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RESEARCH

Association Mapping of Hagberg Falling Number in Hard White Spring Wheat

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ABSTRACT

Hagberg falling number (FN) is an important quality trait used for grain grading. The FN test can only be measured in the late stages of variety development when progeny are homogeneous and sufficient grain is available for testing. The FN is strongly influenced by environmental conditions during reproductive growth stages, including excess moisture, extreme temperature, and biotic and abiotic stresses. The objective of this study was to identify potential genomic regions that influence FN in a subset of 110 hard white spring (HWS) wheat (*Triticum aestivum* L.) accessions from the National Small Grain Collection (NSGC) using genome-wide association mapping. The FN tests were conducted using grain flour samples of these accessions grown in five environments. A total of 1740 single nucleotide polymorphism (SNP) markers were used to detect SNP–FN associations using both the general linear model (GLM) and the mixed linear model (MLM). Thirteen quantitative trait loci (QTL) located in nine chromosomal regions were identified in both GLM and MLM approaches. Pyramiding these QTL could explain up to 45% of the phenotypic variation of FN. The present study identified several potential markers for use in marker-assisted selection for high FN lines in wheat breeding.

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Abbreviations: AM, association mapping; BLUP, best linear unbiased predictors; E1, an irrigated environment with normal nitrogen; E2, a terminal drought environment with normal nitrogen; E3, a terminal drought environment with deficient nitrogen; ELISA, Enzyme Linked Immunosorbert Assay; FDR, false discovery rate; FN, Hagberg falling number; G1, the first population group, mainly from south Asia; G2, the second population group, mainly from west Asia; G3, the third population group; GLM, general linear model; HWS, hard white spring; *K*, hypothetical number of subpopulations; Δ*K*, an ad hoc quantity statistic based on the rate of change; K matrix, the marker based kinship matrix; LD, linkage disequilibrium; FDR, false discovery rate; LMA, late-maturity α-amylase; MLM, mixed linear model; NSGC, National Small Grain Collection; PC, principal component; PCA, principal component analysis; PHS, pre-harvest sprouting; pI, isoelectric point; Q matrix, population structure from the STRUCTURE analysis; QTL, quantitative trait locus/loci; R^2, total explained phenotypic variation; SNP, single nucleotide polymorphism; TCAP, Triticeae Cooperative Agricultural Projects; Y11E1, E1 in year 2011; Y11E2, E2 in year 2011; Y11E3, E3 in year 2011; Y12E1, E1 in year 2012; Y12E2, E2 in year 2012.

The FN is an important quality characteristic for wheat (Hagberg, 1960; Perten, 1964). It has been widely used as a measure of grain quality for wheat and other cereals (Belitz et al.,

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2004). Grain flour with low FN produces dough that is sticky and difficult to process and loaves that are discolored and poorly structured (Chamberlain et al., 1982). The FN test measures the rheological properties of starch and the degree of starch damage. Preharvest sprouting (PHS) can increase α-amylase activity, causing starch breakdown, which results in lower FN. Pre-harvest sprouting is usually caused by a premature break in seed dormancy under humid and wet conditions before harvest, by excessive rain, and sometimes by lodging (Barnard, 2001; Gooding et al., 2012). Red-seeded wheat usually has longer seed dormancy and better PHS resistance than white wheat (Liu et al., 2008). Measuring spike germination and testing α-amylase activity are commonly used to assess PHS, but both are time-consuming and expensive.

Low FN values can also be associated with late-maturity α-amylase (LMA), a genetic defect involving the synthesis of high isoelectric point (pI) isozymes of α-amylase during the later stages of grain development in the absence of germination, resulting in mature grain with high pI α-amylase (Mares and Mrva, 2008). Assessing LMA is more difficult than assessing PHS. Expression of LMA is usually triggered by cool temperature shock during the middle stages of grain development (around 25–30 d postanthesis) (Mrva and Mares, 2001; Mrva et al., 2006). Screening for LMA requires a controlled environment such as a growth chamber. Isoelectric focusing (Gale and Ainsworth, 1984; Mares and Gale, 1990) and an Enzyme Linked Immunosorbent Assay (ELISA; Mrva et al., 2006) have been used to detect and quantify high-pI α-amylase. Both assays are time-consuming and expensive. In addition, the ELISA format is patented and not yet commercially available.

Molecular markers associated with FN would be useful in selecting against lines with low FN in early breeding generations. Kunert et al. (2007) assessed the FN of two advanced backcross populations in two environments and detected seven QTL on chromosomes 1B, 2D, 4B, 7BS, 7BL, 3A, and 6B. Rasul et al. (2009) and Fofana et al. (2009) tested FN using artificially weathered kernels, so their FN experiments were more related to PHS. Rasul et al. (2009) identified two QTL for FN on chromosomes 4A (linked to marker *Xwmc48*) and 4B (linked to marker *Xwmc349*) that were colocated with QTL associated with PHS. The QTL on 4A was possibly the seed-dormancy QTL reported by Kato et al. (2001). Fofana et al. (2009) identified two QTL for FN on chromosomes 3BL (located in the interval of markers *Xbarc77* and *Xwmc307*) and 3DL (located in the interval of markers *Xwmc552* and *Xwmc533*), and both QTL colocated with QTL for PHS and seed coat color.

Most QTL mapping studies on FN or PHS have been conducted using biparental populations, which can only detect the genetic variation between the two parents. Recently, linkage disequilibrium (LD) based association mapping (AM) has received increasing attention and has been used in several QTL mapping studies in wheat (Breseghello and Sorrells, 2006; Emebiri et al., 2010; Jaiswal et al., 2012; Kulwal et al., 2012; Reif et al., 2011). Association mapping can use germplasm collections to identify trait–marker associations without the need to make crosses and develop mapping populations. Because germplasm collections (either exotic germplasm or elite wheat germplasm) have high genetic variation, AM should identify more alleles than biparental mapping. Although AM studies on PHS (Jaiswal et al., 2012; Kulwal et al., 2012) and LMA (Emebiri et al., 2010) have been reported, no previous AM studies have sought to map QTL associated with FN.

