AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Folate derivative profiling and associated molecular markers in rice (*Oryza sativa* L.)

Saran Cheenacharoen[a,b], Sirirat Phaisansuthichol[c], Krisana Lanumteang[d], Passorn Wonnapinij[e], Yuppayao Kophimai[f,*]

[a] *Department of Biology, Faculty of Science and Technology, Chiang Mai Rajabhat University, Chiang Mai 50300, Thailand*
[b] *Centre of Excellence on Biodiversity Research and Implementation for Community, Chiang Mai Rajabhat University, Chiang Mai 50300, Thailand*
[c] *Program in Chemistry, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand*
[d] *Program in Statistics and Information Management, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand*
[e] *Department of Genetics, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand*
[f] *Program in Genetics, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand*

Article Info

Article history:
Received 21 October 2024
Revised 3 October 2025
Accepted 15 October 2025
Available online 21 November 2025

Keywords:
Folate biosynthesis pathway,
Folate derivatives,
High-performance liquid chromatography (HPLC),
Molecular marker,
Rice (*Oryza sativa* L.)

Abstract

Importance of the work: Folate is essential for human health, with rice being a staple food and a potential source of folate in Asia. Identifying high-folate rice varieties and developing molecular markers within genes in the folate biosynthesis pathway that are linked to folate content would support breeding for increased folate levels.

Objectives: To quantify the folate content and assess the association between target molecular markers and folate levels.

Materials and Methods: Folate in 65 rice samples was quantified as 5-methyltetrahydrofolate, 10-formyl folic acid, tetrahydrofolate and folic acid using high-performance liquid chromatography. Molecular markers were developed and tested for their correlation with the folate content.

Results: The total folate content in the sampled rice was in the range 10.27–53.73 µg/100 g. The primary derivative found was 5-methyltetrahydrofolate. When grouped by folate content, 16 varieties with high folate levels were identified: RD14, RD21, San-Pa-Tong, Dor Kuem, Riceberry, RD10, Pathum Thani 80, Jao Daeng, Hantra 60, Niao Dtum, Homnil, Plai Ngahm Prachin Buri, Khow Yai (Purple stem), RD15, Nipponbare and RD35. Five main gene-linked molecular markers associated with folate content were identified. The microsatellite marker RM6082, located near the *Aminodeoxychorismate lyase* gene, was associated with the folic acid levels. Microsatellite marker RM2482, located near the *Dihydroneopterin aldolase* gene, was associated with the 5-methyltetrahydrofolate levels. The single nucleotide polymorphism marker within the *Dihydroneopterin aldolase* gene was associated with both the 10-formyl folic acid and folic acid levels. An insertion-deletion (InDel) marker within the *Aminodeoxychorismate lyase* gene was associated with the folic acid levels. Additionally, an InDel marker within the *Dihydrofolate reductase-thymidylate synthase* gene was associated with the total folate levels.

Main finding: Rice varieties with high folate contents and five gene-linked molecular markers are promising candidates for breeding based on marker-assisted selection.

* Corresponding author.
E-mail address: yuppayao@mju.ac.th (Y. Kophimai)

https://doi.org/10.34044/j.anres.2025.59.6.10

Introduction

Folate, or vitamin B9, is a substance produced in plants and microorganisms (Mastella et al., 2022). Humans and animals obtain folate through their diet, with folate being crucial for human life, as it is essential for the body's metabolic processes. For example, it plays a role in nucleotide synthesis, methylation processes, serves as a cofactor for enzymes, contributes to red blood cell formation, aids in cell division in pregnant women and infants, helps prevent various cancers, supports sperm production in males and promotes the proper development of eggs in females (Wakeel et al., 2018). Inadequate folate intake can lead to several disorders, such as autism, Alzheimer disease, neural tube defects, megaloblastic anemia, increased risk of cardiovascular diseases and various cancers. Adults require 400 µg of folate/d, while pregnant women need 600 µg/d (Blancquaert et al., 2014). However, the folate content is very low in staple foods such as rice, wheat, corn and potatoes (Strobbe and Van Der Straeten, 2017) and folate levels decrease with cooking and storage. For example, the folate content in rice stored for 1 yr decreased by 23% and the folate content in cooked rice decreased by nearly 50% (Dong et al., 2011). Consequently, folate deficiency is prevalent in both developed and developing countries (Blancquaert et al., 2014).

Rice is an important economic crop in Thailand and a staple food for many Asians (FAO, 2023). However, most rice contains very little folate, with approximately 6.11–77.7 µg/100 g in polished white rice (Pfeiffer et al., 1997; Dong et al., 2011; Blancquaert et al., 2014) and 9.7–111.4 µg/100 g in brown rice (Dong et al., 2011; Aiyswaraya et al., 2017; Ashokkumar et al., 2018; Changkai et al., 2025). Only four rice varieties have been reported to contain folate levels exceeding 100 µg/100 g (Dong et al., 2011). Folate is distributed in both the endosperm and the bran layers of the rice grain; therefore, unpolished (brown) rice retains significantly higher folate content than polished white rice (Dong et al., 2011; Tiozon Jr et al., 2021; Akhter et al., 2023).

Populations that consume primarily rice, such as those in Cambodia, Bangladesh, Myanmar, Thailand and China, may often have insufficient folate to meet their health needs. This is especially critical for pregnant women, who require more folate than the general population, as inadequate folate intake during pregnancy can lead to congenital disabilities (Blancquaert et al., 2014). Currently, dietary supplements in the form of folic acid are available to compensate for dietary folate. However, there are concerns and drawbacks associated with folic acid supplementation. As folic acid needs to be converted into its active form (5-methyltetrahydrofolate). Excessive intake of folic acid may result in incomplete conversion, potentially affecting health in various ways, including an increased risk of prostate and colorectal cancer, interference with the effectiveness of antifolate drugs used in treating cancer, rheumatoid arthritis and psoriasis and could promote tumor growth and pernicious anemia. In addition, there are concerns about changes in DNA methylation (Ghorbani et al., 2020). In contrast, folate obtained from natural sources does not have these adverse health effects due to differences in metabolic processes compared to synthetic folic acid (Blancquaert et al., 2014; Warzyszynska and Kim, 2014).

Consequently, improving rice varieties to increase their folate content is crucial for the health of Asians. It is necessary to identify rice varieties with high folate levels to serve as donors for breeding programs. The use of molecular markers for selection (MAS) can enhance the precision of rice breeding, shorten the breeding cycle and reduce the labor and field area required for each generation (Hasan et al., 2015; Niu et al., 2023). However, although three quantitative trait loci (QTLs) related to folate content in rice have been reported on chromosome 3 (*qQTF 3-1*, *qQTF 3-2, qQTF 3-3*), these QTLs are located in non-gene regions unrelated to the folate synthesis pathway and account for only 11–25% of the variance in folate levels (Dong et al., 2014a). Therefore, identifying molecular markers that are associated with or linked to genes involved in the folate synthesis pathway is important and necessary for making breeding programs faster and more accurate.

