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Effects of Genotype, Season, and Nitrogen Nutrition on Gene Expression and Protein Accumulation in Wheat Grain

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Supporting Information

ABSTRACT: Six commercial U.K. cultivars of winter wheat selected to represent different abilities to partition nitrogen into grain protein were grown in replicated field trials at five different sites over three seasons. The proportion of LMW glutenin subunits decreased and the proportion of gliadins increased during grain development and in response to N application. Differences were observed between the proportions of LMW glutenin subunits and gliadins in low- and high-protein grain, these two fractions being decreased and increased, respectively. There was little effect of grain protein content on the proportions of either the HMW glutenin subunits or large glutenin polymers, which are enriched in these subunits, with the latter increasing during development in all cultivars. The proportion of total protein present in polymers in the mature grain decreased with increasing N level. Correlations were also observed between the abundances of gliadin protein transcripts and the corresponding proteins.

KEYWORDS: *wheat, grain quality, grain protein, flour functionality*

INTRODUCTION

The relationship between fertilizer inputs and harvest outputs is of vital importance for all crops, particularly in the context of increasing pressure to reduce nitrogen usage. The availability of nitrogen in the environment has an effect on the protein content and composition of wheat, which in turn affects the processing properties. The application of nitrogen fertilizer is required to achieve the 13% dry weight protein specification for breadmaking wheat in the U.K. Such applications are costly and may have a negative environmental footprint. There is therefore a need to develop new varieties that are more efficient in their uptake and utilization of nitrogen. Several studies have identified differences in the nitrogen use efficiency of modern and older cultivars, including high-quality breadmaking cultivars such as Xi19, Hereward, and Malacca.[1] Furthermore, whereas an inverse correlation is generally observed between the yield and grain protein content of bread wheat,[2] some cultivars consistently accumulate higher protein contents than would be expected based on their yields. These have been defined as exhibiting grain protein deviation[3] and may provide a basis for the future development of wheat cultivars that require less nitrogen fertilization to achieve acceptable protein contents for breadmaking. However, protein content is only one factor affecting processing quality, with protein quality (composition) also being important. It is, therefore, important to understand the relationship between N nutrition, grain protein content, grain protein composition, and processing properties.

The relationships between genetically determined differences in gluten protein composition and grain processing properties are well established.[4,5] However, wheat grain quality is also known to be highly sensitive to environmental conditions, although the basis for these effects is poorly understood.[6] Unravelling the interactions between genotype, environment, and nutrition is essential to facilitate the development of new wheat varieties having high stability to environmental fluctuations and, in particular, to maintain quality across a range of nitrogen inputs.

This paper describes multisite trials of six wheat cultivars with different levels of nitrogen over three years, to investigate the content and composition of gluten storage proteins during development and at maturity (using SDS-PAGE and size exclusion HPLC). Furthermore, transcriptome profiling of the developing caryopses at 21 days post-anthesis allowed the gliadin protein accumulated to be related to the expression levels of the corresponding genes.

MATERIALS AND METHODS

Growth and Harvesting of Wheat. Five U.K. breadmaking wheat cultivars (Cordiale, Hereward, Malacca, Marksman, and Xi19) and one feed wheat cultivar (Istabraq) were grown in randomized block with three-blocks (replicated) field trials in 2009, 2010, and 2011 at five U.K. sites: Rothamsted Research (Harpenden, Hertfordshire), RAGT (Ickleton, Cambridgeshire), KWS (Thriplow, Cambridgeshire), Syngenta (Duxford, Cambridgeshire), and Limagrain (Woolpit, Bury St Edmunds, Suffolk). Nitrogen was applied at three levels (100, 200, and 350 kg/ha in total, split in up to three applications) before anthesis. Mature grain was sampled in all years. In 2009 and 2010 at Rothamsted, main stem ears were tagged at anthesis and whole