The objectives of the present study were to examine genetic variation for grain FN in the absence of spouting in 110 HWS wheat accessions from NSGC and to conduct genome-wide association mapping of FN using SNP markers.

MATERIALS AND METHODS

Plant Materials

Through the Triticeae Cooperative Agricultural Projects (TCAP), a total of 110 HWS wheat accessions (Supplemental Table S1) from the USDA-ARS NSGC were selected from 540 accessions of all classes evaluated in 2011 and 2012.

These 540 accessions were selected on the basis of the heading date, plant height, and seed availability from a 2010 seed increase experiment at Aberdeen, ID (42.96° N, 112.83° W, elevation 1342 m). Accessions with heading dates from 58 to 75 d after planting were selected, and accessions with severe lodging were excluded.

Experiment Design

The experiment was part of a large study to evaluate water and nitrogen use efficiency, and the 540 accessions with replicated checks were evaluated in three environments: (i) an irrigated environment with normal nitrogen (E1), (ii) a terminal drought environment with normal nitrogen (E2), and (iii) a terminal drought environment with deficient nitrogen (E3).

The 540 accessions and 5 checks were planted using an augmented design (Federer, 1956) with only checks replicated in three field environments in year 2011 (Y11E1, Y11E2, Y11E3) and two environments in year 2012 (Y12E1 and Y12E2) in Aberdeen, ID. Accessions were divided into three maturity groups (3 large blocks) on the basis of heading date: early (58–66 d), medium (66–70 d), and late (70–75 d) with 180 lines in each group. Each large block was further divided into four small blocks (2 by 2). The 5 checks were replicated 12 times by random planting in each of the 12 small blocks. A total of 600 plots were planted, with 20 plots wide and 30 plots deep for each environment. Plots in both years were 1.5-m wide by 1.8-m long with seven rows sown at 225 seeds m^{-2}.

In 2011, plots were planted on 25 Apr. and harvested on 31 Aug. Precipitation from April through August was 66 mm. In 2012, plots were planted on 11 Apr. and harvested on 20 Aug. Precipitation from April through August was 46 mm. The average daily maximum and minimum temperature from April to August in 2011 was 23.0 and 5.2°C, respectively. The

average daily maximum and minimum temperature from April to August in 2012 was 25.4 and 6.5°C, respectively.

The field was irrigated with a drip tape system for precise control of irrigation for each environment. Water was applied from canopy closure to physiological maturity for the irrigated environments (Y11E1 and Y12E1). For the terminal drought environments (Y11E2, Y11E3, and Y12E2), water application was stopped when 95% of the plots in a maturity group had headed and begun flowering. Irrigated trials received an average of 1063 mm of water in addition to natural precipitation. Early, medium, and late maturing plots in the terminal drought environments received respectively 354, 236, and 117 mm less water each year than the irrigated plots. Herbicides and fungicides were applied to control weeds and diseases when necessary (data not shown). Nitrogen was applied before planting on the basis of soil nitrogen level and a yield target (6.73 tons ha^{-1}). The nitrogen-deficient environment Y11E3 received 30% less nitrogen fertilizer than the other four environments.

Plots were harvested using a Wintersteiger Classic small plot combine (Wintersteiger Inc., Salt lake City, UT) equipped with a Harvest Master weighing system (Juniper Systems, Inc., Logan, UT). Seeds from each plot were cleaned and stored in a cool and dry environment before FN testing.

FN Test

The FN tests were conducted 1 mo after harvest in both years for the 110 selected HWS wheat accessions. A total of 200 g of clean seeds of each accession was ground using a Perten Lab Mill 3100 with a 0.8-mm screen (Perten Instruments, Inc. Springfield, Illinois). The FN was measured using a Perten Falling Number 1700 (Perten Instruments, Inc. Springfield, Illinois) according to ICC standard No. 107/1 (1995) and AACC Method 56–81B (1992). Two 7 g meal samples (adjusted for meal moisture) per accession were weighted and mixed with 25 mL distilled water in a FN tube. The tube was shaken vigorously for 3 s to form a homogeneous suspension using a Shakematic shaker (Perten Instruments, Inc., Springfield, Illinois). A viscometer-stirrer was added to the tube and the tube was placed in the Perten Falling Number 1700. The FN (s) includes a 5-s period of standing in boiling water, 55 s of stirring, and the time for the stirrer to fall to the bottom of the tube. The FN readings were adjusted for altitude. The mean of the two tubes per accession was calculated and used in the analysis.

Marker Data

Single nucleotide polymorphism markers used in the present study were generated by an Illumina Infinium 9K iSelect platform (Illumina, Inc.) through the TCAP. The SNP names were coded with IWA (Illumina wheat Design A) and the reference number for each SNP (e.g., IWA4351). SNP markers with a minor allele frequency <0.05 or missing data >10% were removed before analysis. On the basis of the SNP reference map from TCAP (Cavanagh et al., 2013), markers with unknown chromosome positions were excluded from the analyses. Also, only a single SNP was included in the analysis for loci with more than one marker.

