J. Trop. Agric. and Fd. Sc. 53 (2)(2025): 77 – 85

Identification of key genes involved in rice folate biosynthesis pathway

Sew, Y. S., Abd Rashid, M. R., Ahmad, M. A., AbuBakar, N., Zainal Abidin, R. A., Ab Razak, M. S. F. and Simoh, S.

Biotechnology and Nanotechnology Research Centre, MARDI Headquarters, 43400, Serdang, Selangor, Malaysia

Abstract

Folate (vitamin B9) is vital for growth and development, but its deficiency especially in populations relying heavily on low-folate staple foods like polished rice poses significant health risks, making rice biofortification with natural, more bioavailable folates a crucial strategy for improving global nutrition. This study aimed to identify key functional genes involved in the folate biosynthesis pathway for rice biofortification. We assessed the antioxidant activities of four pigmented rice varieties (PH 9, BALI, MRQ 100, and MRM 16) and two non-pigmented rice varieties (MRQ 76 and MR 297) using antioxidant assays (i.e. TPC, DPPH and FRAP), along with measurements of folate biosynthesis gene expression levels using quantitative PCR method. Antioxidant activities were significantly higher in the pigmented than the non-pigmented ones (p <0.05). One-way ANOVA with post-hoc analysis revealed that four folate biosynthesis genes, namely Os01g0238500, Os06g0699700, Os07g0618500, and Os12g0623800 were differentially expressed between rice with distinct pigmentation (p <0.05). Spearman's rho correlation analysis indicated a significant and positive correlation between antioxidant activities and the expression levels of aminodeoxychorismate synthase/glutamine amidotransferase (ADCS) (Os06g0699700) and dihydropterin pyrophosphokinase/ dihydropteroate synthase (HPPK/ DHPS) (Os07g0618500) (p <0.05). These findings suggest that these two genes play a crucial role in folate biosynthesis in rice and may serve as promising targets for enhancing folate content through biofortification.

Keywords: *antioxidant activity, biofortification, folate, gene expression, rice*

Introduction

Folate, also known as vitamin B9, is an essential water-soluble vitamin that plays a key role in DNA synthesis, cell division, and overall growth and development. It is particularly important during periods of rapid growth such as pregnancy and infancy. Rice, being a staple food for over half the world's population, especially in Asia and parts of Africa is a crucial target for nutritional enhancement. However, natural folate levels in rice grains are generally low, particularly in polished white rice, which is the most commonly consumed form. Folate deficiency remains a major global health concern, particularly in low- and middle-income countries. Insufficient folate intake in folate can lead to serious health issues, including neural tube defects in newborns, anemia, cognitive impairments, immune dysfunction and an increased risk of cardiovascular disease (Yingngam 2024). While many high-income countries have implemented mandatory folic acid supplementation and fortification programs to combat folate deficiency, the bioavailability of synthetic folic acid remains a concern. Studies suggest that excess unmetabolised folic acid in the bloodstream may mask vitamin B12 deficiency and has potential links to cancer (Bailey & Berry, 2005). Conversely, natural folates found in crops like rice are more bioavailable than synthetic folic acid, which is essential for long-term health improvement.

Folate biosynthesis in rice is localised in three subcellular compartments: plastid, cytosol and mitochondria (*Figure 1*). The process begins with the enzyme GTP cyclohydrolase I present in cytosol that converts GTP (guanosine triphosphate) into dihydroneopterin triphosphate (DHN-P3), then 7,8-dihydroneopterin (DHN-P) and dihydroneopterin (DHN). The next step involves the enzyme dihydroneopterin aldolase (DHNA), which catalyses the formation of hydroxymethyldihydropterin (HMDHP). This compound is next converted to 6-hydroxymethyldihydropterin pyrophosphate

Article history
Received: 4.6.2025
Accepted: 12.8.2025

Authors' full names: Yun Shin Sew, Muhamad Ridzuan Abd Rashid, Muhamad Aizuddin Ahmad, Norliza Abu Bakar, Rabiatul Adawiah Zainal Abidin, Mohd Shahril Firdaus Ab Razak and Sanimah Simoh
Corresponding author: sewyshin@mardi.gov.my