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Table 1. Protein Composition (SDS-PAGE and SE-HPLC) and Gene Expression Data Present for All Six Cultivars (Cordiale, Hereward, Istabraq, Malacca, Marksman, and Xi19) for Combinations of Sites, Years, Time Points and N Levels[a]

analysis	year	site	N levels (kg/ha)			time points (DPA[b])				
protein composition[c]	2009	Rothamsted	100	200	350	21	28	35	42	M[d]
	2010	Rothamsted	100	200	350	21	28	35	42	M
		RAGT	100	200	350	21[e]				M
		Limagrain	100	200	350					M
		KWS	100	200	350					M
		Syngenta	100	200	350					M
	201	Rothamsted	100	200	350	21[e]				M
		RAGT	100	200	350	21[e]				M
		Limagrain	100	200	350					M
		KWS	100	200	350					M
		Syngenta	100	200	350					M
gene expression[f]	2009	Rothamsted	100	200	350	21				
	2010	Rothamsted	100	200	350	21				

[a]The sites were Rothamsted Research (Harpenden, Hertfordshire), RAGT (Ickleton, Cambridgeshire), KWS (Thriplow, Cambridgeshire), Syngenta (Duxford, Cambridgeshire), and Limagrain (Woolpit, Bury St Edmunds, Suffolk). [b]Days post-anthesis. [c]Nitrogen SDS-PAGE data: three biological replicates, two technical replicates; SE-HPLC data: one biological replicate, one technical replicate. [d]M = maturity. [e]21 DPA samples for 200 kg N/ha only. [f]Gene expression data: three biological replicates, one technical replicate.

caryopses were harvested from the middle part of the ear at 21, 28, 35, and 42 days post-anthesis (DPA). In 2010 and 2011, caryopses were harvested at 21 DPA from the RAGT site.

Total Nitrogen Determination. Total nitrogen was determined in mature grain using the Dumas combustion method,[7] using a CNS (carbon, nitrogen, sulfur) combustion analyzer (Leco Corp., St. Paul, MN, USA).

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples (35 mg) of wholemeal flour milled in a hand-operated grinder were extracted with 1 mL of 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 1.5% (w/v) dithiothreitol, 10% (v/v) glycerol, and 0.002% (w/v) bromophenol blue. Gels (NuPAGE 12% Bis-Tris, Life Technologies, Paisley, UK) were stained with Coomassie brilliant blue and then scanned and analyzed using Total Lab TL120 version 2006F (Nonlinear Dynamics, Newcastle upon Tyne, UK) with an optical density curve calibrated using a Kodak T14 control scale (Tiffen, LLC, Rochester, NY, USA). Values for band optical density and band percent as a proportion of the total lane optical density were analyzed. Analysis was carried out for three biological replicates and two technical replicates of each gel that were run together on the same day and stained and destained together.

SE-HPLC. Size exclusion high-performance liquid chromatography (SE-HPLC) was used to determine the protein polymer size distribution of white flour samples milled using a Chopin CD 1 laboratory mill (Chopin Technologies, Villeneuve-la-Garenne Cedex, France). The analysis was performed according to the Profilblé method developed jointly by ARVALIS and l'Institut National de Recherche Agronomique.[8] Flour (160 mg) was mixed with 20 mL of 1% SDS (w/v) in 0.1 M phosphate buffer (pH 6.9), sonicated (Misonix Microson XL2000, Qsonica, LLC, Newtown, CT, USA) to solubilize the polymeric gluten proteins, and then centrifuged for 10 min at 4500*g*. An aliquot of the supernatant was sealed in a HPLC vial ready for analysis. SE-HPLC was conducted using a Jasco (Jasco Ltd, Great Dunmow, Essex, UK) system operating with a TSK gel G 4000SW column (30 cm × 7.5 mm) and a TSK gel SK guard column (7.5 cm × 7.5 mm). The flow rate was 0.7 mL/min, and detection was performed at 214 nm. Samples from the three biological replicates were pooled prior to analysis. The chromatograms were integrated using a combination of automated algorithms and manual rules developed as part of the Profilblé method. Peak ratios were calculated as reported by Millar (2003).[9]

RNA Extraction. Twenty-one DPA caryopses for RNA extraction were frozen at −80 °C until required. RNA was extracted using a method based on Chang et al. (1993).[10,11] About 1.5 g of whole caryopses was ground in a cooled mill, and RNA was extracted in CTAB buffer (2% CTAB, 2% PVP K30, 100 mM Tris-HCl, pH 8.0, 25 mM EDTA, 2.0 M NaCl, 0.5 g/L spermidine, 2% (w/v) 2-mercaptoethanol) with chloroform/isoamyl alcohol (24:1) to remove proteins. The RNA was precipitated by incubation with 10 M LiCl on ice overnight and then dissolved in SSTE buffer (1.0 M NaCl, 0.5% (w/v) SDS, 10 mM Tris HCl pH 8.0, 1 mM EDTA) to remove polysaccharides and extracted once with chloroform/isoamyl alcohol. After ethanol precipitation, total RNA was dissolved in DEPC-treated water and stored at −80 °C.