Population Structure Analysis

The population structure of the 110 HWS wheat accessions was analyzed using STRUCTURE 2.3.4 (Pritchard et al., 2000) with a model allowing for admixture and correlated allele frequencies (Falush et al., 2003). A subset of 498 SNP markers spaced at intervals of at least 5 cM was used in the analysis to reduce the run time. The length of the burn-in period was set to 10,000 and the number of iterations was set to 100,000. The hypothetical number of subpopulations (K) was tested from 1 to 8 with 5 independent runs for each K. The output from STRUCTURE was analyzed in STRUCTURE HARVESTER (Earl and vonHoldt, 2012). An ad hoc quantity statistic based on the rate of change (ΔK) in the logarithm of the probability of likelihood [$LnP(D)$] value between successive K values (Evanno et al., 2005) was used to predict the greatest possible number of subpopulations. CLUMPP (Jakobsson and Rosenberg, 2007) and Distruct (Rosenberg, 2004) were used to sort the cluster labels and graphically display the STRUCTURE results. Principle component analysis (PCA) was also conducted to interpret the population structure.

Statistical Analysis

The FN data of each accession in each environment were adjusted on the basis of augmented design using the function "DAU.test" in R package "agricolae" (R Core Team, 2012; de Mendiburu, 2012). The broad sense heritability (h_B^2) and BLUP of the FN data across the five environments were computed using R package "lme4" (Bates et al., 2012) treating genotypes as random effect and environments as fixed effect. If not otherwise mentioned, all the analyses were conducted in R (R Core Team, 2012).

Association Mapping

The adjusted FN of individual environments and the BLUP of the five environments were used in association mapping. Tassel 3.0 (Bradbury et al., 2007) was used for genome wide association mapping. Both the GLM (Q model) and the MLM (Q + K model) approaches were used to identify significant marker–trait association. A total of 1740 filtered SNP markers were used in the analysis. The Q matrix from the STRUCTURE analysis was used to account for the population structure. The marker based kinship matrix (K matrix) was computed in Tassel and used in Q + K analysis to account for both family and population structure. P values of each marker in the output of GLM were adjusted using Bonferroni method ($\alpha = 0.1$). Significance level for the MLM output was set as $\alpha = 0.01$ (without adjustment). A modified FDRcriterion (Storey, 2002) was also used to test significant associations using the qvalue package (Dabney et al., 2011) in R.

For the markers identified both in GLM and MLM procedures, stepwise multiple regression with the Q matrix as covariance was conducted to check marker contribution in each environment; to simplify the model, markers that were kept in the model for more than 3 environments were used to calculate the total explained phenotypic variation (R^2) by stepwise multiple regression again; if Q was left in the model, its proportion was removed from the total R^2.

Table 1. Descriptive statistics for Hagberg falling number (FN) measured in seconds in 110 hard white spring wheat accessions over five environments.

Environment†	Mean‡	SD	Min.	Max.	No. of accessions with FN < 300 sec
Y11E1	351c	70	102	500	25
Y11E2	404a	64	221	556	9
Y11E3	392ab	61	194	515	8
Y12E1	397a	51	274	502	2
Y12E2	382b	51	264	479	8

† Y11E1, full irrigation environment (E1) in 2011; Y11E2, terminal drought (E2) in 2011; Y11E3, terminal drought and nitrogen deficient environment (E3) in 2011; Y12E1, E1 in 2012; Y12E2, E2 in 2012.

‡ Honestly Significant Difference test of the FN mean in each environment; means with the same letter are not significantly different.

RESULTS

Phenotypic Variation of FN in 110 HWS Accessions across Five Environments

The FN data of the five individual environments (Table 1 and Fig. 1) and the BLUP (Fig. 1) data across the five environments showed wide variation (102–556 s) and exhibited normal distributions with some skewness. The FN of the three environments in 2011 varied more than the FN in the two environments in 2012 (Table 1 and Fig. 1). An FN value <300 s is used as a basis for discounting or rejecting grain when tested at grain elevators (Sologuk and Sorenson, 2005), so low FN in the present study was defined as <300 s. A total of 25, 9, 8, 2, and 8 accessions had low FN in Y11E1, Y11E2, Y11E3, Y12E1, and Y12E2, respectively. Of the 25 accessions showing low FN in 2011 (Table 2), two accessions (PI428421 and PI613289) had low FN in four to five environments without lodging, and three accessions (PI314941, PI384346, and PI429318) had low FN in all three environments in 2011 without lodging. These accessions with consistent low FN could be potentially used in future genetic studies of FN. Of the 25 accessions with low FN in Y11E1, ten had severe lodging (score >6) in that trial, but these rarely showed low FN in the other four environments (Table 2). Also, most of the accessions with low FN in more than one environment did not lodge (Table 2), suggesting that the lodging effect on FN was minimal in the present study.

Broad sense heritability was very high (90%) across the five environments, suggesting that the FN is relatively consistent among environments in the absence of PHS. Correlation coefficients among the five individual environments and between the five environments with the BLUP data were high, ranging from 0.43 to 0.91 (Table 3).

Population Structure and its Relation to Geographic Origin

On the basis of the ΔK value of the STRUCTURE results (Evanno et al., 2005), two subpopulations ($K = 2$) were determined. One subpopulation was mainly comprised

