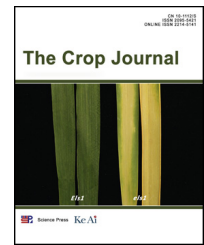


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# Genome-wide association mapping of vitamins B1 and B2 in common wheat<sup>☆</sup>

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## ABSTRACT

Vitamin B is essential for maintaining normal life activities in humans and animals who have to intake the microelement from the outside, especially from cereal products. In the present study 166 Chinese and foreign wheat cultivars planted in two environments were characterized for variation in vitamin B1 and B2 contents. A genome-wide association study (GWAS) using the wheat 90 K SNP assay identified 17 loci for vitamin B1 and 7 for vitamin B2 contents. Linear regression analysis showed a significantly positive correlation of the number of favorable alleles with vitamin B1 and B2 contents. Marker-trait associations (MTAs) at *IWB43809* (6AS, 0 cM) and *IWB69903* (6AS, 13 cM) were new and stable, and significantly associated with vitamin B1 content across two environments. The loci identified in this study and associated SNP markers could be used for improvement of vitamin B1 and B2 contents to obtain superior quality along with grain yield in wheat.

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## 1. Introduction

Vitamin B, one of the important microelements, is essential for maintaining normal life activities in human and animals. The vitamin B complex comprises eight water-soluble components, viz. thiamin (vitamin B1), riboflavin (vitamin B2), pantothenic acid (vitamin B3), nicotinic acid (vitamin B5), pyridoxine (vitamin B6), biotin (vitamin B7), folic acid (vitamin B9), and cobalamine (vitamin B12) that play important roles in the metabolism of carbohydrates, proteins and fats. Thiamin deficiency is associated with neurological problems, including

Alzheimer's disease, cognitive deficit and encephalopathy [1–2]. Riboflavin deficiency destroys mucosal membranes in the digestive system and can lead to cardiovascular disease and colorectal cancer [3–4]. Instead of biosynthesizing these vitamins within their own bodies, humans and animals must obtain them from external sources in order to remain healthy.

Vitamins B1 and B2 often occur together in the same foods and were initially regarded as a single component. In cereals, the most important staple food sources, the complex vitamin B complex is concentrated in the bran and germ, with 32% to 64% of the vitamin B1 and 26% to 37% of the vitamin B2 being

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present in the aleurone layer and embryo, respectively [5–6]. A number of studies of vitamin B1 and B2 contents in wheat have been reported. For example, Davis et al. [7] evaluated 231 cultivars grown at 49 locations over three years and determined variation in vitamins B1 and B2 levels ranging from 3.3 to 6.5  $\mu\text{g g}^{-1}$  and 1.0 to 1.7  $\mu\text{g g}^{-1}$ , respectively. Batifoulou et al. [8] determined the variation in vitamin B1 (2.6–6.1  $\mu\text{g g}^{-1}$ ) and vitamin B2 (0.5–1.1  $\mu\text{g g}^{-1}$ ) contents in 49 wheat cultivars. Shewry et al. [9] showed that there were large and significant variations in B1 and B2 contents among 24 wheat cultivars, ranging from 5.5 to 13.6  $\mu\text{g g}^{-1}$  for vitamin B1, and from 0.8 to 1.4  $\mu\text{g g}^{-1}$  for vitamin B2. Davis et al. [7] indicated that the total contents of vitamins B1 and B2 were influenced by genotype, environment and genotype  $\times$  environment (G  $\times$  E). However, there are no reports on QTL mapping and genome-wide association studies (GWAS) of the genetic bases of variation in vitamin B1 and B2 contents to date.

GWAS is an efficient approach to identify associations between genotypes and phenotypes in plants [10–11]. For example, Rasheed et al. [12] identified 44 marker-trait associations (MTAs) for nine yield and related traits using a GWAS of 123 wheat cultivars and 14,960 SNP markers. Dong et al. [13] detected 52 MTAs for stem water-soluble carbohydrate in 166 bread wheat cultivars using GWAS based on data obtained with the wheat 90 K SNP array. These studies were carried to determine genetic factors affecting complex traits. In the present study, a GWAS of vitamin B1 and B2 contents was performed using the same panel of 166 Chinese and foreign bread wheat cultivars and the wheat 90 K iSelect assay. The aim was to identify loci associated with vitamins B1 and B2 for quality improvement in bread wheat.