Gene Expression Profiling. Transcriptome profiling was performed using the Affymetrix GeneChip Wheat Genome Array and the standard one-cycle cDNA synthesis protocol and hybridization (as described in the GeneChip Expression Analysis Technical Manual) at the University of Bristol. Transcriptome data were analyzed using the GeneSpring version 11 (Agilent Technologies Inc., Santa Clara, CA, USA) package. The standard workflow was followed for two-factor experiments (nitrogen level, variety). Gene expression analysis was carried out for each of three biological replicates of whole caryopses harvested at 21 DPA from one site (Rothamsted Research) only, in 2009 and 2010.

Statistical Methods. Protein composition and gene expression data were determined for all six cultivars for each of different combinations of sites, years, time points, and N levels, as described in Table 1.

SDS-PAGE data were analyzed by residual maximum likelihood (REML) using GenStat (14th edition, VSN International Ltd, Hemel Hempstead, UK) to fit a linear mixed model consisting of random (design) and fixed (treatment) effects. The main effects and interactions between cultivar, N level, and time point factors formed the fixed part of the model. The effects of sites and years and further nested effects relating to blocking at the field (blocks and plots) and laboratory (gels and lanes within gels) stages were taken as design features, so that results for the combinations of cultivar, N level, and time point were effectively without issues of unequal representation across different sites and years. Data were subjected to $\log_e$ transformation to ensure a Normal distribution and homogeneity of variance. Comparisons of predicted means of statistical ($p < 0.05$, F-test) relevance from the model were made using estimated standard error of the difference values on the corresponding degrees of freedom, thus providing least significant difference (LSD) values at the 5% ($p = 0.05$) level of significance.

SE-HPLC data were transformed by using a $\log_e$-centering transformation[12] due to their compositional nature. To give an overall description and to afford a separation of the profiles of the samples in the absence of biological replication due to pooling, a multivariate method, principal coordinates analysis (PCO),[13] was applied.

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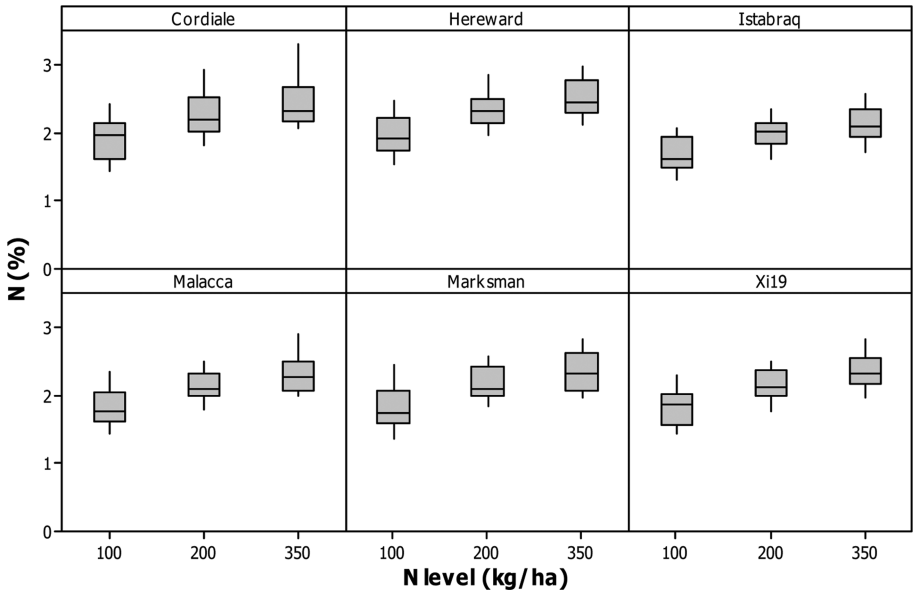

Figure 1. Box plots of the wholegrain N content (%) at maturity for each of six wheat cultivars grown at three different levels of N fertilization (100, 250, or 350 kg N/ha. Data displayed represent the distribution of means of samples collected over combinations of three years and five sites with three biological replicates (N = 594 in total, n = 11 per box plot).

