABLES

Table 1.1	Total folate contents in selected cereals and cereal products according to U.S. and UK Food composition databases ( $\mu\text{g}/100\text{g}$ edible part)	16
Table 2.1	Internal and Folate Standards Used for LC-MS/MS analysis	38
Table 2.2	Extinction coefficients of monoglutamated folates	39
Table 2.3	Extraction methods used for LC-MS/MS folate measurement in rice grain	40
Table 2.4	Retention times, cone voltages, and collision voltages used to identify and quantify analytes using MRM with the LC-MS/MS method	51
Table 2.5	Recovery of folate metabolites from various extraction methods	52
Table 2.6	Individual folate recovery from the rice grain matrix spiked with 5- $\text{CH}_3/\text{CHO}-\text{H}_4\text{PteGlu}_{1-8}$ and $\text{PteGlu}_n$ standards	54
Table 2.7	Intra- and inter-day precision ( $n=5$ ) and accuracy results for the determination of folates in rice matrix using LC-MS/MS	57
Table 2.8	Calibration data for analysis of folates using LC-MS/MS	59
Table 2.9	Limit of detection (LOD) and lower limit of quantification (LLOQ) for individual folates measured using LC-MS/MS	60
Table 2.10	Ion suppression of individual folates. Matrix effect% = $(\text{B}-\text{C})/\text{A} \times 100$ with (A) as the peak area of the neat standards in extraction buffer, (B) the peak area of rice matrix spiked with the 500 nM standards and (C) the peak area of the endogenous amount of folates present in the rice matrix	60
Table 2.11	Folate species in CRM 121 as measured using LC-MS/MS. Values are mean of triplicates with error bars (standard deviation)	62
Table 3.1	Rice materials screened for total folate concentration using microbiological assay	71
Table 3.2	Materials for growing rice in pots in the greenhouse	73
Table 3.3	Microbiologically determined folate contents of different rice varieties	84
Table 3.4	Folate contents in unpolished rice ( $\mu\text{g}/100\text{g}$ ), determined by dienzymic method	90
Table 3.5	Recovery of dienzymic method for folate determination in unpolished rice grain using HPLC-UV/FLD	91
Table 3.6	Some data comparison of total folate in brown rice grain (results obtained from three methods of analysis, $\mu\text{g}/100\text{g}$ )	98



Table 4.1	Total folate levels and folate derivatives in leaf and seed from Dongjin wild type and <i>FPGS Os03g02030</i> mutant	116
Table 4.2	Folate forms and total concentration in unpolished rice grains with over-expressed <i>FPGS</i> genes, cFBP and GNMT (mean $\pm$ SD; $n=3$ ) compared to the WT (Nipponbare)	121

## ABBREVIATIONS

H <sub>4</sub> PteGlu	tetrahydrofolate
5,10-CH <sup>+</sup> -H <sub>4</sub> PteGlu	5,10-methylene-tetrahydrofolate
5-CH <sub>3</sub> -H <sub>4</sub> PteGlu	5-methyl-tetrahydrofolate
CHO-H <sub>4</sub> PteGlu	formyl-tetrahydrofolate
5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>4</sub>	5-methyl-tetraglutamate
5-CH <sub>3</sub> -H <sub>4</sub> PteGlu <sub>5</sub>	5-methyl-pentaglutamate
CHO-PteGlu <sub>5</sub>	formyl-pentaglutamate
APCI	atmospheric pressure chemical ionisation
cFBP	cow's folate binding protein
CRM	certified reference material
DHPS	dihydropteroate synthase
DMHA	dimethylhexylamine
EPI	Enhanced product ion
FPGS	folylpolyglutamate synthetase
GNMT	glycine N-methyltransferase
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
LLOQ	lower limit of quantification
MA	microbiological assay
MRM	multiple reaction monitoring
MTX	methotrexate
pABA	<i>para</i> aminobenzoate
PteGlu	folic acid
WCV	wide compatibility variety