## 2. Materials and methods

### 2.1. Plant materials

A collection of 166 bread wheat cultivars and advanced lines from the Yellow and Huai Valley Facultative Region and foreign countries was used for the study (Table S1); 144 of them were from China, nine from Italy, seven from Argentina, four from Japan, one from Australia and one from Turkey. Field trials were conducted in randomized complete blocks with three replicates in Anyang (Henan province) and Suixi (Anhui province) during the 2015–2016 cropping season, providing data for two environments. Each plot contained three 2 m rows spaced 20 cm apart.

### 2.2. Genotyping and quality control

Genomic DNA was extracted by a modified method according to Lagudah et al. [15]; samples were sent to CapitalBio Corporation (Beijing, China; <http://www.capitalbio.com/>) for genotyping with the high-density illumina 90 K infinium SNP array [16]. PowerMarker V3.2.5 was used to calculate gene diversity, minor allele frequency (MAF) and polymorphism information content (PIC). Genotyping and quality control was described in our previous study [13]. Markers were removed if their locations in chromosomes were unknown, there were

>30% missing values, they showed a MAF of <5%, or were represented by >10% heterozygosis.

### 2.3. Milling

Thirty g kernel samples were milled using a Cyclotec 1093 Mill (Foss Tecator). The ground whole meal was stored at  $-20\text{ }^{\circ}\text{C}$  prior to analysis. The water contents of the whole meal samples were measured in a drying oven at  $130\text{ }^{\circ}\text{C}$  for 1 h after freezing.

### 2.4. Vitamin B1 and B2 extraction and determination

Vitamins B1 and B2 were extracted following Ndaw et al. [17] with minor modifications, in which the extraction solvent contents were reduced by 50%. A finely ground sample (2.5 g) was weighed into a 100 mL reagent bottle. Twenty-five ml of  $0.05\text{ mol L}^{-1}$  sodium acetate (pH 4.5) were added to the sample, followed by a mixture of papain (50 mg), 1% glutathione (250  $\mu\text{L}$ ), acid phosphatase (10 mg) and  $\alpha$ -amylase (5 mg). The sample was mixed completely and incubated in a shaker at  $37\text{ }^{\circ}\text{C}$  for 20 h, then diluted with distilled water in a 50 mL volumetric flask. The supernatant was filtered through filter paper. The filtrate obtained after a second filtration through a cellulose acetate filter (0.2  $\mu\text{m}$ ) was used for chromatographic determination of vitamin B2. An aliquot of the first filtrate (2 mL) was added to a 10 mL tube with an alkaline solution of potassium ferricyanide (2 mL). The mixture was agitated and then left to stand for exactly 5 min. Two mL of butanol was added with vortexing, and the tube was stood for stratification. The supernatant fluid filtered through a cellulose acetate filter (0.2  $\mu\text{m}$ ) was used for the chromatographic determination of vitamin B1.

### 2.5. Chromatographic determination

A high performance liquid chromatography (HPLC) system (a 2010 Shimadzu Model) and an RF10AxI fluorescence detector (Shimadzu, Shimane, Japan) was used to determine vitamins B1 and B2 contents. Separation by HPLC was accomplished using a XTerra RP18 column (150.0 mm  $\times$  4.6 mm, 5  $\mu\text{m}$ , Waters Corporation, Massachusetts, USA) following Arella et al. [18].

### 2.6. Statistical analyses

Analysis of variance (ANOVA) was performed using PROC GLM in SAS software version 9.2 (SAS Institute Inc., Cary, NC, USA). Least square means were calculated for each parameter and used to test the significance of differences ( $P < 0.001$ ) between samples. Broad-sense heritabilities ( $h^2$ ) were calculated following Lin and Allaire [19].