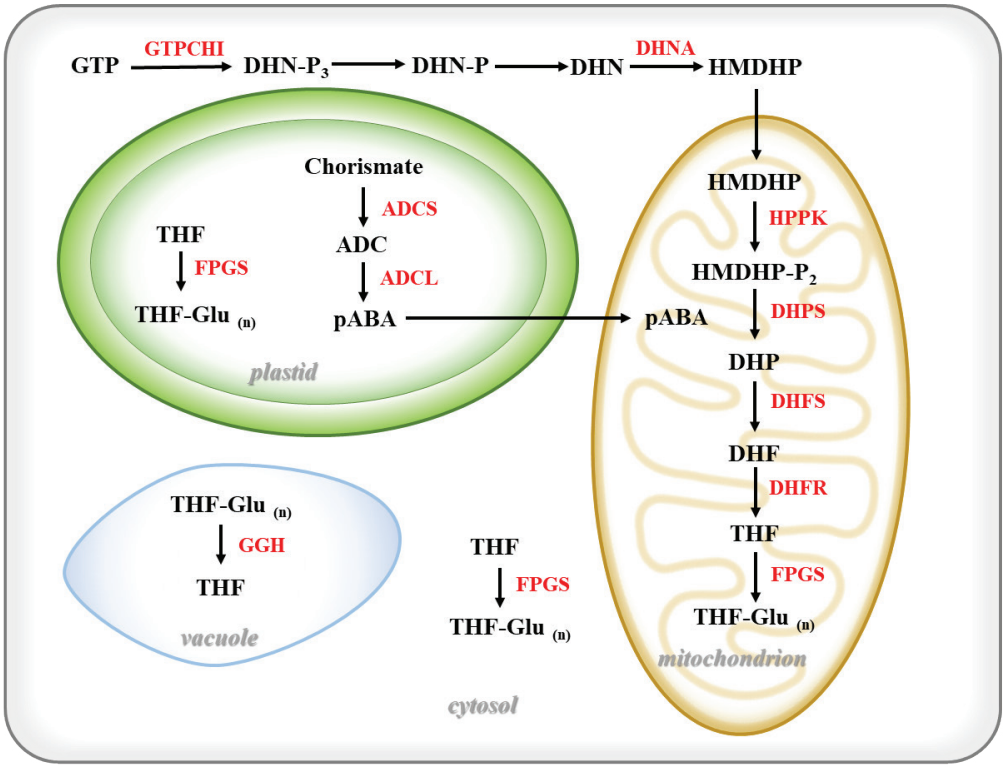

Figure 1. Rice folate biosynthesis: Precursors: GTP, guanosine triphosphate; DHN-P3, dihydroneopterin triphosphate; DHN-P, 7,8-dihydroneopterin; DHN, dihydroneopterin; HMDHP, hydroxymethyldihydropterin; HMDHP-P2, 6-hydroxymethyldihydropterin pyrophosphate; DHP, dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate; THF-Glu(n), tetrahydrofolate polyglutamate; ADC, aminodeoxychorismate; pABA, para-aminobenzoic acid. Enzymes: GTPCHI, GTP cyclohydrolase 1 (Os04g0662700/ LOC_Os04g56710); DHNA, dihydroneopterine aldolase (Os08g0556200/ LOC_Os05g44130); HPPK, HMDHP pyrophosphokinase (Os07g0618500/ LOC_Os07g42632); DHPS, dihydropteroate synthase (Os07g0618500/LOC_Os07g42632); DHFS, dihydrofolate synthetase (Os12g0623800/LOC_Os12g42870); ADCS, aminodeoxychorismate synthase (Os06g0699700/ LOC_Os06g48620); ADCL, aminodeoxychorismate lyases (Os01g0238500/ LOC_Os01g13690 and Os05g0244700/ LOC_Os05g15530); DHFR, dihydrofolate reductase (Os11g0484400/LOC_Os11g29390); FPGS, folylpolyglutamate synthetase (Os03g0111100/ LOC_Os03g02030); GGH, gamma-glutamyl hydrolase (Os05g0517500/LOC_Os05g44130)

(HMDHP-P2) by HMDHP pyrophosphokinase (HPPK) in mitochondria. Parallel to this, the p-aminobenzoate (pABA) branch of the pathway begins with chorismate, which is converted to pABA by aminodeoxychorismate synthase (ADCS) and aminodeoxychorismate lyase (ADCL). The pABA and dihydropterin intermediates HMDHP-P2 are then combined by the enzyme dihydropteroate synthase (DHPS) to form dihydropteroate (DHP). Dihydropteroate is subsequently converted to dihydrofolate by the addition of glutamate residues, a reaction catalysed by dihydrofolate synthetase (DHFS), before converting to tetrahydrofolate (THF) by dihydrofolate reductase (DHFR). While the polyglutamation of folate is catalysed by the enzyme folylpolyglutamate synthetase (FPGS), and depolyglutamation is through the hydrolysis process by the enzyme gamma-glutamyl hydrolase (GGH). Several forms of natural folates found available in rice grains. Those are known to be folic acid and its derivatives such as 10-formylfolic acid, tetrahydrofolate, 5-methyltetrahydrofolate and 5, 10-methenyltetrahydrofolate (Ashokkumar et al. 2018). These rice folates possess strong antioxidant properties (Gliszczyńska-Świgło 2007), which are closely linked to its protective role against cardiovascular, hematological, and neurological diseases, as well as cancer (Rezk et al. 2003).

Pigmented rice varieties, characterised by darker pericarp colour (e.g. black, purple, red, brown) and distinctive flavours, have higher antioxidant properties than non-pigmented (white) rice, making them valuable as functional foods (Goufo & Trindade 2014). Of particular interest, the total folate content in pigmented rice varieties is significantly higher than in non-pigmented ones, as reported by Ashokkumar et al. (2018). Despite decades of research on rice folate, most studies have focused primarily on the functional roles of specific folate-related genes. However, the relationship between folate gene expression and the antioxidant properties of folates in rice with varying pericarp colours remains poorly understood, warranting further investigation. Therefore, the present study explores this relationship using molecular and statistical approaches, aiming to identify key genes

involved in the rice folate biosynthesis pathway. These genes could potentially serve as targets for rice folate biofortification through various strategies.