Following regression analysis of the original variables on the two coordinate vectors (PCo1 and PCo2) retained, the variables were ranked, via the regression F-statistics, in order of the importance of their contribution to the principal coordinate vectors and therefore to any discrimination seen in the two dimensions. The means for the SE-HPLC data across combinations of years and sites were calculated to allow visualization of general trends over development, with standard error based on variation across years and sites.

Gene expression values were subjected to $\log_e$ transformation to ensure a Normal distribution and homogeneity of variance. The data were analyzed by REML using a model with the main effects and interaction between cultivar and N level as the treatment structure and with the field blocking (blocks and plots) nested within the two years (2009 and 2010) as the design structure.

Pearson correlations between gene expression data for the Rothamsted Research site at 21 DPA in the two years (2009 and 2010) and for all varieties and N levels, with the protein variables from either SDS-PAGE (with replication) or SE-HPLC (without replication), were calculated and tested for significance (F-tests).

RESULTS

Determination of Protein Content and Composition. The percentage N content of the wholegrain samples increased with increasing N fertilization for all varieties, from a mean of 1.83% (approximately 10.43% protein, conversion factor = 5.7) at 100 kg N/ha to 2.34% (approximately 13.34% protein) at 350 kg N/ha (Figure 1). Istabraq had the lowest protein content and Hereward the highest at all N-fertilization levels.

The content and composition of gluten proteins in the samples were determined using both SDS-PAGE and SE-HPLC.

SDS-PAGE Analysis. Quantitative gel scanning of SDS-PAGE separations of fractions extracted under reducing conditions was used to determine the proportions of bands corresponding to the major groups of gluten protein subunits: the HMW subunits of glutenin (HMW GS), LMW subunits of glutenin (LMW GS), and gliadins. Combining data from the SDS-PAGE analyses of samples from three years and five sites (with three biological and two technical replicates of each) showed broadly similar patterns for these protein groups for the six cultivars, but with some statistically significant (p < 0.05, LSD) differences (Figure 2).

The HMW GS (Figure 2A) accounted for between about 8% and 18% of the total gluten protein fraction, depending on the cultivar, stage of development, and N level (p = 0.001, F-test). Generally, the proportions of HMW GS were not strongly influenced by the N level. However, Marksman contained the highest proportion of HMW GS at all N levels and also showed a small overall increase in response to N. The LMW GS (Figure 2B and C) and gliadins (Figure 2D and E) were the major groups, accounting for between about 45% and 55% and between 30% and 40% of the total gluten protein fraction, respectively, at 21 DPA. These two fractions decreased or increased, respectively, as a proportion of the total protein during grain development.

The significance (p-values) of terms in the model fitted using REML are given in Table 2. For gliadins, the interactions between cultivar and time point and between N level and time point were significant (p < 0.001, F-test), but the interaction between cultivar and N level was not significant (p = 0.153, F-test), indicating that each cultivar responded in a similar way to increased N fertilization. During development the proportion of gliadins increased (means are presented in Figure 2D and E), with the increase being greater in Cordiale, Marksman, and Xi19 than in the other three cultivars. For all cultivars, there was a small increase between 28 and 35 DPA compared to rapid changes between 21 and 28 DPA (particularly at 200 kg N/ha) and between 35 and 42 DPA. Malacca contained the lowest proportion of gliadins throughout development and at maturity. The proportion of gliadins was lowest at the lowest N level, with little difference between the proportions at 200 and 350 kg N/ha.