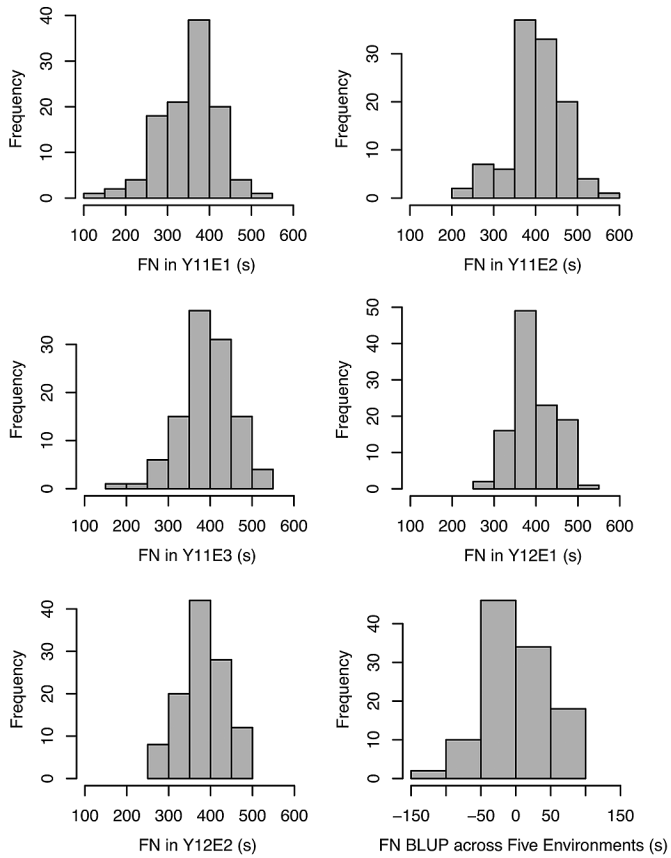

Figure 1. Histogram of Hagberg falling number (FN) in five environments and the best linear unbiased predictors (BLUP) of the 110 hard white spring wheat accessions across five environments: Y11E1, irrigated(E1) in 2011; Y11E2, terminal drought (E2) in 2011; Y11E3, terminal drought and nitrogen deficiency environment (E3) in 2011; Y12E1, E1 in 2012; Y12E2, E2 in 2012; BLUP, best linear unbiased predictors of FN over all 5 environments.

of accessions from south and west Asia (green in Fig. 2) and the other subpopulation was comprised of accessions from several different regions and countries (red in Fig. 2). When the population structure was analyzed by PCA, the first PC explained 14.1% of the genetic variation in FN and the second PC explained 8.8% (Fig. 3). The first PC splits the population into 2 groups, which are consistent with the STRUCTURE result. The first two principal components (PCs) split the population into 3 subgroups (Fig. 3): the first group (G1) is mainly from south Asia, the second group (G2) from west Asia, and the last group (G3) from the rest of the countries (about half in west Asia). Analysis of variance using means of FN from the five environments showed significant differences between the three groups: G2 had significantly higher FN (423 s) than G1 (380 s) and G3 (361 s) on the basis of Honestly Significant Difference test (data not shown). ANOVA of the improvement status of the 110 accessions showed that landraces had significantly higher FN (402 s) than cultivars and breeding lines (about 368 s). In addition, landraces were concentrated in G1 and G2, and cultivars and breeding lines in G3 (Fig. 3).

Table 2. Hagberg falling number (FN) and lodging over five environments for the 24 accessions (ACNO) showing low FN (<300 s) in environment Y11E1.

	FN‡					Lodging§				
ACNO†	Y11 E1	Y11 E2	Y11 E3	Y12 E1	Y12 E2	Y11 E1	Y11 E2	Y11 E3	Y12 E1	Y12 E2
PI165700	*272*	379	402	378	385	9	9	9	7	0
PI183528	*284*	293	275	359	344	7	0	0	0	0
PI294970	*260*	400	428	441	386	8	9	9	4	0
PI366057	*265*	326	355	399	368	9	0	0	0	0
PI366059	*235*	365	323	405	364	7	2	0	0	0
PI623378	*268*	431	423	459	443	9	0	7	0	0
PI623426	*268*	420	458	473	458	9	9	9	9	5
PI624979	*250*	417	460	379	444	9	8	9	0	0
PI625725	*102*	435	311	*277*	404	8	9	9	8	6
PI626003	*169*	420	427	416	406	9	7	8	4	0
Cltr14246	*275*	*276*	321	330	349	0	0	0	0	0
PI201414	*296*	402	359	364	369	0	0	0	0	0
PI314941	*232*	*260*	*285*	338	314	0	0	0	8	3
PI378917	*280*	315	319	361	380	0	0	0	0	0
PI384346	*281*	*288*	*289*	317	325	0	0	0	0	0
PI406529	*255*	*275*	*248*	316	320	0	0	0	0	0
PI428421	*182*	*221*	*194*	*274*	*295*	0	0	0	0	0
PI429318	*293*	*268*	*281*	342	339	0	0	0	0	0
PI445734	*267*	305	309	322	324	0	0	0	0	0
PI520377	*200*	*221*	311	317	316	0	0	0	5	0
PI525284	*287*	375	346	384	*276*	0	0	0	0	0
PI537060	*287*	386	337	360	351	1	6	0	0	0
PI613289	*214*	*296*	*289*	317	*278*	0	0	0	0	0
PI625764	*279*	396	392	396	400	0	7	8	5	0
PI74801	*284*	322	359	363	341	0	0	0	0	0

† The first 10 accessions with FN < 300 sec and lodging score > 6 for environment Y11E1; the last 15 accessions with FN < 300 and lodging score ≤ 6 for environment Y11E1.

‡ FN < 300 s in italic; Y11E1, full irrigation environment (E1) in 2011; Y11E2, terminal drought (E2) in 2011; Y11E3, terminal drought and nitrogen deficiency environment (E3) in 2011; Y12E1, E1 in 2012; Y12E2, E2 in 2012.

§ 0 is no lodging and 9 is completely lodged.

Table 3. Correlation coefficients for Hagberg falling number (FN) for 110 hard white spring wheat accessions grown in five environments.

