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ARTICLE

Effects of Environment and Genotype on Folate Contents in Wheat in the HEALTHGRAIN Diversity Screen†

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This study examined the environmental and genetic variation in folate contents and compositions of wheat genotypes. The selected genotypes, 24 from winter wheat and 2 from spring wheat, were grown in Martonvásár, Hungary, for three consecutive years as well as at four locations (Hungary, France, United Kingdom, and Poland) in one year. Total folate contents were determined by microbiological assay, and folate vitamers were determined for selected genotypes by high-performance liquid chromatography. Statistically significant differences in folate content arose among both harvesting years and growing locations. Grains grown in Hungary had the highest average folate content and those from Poland the lowest. Altogether, a 2.8-fold difference in total folate content appeared, ranging from 323 ng/g of dm (Chinese Spring, grown in Hungary in 2005) to 889 ng/g of dm (Riband, grown in Hungary in 2007). In general, the total folate content varied more greatly among the four growing locations than among the three harvesting years. Environmental factors affected folate content more strongly than genetic factors. In addition, small grains with high bran yield and low thousand kernel weight had high folate contents. The dominant vitamer in wheat genotypes was 5-HCO-H_4folate. Other formylated folates and 5,10-CH^+-H_4folate also existed in significant amounts. Variation in the proportions of 5-HCO-H_4folate and 5-CH_3-H_4folate were mainly responsible for the variation in total folate content: samples with high total folate content had a high proportion of 5-CH_3-H_4folate and a low proportion of 5-HCO-H_4folate. Genotypes with both low and high folate contents, as well as with narrow or broad range, were identified. Thus, the study produced important data for plant breeding to select lines with stable folate contents.

KEYWORDS: Folate; folate vitamers; cereals; wheat; wholegrain; genotype; variation; environment; year; location

INTRODUCTION

Wholegrain cereals are good sources of folate, a B-vitamin that is essential for nucleotide synthesis and several reactions including transfer of one-carbon units. It is well established that folate deficiency may lead to megaloblastic anemia and that folate efficiently prevents neural tube defects in the developing fetus (*1*, *2*). Folate, however, has recently been intensively studied for several other health-promoting activities: prevention of cardiovascular disease and stroke (*3*, *4*); protection against certain cancers such as colorectal cancer (*5*), pancreatic and esophageal cancers (*6*); and its role in cognitive functions (*7*, *8*). High intakes of folate may also promote tumorigenesis among individuals with neoplasms, but it remains to be studied whether this effect is limited to synthetic folic acid as opposed to natural folate forms (*9*).

Daily folate intake falls below recommendations in many countries. Especially in countries without mandatory folic acid fortification, good dietary sources of folate and means to enhance natural folate contents by careful selection of raw materials and tailored food processing need to be studied. Cereal products are the main sources of dietary folate; for instance, in the Finnish diet they contribute approximately one-third of the daily folate intake (*10*). Estimates of the bioavailability of natural folates from food sources vary. As shown by Fenech et al. (*11*) and Vahteristo et al. (*12*), however, endogenous folate in cereal products is readily bioavailable and may improve folate status as effectively as folic acid.

Folate in wheat consists of several vitamers. Their bioavailabilities are unlikely to differ much, but their different stabilities may affect the way they can mediate and pursue the vitamin activity (*13*). The majority of the folate in cereals often exists as formyl and methyl derivatives (*14*). Folates are unevenly distributed in the grain, with high concentrations typically found in outer layers of the kernel and in the germ. For instance, aleurone flour has been reported to contain 5150 ng/g folate (*11*), and according to Arcot et al. (*15*), wheat bran contained more than twice as much folate (1600 ng/g of dry matter) as the grains. Brans from different wheat classes reportedly had high folate contents, from 1820 to 4140 ng/g of dry matter (*16*). The use of grains often requires milling and fractionation that may have significant effects on folate content. Hegedüs et al. (*17*) showed that with

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an extraction rate of 87% the folate concentration of wheat flour had reduced to 79% of that in wholemeal flour, and in flour with an extraction rate of 66% the folate content was only 10% of that in wholemeal flour. Likewise, Patring et al. (*18*) reported folate contents of 220−530 ng/g of dm in Swedish and Norwegian wholegrain wheat flours but only 46−220 ng/g of dm in sifted wheat flours with extraction rates of 70−80%. Folate contents in wheat fractions can thus easily vary 10-fold. The large range in folate contents may be explained especially by differences in the proportion of the folate-rich aleurone layer.

Key genes of folate biosynthesis in wheat have been isolated and identified, and it seems that the seed is capable of effectively replenishing its folate pool throughout its life cycle (*19*). Folate contents in cereals differ markedly according to the grain species and presumably also to genotypes and growing conditions. The magnitude of the variation as well as the underlying factors, however, have not been thoroughly studied. Keagy et al. (*20*) reported significantly lower folate contents in wheat flours derived from soft wheat classes than in those derived from hard classes, whereas Mullin and Jui (*16*) found that folate contents in brans derived from soft wheat classes were approximately 50% higher than in brans derived from hard classes. According to Patring et al. (*18*), wholegrain wheat flours consumed in Sweden and Norway contained 220−430 and 380−530 ng of folate/g, respectively. For 12 Australian wheats from different growing locations folate contents varied from 799 to 1143 ng/g of dm (*15*), and in four Polish wheat cultivars harvested in the same year at the same location folate contents varied from 336 to 403 ng/g of dm (*21*). In our previous study (*22*) on various wheat genotypes grown at the same location in the same year, the average folate content of 130 winter wheat genotypes was 561 ± 102 ng/g of dm, ranging from 364 to 774 ng/g of dm. The average folate content and range for 20 spring wheat genotypes were similar (551 ± 108 and 323−741 ng/g of dm, respectively). The large range in the reported folate contents reflects the effects of genotype and growing conditions (climate, soil type, weather conditions, etc.). Nevertheless, a substantial part of the variation may be explained by differerences in postharvest treatments, sampling, and analytical methods. A reliable evaluation of variation in folate contents thus requires well-controlled trials with a comprehensive number of genotypes grown over several years. Determination of cereal folate is challenging because the concentrations are relatively low and folate may be physically entrapped in the matrix. Folates are susceptible to heat, light, and oxygen. Thus, the critical steps may take place before the actual quantification, for instance, during sampling, extraction, and purification.