Materials and method

Rice seeds samples and extraction

A total of 6 rice varieties (*Oryza sativa* L.) were studied, comprising 4 pigmented varieties and 2 non-pigmented varieties. The pigmented varieties included black glutinous rice (PH9), black rice (BALI), red rice (MRM 16 and MRQ 100). The non-pigmented varieties were fragrant rice (MRQ 76) and white rice (MR 297). All varieties were sourced from the MARDI rice germplasm. The rice seeds were dehusked and ground into powder, followed by freeze-drying prior to metabolite extraction as described previously in Simoh et al. (2018) with minor modifications.

Evaluation of rice antioxidant activities

Total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing power (FRAP) assays were used to evaluate the antioxidant activities of all rice extracts. TPC was determined using the Folin-Ciocalteu's reagent (Singleton et al. 1999). The TPC was calculated as mg gallic acid equivalent per g dry weight of plant (mg GAE/ g dry weight sample). The DPPH assay was performed according to the previous described method by Brand-Williams et al. (1995) with some modifications. 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a violet-coloured free stable radical and it was used as scavenger for many other radicals. The violet colour of DPPH faints into the yellow colour of its reduced congener (DPPH-H), which can be detected in the visible spectra (from 520 nm to 330 nm) (Ionita, 2005). For DPPH assay, the antioxidant activity was expressed in mg Trolox equivalent per g dry weight of plant (mg TE/ g dry weight sample). FRAP method measures the ability of a solution to reduce a ferric-tripyridyltriazine complex (Fe^{3}+-TPTZ) to the ferrous form, Fe^{2+} producing a blue colour with the absorption at 593 nm. The ability to reduce ferric ions was measured using method as described by Benzie and Strain (1996) with some modifications. The antioxidant activity was expressed in mM $FeSO_4$/g dry weight of sample for FRAP assay. All assays were performed in triplicates.

Gene expression analysis io folate biosynthesis genes

Total RNA was extracted from individual whole rice grain samples of PH 9, BALI, MRQ 100, MRM 16, MRQ 76, and MR 297 using the MLT method (Mornkham et al. 2013). First-strand cDNAs were synthesized using the Quantitect Reverse Transcription kit (Qiagen) following the manufacturer's instructions. PCR primer pairs for folate and reference genes were designed and purchased from Integrated DNA Technologies (IDT Singapore Pte Ltd). Ubiquitin 5 (UBQ5) was used as the reference gene for Real-Time PCR data normalisation, as it was found to be the most stably expressed endogenous gene in both pigmented and non-pigmented varieties. Real-Time PCR assays were performed using the designed qPCR primers and rice cDNAs from different varieties with qPCRBIO SyGreen mix (PCR Biosystems, London, UK) in an ABI StepOnePlus Real-Time PCR system (Applied Biosystems). The reactions were performed in triplicate, using 5 µL of Master Mix, 0.25 µM of each primer, 1 µL of diluted cDNA, and DNase-free water to reach a final volume of 10 µL. The PCR amplification protocol included an initial cycle of 5 minutes at 95°C, followed by 40 cycles of 15 seconds of denaturation at 95°C, 10 seconds of annealing at 60°C, and 15 seconds of elongation at 72 °C. This was followed by a melting curve analysis with continuous fluorescence data acquisition during the 60 − 95°C melting phase. The melt curve analysis indicated that only one product was formed for each gene primer reaction. The relative changes in rice gene expression were calculated using the $2^{-\Delta\Delta}$CT method (Livak & Schmittgen 2001).

Statistical analysis

All data obtained from antioxidant activity and real-time PCR assays were expressed as means ± standard errors from three replicate determinations. Spearman's correlation analysis was performed to examine the relationship between antioxidant activities and gene expression data. ANOVA followed by Tukey's Honest Significant Difference (HSD) analysis was used to compare pigmented and non-pigmented rice varieties.

Results and discussion

Antioxidant activities between pigmented and non-pigmented rice varieties

Figure 2 shows the results of antioxidant activity assays (TPC, DPPH and FRAP) tested across all selected rice varieties. The results of all assays indicated a common trend, where the average antioxidant activities were highest in black pigmented rice (PH9 and BALI), followed by red pigmented rice (MRQ 100 and MRM 16), and lowest in white rice (MRQ 76 and MR 297). These findings suggest that the intensity of rice pericarp pigmentation has a significant impact on antioxidant activity. When all rice varieties were categorised into black, red and white rice, all groups showed significant differences from one another (p <0.01). In general, pigmented rice varieties (i.e., black and red rice) exhibited significantly higher antioxidant activities compared to non-pigmented varieties (white rice). This finding is consistent with previous reports that pigmented rice varieties exhibit stronger antioxidant activities compared to non-pigmented ones (Nam et al. 2006; Laokuldilok et al. 2011)