The current experiment measured the folate content in 65 rice samples using high-performance liquid chromatography (HPLC), which has the advantages of being accurate, fast and capable of separating different folate derivatives (Arcot and Shrestha, 2005; Gupta et al., 2022). The aim was to identify rice varieties with a high folate content, which could be beneficial for improving other rice varieties by increasing folate levels in the future. Additionally, this study tested the association between the folate content and the molecular markers of the genes involved in the folate biosynthesis pathway. This approach aimed to discover molecular markers that were different from those reported elsewhere, as these newly discovered markers would likely have a major impact on the folate content because they are located in genes directly involved in the folate biosynthesis pathway, which could aid in future rice selection and improvement.

Materials and Methods

Plant materials

The 65 rice samples, including both improved and landrace varieties with variation in seed coat color and starch type, were cultivated in a greenhouse during the wet season of 2020. To ensure uniform environmental conditions for all samples, the rice plants were grown in plastic pots (approximately 30 cm in diameter) filled with homogenized topsoil taken from the same source. A compound NPK fertilizer (15-15-15) was applied every 2 wk and the amount of fertilizer was standardized using a measuring scoop. In addition, watering was standardized by filling each pot to the same level. The pots were arranged in a completely randomized design with three replicates per rice sample. Once the seeds had matured, they were harvested, dried at 50°C for 5 d, pooled by sample and processed into brown rice. The rice samples used in this study are detailed in Supplementary Table S1.

Measurement of folate content using high-performance liquid chromatography technique

Sample preparation and standard solution

The brown rice sample was ground into a powder using a stone mortar, sieved through an 80-mesh screen and then dried at 60°C for 6 hr. A 0.500 gram portion of the sample was weighed and placed in a microcentrifuge tube. The sample was extracted with 1 mL of deionized water, then shaken for 30 min using a vortex mixer. It was left to stand for 12 hr and then centrifuged at 12,000 revolutions per minute for 10 min. The sediment was removed and the supernatant filtered using a 0.22 µm membrane filter before being analyzed using HPLC.

Four standard compounds were used, all obtained from Sigma-Aldrich (St. Louis, MI, USA): 5-methyltetrahydrofolate (5-MTHF), tetrahydrofolate (THF), 10-formyl folic acid (10-CHOFA) and folic acid (FA). Standard solutions were prepared at concentrations of 1 mg/L, 5 mg/L, 10 mg/L, 20 mg/L, 30 mg/L, 50 mg/L and 75 mg/L. Then, the mixed standard solutions were filtered using a 0.22 µm membrane filter before being analyzed using HPLC.

Optimal conditions for folate measurement

Analyses were carried out on a HPLC with diode array detection (HP 1100 system; Agilent Technologies; Palo Alto, CA, USA) and a C18 column with dimensions of 250 mm × 4.6 mm × 5 µm (Thermo Quest; Kleinosteim, Germany). The detector was a photodiode array. The mobile phase consisted of 0.10% formic acid in acetonitrile (85:15 volume per volume). The flow rate was 0.70 mL/min and the injection volume of the sample was 20.0 µL. The detection wavelength was set at 267 nm. The retention times for 5-MTHF, THF, 10-CHOFA and FA were 4.892 min, 5.367 min, 7.299 min and 9.165 min, respectively. Each sample was analyzed with duplicate injections, with a total analysis time of 10 min per run.

Genotyping

The DNA markers used to detect gene variation in the studied rice samples were sourced from three areas:

Microsatellite markers

The positions of the genes involved in the folate synthesis pathway in rice were identified using the KEGG (Kyoto encyclopedia of genes and genomes) database. Microsatellite markers located near those genes were found in Table 18 of International Rice Genome Sequencing Project (2005). Only markers that showed variation in the studied rice population were selected, resulting in a total of 15 markers (Supplementary Table S2).

Gene-specific markers

Genes involved in the folate biosynthesis pathway in rice were selected based on the reports by Anukul et al. (2010) and Dong et al. (2014a). Sequence variation within these genes was investigated using whole-genome sequence data from 14 rice varieties, focusing on the 5′ upstream regions, which may influence gene expression and on exonic regions, which result in amino acid changes. When polymorphisms were identified in these regions, primers were designed to amplify the target loci in all rice samples using the Primer3Plus program (Untergasser et al., 2012). The resulting molecular markers were both single nucleotide polymorphism (SNP) and insertion-deletion (InDel) markers. In total, seven genes with nucleotide sequence polymorphisms were identified (Supplementary Table S2). For SNP markers, allele-specific fluorescent labeling was used to distinguish each allele, enabling genotyping using real-time polymerase chain reaction (PCR).

DNA markers in quantitative trait loci regions associated with folate content in rice

The QTL regions that had been reported to be associated with folate content in rice (Dong et al., 2014a) were studied. These included markers RM 16, RM 156 and genes located near the QTL regions, such as the *OsmSHMT* gene. Primers were designed to investigate these regions and only markers showing variation in the studied rice population were selected, yielding a total of four markers (Supplementary Table S2).

The sizes of DNA fragments for the microsatellite and InDel markers were determined using the PCR amplification method involving: template DNA (10–20 ng/µL), 1 µL; exTEN 2X PCR Master Mix (1st BASE; Singapore), 5 µL; each primer (5 µM), 0.5 µL; and water to a total volume of 10 µL. The PCR conditions were: 1) initial denaturation at 95°C for 3 min; 2) denaturation at 95°C for 30 s; 3) annealing at the appropriate temperature for each primer pair for 30 s; 4) extension at 72°C for 1 min, with steps 2 to 4 repeated for 35 cycles; and 5) a final extension at 72°C for 8 min. DNA fragment sizes were checked using agarose gel electrophoresis in 0.5X tris-borate-ethylenediaminetetraacetic acid buffer at 135 V.

The PCR allele competitive extension (PACE) technique was used for SNP variation detection. The PCR amplification components were: template DNA (10–20 ng/µL), 1 µL; 2X PACE GMM (3cr Bioscience; HW, UK), 5 µL; Assay mix (including allele-specific primer 1 at 100 µM, 1.2 µL; allele-specific primer 2 at 100 µM, 1.2 µL; and common reverse primer at 100 µM, 3 µL, adjusted to a total volume of 10 µL with water), 0.15 µL; and water, 3.85 µL. The PCR conditions were: 1) enzyme activation at 94°C for 15 min; 2) template denaturation at 94°C for 20 s; 3) annealing and extension at 65–57°C for 60 s, with steps 2 and 3 repeated for 10 cycles, decreasing the annealing temperature by 0.8°C each cycle; 4) denaturation at 94°C for 20 s; 5) annealing and extension at 57°C for 60 s, with steps 4 and 5 repeated for 30 cycles; 6) allele detection based on observing fluorescence from Hex and FAM dyes at 37°C for 60 s.