Environment†	Y11E1	Y11E2	Y11E3	Y12E1	Y12E2
Y11E2	0.64 ‡				
Y11E3	0.66	0.80			
Y12E1	0.57	0.76	0.78		
Y12E2	0.43	0.70	0.67	0.72	
BLUP	0.79	0.91	0.91	0.88	0.80

† Y11E1, full irrigation environment (E1) in 2011; Y11E2, terminal drought (E2) in 2011; Y11E3, terminal drought and nitrogen deficiency environment (E3) in 2011; Y12E1, E1 in 2012; Y12E2, E2 in 2012; BLUP, best linear unbiased predictors of FN over all 5 environments.

‡ All correlation coefficients are significant at $\alpha = 0.001$.

Association Mapping of FN

Using the Bonferroni method to adjust the *p* value of the markers from the GLM (Q model), a total of 48 SNP markers (40 QTL) from 14 chromosomes were significant (adjusted $p < 0.1$) and these markers explained 10 to 20% of total phenotypic variation of FN in the five environments (Supplemental Table S2). Of the 40 QTL associated with FN, three were identified in Y11E1, 16 in Y11E2, 17 in Y11E3, 14 in Y12E1, one in Y12E2, and 17 for the BLUP data. Eleven QTL were identified in more than two environments (Supplemental Table S2). The phenotypic variation explained by the Q matrix was 16.7% for Y11E2, 17.6% for Y11E3, 25.7% for Y12E1, 26.1% for Y12E2, and 19.8% for the BLUP. The Q matrix effect (2%) was not significant for Y11E1.

For the MLM (Q + K model), a total of 57 markers (48 QTL) from 15 chromosomes were significant (unadjusted $p < 0.01$; Supplemental Table S3). Of the 48 QTL, 16 were associated with FN in Y11E1, 13 in Y11E2, 7 in Y11E3, 9 in Y12E1, 15 in Y12E2, and 14 for the BLUP data. Six out of the 48 QTL were identified in more than 2 environments (Supplemental Table S3). However, none of these 57 markers were significant if the FDR ($\alpha = 0.1$) or Bonferroni ($\alpha = 0.1$) criteria were used.

A total of 13 SNP markers on nine chromosomal regions were identified in both Q model and Q + K model (Table 4) and each explained the phenotypic variation from 11% to 20% in different environments (Table 4). Six out of the 13 markers had significant contributions to FN in more than three environments in stepwise multiple regression (data not shown). Using stepwise multiple regressions with the Q matrix as covariance, the six markers together explained the total phenotypic variation of 24.3% for Y11E1, 45.1% for Y11E2, 38.5% for Y11E3, 32.3% for Y12E1, 25.8% for Y12E2, and 43.7% for BLUP (Table 5). On the basis of the consensus SNP map (Cavanagh et al., 2013), the six SNP markers were located on 1B, 2A, 2B, 4B, 6B, and 7B (Table 5).

DISCUSSION

Evaluation of FN in Diverse Environments

The FN has been used in grain grading (Belitz et al., 2004) and is affected by several factors (Wang et al., 2008). It is known that PHS (Wang et al., 2008) and LMA (Mares and Mrva, 2008; Wang et al., 2008) are associated with low FN in harvested grain. The present study focused on the evaluation of FN variation in a diverse group of 110 HWS wheat accessions from the NSGC grown in five environments without separating the effects of PHS and LMA. Our results suggest that FN variation was affected by accession, geographic origin of the accession, and environment.

The present study was conducted in five different environments and the environment effect was significant for FN, but environment Y11E1 was quite different from the other environments, having the lowest mean FN. The low FN for ten of the accessions in Y11E1 was probably related to lodging (Table 2), possibly related to break of seed dormancy due to the high moisture in the lodged plots. The low FN for the other 15 accessions might be caused either by

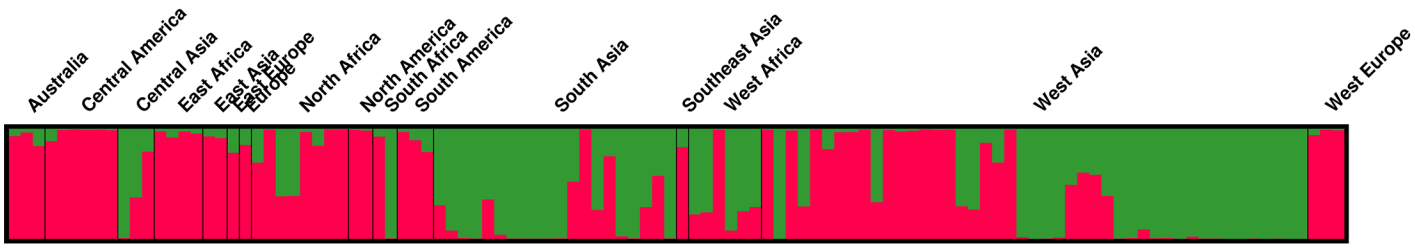

Figure 2. Population structure plot (K = 2) of 110 hard white spring wheat accessions on the basis of 498 single nucleotide polymorphism markers using STRUCTURE 2.3.4 (Pritchard et al., 2000). Each color represents a subpopulation, each vertical bar represents a genotype, and each block represents a geographic origin.