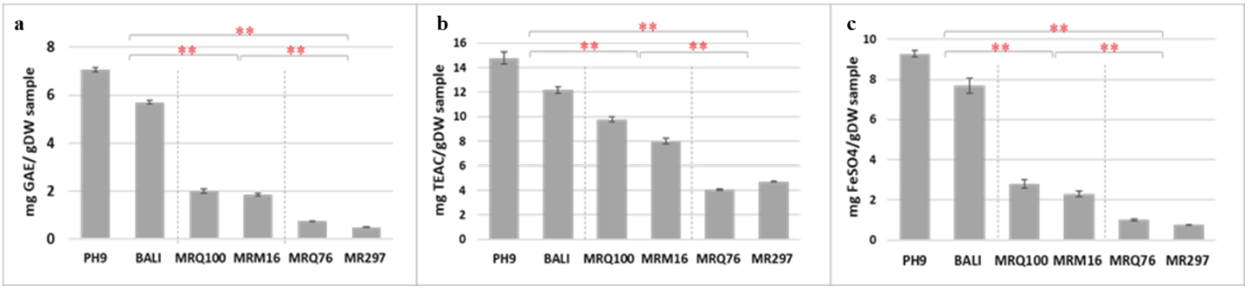

Figure 2. Antioxidant activity analysis of pigmented and non-pigmented rice varieties. a) TPC, b) DPPH and c) FRAP, mean ± SE, n=3. Rice varieties are categorised according to their pigmentation, i.e. black (PH 9 and BALI), red (MRQ 100 and MRM 16) and white (MRQ 76 and MR 297). Significant differences between rice groups are compared using one-way ANOVA HSD analysis, asterisk (**) indicate p <0.01

Earlier studies have shown the antioxidant activities in rice grains are collectively contributed by bioactive compounds such as anthocyanins, phenolic compounds, flavonoids, tocopherols as well as folates and their derivatives (Abdel-Aal et al. 2006; Biswas et al. 2018; Goufo & Trindade 2014). Notably, folates exhibit antioxidant activities comparable to vitamins C and E (Antony, 1996; Kaliora et al. 2006). Folates exhibit antioxidant capacity through various in vivo mechanisms, including scavenging free radicals, enhancing the synthesis of glutathione, working synergistically to neutralize reactive oxygen species, protecting cells from oxidative stress, and supporting DNA repair and synthesis (Lu, 2009). While folic acids supplementation in plants have effectively enhanced the antioxidant activity, resulting in combating adverse effects of salinity (Al-Elwany et al. 2022) and resistance against abiotic stress caused by *Pseudomanas syringae* (Wittek et al. 2015).

Gene expression measurements on rice folate biosynthesis genes

Folate biosynthesis genes were data mined from in-house rice transcriptome data and public databases. These genes encode for enzymes along rice folate biosynthesis pathway were identified via KEGG pathway analysis with their individual Enzyme Commission (EC) and NCBI accession number as listed in *Table 1*. The primer sequences for each folate biosynthesis gene including the UBQ5 reference gene are as listed in *Table 2*.

The gene expression values of each rice folate biosynthesis gene across varieties with varying pigmentations were obtained, and the relative gene expression levels were calculated with reference to the variety MR 297 (white rice) (*Figure 3*). Folate biosynthesis genes show several significant differences in gene expression between rice varieties with different pericarp pigmentations include Os07g061850, Os12g0623800, Os06g0699700, Os01g0238500, Os05g0244700 and Os11g048400. Notably, the expression levels of certain folate genes such as Os07g0618500, Os06g0699700 and Os11g048400 are significantly higher in PH9 (black rice) compared to MR297 (white rice) ($p < 0.05$). These findings indicated that a significantly higher mRNA level of those genes expressed in rice with darker pericarp. In contrast, the expression of Os01g238500 was found to be consistently downregulated in pigmented compared to non-pigmented varieties.

Table 1. List of genes involved in rice folate biosynthesis

No.	Gene ID (RAP)	Gene ID (MSU)	KEGG pathway ID	Gene name	Accession number
1	Os04g0662700	LOC_Os04g56710	EC:3.5.4.16	GTP cyclohydrolase 1 (GTPCHI)	AK069126
2	Os08g0556200	LOC_Os08g44210	EC:4.1.2.25	Dihydroneopterin aldolase (DHNA)	AK121222
3	Os07g0618500	LOC_Os07g42632	EC:2.7.6.3; EC:2.5.1.15	Dihydropterin pyrophosphokinase/ dihydropteroate synthase (HPPK/DHPS)	AK068210
4	Os12g0623800	LOC_Os12g42870	EC:6.3.2.12	Dihydrofolate synthase (DHFS)	AK063876
5	Os06g0699700	LOC_Os06g48620	EC:2.6.1.85	Aminodeoxychorismate synthase/glutamine amidotransferase (ADCS)	AK059492
6	Os01g0238500	LOC_Os01g13690	EC:4.1.3.38	Aminodeoxychorismate lyase (ADCL)	AK067076
7	Os05g0244700	LOC_Os05g15530	EC:4.1.3.38	Aminodeoxychorismate lyase (ADCL)	AK073635
8	Os11g0484400	LOC_Os11g29390	EC:1.5.1.3	Bifunctional dihydrofolate reductase-thymidylate synthase (DHFR)	AK242642
9	Os03g011110	LOC_Os03g02030	EC:6.3.2.17	Folylpolyglutamate synthetase (FPGS)	AK102025
10	Os05g0517500	LOC_Os05g44130	EC 3.4.19.9	Gamma-glutamyl hydrolase (GGH)	AK065245