### 2.7. Population structure analysis

Population structure was described in our previous study [13]. Briefly, Structure v. 2.3.4 was used to estimate population structure based on 5624 SNP markers distributed evenly across the entire genome using Bayesian cluster analysis; markers were chosen on the basis of a MAF of over 5% and

**Table 1 – Analysis of variance of vitamin B1 and B2 contents in 166 cultivars grown in two environments.**

Source of variation	df	Vitamin B1			Vitamin B2		
		MS	F-value	R <sup>2</sup> (%)	MS	F-value	R <sup>2</sup> (%)
Genotype	165	27.43	3.41 ***	66.82	0.01	1.45 **	49.11
Environment	1	441.91	54.94 ***	6.52	0.03	3.69	0.89
Genotype × environment	165	10.07	1.25 *	24.53	0.01	0.74	49.11
Residual error		8.04		0.12	0.01		0.3

\* Significant at P = 0.05.  
\*\* Significant at P = 0.01.  
\*\*\* Significant at P = 0.001.

<30% missing data, and showed heterozygosity of <10% [20]. Each K value was run repeatedly and independently to ensure the sampling variance of inferred population structure. A range of K from 1 to 10 was based on admixture and correlated allele frequencies models. Each run was carried out with 10,000 replicates for the burn-in period and 100,000 replicates during analysis. The optimum value of K was chosen by the highest  $\Delta K$  [21].

### 2.8. Association analysis

Vitamin B1 and B2 contents, genotype and population structure (Q-matrix) were implemented in TASSEL software version 5.0 using the mixed linear model (MLM) for association analysis. The significance of SNP markers was determined by a threshold P-value of 0.001 [13,14], and MTAs within 5-cM intervals were declared to be the same loci according to Wang et al. [16]. The distributions of observed and expected P-values. Manhattan plots were used to map SNP markers significantly associated with vitamin B1 and B2 contents. Both the Quantile-Qantile and Manhattan plots were drawn in R Language (R version 3.1.2; <http://www.r-project.org/>).

## 3. Results

### 3.1. Phenotypic variation

There was continuous variation for vitamin B1 and B2 contents in both environments (Fig. S1). The averaged vitamin B1 and B2 contents among cultivars ranged from 5.34 to 16.74  $\mu\text{g g}^{-1}$  and from 0.48 to 0.74  $\mu\text{g g}^{-1}$ , respectively. Cultivars Lumai 23, Xiaoyan 22, Zhengmai 366, Zhoumai 16, Nidera Baguette 10, and Nidera Baguette 20 had the highest vitamin B1 contents, whereas Aifeng 3, Xinmai 19, Xiaoyan 54, Bima 1, and Zhengzhou 3 had the highest vitamin B2 contents (Table S1). ANOVA showed significant differences for vitamins B1 ( $P < 0.001$ ) and B2 contents ( $P < 0.01$ ) among genotypes (Table 1). No significant differences were identified among three sub-populations for vitamin B2 content, whereas the mean value of vitamin B1 content of sub-population 3 was significantly ( $P < 0.05$ ) less than those of sub-populations 1 and 2 based on multiple comparison analysis (Table S2). The heritabilities of vitamin B1 and B2 contents were 0.83 and 0.77, respectively.

**Table 2 – SNP markers significantly associated with vitamin B1 content in the association panel.**

SNP ID	Environment	Chromosome	Position <sup>a</sup>	SNP <sup>b</sup>	MAF <sup>c</sup>	P-value	R <sup>2</sup> (%)
IWB12483	Suixi	1DL	100	<u>A/G</u>	0.16	6.05E-04	10.6
IWB6046	Suixi	2AS	156	<u>A/G</u>	0.09	6.71E-04	10.3
IWB1795	Suixi	2BL	146	<u>A/G</u>	0.12	7.95E-04	9.2
IWB49825	Anyang, Average <sup>d</sup>	2DL	81	<u>A/C</u>	0.13	3.94E-04	7.6
IWB11577	Suixi	3B	33	<u>A/G</u>	0.19	7.68E-04	8.6
IWB13323	Anyang	4AS	37	<u>A/G</u>	0.16	6.09E-04	7.3
IWB64353	Anyang	4AL	137	<u>A/G</u>	0.31	5.60E-04	7.8
IWA1505	Suixi	4AL	145	<u>A/C</u>	0.21	2.17E-04	8.4
IWB53093	Anyang	4BS	62	<u>A/C</u>	0.17	8.18E-04	6.9
IWB48019	Suixi	5BL	105	<u>A/C</u>	0.45	3.91E-04	8.6
IWB28597	Anyang	5BL	218	<u>A/G</u>	0.10	3.76E-04	7.6
IWB43809	Anyang, Suixi, Average	6AS	0	<u>A/G</u>	0.17	3.92E-04	7.7
IWB69903	Anyang, Suixi, Average	6AS	13	<u>A/G</u>	0.18	1.90E-04	8.4
IWA5508	Suixi	6AS	31	<u>A/G</u>	0.41	5.88E-04	7.1
IWA2479	Suixi, Average	6BS	0	<u>A/G</u>	0.34	2.49E-04	6.9
IWB33834	Anyang	6BL	72	<u>A/G</u>	0.19	9.70E-04	6.5
IWB41653	Anyang, Average	7BL	134	<u>A/G</u>	0.08	5.30E-04	7.3