Statistical analysis

Nonparametric statistics were used to analyze the data because the amount of folate was not normally distributed (Shapiro Wilk test, $p < 0.05$). The Mann-Whitney test was applied to compare differences in medians between two groups. Additionally, the Kruskal-Wallis test was used to compare median differences among more than two groups using the R software package, incorporating a programming language for statistical computing and graphics (R Core Team, 2021). If the test results indicated a significant difference in medians across groups, it could be concluded that the folate contents in the rice and associated molecular markers were significantly different among the examined groups, suggesting that specific molecular markers might be associated with differences in the folate content in the rice, potentially providing valuable insights for genetic studies and breeding programs aimed at enhancing folate levels.

The correlations test between the amounts of each compound were calculated using Spearman's rank correlation, utilizing the corrplot package (Wei and Simko, 2024) in the R software package. Histograms and bar graphs were created using Excel (Microsoft Corporation, 2018). The relationship among samples was constructed from data on the four folate derivatives and the total folate content using the PAST software 4.17 (Hammer et al., 2001), based on the unweighted pair group method with arithmetic mean (UPGMA) algorithm and displayed in a dendrogram. The similarity matrix, on which the clusters were based, was calculated using the Euclidean distance.

Results and Discussion

Folate content

Based on the measurement results of the 65 rice samples, the folate content varied significantly across different varieties, reflecting genetic diversity. The total content of all derivatives combined was in the range 10.27–53.73 µg/100 g (mean ± SD 26.93 ± 9.57 µg/100 g), as shown in Supplementary Table S1 and Fig. 1. This wide range suggested that certain rice varieties had a naturally higher capacity for folate accumulation, making them valuable candidates for biofortification programs. Among the folate derivatives, 5-MTHF was the most abundant in all samples (7.22–42.36 µg/100 g, mean ± SD 22.35 ± 7.52 µg/100 g). This was followed by 10-CHOFA (0.00–10.22 µg/100 g, mean ± SD 3.14 ± 2.87 µg/100 g). The THF content was in the range 0.00–3.34 µg/100 g, mean ± SD 0.94 ± 0.79 µg/100 g) and the FA content was in the range 0.00–5.55 µg/100 g (mean ± SD 0.50 ± 0.86 µg/100 g).These results were consistent with Pfeiffer et al. (1997) and Ashokkumar et al. (2018), who measured the folate content in polished and brown rice, respectively and reported that the main derivative in their rice samples was 5-MTHF, followed by 10-CHOFA. The total folate content and the amount of each folate derivative in the current study were within the range reported by Ashokkumar et al. (2018).

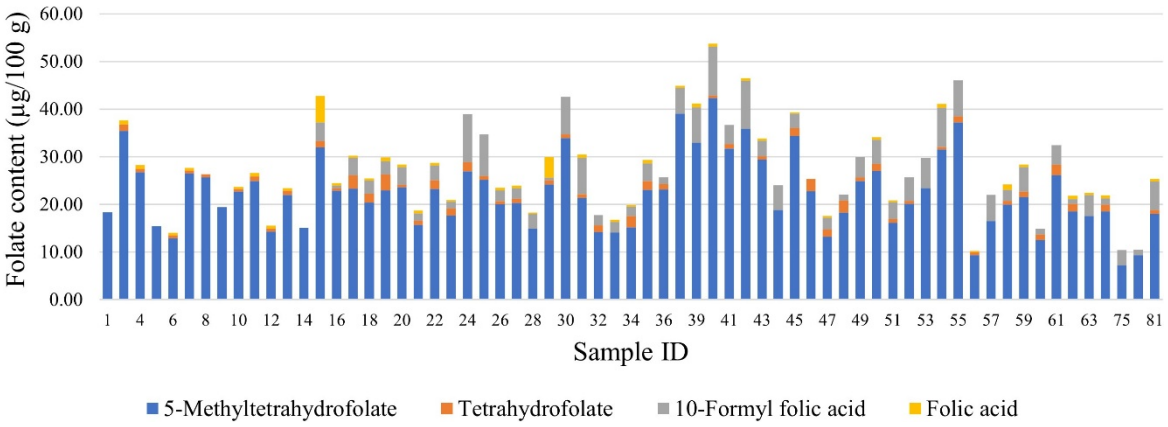

Fig. 1 Folate content in each sample, partitioned by derivative (5-methyltetrahydrofolate, tetrahydrofolate, 10-formyl folic acid, and folic acid).

However, the current amounts of FA detected were lower than those reported by Aiyswaraya et al. (2017), with the difference perhaps due to variations in the sample preparation method, the folate extraction technique, the rice varieties analyzed and the method used for folate quantification (Pfeiffer et al., 1997; Czarnowska-Kujawska et al., 2017; Ashokkumar et al., 2018). The low levels of 10-CHOFA, THF and FA (Supplementary Table S1 and Fig. 1) may be attributed to the instability of certain folate forms during post-harvest processing and storage (Dong et al., 2011).

The correlation test between the amounts of each compound, using Spearman's rank correlation, revealed five significant positive correlations, as shown in Fig. 2.

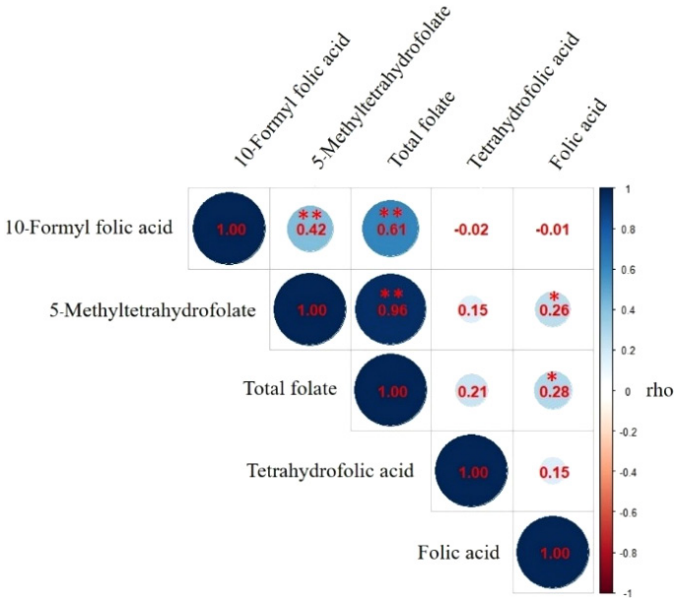

Fig. 2 Correlations among the amounts of folate derivatives (Spearman's ρ).
* indicates significant at $p < 0.05$ and ** indicates $p < 0.01$.