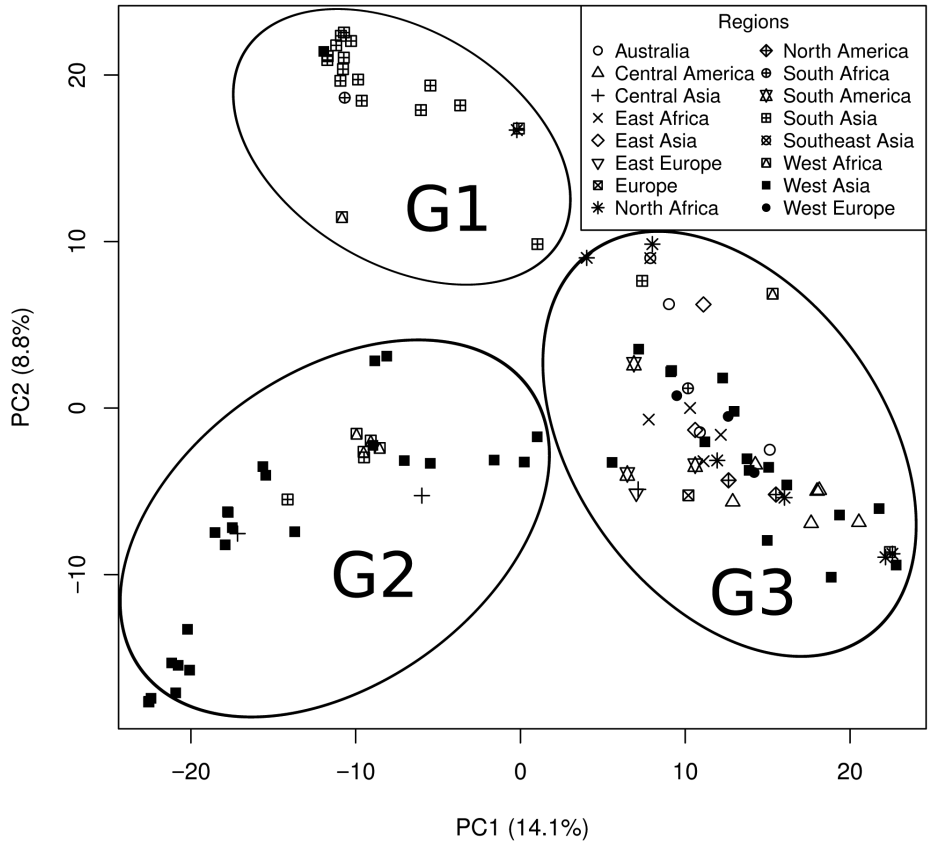

Figure 3. Biplot of principal component analysis using the first two principal components (PCs). The numbers in parentheses are the proportion of variance explained by each PC. Legend shows the geographic origin of each accession. G1, G2, and G3 represent the first, second, and third groups, respectively.

genetic effects (for those showing low FN in several environments) or by the environmental effect of Y11E1 relating to irrigation plus high precipitation in August, 2011.

Effect of Population Structure in Association Mapping

The presence of population structure can influence association mapping results and lead to false positive trait-marker associations (Yu et al., 2006; Zhu et al., 2008). Therefore, the population structure should be analyzed as part of the association studies. In the present study, the population structure was simple because two subpopulations were optimal according to the STRUCTURE results and only two PCs were significant according to the scree plot of the PCA result. Even though the population structure was simple, false positives were greatly reduced after including the Q matrix in the analysis. A total of 720 SNP markers were significant without population structure correction (data not shown), whereas only 81 SNP markers were significant when the Q matrix was included in the analysis (Supplemental Table S2). This conclusion is supported by the large amount of phenotypic variation explained by the Q matrix in each treatment: about 17% for Y11E2 and Y11E3, and about 26% for Y12E1 and Y12E2. However, the Q matrix for Y11E1 was not significant (2% of the phenotypic variation), possibly because accessions with low FN were distributed in all three subpopulations in Y11E1 (data not shown), which reduced the FN difference

Table 4. Markers significantly associated with Hagberg falling number (FN) in both Q model and Q + K model, their chromosomal positions, and associated *p* values.

Marker	Short Name	Chr†	Y11E1‡			Y11E2			Y11E3			Y12E1			Y12E2			BLUP		
			-log10(*p*)			-log10(*p*)			-log10(*p*)			-log10(*p*)			-log10(*p*)			-log10(*p*)		
			Q	Q+K	R^2§	Q	Q+K	R^2	Q	Q+K	R^2	Q	Q+K	R^2	Q	Q+K	R^2	Q	Q+K	R^2
wsnp_Ex_c4808_8584651	IWA4007	1B	-¶	–	–	5.1	–	0.14	5.1	–	0.14	4.5	–	0.11	–	2.0	–	4.7	–	0.12
wsnp_CAP12_c1337_682282	IWA919	1B	–	–	–	4.4	2.4	0.11	–	–	–	–	–	–	–	2.1	–	–	–	–
wsnp_JD_c2578_3489735	IWA5959	2A	–	–	–	5.3	2.0	0.14	5.2	2.2	0.14	4.9	–	0.11	–	2.1	–	6.3	–	0.16
wsnp_CAP12_c197_110707	IWA933	2B	–	–	–	7.7	2.8	0.20	6.9	3.1	0.18	–	–	–	–	–	–	6.2	2.5	0.16
wsnp_Ex_c3130_5789888	IWA3331	3B	–	–	–	–	–	–	–	–	–	–	–	–	4.6	2.9	0.11	–	–	–
wsnp_Ex_c37502_45236634	IWA3615	4B	–	–	–	4.4	2.1	0.11	–	–	–	–	2.4	–	–	–	–	–	–	–
wsnp_BE444644A_Ta_2_1	IWA154	5A	–	2.5	–	–	2.2	–	–	–	–	–	–	–	–	2.6	–	4.9	2.9	0.12
wsnp_BF293311B_Ta_2_1	IWA450	6B	4.3	–	0.14	–	2.2	–	–	–	–	–	2.1	–	–	2.4	–	4.6	2.4	0.12
wsnp_Ex_c34011_42398664	IWA3460	6B	–	–	–	4.8	–	0.12	5.1	–	0.13	–	–	–	–	2.9	–	5.2	–	0.13
wsnp_Ex_c34123_42489621	IWA3464	6B	–	3.1	–	–	–	–	–	–	–	4.5	2.7	0.11	–	–	–	4.7	2.2	0.12
wsnp_Ku_c139_279238	IWA6507	7A	–	–	–	–	–	–	–	–	–	4.7	2.2	0.11	–	–	–	–	–	–
wsnp_CAP11_c639_424134	IWA795	7A	–	–	–	4.3	–	0.11	4.7	2.4	0.12	–	–	–	–	–	–	–	–	–
wsnp_BF291608B_Ta_2_1	IWA436	7B	–	–	–	–	–	–	–	–	–	4.6	2.3	0.11	–	2.3	–	–	2.1	–

† Chr, chromosome.