The aim of the HEALTHGRAIN Integrated Project (European Union Sixth Framework Program) is to improve the well-being of consumers and to reduce the risk of metabolic diseases by increasing the intake of protective compounds in grains. This is achieved through development of health-promoting, safe, and high-quality cereal foods and ingredients. The objective of this substudy was to examine the environmental and genetic variation in folate contents and compositions of diverse wheat genotypes. This was performed by growing the genotypes in Hungary for three consecutive years, 2005−2007, as well as at three other locations (France, United Kingdom, and Poland) in 2007 in a controlled manner. Folate vitamer distributions were also determined for selected genotypes to elucidate their potential in explaining the variation.

MATERIALS AND METHODS

Samples. The samples included 24 winter wheat and 2 spring wheat (*Triticum aestivum* var. *aestivum*) genotypes (**Table 1**). The selection of the genotypes was based on the results of a previous experiment in 2005 studying bioactive constituents in cereals (*23*). The folate contents of 150 wheat genotypes grown in Hungary in 2005 have been reported in our earlier publication (*22*). Three of the selected genotypes for the present study had a total folate content below 450 ng/g of dm and 12 were above 650 ng/g of dm in 2005. In addition, several of the genotypes were ranked high with respect to their total phytochemical or fiber contents, and genotypes CF99105 and Disponent were characterized by both high phytochemical and high fiber contents. Genotype CF99105 also had a high thousand kernel weight and low bran yield, indicating good milling performance with high yield (*23*).

To study the extent of environmental variation in folate content, the genotypes were grown in Martonvásár (Hungary) for three years, 2005, 2006, and 2007, as well as in Enchantillon (France), Woolpit (United Kingdom), and Choryn (Poland) in 2007. The two spring wheat genotypes, Chinese Spring and Cadenza, however, were not grown in Poland. In addition, two winter wheat genotypes, Crousty and Tiger, that were chosen to be used as starting materials in the processing module of the HEALTHGRAIN project were grown in Hungary in 2007 only and were thus excluded from the comparison of folate contents among years. After harvesting, the wheat grains were transported to Martonvásár, Hungary, where they were milled to wholemeal flours of 0.5 mm particle size and stored in sealed plastic bags in the dark at −18 °C until shipping to the laboratory for folate analysis (*23*).

Two batches of winter wheat genotype MV Emese, grown in Hungary, were used as an in-house reference for routine quality control of the method of analysis. The batch harvested in 2005 was used during analyses of samples grown in 2005−2006 and the batch harvested in 2007 during analyses that year.

The samples were well characterized for their agronomic and quality parameters, including the geographical origins of the genotypes, type and age of the germplasm, cultivation and weather conditions, dry matter contents, bran yields, and thousand kernel weights (*23*, *24*). These data were obtained from other HEALTHGRAIN project partners.

Folate Analysis. The sample preparation procedure included heat extraction (with a buffer-to-sample ratio of ca. 15) followed by trienzyme treatment with α-amylase (EC 3.2.1.1, Sigma, St. Louis, MO), hog kidney or chicken pancreas conjugase (Difco, Sparks, MD), and protease (EC 3.4.24.31, Sigma, St. Louis, MO) (*22*, *25*, *26*). Chicken pancreas conjugase hydrolyzes naturally occurring folylpolyglutamates to shorter chain compounds, mainly to folyldiglutamates, and is suitable for the microbiological assay, whereas hog kidney conjugase is needed to hydrolyze folylpolyglutamates to monoglutamates that can then be determined by HPLC. Total folate contents were determined in duplicate with a microbiological assay on microtiter plates using *Lactobacillus rhamnosus* ATCC 7469 as the growth organism and 5-formyltetrahydrofolate as the calibrant (*26*). Method performance of the microbiological assay was confirmed by analyzing a blank sample as well as certified reference material CRM 121 (wholemeal flour) or in-house reference (wholemeal flour from genotype MV Emese) in each set of samples. Two batches of the reference were similarly prepared and used in the study: the first batch was used for samples harvested in 2005 and 2006 and the second batch for samples harvested in 2007. Action limits in the control charts were 500 ± 70 ng/g of dm (the certified value) for CRM 121 and average ± 1.5 × standard deviation for the in-house reference: 470 ± 74 ng/g for the first batch and 585 ± 87 ng/g for the second batch. In addition, total folate contents of duplicate samples were not allowed to differ by >10%.