<sup>a</sup> Position from the wheat 90 K SNP consensus map [16].

<sup>b</sup> Favorable alleles for increasing vitamin B1 content are underlined.

<sup>c</sup> Minor allele frequency.

<sup>d</sup> Average indicates the MTA based on the mean phenotypic data from both environments.

**Table 3 – SNP markers significantly associated with vitamin B2 content in the association panel.**

SNP ID	Environment	Chromosome	Position <sup>a</sup>	SNP <sup>b</sup>	MAF <sup>c</sup>	P-value	R <sup>2</sup> (%)
IWB11044	Suixi	1AS	51	<u>A/C</u>	0.31	5.55E-04	7.1
IWB23596	Suixi	1DS	68	<u>A/G</u>	0.19	3.00E-05	10.5
IWB58793	Anyang	3B	62	<u>A/G</u>	0.28	6.53E-04	6.6
IWB56921	Suixi	4AL	75	<u>A/C</u>	0.38	3.08E-04	7.7
IWA8005	Suixi	5BL	49	<u>A/G</u>	0.07	1.01E-04	9.0
IWB58995	Suixi	6AS	59	<u>A/G</u>	0.10	2.80E-04	7.8
IWB65016	Suixi	7BL	159	<u>A/G</u>	0.08	5.98E-05	9.7

<sup>a</sup> Position from the wheat 90 K SNP consensus map [16].

<sup>b</sup> Favorable alleles for increasing vitamin B2 content are underlined.

<sup>c</sup> Minor allele frequency.

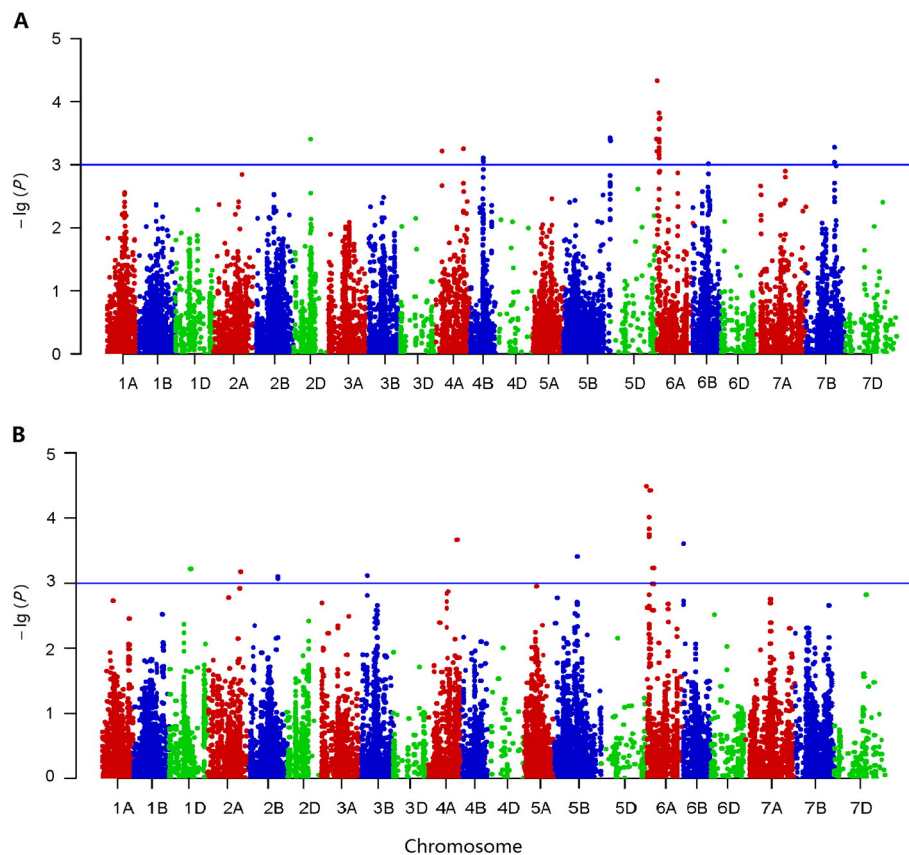
### 3.2. Analysis of SNP markers and population structure