Since the amounts of 5-MTHF, 10-CHOFA and FA are related to the total folate amount, measuring just these three derivatives may be sufficient to assess folate levels in rice. Alternatively, in cases of major budgetary constraints, measuring only 5-MTHF could be sufficient, as it was the most abundant form in all rice samples and was also correlated with the amounts of 10-CHOFA, FA and total folate. However, measuring only the FA content, as was done in the study by Aiyswaraya et al. (2017), would have been unlikely to provide an accurate estimate of the total folate content in the rice.

Comparisons involving the folate contents in the rice types grouped by variety (improved versus landrace), seed coat color (colored versus white) and grain type (glutinous versus nonglutinous) indicated that the amounts of THF and FA were significantly higher in the improved varieties than in the landrace varieties, as shown in Table 1. However, the levels of THF and FA were relatively minor components in the rice. These findings suggested that modern breeding efforts may have inadvertently selected for traits that had enhanced folate biosynthesis or retention. Notably, despite the differences in THF and FA, the total folate content was not significantly different between the two groups, suggesting that the predominant derivative (5-MTHF), which is the main form of folate in rice, may be similarly distributed across both the improved and landrace varieties. Therefore, while improved varieties may show advantages in certain folate forms, landraces still hold potential for contributing to folate biofortification through hybridization and marker-assisted selection.

Table 1 Folate derivative contents in improved and landrace rice varieties (mean, median, SD; µg/100 g) and results of statistical comparisons between variety types.

Derivative	Variety type	Mean (µg/100 g)	Median (µg/100 g)	SD (µg/100 g)	Number of plants	*p* value
Tetrahydrofolic acid	Improved	1.13	0.96 [a]	0.85	36	0.015
	Landrace	0.70	0.59 [b]	0.65	29	
Folic acid	Improved	0.59	0.50 [a]	0.90	36	0.009
	Landrace	0.38	0.00 [b]	0.80	29	

Different lowercase superscripts indicate significant ($p < 0.05$) differences between improved and landrace rice within each derivative.

Although pigmented rice has been reported to contain higher levels of beneficial compounds, such as flavonoids, oligomeric proanthocyanidin and proteins, than white rice (Zhu et al., 2024) and that protein expression differs between the two groups (Sew et al., 2023), the current experiment showed that the folate content in both groups did not differ significantly. This suggested that folate accumulation may be regulated independently of pigmentation pathways. However, further metabolomic and transcriptomic studies could help to clarify whether shared regulatory mechanisms exist between folate biosynthesis and pigment production.

The relationships among the samples were depicted in a dendrogram, with grouping based on the correlation of all four folate derivatives and the total folate content. The samples were categorized into three major groups (Fig. 3): Group 1, Group 2A and 2B and Group 3. Rice samples in the same group had similar values for the total folate content, with Group 3 having the highest value (32.43–53.73 µg/100 g). This group contained RD14, RD21, San-Pa-Tong, Dor Kuem, Riceberry, RD10, Pathum Thani 80, Jao Daeng, Hantra 60, Niao Dtum, Homnil, Plai Ngahm Prachin Buri, Khow Yai (Purple stem), RD15, Nipponbare and RD35. These rice varieties

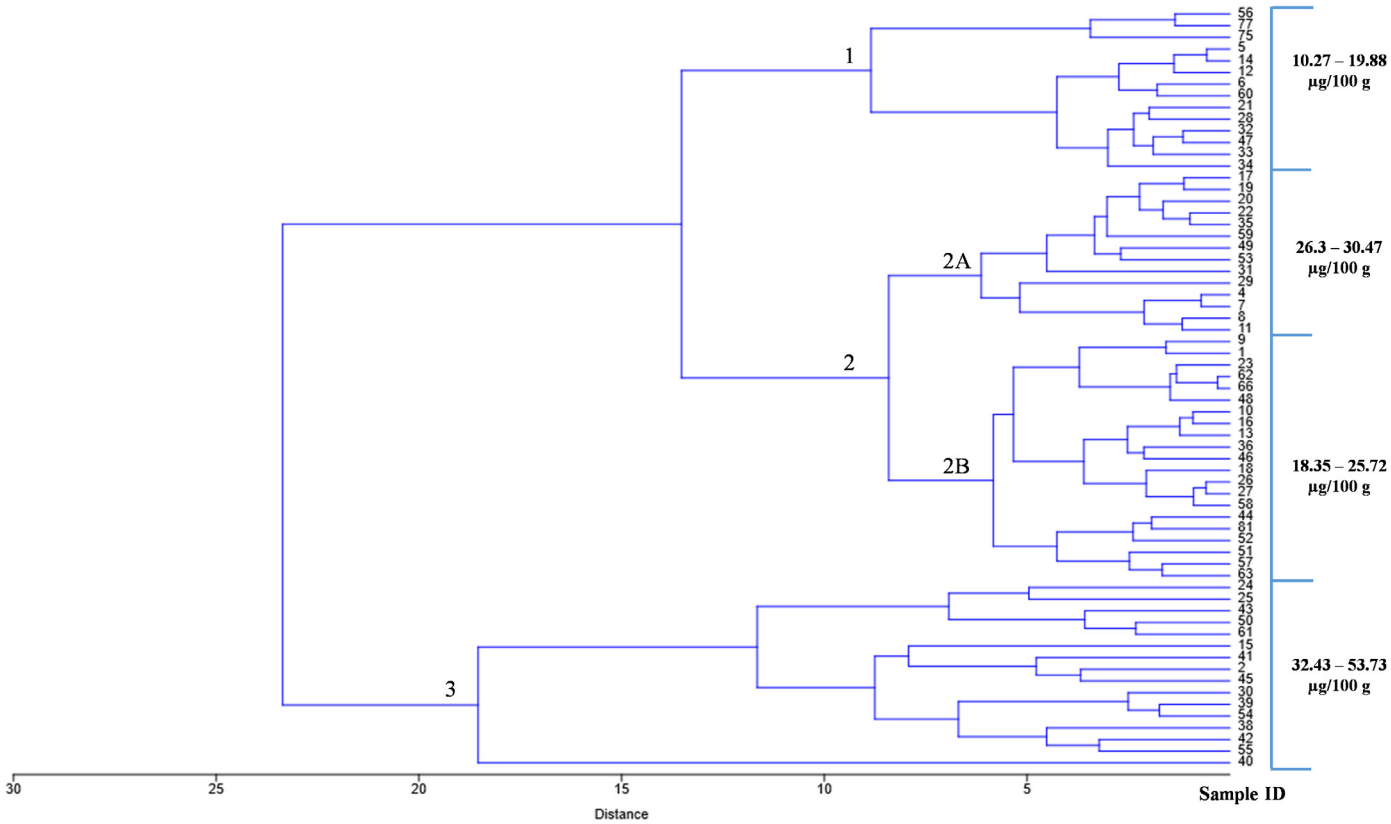

Fig. 3 Dendrogram of 65 rice samples generated by hierarchical cluster analysis (unweighted pair group method with arithmetic mean, UPGMA), based on the contents of four folate derivatives and total folate. Samples were grouped into four clusters (1, 2A, 2B, and 3).