‡ Y11E1, full irrigation environment (E1) in 2011; Y11E2, terminal drought (E2) in 2011; Y11E3, terminal drought and nitrogen deficiency environment (E3) in 2011; Y12E1, E1 in 2012; Y12E2, E2 in 2012; BLUP, best linear unbiased predictors of FN over all 5 environments.

§ R^2, phenotypic variation explained by the marker in Q model.

¶ '-', not significant.

between groups. In contrast, accessions with low FN almost all came from groups 1 and 3 in the other environments (data not shown).

Association Analysis using Q and Q + K Models

Two models, the Q model and the Q + K model, were used to explore the marker–trait associations in the present study. The Q model used the population structure correction, whereas the Q + K model considered both population structure (Q matrix) and the relative kinship (K matrix) between plant materials. The Q + K model can control the population structure more effectively than the Q model (Yu et al., 2006). To control the probability of false positives, three methods are commonly used: permutation, Bonferroni correction, and FDR correction (Benjamini and Hochberg, 1995; Churchill and Doerge, 1994; Dunn, 1961; Storey, 2002). The Bonferroni method is more conservative than FDR (Benjamini and Hochberg, 1995). Compared with Bonferroni and FDR methods, the permutation method (usually 1000 times) is time consuming. The current study used both Q and Q + K models and the three correction methods. In our results using the Q model, the Bonferroni correction at $\alpha = 0.1$ had almost the same effect as 1000 permutation in controlling the error rate, while FDR correction at $\alpha = 0.05$ gave too many significant markers compared with the other two methods (data not shown). Therefore, the Bonferroni correction at $\alpha = 0.1$ was used to adjust the *p* values in the Q model of association mapping in the present study. In the analysis using the Q + K model, marker *p* values were two or more orders of magnitude lower than those from the Q model (Table 4), so the Q + K model already controlled the false positives effectively compared with Q model (Yu et al., 2006). Therefore, it might be redundant to use the Bonferroni or FDR methods to correct the *p* values in the Q + K model, and doing so will increase the false negative rate and filter most or all of potential positive markers. The potential drawback of using FDR to adjust the *p* values from the Q + K model has been discussed in several previous studies (Ghavami et al., 2011; Jaiswal et al., 2012; Kulwal et al., 2012; Mulki et al., 2013). QTL that were detected in more than one analysis or in more than one environment are most likely to be true QTL, as suggested by Moncada et al. (2001).

Comparisons with Previous Studies

Few previous studies have been conducted for FN in the absence of the PHS effect. Most studies focused on PHS and, more recently, on LMA, but it is possible that there are some shared QTL for FN, PHS, and LMA due to their close relationship. Quantitative trait loci for PHS have been mapped on all 21 chromosomes of

Table 5. Total phenotypic variation (R^2) explained by six single nucleotide polymorphism markers that had significant contribution in more than three environments.

Marker	Short Name	Chr†	Y11E1‡	Y11E2	Y11E3	Y12E1	Y12E2	BLUP
wsnp_Ex_c4808_8584651	IWA4007	1B		x	x	x	x	x
wsnp_JD_c2578_3489735	IWA5959	2A	x§	x	x	x	x	x
wsnp_CAP12_c197_110707	IWA933	2B		x	x	x	x	x
wsnp_Ex_c37502_45236634	IWA3615	4B	x	x	x	x		x
wsnp_BF293311B_Ta_2_1	IWA450	6B	x	x	x	x	x	x
wsnp_BF291608B_Ta_2_1	IWA436	7B		x		x	x	x
Total R^2 (%)¶			24.3	45.1	38.5	32.3	25.8	43.7

† Chr, chromosome.

‡ Y11E1, full irrigation environment (E1) in 2011; Y11E2, terminal drought (E2) in 2011; Y11E3, terminal drought and nitrogen deficiency environment (E3) in 2011; Y12E1, E1 in 2012; Y12E2, E2 in 2012; BLUP, best linear unbiased predictors of FN over all 5 environments.

§ x indicates the marker was kept in the model of the stepwise multiple regression.

¶ Phenotypic variation explained by all markers kept in the model on the basis of multiple regression analysis.

bread wheat, but have been located mainly on chromosomes 2B, 2D, 3A, 3B, 3D (the red kernel color gene "*R*" and the viviparous gene *TaVp1*), and 4AL ("*Phs*" gene and seed dormancy; Jaiswal et al., 2012; Kulwal et al., 2012). QTL for LMA have been identified in chromosomes 7BL and the centromeric regions of chromosomes 3B (Emebiri et al., 2010; Mrva and Mares, 2001, 2002) and 6B (possibly corresponding to the α-*Amy-B1* gene; Emebiri et al., 2010). For FN, QTL have been reported on 1B, 2D, 3A, 3B, 3D, 4A, 4B, 7BS, 7BL (possibly corresponding to the α-*Amy-B2* gene), and 6B (possibly corresponding to the α-*Amy-B1* locus; Fofana et al., 2009; Kunert et al., 2007; Rasul et al., 2009). From these studies it seems the *Rht* gene on 4B and the α-*Amy-B1* gene on 6B had effects on both LMA and FN without effects from sprouting and LMA. Gibberellin insensitive *Rht* alleles (*Rht-B1b*, *Rht-B1c*, *Rht-D1b*, *Rht-D1c*) could reduce the incidence of PHS and LMA, reduce the grain α-amylase activity, and increase FN (Flintham et al., 1997; Gooding et al., 1999; Mares and Mrva, 2008).