Distributions of folate vitamers were determined for 17 samples, 15 winter wheat genotypes and 2 spring wheat genotypes, harvested in the four locations in 2007, by HPLC after affinity chromatographic purification. Details on the method validation have been provided elsewhere (*22*, *25*, *26*). Samples were analyzed in duplicate. (6*S*)-Tetrahydrofolate (H_4folate, sodium salt), (6*S*)-5-methyltetrahydrofolate (5-CH_3-H_4folate, calcium salt), and (6*S*)-5-formyltetrahydrofolate (5-HCO-H_4folate, sodium salt) were obtained from Merck Eprova (Schaffhausen, Switzerland). 10-Formylfolic acid (10-HCO-PGA) and folic acid (PGA) were obtained from Dr. Schirck's Laboratories (Jona, Switzerland). 10-Formyldihydrofolate (10-HCO-H_2folate) was synthesized from 5,10-methenyltetrahydrofolate as described by Kariluoto et al. (*26*). Calibrants were dissolved, spectrophotometrically checked for their purities, and stored as described by Kariluoto et al. (*26*). 5,10-Methenyltetrahydrofolate ([6*R*]-5,10-CH^+-H_4, chlorine hydrochloride) was obtained from Merck Eprova. It was weighed

Table 1. Total Folate Contents of Wheat Genotypes Grown in Three Years and at Four Locations

	total folate content (ng/g of dm)									
genotype	Hungary 2005	Hungary 2006	Hungary 2007	France 2007	United Kingdom 2007	Poland 2007	mean (ng/g of dm)	CV (%)	range (ng/g of dm)	homogenous groups[b]
Campari	651	685	745	502	418	427	571	25	418−745	a−e
Herzog	657	723	673	487	532	524	599	16	487−723	b−g
Disponent	665	699	735	595	523	524	623	14	523−735	c−g
Tommi	669	700	690	479	442	550	588	19	442−700	a−g
Tremie	573	711	721	527	541	458	588	18	458−721	a−g
CF99105	684	654	840	537	509	477	617	22	477−840	c−g
Valoris	585	518	588	412	388	367	476	21	367−588	a
Isengrain	488	630	668	511	471	505	546	15	471−668	a−d
Claire	510	649	808	501	540	459	578	22	459−808	a−f
Maris Huntsman	696	630	785	501	607	533	625	17	501−785	c−g
Lynx	721	703	870	579	648	533	676	18	533−870	e−g
Malacca	701	796	757	635	569	514	662	17	514−796	d−g
Rialto	751	762	766	532	552	541	650	18	532−766	c−g
Riband	704	712	889	609	630	626	695	15	609−889	f,g
Avalon	533	564	653	565	495	483	549	11	483−653	a−d
San Pastore	438	483	739	469	599	496	537	21	438−739	a−c
Estica	757	701	881	735	570	573	703	17	570−881	g
Gloria	460	702	705	618	618	532	606	16	460−705	b−g
Spartanka	364	713	706	574	459	504	553	25	364−713	a−d
Obriy	534	605	739	499	613	447	573	18	447−739	a−e
Atlas 66	680	680	769	658	555	559	650	13	555−769	c−g
Crousty			770	554	616	573	628	9	554−770	c−g
Tiger			538	456	471	441	477	16	441−538	a,b
MV Emese	469	621	686	534	537	451	550	16	451−686	a−d
Chinese Spring[a]	323	533	532	425	536		470	20	323−536	a
Cadenza[a]	565	713	676	638	518		622	13	518−713	c−g
mean (ng/g of dm)	591	662	728	543	537	504				
CV (%)	21	12	13	14	13	11				
range (ng/g of dm)	323−757	483−796	532−889	412−735	388−648	367−626				

[a] Spring wheat genotype. [b] Homogenous groups of wheat genotypes are marked with the same letter. Groups were identified by multiple-range test with total folate contents of the 26 genotypes over the six different environmental conditions ($p < 0.05$).

and dissolved as described by van den Berg et al. (*27*) and stored in 0.01 M acetate buffer containing 1% (w/v) ascorbic acid (pH 4.9) at −20 °C. For the purity check the stock solution was diluted into 0.1 M K_2HPO_4 (pH adjusted to 1.0 with HCl), and the purity was confirmed using a molar absorptivity coefficient at pH 1.0 ($\varepsilon_{352} = 25 \times 10^3\ M^{-1}\ cm^{-1}$) (*28*). The detection was carried out by an UV detector set at 290 nm and by a fluorescence detector set at 290 nm excitation and 356 nm emission wavelengths for reduced folates and at 360/460 nm for 10-formylfolic acid. Peaks were identified by their retention times, and identities were confirmed by spiking and by comparing ratios of UV and fluorescence peaks.

Statistical Analyses. Multifactor analysis of variance (ANOVA) was used to evaluate the differences between the means of total folate contents of different genotypes among harvesting years and growing locations. The multiple-range test was used to determine the means significantly different from the others, with Fisher's least significant difference procedure to discriminate among the means. To relate total folate content to kernel characteristics, Pearson correlation coefficients were calculated to examine the correlation between total folate content and bran yield or thousand kernel weight. Statgraphics Plus 4.0 software (Manugistics Inc., Rockville, MD) was used in statistical analyses, and principal component analysis (PCA) was performed using Unscrambler v. 9.0 software (Camo Software AS, Oslo, Norway).

RESULTS AND DISCUSSION

Overall Variation in Total Folate Content. A 2.8-fold difference emerged in total folate content of the genotypes ranging from 323 ng/g of dm (Chinese Spring, grown in Hungary in 2005) to 889 ng/g of dm (Riband, grown in Hungary in 2007; **Table 1**). For individual genotypes, the difference between the highest and lowest folate contents among all harvesting years and growing locations was on average 1.5-fold, the smallest difference being for Tiger (1.2-fold) and the highest for Spartanka (2.0-fold). Analysis of variance showed statistically significant differences in total folate content averages among the genotypes grown under the six environments, that is, harvesting years and growing locations ($F(25,149) = 1.96$, $p = 0.0084$). The multiple-range test identified 7 homogeneous groups based on average total folate contents of the 26 genotypes over the different environments ($p < 0.05$; **Table 1**). High and low folate levels, as well as narrow and wide ranges in folate contents, existed among both old and modern genotypes.

PCA with folate content and composition, bran yield, and thousand kernel weight produced a loading plot (**Figure 1**) in which the two first principal components explained 35 and 27% of the total variation of the 150 samples, accounting for 62% of the total variation. Because the closely located total folate content and proportion of 5-CH_3-H_4folate were located far from the proportion of 5-HCO-H_4folate along the first principal component, it can be assumed that the first component differentiated the samples by their folate contents and compositions. The score plot in **Figure 2** with growing locations coded from 1 to 4 shows that grains grown in Hungary were mainly located in the left side of the *y*-axis, indicating that the second principal component separated the samples by growing location.