Among the 81,587 SNP markers in the 90 K array, 40,267 (49.4%) were mapped to individual chromosomes [16]. Finally, 18,207 (22.3%) markers were selected after a strict quality control in our association panel and were integrated into a linkage map involving all 21 wheat chromosomes. These markers covered a genetic distance of 3700 cM, with an average density of one marker per 0.2 cM. The marker density was much lower for the D genome (254.4 markers per chromosome) compared to the A (1007.7 markers per

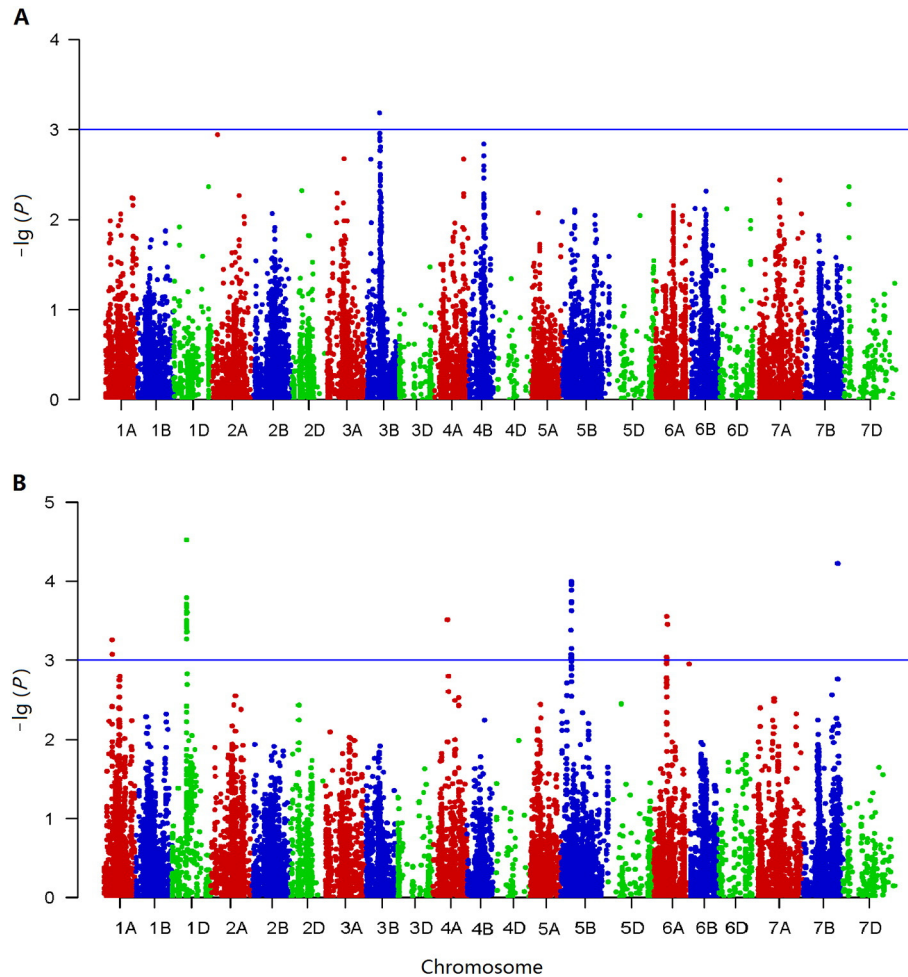
chromosome) and B (1338.9 markers per chromosome) genomes. Among D genome chromosomes, and 4D had the lowest (50). The average SNP diversity (*H*) and PIC values were 0.35 and 0.29, respectively.