are particularly suitable for individuals seeking high-folate diets. However, it is recommended to consume brown rice instead of white rice, as brown rice contains significantly higher folate levels (Dong et al., 2011; Tiozon Jr et al., 2021; Akhter et al., 2023). In addition, varieties with folate levels exceeding 40 µg/100 g (RD14, Riceberry, Dor Kuem, RD10, Pathum Thani 80, RD21, Jao Daeng and San-Pa-Tong) could serve as donor parents in breeding programs aimed at enhancing the folate content in commercial rice. However, folate levels decrease by approximately one-half due to the heat during cooking, as well as declining due to storage (Dong et al., 2011). Therefore, breeding for a high folate content should be complemented by strategies to preserve the folate level during storage, such as improved packaging techniques. Furthermore, rice biofortification should be complemented by dietary diversification. It is important to consume a variety of other foods rich in folate, such as spinach, beans, lentils, liver, to ensure adequate folate intake (Strobbe and Straeten, 2017).

Molecular markers associated with folate content

An analysis of the differences in the folate content between alleles of the 26 molecular markers revealed that five markers had significant differences in the folate content between their respective alleles (Table 2). First, the microsatellite marker RM2482 (located near the *Dihydroneopterin aldolase* (*DHNA*) gene on chromosome 9) associated with the 5-MTHF content (Figs. 4A and 4B). Second, an SNP marker within the *DHNA* gene (on chromosome 6) linked to 10-CHOFA content (Figs. 4C and 4D) and the FA content (Figs. 4E and 4G). The *DHNA* gene, which is a member of a gene family and therefore was found on multiple chromosomes (Sakai et al., 2013). Third, the InDel marker within the *ADCL* gene (on chromosome 5) associated with the FA content (Figs. 4E and 4H). Fourth, the InDel marker within the *Dihydrofolate Reductase-Thymidylate Synthase* (*DHFR-TS*) gene (on chromosome 12) associated with the total folate content (Figs. 4I and 4J). Fifth, the microsatellite marker RM6082 (located near the *Aminodeoxychorismate lyase* (*ADCL*) gene on chromosome 5) associated with the FA content (Figs. 4E and 4F). Therefore, the three genes most likely influencing the folate content in the rice samples studied were *ADCL*, *DHNA* and *DHFR-TS*.

Table 2 Molecular markers/genes associated with folate derivatives and total folate content in the studied rice varieties (mean, median, SD; µg/100 g).

Number	Marker/Gene	Derivative	*p* value	DNA size	Mean (µg/100 g)	Median (µg/100 g)	SD (µg/100 g)	Number of plants*
1	RM6082 (Located near the *Aminodeoxychorismate lyase (ADCL)* gene on chr. 5)	Folic acid	0.007	150	0.53	0.55 ^{a}	0.28	17
				170	0.47	0.29 ^{b}	1.01	46
				150 and 170 (heterozygous)	0.69	0.69 ^{a}	0.12	2
2	RM2482 (Located near the *Dihydroneopterin aldolase (DHNA)* gene on chr. 9)	5-Methyltetra hydrofolate	0.028	290	25.36	23.29 ^{a}	8.47	16
				300	18.73	19.37 ^{ab}	8.05	8
				320	20.08	20.09 ^{ab}	7.85	5
				390	24.00	23.28 ^{a}	6.59	26
				400	17.26	17.27 ^{b}	4.10	10
3	*Dihydroneopterin aldolase (DHNA)* gene (Chr. 6)	10-Formyl folic acid	0.041	SNP C	2.19	1.02 ^{b}	2.79	19
				SNP A	3.60	3.10 ^{a}	2.83	45
		Folic acid	0.018	SNP C	0.93	0.53 ^{a}	1.45	19
				SNP A	0.31	0.34 ^{b}	0.31	45
4	*Aminodeoxychorismate lyase (ADCL) gene* (Chr. 5)	Folic acid	0.003	257	0.19	0.00 ^{b}	0.74	20
				266	0.63	0.50 ^{a}	0.79	44
5	*Dihydrofolate reductase-thymidylate synthase (DHFR-TS)* gene (Chr. 12)	Total folate	0.039	140	31.60	29.41 ^{a}	9.43	12
				153	25.80	24.12 ^{b}	9.44	52

* indicates that the heterozygote sample was excluded from the analysis because duplicate measurements were not available.

Different lowercase superscript letters within each marker indicate significant differences ($p < 0.05$) among allelic classes/DNA sizes; *p* values are shown for each association test.