Table 2. The primer sequences used in qPCR assays

	Gene name	Gene ID	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
1	GTP cyclohydrolase 1 (GTPCHI)	Os04g0662700	CCATAGCTTGGTGCCCTTTA	GAGGGAGCTGGTACTGATTTG
2	Dihydroneopterin aldolase (DHNA)	Os08g0556200	CTGCTCAAGTTCCCTCAGATT	ATATCTCCACGCCCAAATAGTC
3	Dihydropterin pyrophosphokinase/ dihydropteroate synthase (HPPK/DHPS)	Os07g0618500	CCATTATTTGGTGGGTTGCTTC	GAACTTGGTGCCTTGCTTATTC
4	Dihydrofolate synthase (DHFS)	Os12g0623800	GCCCAAGACCAGATGTTGTA	CAGCCATCCATATCTCCTTCAG
5	Aminodeoxychorismate synthase/glutamine amidotransferase (ADCS)	Os06g0699700	AAGTCGCAGCACACCTATTC	CTGGCATAGGCACCTTTCT
6	Aminodeoxychorismate lyase (ADCL)	Os01g0238500	GAGCGAGCTGAGATGGATATG	CGTTGACGACACGTACAGATAA
7	Aminodeoxychorismate lyase (ADCL)	Os05g0244700	AGCTAGACGACGACTCATAGA	GACGGATCCTCCACTCATAATAC
8	Bifunctional dihydrofolate reductase-thymidylate synthase (DHFR)	Os11g0484400	CCTGTGGTAGAGAGCAACATTAG	GCTGATTCCTTGCCATTTGAG
9	Folylpolyglutamate synthetase (FPGS)	Os03g0111100	GGACCAAAGGAAAGGGTTCA	GCTCCCTGACATCCATCAAA
10	Gamma-glutamyl hydrolase (GGH)	Os05g0517500	CCCAAGACATTGCGAGAGAA	CTTGCACGGTTGAGACATAGA
11	Ubiquitin 5 (UBQ5)	Os01g0328400	ACCACTTCGACCGCCACTACT	ACGCCTAAGCCTGCTGGTT

To identify genes that are differentially expressed according to distinct pericarp pigmentation (i.e., black, red, and white rice), the expression data of folate biosynthesis genes in these designated groups were subjected to one-way ANOVA followed by Tukey's HSD post-hoc analysis (*Table 3*). Genes that were differentially expressed between black and red rice include Os12g0623800 and Os06g0699700; whereas Os07g0618500, Os12g0623800, Os06g0699700 and Os01g0238500 expressed significantly different between black and white rice. Notably, Os07g0618500 and Os01g0238500 are those differential expressed genes between red and white rice.

Spearman's rho correlation analysis

The relationship between the two data sets of antioxidant activities and gene expression levels of folate genes was next investigated. Considering these two sets of variables are independent and monotonic, a Spearman's rho correlation analysis was conducted (*Table 4*). Spearman's Rho correlation analysis, also known as Spearman's rank correlation coefficient, is a statistical method used to measure the strength and direction of association between two ranked variables. The expression levels of three folate genes Os01g0238500, Os06g0699700 and Os07g0618500 were significantly correlated with their antioxidative characteristics as consistently revealed by all antioxidant assays performed (i.e. TPC, DPPH and FRAP) (p <0.05). The strongest and positive relationship was observed for Os06g0699700, with correlation coefficients more than 0.8. Moderate correlation was observed for Os07g0618500 (r >0.60). In converse, a weak and negative correlation was observed for Os01g0238500 (r >-0.50). Although Os12g0623800 was also negative correlated with antioxidant activity (r >-0.40), but it was only deemed to be significant by FRAP assay.

The current study demonstrates that the expression levels of specific folate biosynthesis genes particularly Os07g0618500 and Os06g0699700 are significantly higher in pigmented rice varieties. Furthermore, a strong positive correlation was observed between gene expression and antioxidant activity in rice. These results collectively suggest that these two genes are potential key regulators of folate accumulation in rice seeds and contribute significantly to antioxidant activity. The rice gene Os07g0618500 (dihydropterin pyrophosphokinase/ dihydropteroate synthase) encodes bifunctional enzymes which catalyse sequential reactions of HPPK and DHPS in the folate biosynthesis pathway. An elevated level of folate content was observed in rice plants expressing the wheat HPPK/DHPS pyrophosphokinase gene (Gillies et al. 2008; McIntosh & Henry, 2008), suggesting a key role for this gene in folate biosynthesis, consistent with the findings of the present study. Similarly, an increase in gene expression of HPPK/DHPS has led to up to a 12-fold increase in folate levels in transgenic

potato (De Lepeleire et al. 2018). Likewise, there have been several studies showing the increased in expression level of aminodeoxychorismate synthase/ glutamine amidotransferase (ADCS) (Os06g0699700) were able to boost the folate accumulation in plants. For instance, transgenic tomato with overexpression of *Arabidopsis* aminodeoxychorismate synthase found to elevate as high as 20-fold the production of p-aminobenzoate (PABA), the precursor for folate biosynthesis (Díaz de la Garza et al. 2007). Furthermore, a study conducted by Dong et al. (2014) revealed that the overexpression of Arabidopsis ADCS in the japonica rice variety 'Kitaake' significantly boosted folate content in the seeds. Their research highlighted the individual overexpression of either GTP cyclohydrolase I (GTPCHI) or ADCS increased folate levels, combining GTPCHI with other genes (excluding ADCS) actually led to reduced folate accumulation compared to GTPCHI alone. These findings indicate that the availability of PABA, which is regulated by ADCS, is a key limiting factor in folate biosynthesis. Clearly, both HPPK/DHPS and ADCS genes play a crucial role in governing the accumulation of folate content. Hence, it is anticipated that by simultaneously enhancing both upstream (ADCS) and downstream (HPPK/DHPS) steps, plants such as rice can achieve a substantial increase in folate content, offering a promising strategy to combat folate deficiency through biofortified crops.