Association of Total Folate Content with Kernel Characteristics. PCA showed that both bran yield and thousand kernel weight explained the variance, because they were located within the two ellipses representing 50 and 100% explanation rates. Total folate contents were

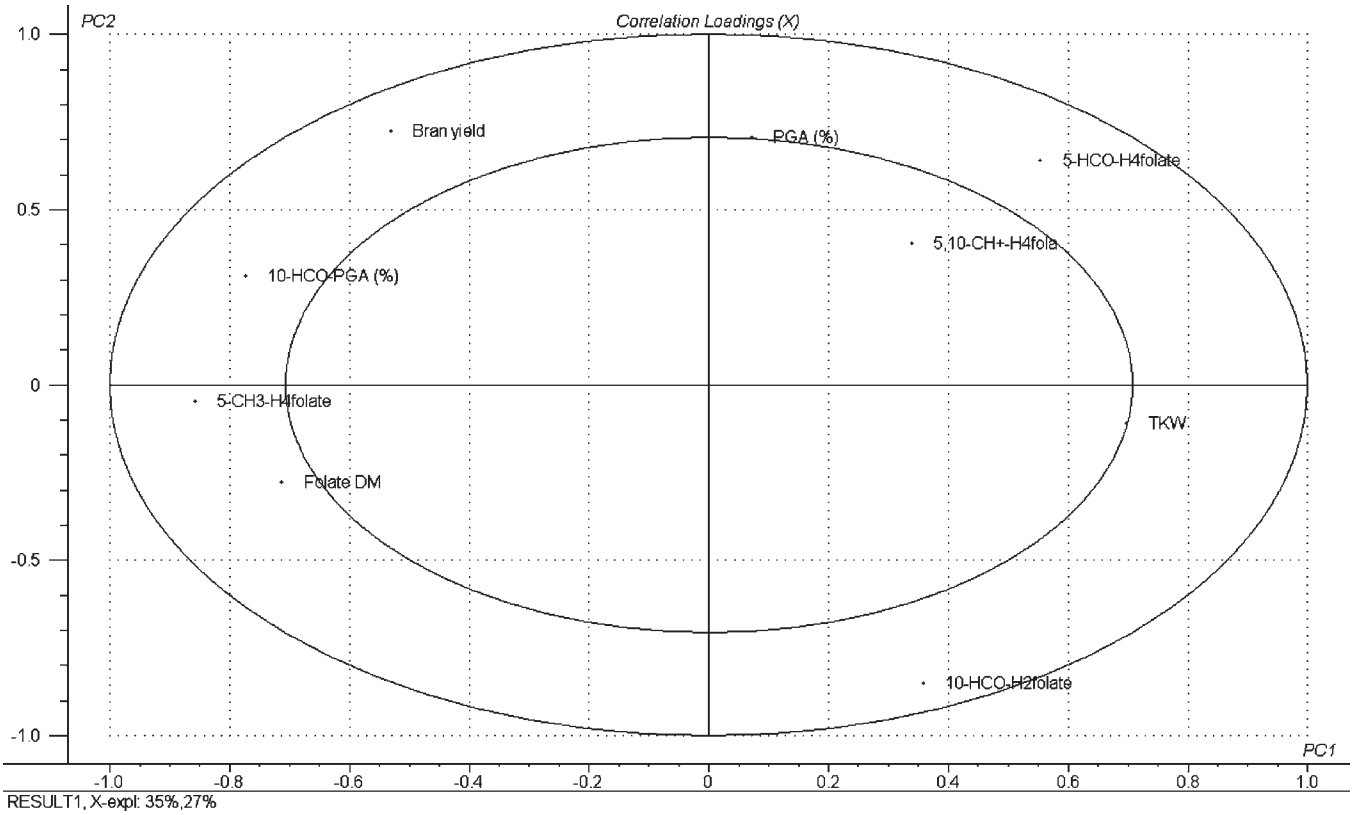

Figure 1. Principal component analysis of total folate contents (ng/g of dm) and proportions of vitamers, bran yields, and thousand kernel weights (TKW) of wheat grains grown under six different environments (N = 150).