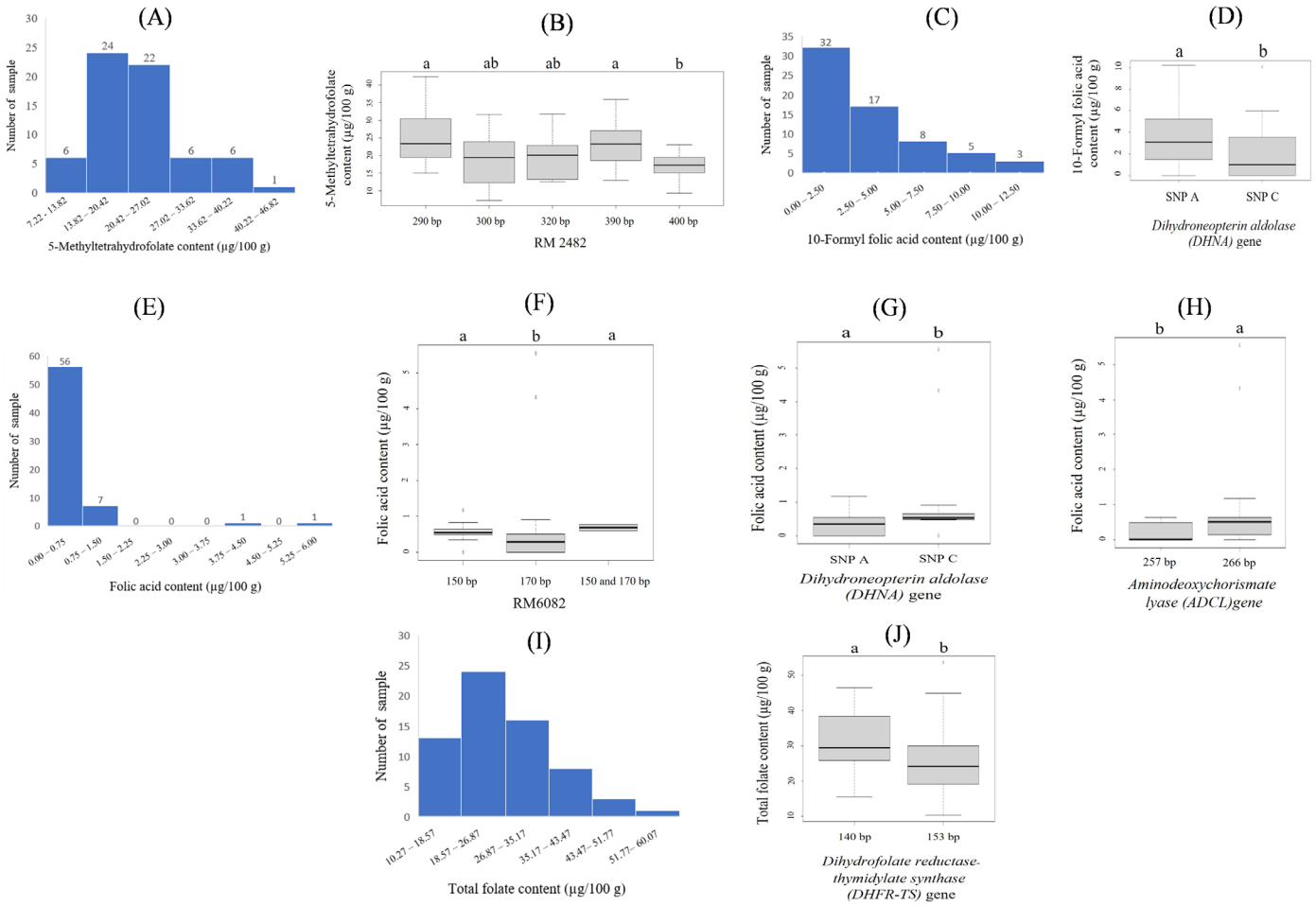

Fig. 4 Distribution of folate derivatives and total folate across rice samples, and boxplots showing associations with selected molecular markers: (A–B) 5-methyltetrahydrofolate; (C–D) 10-formyl folic acid; (E–H) folic acid; and (I–J) total folate. In boxplots, the bold horizontal line indicates the median, boxes show the interquartile range, whiskers represent 1.5× IQR, and different lowercase letters indicate significant differences among allelic classes/DNA sizes ($p < 0.05$).

Folate biosynthesis involves three main cellular compartments: 1) cytosol, where pterin synthesis occurs; 2) chloroplast, where p-ABA (para-aminobenzoate) is synthesized; and 3) mitochondria, where the combination of pterin and p-ABA takes place, resulting in the formation of folate. The three genes play a crucial role in the final steps within each of these compartments. The *ADCL* gene functions in the chloroplast, catalyzing the conversion of aminodeoxychorismate to p-ABA, which is the final step in the biosynthesis of p-ABA. The *DHNA* gene operates in the cytosol, converting dihydromonapterin and dihydroneopterin to hydroxymethyldihydropterin, which is the final step in the biosynthesis of pteridine. The *DHFR-TS* gene functions in the mitochondria, converting dihydrofolate to tetrahydrofolate (Bekaert et al., 2008). Therefore, these three genes are essential for the overall process of folate biosynthesis.

The study by Dong et al. (2014a), which aimed to identify QTLs associated with folate content in two rice populations—Lemont × Teqing recombinant inbred lines and Koshihikari/Kasalath × Koshihikari backcross inbred lines—found that *qQTF-3-1*, *qQTF-3-2* and *qQTF-3-3*, located on chromosome 3, were associated with the folate content in rice. However, these QTLs were not located near or within genes involved in the folate biosynthesis pathway. Therefore, the current study is the first to identify molecular markers linked to genes within the folate biosynthesis pathway that are associated with the folate content in rice.

When examining the alleles of five molecular markers associated with folate content in 16 high-folate rice varieties, it was found that each variety carried 2–5 high-folate-associated alleles (Table 3). Therefore, if these varieties are to be used in breeding programs to enhance folate levels, molecular markers that carry high-folate-associated alleles in each specific variety could be considered for marker-assisted selection. Nevertheless, the current study was limited by the small sample size. Before these molecular markers can be applied in marker-assisted selection, they should be validated in the target population, such as by using these markers in an F_2 population and evaluating the correlation between genotypes and phenotypes (Cheenacharoen et al., 2023).

Folate biofortification

An increased folate content in rice may be achieved through environmental modifications, such as using different light colors to stimulate the activity of genes in the folate biosynthesis pathway. For example, Chang et al. (2021) investigated exposing wheat seedlings to different light colors and reported that red light significantly increased the folate content in the seedlings. This was accompanied by increased activity of the enzymes guanosine triphosphate cyclohydrolase 1 (GCH1) and aminodeoxychorismate synthase (ADCS), as well as elevated levels of pterin and p-ABA. Additionally, the expression increased of genes such as *GCH1*, *ADCS*, *hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase* (*HPPK-DHPS*) and *folypolyglutamate synthase* (*FPGS*) when the plants were exposed to red light. Similarly, Xiang et al. (2020) found that maize seedlings exposed to low temperatures (10°C) had an increased folate content, with elevated expression of genes such as *GTP cyclohydrolase* (*GTPCH*), *2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase* (*HPPK*), *dihydrofolate synthase* (*DHFS*), *DHFR* and *FPGS*. However, it is challenging to adjust the environmental conditions to stimulate folate production in large-scale rice farming. Therefore, genetic modification may be a more suitable approach for farmers.

Dong et al. (2014b) introduced nine genes into the folate biosynthesis pathway from *Arabidopsis thaliana—AtGTPCHI*, *AtDHNA*, *AtADCS*, *AtADCL*, *AtHPPK*, *AtDHPS*, *AtDHFS*, *AtDHFR* and *AtFPGS*—into the *japonica* rice variety Kitaake. They found that all the genes had increased expression levels in Kitaake rice; however, only the rice with the *AtGTPCHI* or *AtADCS* gene had a significant increase in the folate content. Rice that received the *AtDHFS* or *AtFPGS* gene had only a slight increase in the folate content compared to the wild type. Similarly, Blancquaert et al. (2013) found that the *japonica* variety Nipponbare, which overexpressed the *GTPCHI* and *ADCS* genes from *A. thaliana*, also had increased folate levels. Additionally, other studies have reported on the genetic engineering of genes to enhance folate levels (Strobbe and Straeten, 2017; Changkai et al., 2025). Furthermore, gene editing techniques, such as CRISPR-Cas9, have been explored to modify rice genes to increase the folate levels in the endosperm, since some folate accumulates in the seed coat, which is removed when rice is polished to produce white rice. Gene editing could help to enhance the folate levels in white rice (Khan et al., 2023).

Table 3 Rice varieties with high total folate content and the molecular markers/genes carrying high-folate-associated alleles.