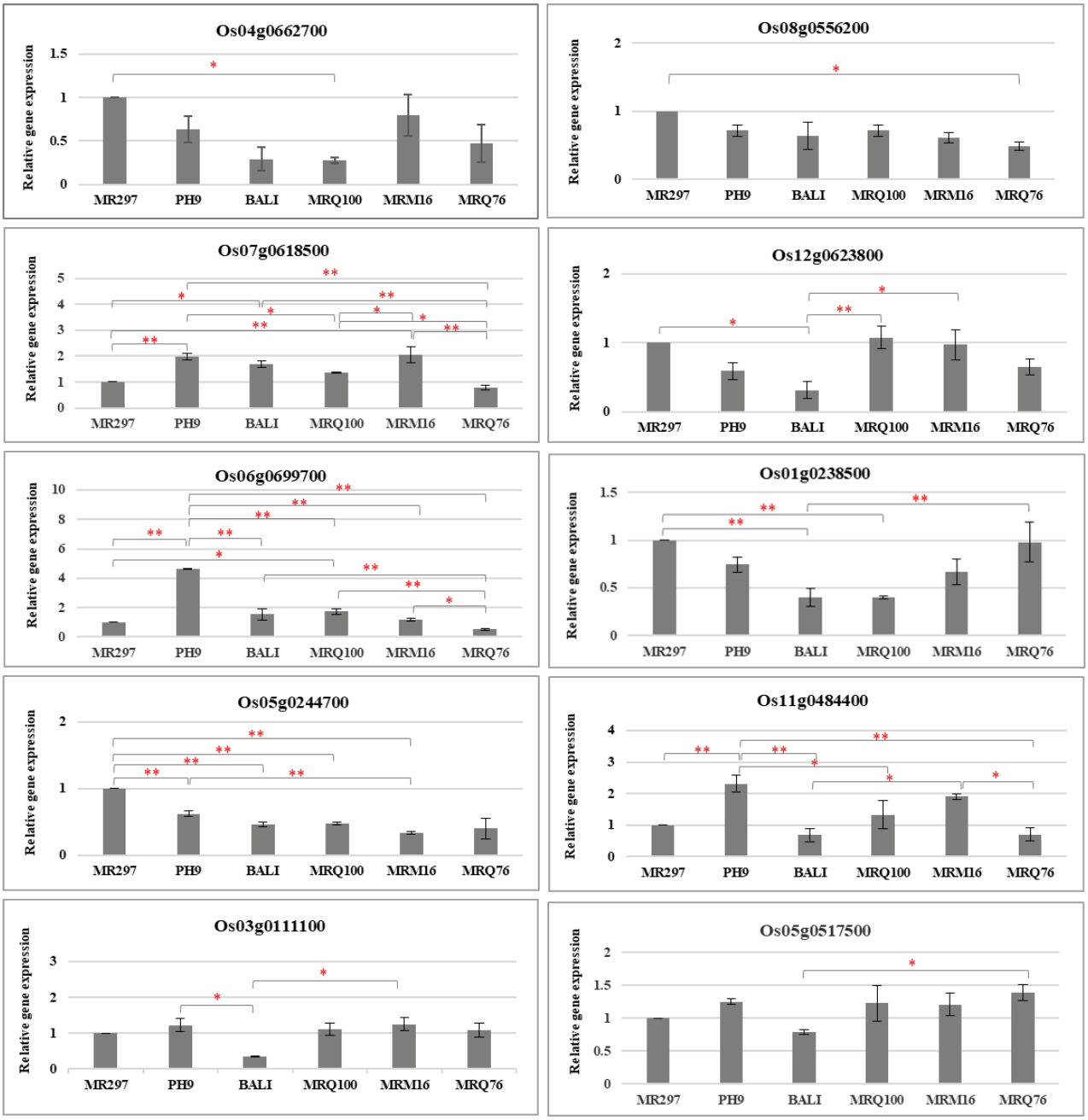

Figure 3. The expression profiles of folate related genes across pigmented and non-pigmented rice varieties. Relative gene expression levels of each gene were expressed as mean (± SE, n=3), UBQ was used as reference gene and MR 297 was used as reference variety. Asterisk (*) and (**) indicate statistically significant differential expression between varieties, p <0.05 and p<0.01, respectively

Table 3. Multiple comparison by ANOVA with Tukey's HSD analysis of folate biosynthesis genes across designated rice groups (black, red and white rice). Significances between groups are indicated in bold where p-values less than 0.05