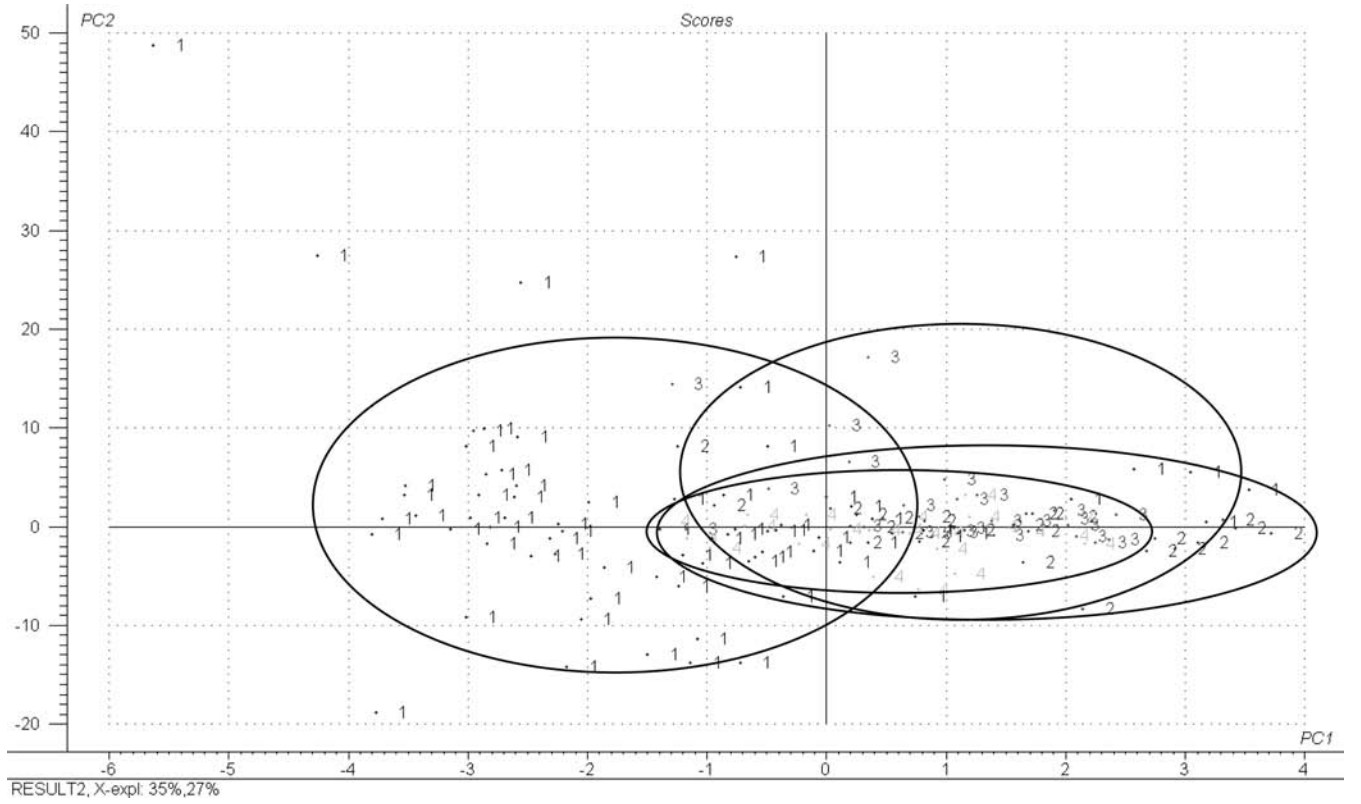

Figure 2. Score plots of grain objects marked by growing location codes (1 = Hungary, 2 = France, 3 = United Kingdom, 4 = Poland).

positively correlated with bran yield and negatively correlated with thousand kernel weight; thus, small grains with low thousand kernel weight and high bran yield predicted high folate contents.

Indeed, Pearson correlation coefficients demonstrated a strong negative relationship between thousand kernel weight and total folate (−0.326323; df = 149) that was even more negative if the

Table 2. Mean Folate Vitamer Contents and Relative Proportions of Wheat Genotypes from Four Locations

	folate vitamer content (ng/g of dm) and % of the vitamer sum						
genotype	5-CH_3-H_4folate	10-HCO-H_2folate	10-HCO-PGA	5-HCO-H_4folate	5,10-CH^+-H_4folate	PGA	sum of vitamers
Tommi	62 ± 24 18%	30 ± 10 9%	60 ± 23 17%	116 ± 25 34%	61 ± 11 18%	11 ± 2 3%	339 ± 37
Tremie	57 ± 22 15%	48 ± 29 12%	67 ± 26 17%	126 ± 38 33%	64 ± 9 17%	17 ± 3 5%	379 ± 63
CF99105	65 ± 28 15%	78 ± 9 18%	71 ± 32 16%	128 ± 10 30%	79 ± 19 18%	13 ± 3 3%	434 ± 89
Valoris	27 ± 11 9%	27 ± 31 9%	54 ± 26 17%	123 ± 7 40%	65 ± 19 21%	15 ± 2 5%	312 ± 41
Claire	73 ± 25 21%	9 ± 7 3%	73 ± 30 20%	140 ± 17 40%	47 ± 28 13%	14 ± 3 4%	356 ± 53
Malacca	97 ± 63 19%	65 ± 10 14%	86 ± 46 17%	161 ± 22 34%	66 ± 14 14%	13 ± 4 3%	487 ± 105
Rialto	70 ± 30 17%	36 ± 41 9%	70 ± 33 16%	147 ± 24 35%	86 ± 7 20%	14 ± 4 3%	423 ± 56
Riband	79 ± 31 14%	104 ± 71 18%	84 ± 36 15%	152 ± 16 31%	97 ± 7 19%	18 ± 3 3%	533 ± 125
Avalon	63 ± 31 18%	25 ± 33 8%	56 ± 27 17%	116 ± 14 36%	56 ± 12 18%	10 ± 2 3%	326 ± 53
Estica	79 ± 25 16%	43 ± 38 9%	75 ± 29 16%	164 ± 32 34%	107 ± 14 22%	15 ± 2 3%	481 ± 22
Gloria	49 ± 31 10%	70 ± 45 14%	62 ± 18 13%	176 ± 7 38%	96 ± 35 21%	14 ± 4 3%	469 ± 87
Spartanka	35 ± 24 11%	56 ± 38 17%	43 ± 11 14%	107 ± 17 34%	67 ± 15 21%	8 ± 2 3%	317 ± 27
Crousty	58 ± 17 13%	98 ± 60 21%	62 ± 16 14%	131 ± 18 30%	85 ± 40 19%	11 ± 2 3%	445 ± 50
Tiger	46 ± 25 13%	84 ± 39 23%	50 ± 19 14%	117 ± 23 33%	58 ± 26 16%	10 ± 4 3%	365 ± 114
MV Emese	52 ± 30 12%	70 ± 19 18%	66 ± 19 15%	158 ± 27 37%	63 ± 15 15%	13 ± 2 3%	427 ± 52
Chinese Spring	35 ± 10 9%	109 ± 22 27%	53 ± 12 13%	125 ± 15 31%	67 ± 21 16%	16 ± 1 4%	406 ± 24
Cadenza	42 ± 21 11%	73 ± 28 19%	50 ± 20 13%	147 ± 35 38%	64 ± 9 17%	11 ± 5 3%	387 ± 80
mean	58 ± 18 14%	61 ± 30 15%	64 ± 12 16%	137 ± 20 35%	72 ± 17 18%	13 ± 3 3%	405 ± 65
CV%	32	49	19	15	23	20	16