No.	Variety	Total folate content (µg/100 g)	Number of high-folate-associated alleles	Marker with high-folate-associated alleles
1	Homnil	37.65	3	RM2482, *DHNA* gene, *ADCL* gene
2	Riceberry	42.79	4	RM2482, *DHNA* gene, *ADCL* gene, *DHFR-TS* gene
3	Niao Dtum	38.96	3	RM2482, *DHNA* gene, *ADCL* gene
4	Khow Yai (Purple stem)	34.70	3	RM2482, *DHNA* gene, *ADCL* gene
5	RD10	42.60	3	RM2482, *DHNA* gene, *ADCL* gene
6	Dor Kuem	44.93	3	RM2482, *DHNA* gene, *ADCL* gene
7	Pathum Thani 80	41.20	3	RM2482, *DHNA* gene, *ADCL* gene
8	RD14	53.73	2	RM2482, *DHNA* gene
9	Plai Ngahm Prachin Buri	36.64	2	*DHNA* gene, *ADCL* gene
10	RD21	46.51	5	RM6082, RM2482, *DHNA* gene, *ADCL* gene, *DHFR-TS* gene
11	RD15	33.88	5	RM6082, RM2482, *DHNA* gene, *ADCL* gene, *DHFR-TS* gene
12	Hantra 60	39.33	3	RM2482, *DHNA* gene, *ADCL* gene
13	Nipponbare	34.11	2	RM2482, *DHNA* gene
14	Jao Daeng	41.11	2	*DHNA* gene, *ADCL* gene
15	San-Pa-Tong	46.05	3	RM2482, *DHNA* gene, *DHFR-TS* gene
16	RD35	32.43	5	RM6082, RM2482, *DHNA* gene, *ADCL* gene, *DHFR-TS* gene

However, in Thai rice, which belongs to the *indica* group, there are challenges related to regeneration after the introduction of external genes (Liang et al., 2021; personal communication). Therefore, genetic modification via gene insertion or CRISPR-Cas9 techniques may not yet be suitable for Thai rice.

The most suitable current approach for improving the folate content in Thai rice is the use of molecular marker-assisted selection. Since some Thai rice varieties already have a high folate content, they can be used as donor varieties. By crossing them with other varieties that have good cooking quality and desirable agronomic traits, it is possible to develop Thai rice varieties with a higher folate content and other favorable characteristics. In particular, if molecular markers linked to the folate content in Thai rice can be identified, the breeding process can be accelerated, as selection can be done at the seedling stage. This would also conserve resources, as only plants carrying the desired alleles would be cultivated. Notably, the current study identified that the molecular markers linked to the *ADCL*, *DHNA* and *DHFR-TS* gene were associated with the folate content in the studied rice samples (Table 2 and Fig. 4), most of which were Thai rice varieties. Therefore, these molecular markers should be promising candidates for further study in Thai rice populations.

Conclusion

By quantifying four folate derivatives—5-methyltetrahydrofolate, 10-formyl folic acid, tetrahydrofolate and folic acid—in 65 rice samples, the total folate content was in the range 10.27–53.73 µg/100 g, with 5-methyltetrahydrofolate being the most abundant form across all samples. In total, 16 rice varieties were identified as high-folate varieties: RD14, RD21, San-Pa-Tong, Dor Kuem, Riceberry, RD10, Pathum Thani 80, Jao Daeng, Hantra 60, Niao Dtum, Homnil, Plai Ngahm Prachin Buri, Khow Yai (Purple stem), RD15, Nipponbare and RD35. Analysis of the association between folate derivatives and the molecular markers located within the folate biosynthesis pathway revealed five markers were significantly correlated with folate levels: RM6082 (associated with folic acid); RM2482 (associated with 5-methyltetrahydrofolate); an SNP within the *Dihydroneopterin aldolase* gene (associated with both 10-formyl folic acid and folic acid); an InDel within the *Aminodeoxychorismate lyase* gene (associated with folic acid); and an InDel within the *Dihydrofolate reductase-thymidylate synthase* gene (associated with total folate content). However, these molecular markers should be validated in segregating populations, such as F_2 or backcross lines, before being applied in breeding programs using marker-assisted selection.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

This research was funded by the Fundamental Fund from Thailand Science Research and Innovation (TSRI) and The Office of Agricultural Research and Extension, Maejo University (Research Project Code MJ.1-65-025).

References

Aiyswaraya, S., Saraswathi, R., Ramchander, S., Jaivel, N., Uma, D., Sudhakar, D., Robin, S. 2017. Evaluation of rice (*Oryza sativa L.*) germplasm for the identification of high folate accession using HPLC. Int. J. Curr. Microbiol. App. Sci. 6: 5328–5346. doi.org/10.20546/ijcmas.2017.611.509

Akhter, K.T., Shozib, H.B., Islam, M.H., et al. 2023. Variations in the major nutrient composition of dominant high-yield varieties (HYVs) in parboiled and polished rice of Bangladesh. Foods. 12: 3997. doi.org/10.3390/foods12213997

Anukul, N., Ramos, R.A., Mehrshahi, P., et al. 2010. Folate polyglutamylation is required for rice seed development. Rice. 3: 181–193. doi.org/10.1007/s12284-010-9040-0

Arcot, J., Shrestha, A. 2005. Folate: Methods of analysis. Trends Food Sci. Technol. 16: 253–266. doi.org/10.1016/j.tifs.2005.03.013

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. Nat. Prod. Res. 32: 1733–1737. doi.org/10.1080/14786419.2017.1392957

Bekaert, S., Storozhenko, S., Mehrshahi, P., et al. 2008. Folate biofortification in food plants. Trends Plant Sci. 13: 28–35. doi.org/10.1016/j.tplants.2007.11.001

Blancquaert, D., De Steur, H., Gellynck, X., Van Der Straeten, D. 2014. Present and future of folate biofortification of crop plants. J. Exp. Bot. 65: 895–906. doi.org/10.1093/jxb/ert483

Blancquaert, D., Van Daele, J., Storozhenko, S., Stove, C., Lambert, W., Van Der Straeten, D. 2013. Rice folate enhancement through metabolic engineering has an impact on rice seed metabolism, but does not affect the expression of the endogenous folate biosynthesis genes. Plant Mol. Biol. 83: 329–349. doi.org/10.1007/s11103-013-0091-7

Chang, J., Xie, C., Wang, P., Gu, Z., Han, Y., Yang, R. 2021. Red light enhances folate accumulation in wheat seedlings. J. Zhejiang Univ. Sci. B. 22: 906–916. doi.org/10.1631/jzus.B2100266

Changkai, L., Shikai, H., Guiai, J., et al. 2025. Enhancing folate content in japonica rice through co-expression of *OsADCS* and *OsGTPCHI* indica alleles. Rice Sci. 32: 353–366. doi.org/10.1016/j.rsci.2025.01.008

Cheenacharoen, S., Suwarat, S., Saengtong, V., et al. 2023. Molecular markers for resistant starch content in Thai rice. Asia-Pac. J. Sci. Technol. 28: 1–11. doi.org/10.14456/apst.2023.101

Czarnowska-Kujawska, M., Gujska, E., Michalak, J. 2017. Testing of different extraction procedures for folate HPLC determination in fresh fruits and vegetables. J. Food Compos. Anal. 57: 64–72. doi.org/10.1016/j.jfca.2016.12.019.