The changes observed for LMW GS were the inverse of those observed for the gliadins, which is to be expected, as these two groups together accounted for over 80% of the total fraction. Thus, the interactions between cultivar and time point and between N level and time point were significant (p < 0.001, F-test), but the interaction between cultivar and N level was not

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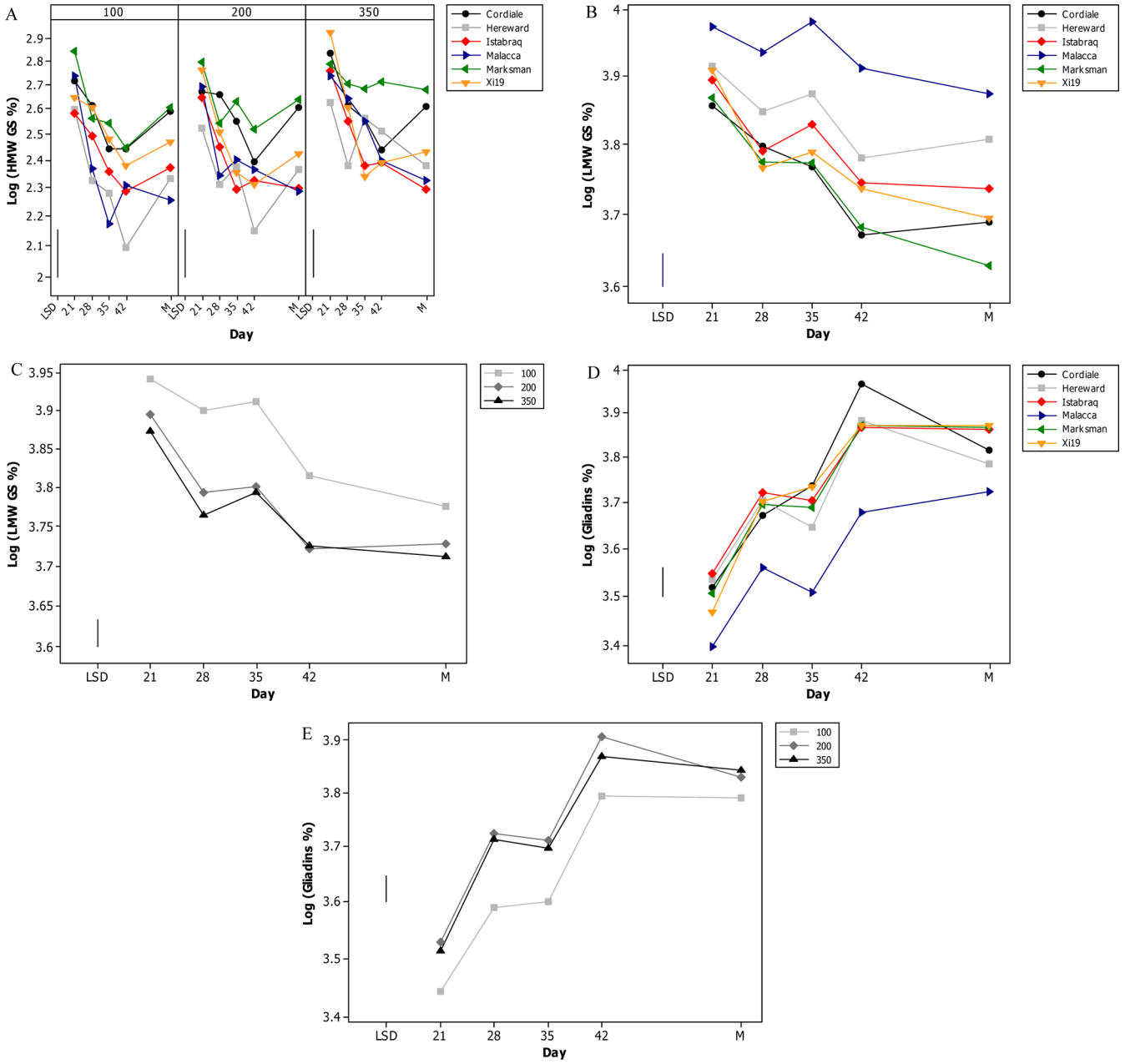

Figure 2. Log_e of the proportion of HMW glutenins (A), LMW glutenins (B, C), and gliadins (D, E) as a percentage of gluten protein in six wheat cultivars grown at three different levels of N fertilization (100, 200, or 350 kg N/ha). Data represent means of samples collected over combinations of three years and five sites with three biological replicates and two technical replicates (N = 1871 in total, n = 11 per mean). LSD (5%) bars are shown to allow comparison between means.