Table 3. Folate Vitamer Distribution (Percent) of Wheat Genotypes (n = 17) Grown at Four Locations (Range in Parentheses)

growing location	5-CH_3-H_4folate	10-HCO-H_2folate	10-HCO-PGA	5-HCO-H_4folate	5,10-CH^+-H_4folate	PGA
Hungary	19 (11−28)	12 (2−35)	20 (14−26)	29 (22−37)	16 (9−22)	3 (2−5)
France	12 (6−18)	15 (3−34)	17 (12−24)	37 (28−46)	16 (10−28)	4 (3−5)
United Kingdom	9 (5−13)	15 (2−28)	12 (8−17)	40 (32−48)	21 (14−34)	3 (2−6)
Poland	17 (8−29)	15 (1−27)	13 (11−16)	33 (24−46)	19 (8−29)	3 (2−5)

two spring wheat genotypes were excluded from the statistical analysis (−0.418152, df = 139). The negative correlation is well in line with previous results (*22*). No statistically significant relationship appeared between bran yield and total folate at the 90% or higher confidence level among the 26 wheat genotypes. If the two spring wheat genotypes were excluded, however, a relatively weak positive relationship manifested between bran yield and total folate among winter wheat genotypes (0.216255; df = 139). In our previous paper (*22*) reporting folate results for 130 winter wheat and 20 spring wheat genotypes, the correlation between bran yield and total folate was also positive for winter wheat genotypes. As the outer layers of the kernel are rich in folate (*11*, *15*, *16*), small kernels (low kernel weight) have higher proportions of outer layers and are thus high in folate.

Folate Vitamer Distribution. Folate results obtained by HPLC were lower than microbiological results. On average, sums of vitamers were 71% of the microbiological values, but for 10 samples were only around 50−60%. HPLC results for folate in food are typically 20−30% lower than total folate results obtained by microbiological assay (*14*, *29*). Nevertheless, HPLC results were well in line with microbiological results in that the sums of folate vitamers were in most cases highest in genotypes grown in Hungary and lowest in genotypes grown in the United Kingdom and Poland.

5-HCO-H_4folate was the most abundant vitamer in both winter and spring wheat genotypes, averaging 35% of the vitamer sum (**Table 2**). Other formylated folates, 10-HCO-H_2folate and 10-CHO-PGA, also existed in significant amounts, accounting for approximately 30% of the vitamer sum. These two vitamers reflect the amount of 10-HCO-H_4folate that cannot be quantitated in our HPLC as such. 5,10-CH^+-H_4folate accounted for 18% and 5-CH_3-H_4folate for 14% of the vitamer sum. Vitamer distributions varied among growing locations (**Table 3**), but this was likely influenced by the total folate content rather than growing location. Unfortunately, H_4folate could not be quantitated reliably, but its proportion was

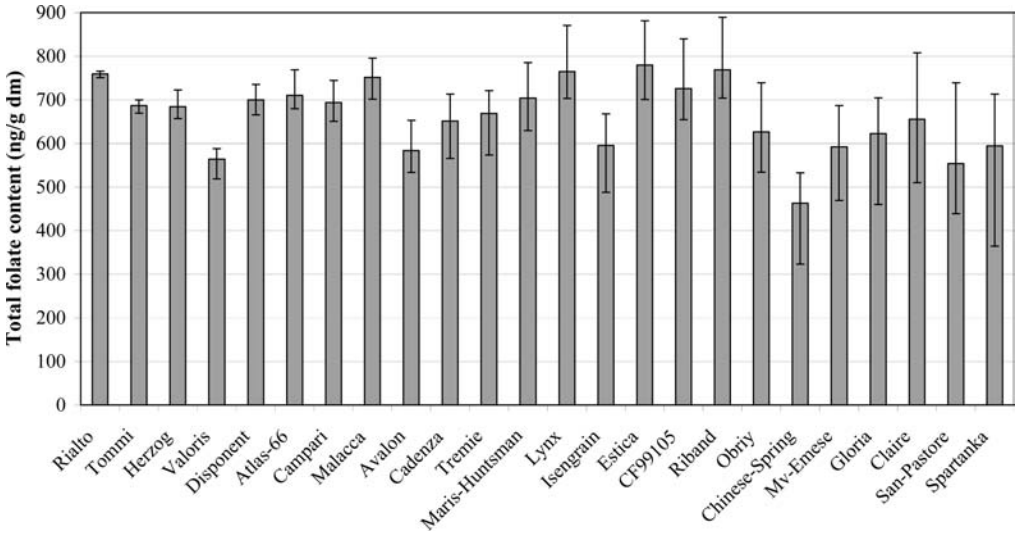

Figure 3. Average total folate contents (ng/g of dm) in wheat genotypes grown in Hungary 2005−2007 in order of increasing variation. Error bars represent the range over three years.

estimated to vary between 5 and 10%. PGA was also found in minor concentrations. It is not synthesized by the plant but formed by oxidation during sample milling and analytical procedures. PGA has been previously determined in rice by a UPLC-MS/MS technique (*30*).