Dong, W., Cheng, Z., Lei, C., et al. 2014b. Overexpression of folate biosynthesis genes in rice (*Oryza sativa* L.) and evaluation of their impact on seed folate content. Plant Foods Hum. Nurt. 69: 379–385. doi.org/10.1007/s11130-014-0450-9

Dong, W., Cheng, Z., Wang, X., et al. 2011. Determination of folate content in rice germplasm (*Oryza sativa* L.) using tri-enzyme extraction and microbiological assays. Int. J. Food. Sci. Nutr. 62: 537–543. doi.org/10.3109/09637486.2011.555476

Dong, W., Cheng, Z., Xu, J., Zheng, T., Wang, X., Zhang, H., Wang, J., Wan, J. 2014a. Identification of QTLs underlying folate content in milled rice. J. Integr. Agr. 13: 1827–1834. doi.org/10.1016/S2095-3119(13)60537-7

FAO. 2023. Crops and livestock products. https://www.fao.org/faostat/en/#data/QCL, 20 September 2025.

Ghorbani, M., Baharara, J., Kerachian, M.A. 2020. Could high serum folate be associated with adverse effects? Iran. J. Basic Med. Sci. 23: 1499–1500. doi.org/10.22038/IJBMS.2020.51870.11762

Gupta, M.K., Ghuge, A., Parab, M., Al-Refaei, Y., Khandare, A., Dand, N., Waghmare, N. 2022. A comparative review on high-performance liquid chromatography (HPLC), ultra performance liquid chromatography (UPLC) & high-performance thin layer chromatography (HPTLC) with current updates. Curr. Issues Pharm. Med. Sci. 35: 224–228. doi.org/10.2478/cipms-2022-0039

Hammer, O., Harper, D.A.T., Ryan, P.D. 2001. Palaeontological statistics software package for education and data analysis. Palaeontol. Electron. 4: 1–9.

Hasan, M.M., Rafii, M.Y., Ismail, M.R., et al. 2015. Marker-assisted backcrossing: a useful method for rice improvement. Biotechnol. Biotechnol. Equip. 29: 237–254. doi.org/10.1080/13102818.2014.995920

International rice genome sequencing project (IRGSP). 2005. The map-based of the rice genome. Nature. 436: 793–800. doi.org/10.1038/nature03895

Khan, A., Pudhuvai, B., Shrestha, A., Mishra, A.K., Shah, M.P., Koul, B., Dey, N. 2023. CRISPR-mediated iron and folate biofortification in crops: Advances and perspectives. Biotechnol. Genet. Eng. Rev. 40: 4138–4168. doi.org/10.1080/02648725.2023.2205202

Liang, Y., Biswas, S., Kim, B., Bailey-Serres, J., Septiningsih, E.M. 2021. Improved transformation and regeneration of Indica rice: Disruption of *SUB1A* as a test case via CRISPR-Cas9. Int. J. Mol. Sci. 22: 6989. doi.org/10.3390/ijms22136989

Mastella, L., Senatore, V. G., Guzzetti, L., Coppolino, M., Campone, L., Labra, M., Beltrani, T., Branduardi, P. 2022. First report on vitamin B9 production including quantitative analysis of its vitamers in the yeast *Scheffersomyces stipitis*. Biotechnol. Biofuels Bioprod. 15: 98. doi.org/10.1186/s13068-022-02194-y

Microsoft Corporation. 2018. Microsoft Excel. https://office.microsoft.com/excel, 15 July 2024.

Niu, F., Chu, H., Sun, B., et al. 2023. Molecular marker assisted breeding of a new Japonica hybrid rice 'Shenyou28'with good quality and disease resistance. Mol. Plant Breed. 14: 1–8. doi.org/10.5376/mpb.2023.14.0002

Pfeiffer, C.M., Rogers, L.M., Gregory, J.F. 1997. Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. J. Agric. Food Chem. 45: 407–413. doi.org/10.1021/jf960633q

R Core Team. 2021. R: A language and environment for statistical computing. https://www.R-project.org/, 20 June 2024.

Sakai, H., Lee, S.S., Tanaka, T., et al. 2013. Rice Annotation Project Database (RAP-DB): An integrative and interactive database for rice genomics. Plant Cell Physiol. 54: e6. doi.org/10.1093/pcp/pcs183

Sew, Y.S., Aizat, W.M., Zainal-Abidin, R.A., Ab Razak, M.S.F., Simoh, S., Abu-Bakar, N. 2023. Proteomic variability and nutrient-related proteins across pigmented and non-pigmented rice grains. Crops. 3: 63–77. doi.org/10.3390/crops3010007

Strobbe, S., Van Der Straeten, D. 2017. Folate biofortification in food crops. Curr. Opin. Biotech. 44: 202–211. doi.org/10.1016/j.copbio.2016.12.003

Tiozon Jr, R.N., Camacho, D.H., Bonto, A.P., Oyong, G.G., Sreenivasulu, N. 2021. Efficient fortification of folic acid in rice through ultrasonic treatment and absorption. Food Chem. 335: 127629. doi.org/10.1016/j.foodchem.2020.127629

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G. 2012. Primer3--new capabilities and interfaces. Nucleic Acids Res. 40: e115. doi.org/10.1093/nar/gks596

Wakeel, A., Arif, S., Bashir, M.A., Ahmad, Z., Rehman, H.U., Kiran, A., Ibrahim, S., Khan, M.R. 2018. Perspectives of folate biofortification of cereal grains. J. Plant. Nutr. 41: 2507–2524. doi.org/10.1080/01904167.2018.1510511

Warzyszynska, J., Kim, Y. 2014. Folate in human health and disease. In: Encyclopedia of life sciences. John Wiley & Sons, Ltd. Chichester, UK. pp. 1–14.

Wei, T., Simko, V. 2024. R package 'corrplot': Visualization of a Correlation Matrix. https://github.com/taiyun/corrplot, 2 March 2025.

Xiang, N., Hu, J., Wen, T., Brennan, M.A., Brennan, C.S., Guo, X. 2020. Effects of temperature stress on the accumulation of ascorbic acid and folates in sweet corn (*Zea mays* L.) seedlings. J. Sci. Food Agric. 100: 1694–1701. doi.org/10.1002/jsfa.10184

Zhu, J., Wang, R., Zhang, Y., Lu, Y., Cai, S., Xiong, Q. 2024. Metabolomics reveals antioxidant metabolites in colored rice grains. Metabolites. 14: 120. doi.org/10.3390/metabo14020120