Dependent variable			Mean difference	Std. error	Sig.
Os04g0662700	Black rice	Red rice	-.07327	.20360	.931
		White rice	-.27179	.20360	.399
	Red rice	Black rice	.07327	.20360	.931
		White rice	-.19851	.20360	.603
	White rice	Black rice	.27179	.20360	.399
		Red rice	.19851	.20360	.603
Os08g0556200	Black rice	Red rice	.018718	.133058	.989
		White rice	-.063120	.133058	.884
	Red rice	Black rice	-.018718	.133058	.989
		White rice	-.081839	.133058	.814
	White rice	Black rice	.063120	.133058	.884
		Red rice	.081839	.133058	.814
Os07g0618500	Black rice	Red rice	.115092	.180669	.802
		White rice	.940615*	.180669	**.000**
	Red rice	Black rice	-.115092	.180669	.802
		White rice	.825523*	.180669	**.001**
	White rice	Black rice	-.940615*	.180669	**.000**
		Red rice	-.825523*	.180669	**.001**
Os12g0623800	Black rice	Red rice	-.567957*	.133782	**.002**
		White rice	-.369809*	.133782	**.036**
	Red rice	Black rice	.567957*	.133782	**.002**
		White rice	.198148	.133782	.327
	White rice	Black rice	.369809*	.133782	**.036**
		Red rice	-.198148	.133782	.327
Os06g0699700	Black rice	Red rice	1.948818*	.582193	**.012**
		White rice	2.644216*	.582193	**.001**
	Red rice	Black rice	-1.948818*	.582193	**.012**
		White rice	.695398	.555099	.443
	White rice	Black rice	-2.644216*	.582193	**.001**
		Red rice	-.695398	.555099	.443
Os01g0238500	Black rice	Red rice	.038941	.114298	.938
		White rice	-.415473*	.114298	**.006**
	Red rice	Black rice	-.038941	.114298	.938
		White rice	-.454414*	.114298	**.003**
	White rice	Black rice	.415473*	.114298	**.006**
		Red rice	.454414*	.114298	**.003**
Os11g0484400	Black rice	Red rice	-.117870	.366504	.945
		White rice	.648151	.366504	.214
	Red rice	Black rice	.117870	.366504	.945
		White rice	.766021	.366504	.126
	White rice	Black rice	-.648151	.366504	.214
		Red rice	-.766021	.366504	.126

Dependent variable			Mean difference	Std. error	Sig.
Os05g0244700	Black rice	Red rice	.136920	.120845	.509
		White rice	-.161461	.120845	.398
	Red rice	Black rice	-.136920	.120845	.509
		White rice	-.298380	.120845	.064
	White rice	Black rice	.161461	.120845	.398
		Red rice	.298380	.120845	.064
Os03g0111100	Black rice	Red rice	-.39642	.22792	.239
		White rice	-.24631	.21622	.513
	Red rice	Black rice	.39642	.22792	.239
		White rice	.15011	.21622	.772
	White rice	Black rice	.24631	.21622	.513
		Red rice	-.15011	.21622	.772
Os05g0517500	Black rice	Red rice	-.200114	.150451	.401
		White rice	-.175032	.150451	.492
	Red rice	Black rice	.200114	.150451	.401
		White rice	.025081	.150451	.985
	White rice	Black rice	.175032	.150451	.492
		Red rice	-.025081	.150451	.985

Table 4. Spearman's rho analysis on the relationship between antioxidant activities of TPC, DPPH and FRAP and the expression levels of folate biosynthesis genes. Asterisk (*) and (**) indicating statistically significant scores, p <0.05 and p <0.01, respectively

	TPC		DPPH		FRAP	
	Correlation Coefficient	Sig. (2-tailed)	Correlation Coefficient	Sig. (2-tailed)	Correlation Coefficient	Sig. (2-tailed)
Os04g0662700	-.315	.202	-.284	.253	-.384	.116
Os08g0556200	-.036	.887	.044	.861	-.204	.417
Os07g0618500	.719**	.001	.717**	.001	.688**	.002
Os12g0623800	-.452	.060	-.369	.132	-.495*	.037
Os06g0699700	.867**	.000	.916**	.000	.848**	.000
Os01g0238500	-.549*	.018	-.557*	.016	-.537*	.022
Os11g0484400	.344	.162	.382	.118	.313	.205
Os05g0244700	.032	.900	.169	.504	-.034	.893
Os03g0111100	.022	.943	.077	.802	.033	.914
Os05g0517500	-.177	.483	-.268	.283	-.092	.716

Conclusion

The current study presents a comprehensive gene expression dataset related to the folate biosynthesis pathway in rice varieties with varying pericarp pigmentation. Leveraging the known antioxidant properties of rice-derived folate, this study identified aminodeoxychorismate synthase/glutamine amidotransferase and dihydropterin pyrophosphokinase/dihydropteroate synthase as likely major contributors to the folate content in rice with significant effects on antioxidant activity. The identified key functional genes lay the foundation for folate biofortification in rice. They can be applied through various biotechnological strategies including marker-assisted breeding, genetic engineering, and genome editing to enhance folate levels in rice.

Acknowledgement

The project was financially supported by MARDI RMK-11 development project (P21003004010001-L) under the framework of main project 21003004010001.

References

Abdel-Aal, E.-S. M., Young, J. C. & Rabalski, I. (2006). Anthocyanin composition in black, blue, pink, purple, and red cereal grains. *Journal of Agricultural and Food Chemistry, 54*(13), 4696-4704.