Interestingly, in general the proportion of 5-CH_3-H_4folate was positively associated with total folate content, whereas samples with lower folate contents were characterized with a high proportion of 5-HCO-H_4folate. In addition, the proportion of 10-CHO-PGA was positively associated with total folate content. The PCA further confirmed these findings (**Figure 1**), showing that variation in the proportions of 5-HCO-H_4folate and 5-CH_3-H_4folate were mainly responsible for the variation of total folate content. The sum of 5-CH_3-H_4folate and 5-HCO-H_4folate was approximately 50% of the vitamer sum (49 ± 5%), which is in good agreement with our previous results (*22*).

Small grains with low thousand kernel weights and high bran yields seemed to contain large proportions of 5-CH_3-H_4folate and 10-CHO-PGA. On the contrary, the thousand kernel weight was positively correlated with the proportion of 5-HCO-H_4folate, indicating that this vitamer may be mostly located in the starchy endosperm. The role of 5-HCO-H_4folate in plants is not clear. It can inhibit serine hydroxymethyltransferase and thereby influence the glycine pool in photorespiration; nevertheless, 5-HCO-H_4folate seems to be well tolerated in plants. It is a very stable vitamer, and it may act as a storage form of one-carbon groups in seeds and fungal spores, that is, in cells that are in a dormant stage (*31*, *32*).

Only a few studies have been published on vitamer distributions in cereal grains. In general, however, it can be summarized that cereal grains contain a wider variety of vitamers than vegetables, in which 5-CH_3-H_4folate typically is the major folate form (*14*, *33*). Gujska and Kuncewicz (*21*) analyzed four wheat varieties for folates and found on average 58% 5-HCO-H_4folate, 23% 5-CH_3-H_4folate, and 20% 10-CHO-PGA. Also in the study by Patring et al. (*18*) on Swedish and Norwegian wholegrain wheat flours, 5-HCO-H_4folate and 10-CHO-PGA dominated, together accounting for 60−90% of the vitamer sum. In our study formyl folates accounted for approximately 65%, which agrees with the results of Patring et al. (*18*). A large disparity still exists in the few results available. We also show that vitamer distributions are not uniform among genotypes or different growing locations. In addition, part of the discrepancy may be explained by different methodologies and folate interconversions caused by, for example, heat treatments and pH changes (*34*). For instance, in our study we determined 5,10-CH^+-H_4folate, which naturally resulted in lower proportions of other vitamers compared to studies in which this vitamer was not determined. 5,10-CH^+-H_4folate is an endogenous vitamer in plants but may also be formed from 10-HCO-H_4-folate during the analysis (*34*). The presence of 5,10-CH^+-H_4folate in rice has been confirmed by UPLC-MS/MS analysis (*30*).

Effect of Environmental Factors on Variation in Total Folate Contents. Folate contents in genotypes grown in three subsequent years in one location (Hungary) varied substantially (**Figure 3**; **Table 1**). Among the harvesting years, an average 1.3-fold difference occurred in the total folate content of individual genotypes, the highest difference being for Spartanka (2.0-fold) and the lowest for Tommi and Rialto (1.0-fold). Although the differences were not striking, there were statistically significant differences in folate content among the harvesting years ($F(2, 73) = 27.43$, $p = 0.0000$) and also among the genotypes ($F(25, 73) = 4.32$, $p = 0.0000$). Multiple-range tests showed that there was a significant difference between each of the years ($p < 0.05$). The total folate content was higher in 2007 than in 2005 for every genotype. Folate contents were also higher in 2006 than in 2005, except for five genotypes (CF99105, Valoris, Maris Huntsman, Lynx, and Estica) and higher in 2007 than in 2006, except for five genotypes (Herzog, Tommi, Malacca, Spartanka, and Cadenza). The average total folate contents of the 26 genotypes were 591 ± 123 ng/g of dm in 2005, 662 ± 77 ng/g of dm in 2006, and 728 ± 92 ng/g of dm in 2007. The coefficient of variation (%CV) clearly declined from 21% in 2005 to 12% in 2006 and 13% in 2007. The grains were harvested during a rainy period in 2005 (*24*), and thousand kernel weights were higher than in subsequent years, which might well have influenced the folate content.

Riband was among the six folate-richest genotypes in all three harvesting years, and Estica, Rialto, Lynx, Malacca, and Maris Huntsman in two of three years. Chinese Spring fell among the six poorest genotypes in all three harvesting years, and Isengrain, MV Emese, San Pastore, Avalon, and Valoris in two of three years. The difference between the highest and the lowest folate contents (Riband 2007 vs Chinese Spring 2005) was 2.8-fold. As seen in **Figure 3** and **Table 1**, for some genotypes such as Rialto, Tommi, and Herzog the ranges of total folate content were narrow, 15−66 ng/g of dm, indicating great stability, whereas for others such as Spartanka, San Pastore, and Claire the ranges were as broad as 297−349 ng/g of dm.

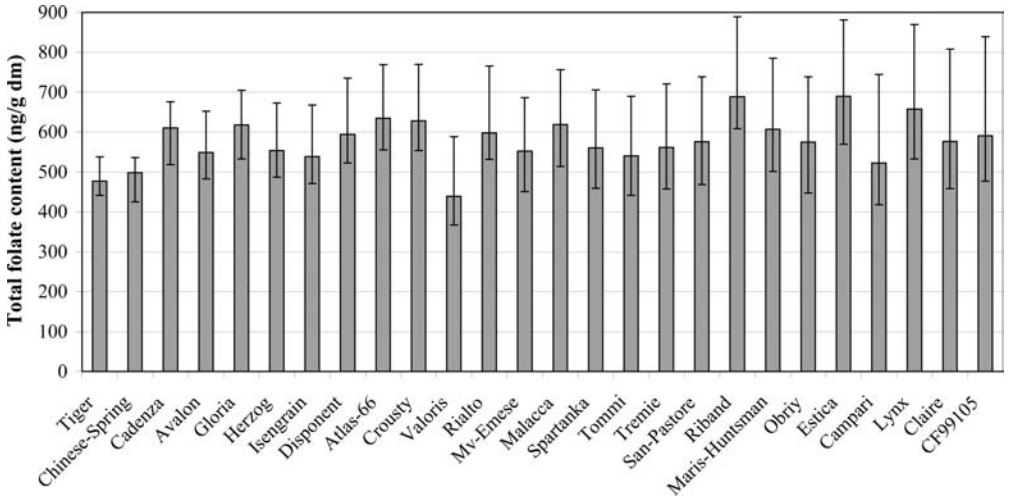

Figure 4. Average total folate contents (ng/g of dm) in wheat genotypes grown at four growing locations in 2007 in order of increasing variation. Error bars represent the range among the four locations.