Saintenac et al. (2013) developed a reference map that integrated SNPs from the 9K iSelect assay, diversity array technology markers, simple sequence repeat markers, and genotyping-by-sequencing markers. By aid of this reference map, several QTL identified in the current study were found to be close to previously-reported QTL related to PHS and LMA. Marker *wsnp_Ex_c3253_5995011* (*IWA3399*) on chromosome 1A identified in GLM is about 4 cM from a seed-dormancy QTL (linked to marker *wPt-4765*) reported by Singh et al. (2010). Marker *wsnp_CAP12_rep_c4278_1949864* (*IWA1007*) on chromosome 4B identified in MLM was about 7 cM from a FN QTL (linked to marker *Xgwm113*) reported by Kunert et al. (2007), which might be corresponding to the semi-dwarf gene *Rht-B1* (Kunert et al., 2007). Marker *wsnp_Ex_c18654_27528399* (*IWA2335*) identified in MLM was about 5 cM from one seed-dormancy QTL on 5B (linked to markers *wPt-4936* and *Xwmc415*) reported by Singh et al. (2010). Marker *wsnp_BF293311B_Ta_2_1* (*IWA450*) on chromosome 6B identified in MLM was about 6 cM from one seed-dormancy QTL (linked to marker *Xgwm518*) reported by Singh et al. (2010), and one LMA QTL (linked to marker *wPt-1730*, might be an α-amylase gene) reported by Emebiri et al. (2010). Marker *wsnp_BF483648B_Ta_2_1* (*IWA507*) on chromosome 7B identified in MLM was colocated with a PHS QTL (linked to marker *wPt-8283*) reported by Kulwal et al. (2012). Cavanagh et al. (2013) found that the LD between neighboring SNPs was on average 5.5 cM in cultivars and 4.9 cM in landraces, but the rate of LD decay varied by location within the genome. Therefore, the significant SNPs close to the reported QTL related to PHS or LMA are possibly the same loci. We were unable to accurately place SNPs not found in the reference map for comparison with previous studies, but chromosome locations of each significant SNP are noted in Supplemental Table S2 and S3.

In addition, after checking the USDA wheat SNP database (USDA wheat SNP database, 2014), marker *wsnp_JD_c2578_3489735* (*IWA5959*) on chromosome 2A was associated with an *Arabidopsis thaliana* serine/threonine protein kinase mRNA; marker *wsnp_Ex_c34011_42398664* (*IWA3460*) on chromosome 6B was associated with *T. aestivum* cryptochrome 2 (*Cry2*) mRNA; marker *wsnp_Ex_c34123_42489621* (*IWA3464*) on chromosome 6B was associated with *T. aestivum* betaine-aldehyde dehydrogenase (BADH) mRNA; and marker *wsnp_Ra_c32055_41111615* (*IWA7860*) on chromosome 3B (identified in Y11E2, Y11E3, and BLUP data in Q model only) was associated with *T. aestivum* boron transporter 2 mRNA. Other markers were associated with unknown mRNAs. Mares and Mrva (2008) mentioned that the LMA QTL on 7B (Emebiri et al., 2010; Mrva and Mares, 2001) was possibly linked in repulsion to a boron tolerance QTL, *Bo1* (Jefferies et al., 2000). Cresswell and Nelson (1973) also found that the RNA level and α-amylase activity in the embryo and endosperm tissue of dormant 2-mo-old kangaroo grass (*Themeda triandra* Forssk.) seed were increased markedly by the presence of boron in the

germination medium. Thus, boron might play an important role in the α-amylase synthesis, and the QTL (marker *wsnp_Ra_c32055_41111615*) on chromosome 3B might be the effect of *T. aestivum* boron transporter 2.

Potential Use of QTL Identified in the Current Study

A total of 13 QTL (SNP markers) associated with FN were identified in both the Q and the Q + K models in the current study (Table 4). Of the 13 QTL identified, six on 1B, 2A, 2B, 4B, 6B, and 7B were associated with FN in three to five environments and the BLUP, and the six QTL together explained 24.3 to 45.1% of the total phenotypic variation in the five environments (Table 5). These QTL may be useful in marker-assisted selection for FN in early generation breeding materials. To determine whether the six QTL identified in the current study relate to PHS or LMA, additional research is needed to characterize the 110 accessions in controlled environments and using specific screening methods for PHS and LMA.

CONCLUSION

The plant materials in the current study showed significant variation for FN without the effect of preharvest sprouting. We identified 13 QTL associated with FN and some of these had similar chromosomal locations with previously-reported QTL associated with PHS and LMA. The six major QTL together can explain up to 45% of FN variation, and can be potentially used in marker-assisted selection for plants with high FN in HWS wheat breeding. Further studies will validate these QTL and associate them with specific factors that affect FN.

Supplemental Information Available

Supplemental information is included with this article.

Supplemental tables provide information for geographic origin and cultivating status of the 110 hard white spring wheat accessions used in the current study and all significant markers identified in the general linear model (GLM, Q model) and in the mixed linear model (MLM, Q + K).

Supplemental Table S1. Geographic origin and cultivating status of the 110 hard white spring wheat (HWS) accessions.

Supplemental Table S2 Significant markers identified in general linear model (GLM, Q model) at $\alpha = 0.1$ after Bonferroni correction of the *p* values.

Supplemental Table 3 Significant markers identified in the mixed linear model (MLM, Q+K) at $\alpha = 0.01$

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