### 3.3. Marker-trait associations

Considering the criteria ( $P < 0.001$ ), 17 loci were significantly associated with vitamin B1 content, and 7 were associated with vitamin B2 content; these were distributed on 12 chromosomes (Tables 2 and 3). The largest numbers of MTAs



**Fig. 1 – Manhattan plots from GWAS for vitamin B1 in two environments. The horizontal line depicts the 1E-03 threshold for significant association. A, Anyang; B, Suixi.**



**Fig. 2 – Manhattan plots from GWAS for vitamin B2 in two environments. The horizontal line depicts the 1E-03 threshold for significant association. A, Anyang; B, Suixi.**

were on chromosomes 4A and 6A, and no MTA was detected on chromosomes 1B, 3A, 3D, 4D, 5A, 5D, 6D, 7A, and 7D (Figs. 1 and 2). MTAs consistently identified in both environments were considered to be stable. Among them, multiple SNP markers associated with vitamin B1 were identified on chromosomes 6AS (0 cM) and 6AS (13 cM) in both environments, explaining 7.7% and 8.4% of the phenotypic variation ( $R^2$ ), respectively (Table 2). There were multiple SNP markers associated with vitamin B2 on chromosomes 1DS (68 cM), 5BL (49 cM) and 6AS (59 cM). QQ plots for the distribution of expected and observed P-values of associated SNP markers are shown in Fig. S2.

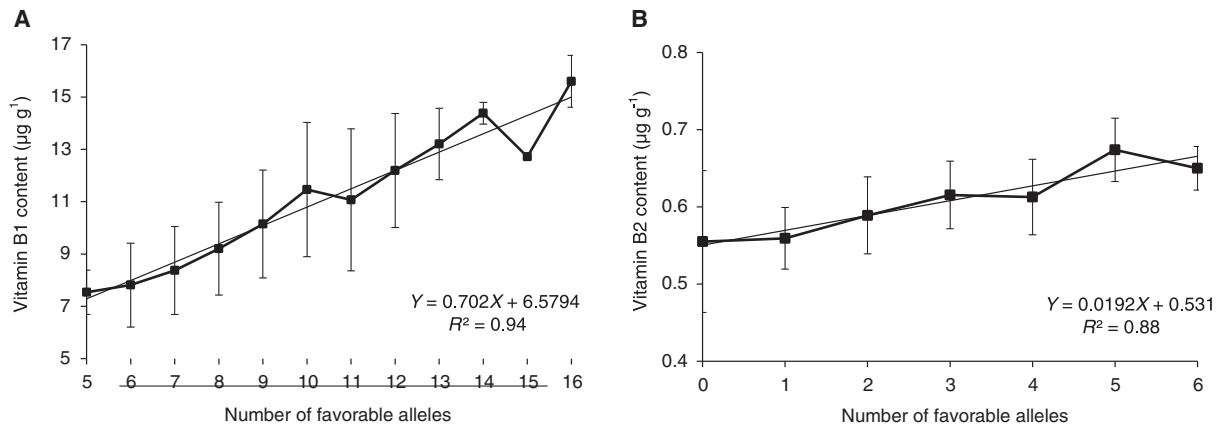
### 3.4. Effects of favorable alleles on vitamins B1 and B2

Alleles with positive effects increasing vitamin B1 and B2 contents were considered to be favorable. Significantly positive correlations were observed between vitamin B1 ( $r = 0.97$ ,  $P < 0.001$ ) or vitamin B2 ( $r = 0.94$ ,  $P < 0.001$ ) contents and the number of favorable alleles (Fig. 3). The numbers of favorable alleles present in a cultivar ranged from 5 to 16 for vitamin B1 content, and from 0 to 6 for vitamin B2 (Table S1).

## 4. Discussion

### 4.1. Marker–trait associations for vitamins B1 and B2

The present results confirmed previous findings [8] that Vitamin B contents of cereal products are controlled mainly by genetic factors [8]. Therefore, identification of QTL associated with vitamins B1 and B2 should be helpful for wheat improvement. Previously, work on vitamin B in cereals primarily focused on extraction [17], content determination [18] and synthesis pathways [22–24]. In the present study, we used a GWAS approach to analyse a panel of 166 bread wheat cultivars by assaying 18,207 SNP markers to identify chromosomal regions associated with vitamin B1 and B2 contents. This is the first attempt to identify genes controlling vitamin B1 and B2 contents in wheat by GWAS. The results provide a basis for improving vitamin B1 and B2 contents. ANOVA indicated that the  $G \times E$  contributed largely phenotypic variance in vitamin B1 (24.53%) and vitamin B2 (49.11%) contents (Table 1), resulting in some inconsistencies in MTA across environments.