Table 2. Significance (F-Statistics and *p*-Values) of the Main Effects and Interactions between the Three Factors Cultivar, Time Point, and N Level for SDS-PAGE Analysis (log_e-Transformed Data)

	source					
	HMW GS		LMW GS		gliadins	
	F	*p*-value	F	*p*-value	F	*p*-value
time point	76.90	<0.001	143.50	<0.001	314.24	<0.001
cultivar	124.79	<0.001	204.75	<0.001	84.26	<0.001
N level	13.10	<0.001	91.84	<0.001	58.04	<0.001
time point·cultivar	5.41	<0.001	5.60	<0.001	5.43	<0.001
time point·N level	3.68	<0.001	4.84	<0.001	4.51	<0.001
cultivar·N level	2.94	0.001	0.90	0.532	1.46	0.153
time point·cultivar·N level	1.86	0.001	0.59	0.981	1.18	0.207

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significant (p = 0.532, F-test). A rapid decrease occurred between 21 and 28 DPA followed by little change between 28 and 35 DPA and a further rapid decrease between 35 and 42 DPA (Figure 2B). Also, Malacca contained a much higher proportion of LMW GS compared to the other cultivars. Finally, the proportion of LMW GS was highest at the lowest N level with little difference between the 200 and 350 kg N/ha levels (Figure 2C).

Consistent differences across years and sites (data not shown) were observed between Cordiale and Marksman (high proportions of HMW GS and low proportions of LMW GS) and Malacca (high LMW GS and low gliadins).

SE-HPLC Analysis. SE-HPLC was used to separate the total grain proteins (monomers and polymers) based on their size distribution, without reduction of disulfide bonds, with five peaks being recognized.[8] The first peak to elute from the column is referred to as F1 and consists of HMW polymers enriched in HMW GS, while the F2 peak comprises LMW polymers and is enriched with LMW GS. The F3 and F4 peaks are composed principally of ω-gliadins and α-, β-, and γ-gliadins, respectively, and the F5 peak comprises LMW proteins including albumins and globulins. The total area under the trace is a measure of the total protein content of the flour and is termed AT.

The SE-HPLC data were analyzed by principal coordinates analysis. The first two PCos accounted for 86.03% (63.82 and 22.21% for PCo1 and PCo2, respectively) of the variation in the distance matrix formed from the similarities between samples, so these two were retained and the data are visualized in Figure 3, with developing and mature samples highlighted in Figure 3A and N treatments highlighted in Figure 3B. The F-statistics resulting from relating the two PCos to the variables are shown in Table 3.

F1 was most important for the separation on the PCo1 axis, followed, to a lesser extent, by F2, F3, and F5, with the mature samples being clearly separated from the immature samples (Figure 3A). F4 and total gluten protein (F1+F2+F3+F4) were important for separation on the PCo2 axis, with the coordinate generally increasing with N level for each year by site by cultivar combination (Figure 3B).

Figure 4 shows changes in the proportions of polymeric gluten proteins, calculated as F1+F2 divided by F3+F4. At all N levels and for all varieties the proportion increased as the grain developed until 28 DPA, followed by a decrease between 28 and 35 DPA and a final increase between 35 DPA and maturity. Istabraq generally had the lowest proportion of polymeric gluten proteins and Hereward the highest. The proportion of polymeric proteins decreased with increasing N level at maturity.

The protein composition also differed between varieties, and these differences were consistent across sites and years (data not shown). Averaging over the site by year combinations, Istabraq had the lowest proportion of the F1 fraction (12.2–12.5% at maturity), while the highest proportion of the F2 fraction and the lowest proportion of the F5 fraction were in Malacca (F2: 25.4–25.5%; F5: 35.7–38.5% at maturity) and Hereward (F2: 25.4–25.9%; F5: 36.4–39.3% at maturity). The lowest proportion of the F3 fraction was in Cordiale (7.1–7.5% at maturity) and Xi19 (7.1–7.4% at maturity).