Al-Elwany, O. A., Hemida, K. A., Abdel-Razek, M. A., El-Mageed, T. A. A., El-Saadony, M. T., AbuQamar, S. F., El-Tarabily, K. A. & Taha, R. S. (2022). Impact of folic acid in modulating antioxidant activity, osmoprotectants, anatomical responses, and photosynthetic efficiency of *Plectranthus amboinicus* under salinity conditions. *Frontiers in Plant Science, 13*, 887091.

Antony, A. C. (1996). Folate receptors. *Annual Review of Nutrition, 16*, 501-521.

Ashokkumar, K., Sivakumar, P. & Saradhadevi, M. (2018). Identification and determination of naturally occurring folates in grains of rice (*Oryza sativa* L.) by UPLC-MS/MS analysis. *Natural Product Research, 32*(14), 1733-1737.

Bailey, L. B. & Berry, R. J. (2005). Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. *The American Journal of Clinical Nutrition, 81*(5), 1213S-1217S.

Benzie, I. F. & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*(1), 70-76.

Biswas, S. K., Kim, D.-E., Keum, Y.-S. & Saini, R. K. (2018). Metabolite profiling and antioxidant activities of white, red, and black rice (*Oryza sativa* L.) grains. *Journal of Food Measurement and Characterization, 12*, 2484-2492.

Brand-Williams, W., Cuvelier, M.-E. & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology, 28*(1), 25-30.

De Lepeleire, J., Strobbe, S., Verstraete, J., Blancquaert, D., Ambach, L., Visser, R. G., Stove, C. & Van Der Straeten, D. (2018). Folate biofortification of potato by tuber-specific expression of four folate biosynthesis genes. *Molecular Plant, 11*(1), 175-188.

Díaz de la Garza, R. I., Gregory III, J. F. & Hanson, A. D. (2007). Folate biofortification of tomato fruit. *Proceedings of the National Academy of Sciences, 104*(10), 4218-4222.

Dong, W., Cheng, Z.-j., Lei, C.-l., Wang, X.-l., Wang, J.-l., Wang, J., Wu, F.-q., Zhang, X., Guo, X.-p. & Zhai, H.-q. (2014). Overexpression of folate biosynthesis genes in rice (*Oryza sativa* L.) and evaluation of their impact on seed folate content. *Plant Foods for Human Nutrition, 69*, 379-385.

Gillies, S. A., McIntosh, S. R. & Henry, R. J. (2008). A transgenic cereal crop with enhanced folate: rice expressing wheat HPPK/DHPS. 58th Australian Cereal Chemistry Conference.

Gliszczyńska-Świgło, A. (2007). Folates as antioxidants. *Food Chemistry, 101*(4), 1480-1483.

Goufo, P. & Trindade, H. (2014). Rice antioxidants: phenolic acids, flavonoids, anthocyanins, proanthocyanidins, tocopherols, tocotrienols, γ□oryzanol, and phytic acid. *Food Science & Nutrition, 2*(2), 75-104.

Ionita, P. (2005). Is DPPH stable free radical a good scavenger for oxygen active species? *Chemical Papers, 59*(1), 11.

Kaliora, A., Dedoussis, G. & Schmidt, H. (2006). Dietary antioxidants in preventing atherogenesis. *Atherosclerosis, 187*(1), 1-17.

Laokuldilok, T., Shoemaker, C. F., Jongkaewwattana, S. & Tulyathan, V. (2011). Antioxidants and antioxidant activity of several pigmented rice brans. *Journal of Agricultural and Food Chemistry, 59*(1), 193-199.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods, 25*(4), 402-408.

Lu, S. C. (2009). Regulation of glutathione synthesis. *Molecular Aspects of Medicine, 30*(1-2), 42-59.

McIntosh, S. R. & Henry, R. J. (2008). Genes of folate biosynthesis in wheat. *Journal of Cereal Science, 48*(3), 632-638.

Nam, S. H., Choi, S. P., Kang, M. Y., Koh, H. J., Kozukue, N. & Friedman, M. (2006). Antioxidative activities of bran extracts from twenty one pigmented rice cultivars. *Food Chemistry, 94*(4), 613-620.

Rezk, B. M., Haenen, G. R., van der Vijgh, W. J. & Bast, A. (2003). Tetrahydrofolate and 5-methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore. *FEBS Letters, 555*(3), 601-605.

Simoh, S., Shin, S. Y., Abd Rahim, F., Aizuddin Ahmad M., Zainal, A. (2018). Comparative analysis of metabolites and antioxidant potentials from different plant parts of *Curcuma aeruginosa* Roxb. *Sains Malaysiana, 47*(12), 3031-3041.

Singleton, V. L., Orthofer, R. & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology* (Vol. 299, pp. 152-178). Elsevier.

Wittek, F., Kanawati, B., Wenig, M., Hoffmann, T., Franz-Oberdorf, K., Schwab, W., Schmitt-Kopplin, P. & Vlot, A. C. (2015). Folic acid induces salicylic acid-dependent immunity in Arabidopsis and enhances susceptibility to *Alternaria brassicicola. Molecular Plant Pathology, 16*(6), 616-622.

Yingngam, B. (2024). Folic Acid Deficiency. In *Causes and Management of Nutritional Deficiency Disorders* (pp. 116-164). IGI Global.