**Fig. 3 – Linear regression analysis for number of favorable alleles and vitamin B. A, vitamin B1; B, vitamin B2.**

Markers IWB12483 (1DL, 100 cM), IWB6046 (2AS, 156 cM), IWB1795 (2BL, 146 cM), IWB11577 (3B, 33 cM), IWB48019 (5BL, 105 cM), IWB43809 (6AS, 0 cM) and IWB69903 (6AS, 13 cM) were significantly associated with vitamin B1 content. Among them, IWB12483 ( $R^2 = 10.6\%$ ), IWB6046 (10.3%) and IWB1795 (9.2%) loci explained the highest phenotypic variations. Notably, MTAs at IWB43809 and IWB69903 on chromosome 6AS were identified in both environments, indicating the QTL were stable. Markers IWB11044 (1AS, 51 cM), IWB23595 (1DS, 68 cM), IWB58793 (3B, 62 cM), IWB56921 (4AL, 75 cM), IWA8005 (5BL, 49 cM), IWB58995 (6AS, 59 cM) and IWB65016 (7BL, 159 cM) were significantly associated with vitamin B2 content. Among the IWB23595 ( $R^2 = 10.5\%$ ), IWA8005 (9.0%) and IWB65016 (9.7%) loci explained the highest phenotypic variations.

#### 4.2. Putative candidate genes

The biosynthetic pathways of vitamins B1 and B2 have been well studied in prokaryotes *Escherichia coli* [25–26] and *Bacillus subtilis* [27–28], but the biosynthesis in eukaryotes is much less understood. The results of the present GWAS study provides a basis for searching for candidate genes involved in vitamin B1 and B2 biosynthesis in wheat.

In bacteria 12 genes involving 11 enzymatic steps are required for vitamin B1 biosynthesis [29–30]; in yeast 19 genes are involved [31]. The functions of several of these genes have been elucidated, e.g., *THI2*, *THI3*, *THI6*, *THI20* and *THI21*. One of the most important genes is *THI3*, which has an important role in pyrimidine biosynthesis and DNA repair [31–33], and has been isolated in *Arabidopsis thaliana* [22] and *Solanum lycopersicum* [34]. However, the homologous gene in common wheat remains unidentified. The biosynthesis of one riboflavin molecule requires one molecule of GTP and two molecules of ribulose 5-phosphate. GTP cyclohydrolase II is the first committed step in biosynthesis of the key enzyme involved in riboflavin, catalyzing the opening of the imidazole ring of GTP and release of pyrophosphate [35–36]. The encoding gene (*rib A*) has been isolated in bacteria [37] and yeast [38], but little information is available in plants. No QTL related to this gene has been identified.

#### 4.3. Potential implications for wheat breeding

Wheat, with a total estimated production of 120 Mt. during 2015–2016 (<http://data.stats.gov.cn/>), is one of the three most important crops in China. It is mainly consumed in human nutrition and is regarded as an important source of vitamins [39]. Markers for MTAs explaining higher phenotypic variation, such as IWB12483, IWB6046, and IWB1795 identified in this study could be used for improvement of vitamin B1 in marker-assisted breeding. Markers IWB23595, IWA8005 and IWB65016 with high phenotypic variation explained could be used for improvement of vitamin B2. The cultivars Lumai 15, Jimai 19, Aifeng 3 and Bima 1 had higher contents of both vitamins B1 and B2, and therefore can be used as parents in breeding programs. There were also multiple MTAs associated with vitamin B1 on chromosomes 4A and 6A, implying that these regions are important for vitamin B1 content only. Germplasm of this type with higher numbers of favorable alleles included Zhoumai 31, Nidera Baguette 10 and Nidera Baguette 20 (Table S1). Likewise, cultivars Lankao 2, Mantol and Funo have higher numbers of favorable alleles for vitamin B2 content, and these can be used as parents to improve vitamin B contents in breeding programs with the expectation of human health benefits.

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#### Appendix A. Supplementary data

Supplementary data for this article can be found online at <https://doi.org/10.1016/j.cj.2017.08.002>.

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