Gene Expression. A total of 55 probe sets on the arrays were identifiable as corresponding to gluten protein transcripts. However, because the gluten proteins showed high polymorphism both within and between cultivars, not all of the probe sets corresponded to genes present within each cultivar. For example, six probe sets corresponded to HMW GS, for genes encoding HMW GS 1Dx3, 1Dx5, 1Bx7, 1Bx14, 1By9, and 1By16, while the HMW subunits expressed in the six cultivars are 1Ax1, 1Bx14, 1By15, 1Dx5, and 1Dy10 in Marksman, 1Bx7, 1By9, 1Dx3, and 1Dy12 in Hereward, 1Ax1, 1Bx17, 1By18, 1Dx5, and 1Dy10 in Cordiale, 1Bx17, 1By18, 1Dx2, and 1Dy12 in Malacca, 1Bx14, 1By15, 1Dx5, and 1Dy10 in Xi19, and 1Bx7, 1By9, 1Dx2, and 1Dy12 in Istabraq (Supplementary Figure S1). Similarly, a high degree of sequence identity within other families of gluten proteins would be expected to have resulted in cross-hybridization with related transcripts. Therefore, Figure 5 shows the mean expression profiles of individual transcripts related to HMW GS (six elements), LMW GS (nine elements), and gliadins (40 elements).

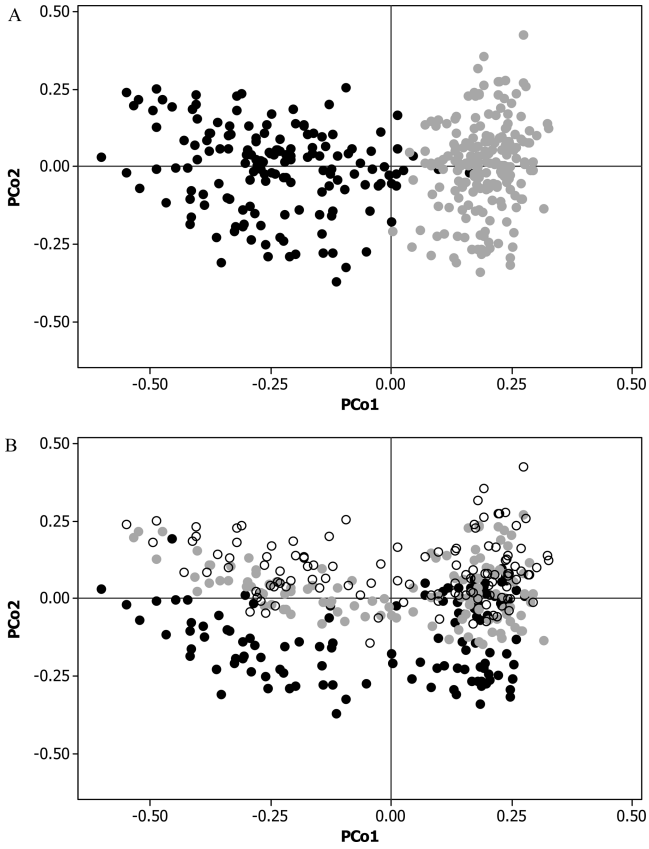

Figure 3. Coordinates for each sample determined by SE-HPLC for the first two PCos (explaining 63.82% and 22.21% of the variation in the distance matrix, respectively) showing the separation of samples according to time point (A; black = immature, gray = mature) or N level (B; black = 100 kg N/ha, gray = 200 kg N/ha, white = 350 kg N/ha).

Table 3. F Statistics from Relating the Two PCos to the ($\log_e$-Transformed) SE-HPLC Variables

variable	PCo1	PCo2
F1	3050.7	1.4
F2	1475.0	0.8
F3	1468.6	37.6
F4	9.0	979.2
F5	1065.5	80.7
gluten protein (F1+F2+F3+F4)	16.4	858.1