Considerable variation was observed in folate contents of the genotypes grown at four locations in 2007 (**Figure 4**; **Table 1**). Among the four growing locations, an average 1.5-fold difference appeared in the total folate content of individual genotypes, the highest differences being for Campari, CF99105, and Claire (1.8-fold) and the lowest for Tiger (1.2-fold). The difference between the highest and the lowest folate contents (Riband Hungary vs Valoris Poland) was 2.4-fold (**Table 1**). Statistically significant differences emerged in folate content both among the growing locations ($F(3, 101) = 103.2$, $p = 0.0000$) and among the genotypes ($F(25, 101) = 5.43$, $p = 0.0000$). Multiple-range tests showed significant differences ($p < 0.05$) in folate contents of grains grown in Hungary compared to France, the United Kingdom, and Poland and also of grains grown in Poland compared to the three other locations. Grains grown in Hungary contained more folate than grains grown at the other locations except for only one genotype, Chinese Spring. The average total folate contents of grains were 728 ± 92 ng/g of dm (Hungary), 543 ± 76 ng/g of dm (France), 537 ± 68 ng/g of dm (United Kingdom), and 504 ± 57 ng/g of dm (Poland).

Riband was among the six folate-richest genotypes at all four growing locations and Estica at three locations, whereas Valoris and Tiger fell among the six poorest genotypes at all four growing locations. The lowest ranges for total folate contents, 97−158 ng/g of dm, over the four growing locations were found for genotypes Tiger, Chinese Spring, and Cadenza (**Figure 4**; **Table 1**). Genotypes Estica, Campari, Lynx, Claire, and CF99105 had the highest ranges, over 300 ng/g of dm and up to 363 ng/g of dm.

The total folate contents in this study, 323−889 ng/g of dm with an average of 504 ng/g of dm, were higher than previously published by Gujska and Kuncewicz (*21*) but lower than the 799−1143 ng/g of dm reported by Arcot et al. (*15*). In our previous study with a diversity of wheat genotypes the ranges were smaller because the grains were grown at the same location during the same year. The range for total folate contents varied from 364 to 774 ng/g of dm for 130 winter wheat genotypes (mean value = 561 ng/g of dm) and from 323 to 741 ng/g of dm for 20 spring wheat genotypes (mean value = 551 ng/g of dm) (*22*).

Two-way analysis of variance showed that both genotype and environmental conditions produced highly significant variation in the total folate contents ($F(25, 149) = 5.84$, $p = 0.0000$; $F(5, 149) = 48.41$, $p = 0.0000$), with environmental factors clearly affecting more than the genotype (mean squares of 187239 and 22603, respectively). In general, the variation of total folate contents for individual genotype was higher among the four growing locations than among the three harvesting years (**Figures 3** and **4**). Judged by the mean squares in the analysis of variance testing the contribution of each of the factors separately, growing location affected total folate content more than harvesting year or genotype (mean squares of 269777, 119918, and 22603, respectively).

Variations among harvesting years and among growing locations were strikingly different for the genotypes Tommi and Rialto. Their folate contents remained very stable among years, but when grown at sites other than Hungary, their folate contents markedly dropped, resulting in high variation among growing locations. On the other hand, there were a few genotypes (such as Gloria, Spartanka, and Chinese Spring) with a larger variation among harvesting years than among growing locations. The difference was caused by the exceptionally low folate content of these genotypes in Hungary in 2005 (rainy period) compared to the other harvesting years and locations. Herzog, with a medium total folate content, had a relatively small variation both between harvesting years and between growing locations. Nevertheless, the overall variation was large due to the higher folate levels in the three harvesting years than at the four locations in 2007.

From genotypes with a wide overall range Crousty, CF99105, Claire, Spartanka, and Campari had medium total folate contents (552−617 ng/g), whereas Lynx had a high folate content (676 ng/g of dm). Riband that had a high folate content also had relatively large variations. From the six genotypes with a narrow overall range, four had a folate content below 550 ng/g of dm (Avalon, Isengrain, Tiger, and Chinese Spring) and only two above 600 ng/g of dm (Cadenza and Disponent). In the previous study with genotypes harvested in Hungary 2005, Disponent had both high total phytochemical and high fiber contents (*23*).

Previous studies indicate a marked variation in folate contents of wheat genotypes (*15*, *16*, *21*). The number of genotypes studied has, however, been relatively small, and often growing conditions have not been similar to allow evaluation of the true genotype effect. The strength of this study, conducted under the HEALTHGRAIN project, lies in inclusion of a relatively large number of genotypes that were grown, harvested, and handled postharvest in a controlled manner. To our knowledge, this is the most extensive study on the inherent variation and the effect of environment on folate content and composition of wheat genotypes. Variation of total folate contents was higher among the four growing locations than among the three harvesting years. Thus, the weather conditions evidently affected folate contents less than the environmental factors

related to the growing location, such as soil type and climatic conditions. Genotype also affected the folate contents, but more weakly than environmental factors. In this study, genotypes with both low and high folate contents, as well as with low or high variation, either overall or among different environmental conditions, could be identified, allowing selection of lines with high and stable folate contents for plant breeding purposes.

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