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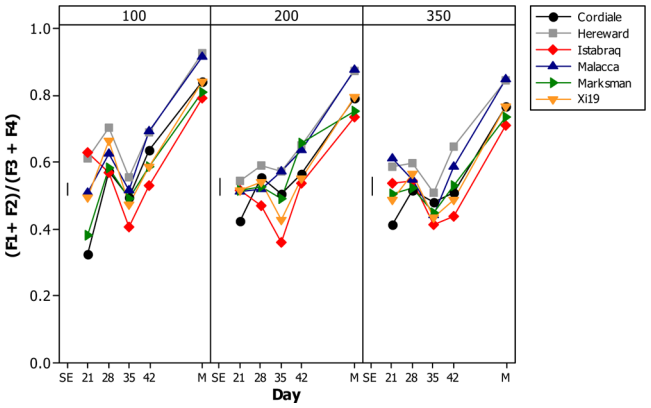

Figure 4. Ratio of polymeric (F1+F2) to monomeric (F3+F4) protein analyzed by SE-HPLC for six wheat cultivars and three levels of N fertilization (100, 200, or 350 kg N/ha). Data are means across year and site combinations. As the data are not replicated, the SEMs are based solely on the variation across years and sites (N = 354 in total, n = 11 per mean). The average SEM is shown as a vertical bar for convenience.

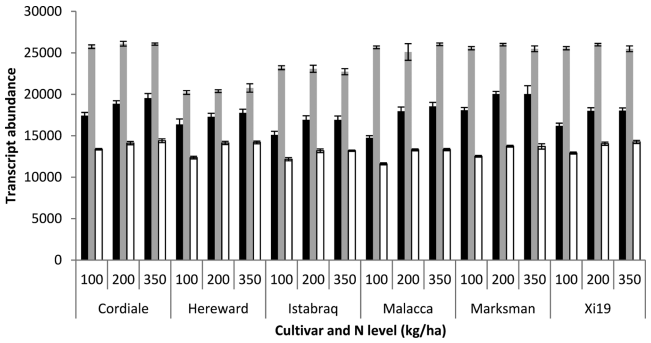

Figure 5. Gene expression values for the mean of all HMW GS (black)-, LMW GS (gray)-, and gliadin (white)-related genes for six wheat cultivars grown at one site (Rothamsted Research) only. Data are from microarray analysis and represent means of samples collected over two years with three biological replicates (N = 108 in total, n = 6 per mean). Error bars represent standard error. See Supporting Information for tables of means for statistical comparison using relevant LSD (5%) values for data on the $\log_e$-transformed scale.

The significance levels (p-values) of the main effects and the interactions between the two factors cultivar and N level are shown in Table 4 (after accounting for sources of design variation). There were independent effects of N level and cultivar (p < 0.001, F-test) for gene expression corresponding to HMW GS, which differs from the SDS-PAGE results for HMW GS, where the cultivar by N level interaction was significant. The relative levels of hybridization to the probe sets suggested that Marksman had the highest level of gene expression, but did not differ significantly (p < 0.05, LSD) from Cordiale (Supplementary Table S1). The other four cultivars appeared to have somewhat lower levels of gene expression, with Istabraq having the lowest. A trend of increasing HMW GS gene expression with increasing N was seen (Supplementary Table S1).

For LMW GS gene expression, only the main effect of cultivar was significant (p < 0.001, F-test). Cordiale, Malacca, Marksman, and Xi19 showed the highest level of LMW GS gene expression and were not significantly (p < 0.05, LSD) different from one another, while Hereward had by far the lowest level (Supplementary Table S2). There was a significant variety by N interaction (p = 0.022, F-test) for gliadin gene expression, which appeared to be lowest for Istabraq and Malacca (Supplementary Table S3). However, the validity of comparisons between cultivars can be questioned, as they may differ in the extent to which the gluten protein genes present correspond to the sequences present in the probe sets. This would result in stronger hybridization signals, indicating greater gene expression.

By contrast, comparisons of hybridization intensity within cultivars are valid, and these all showed significant increases in gene expression with increasing N. For gliadin-related gene expression, Cordiale appeared to display a more linear response with respect to increasing N than the other varieties, having the greatest hybridization intensity of all of the cultivars at 100 kg N/ha. Cordiale was also the only cultivar that showed a marked increase in gliadin gene expression between 200 and 350 kg N/ha (Supplementary Table S3).

Correlation of Gene Expression Data with Protein Composition. The proportions of gliadins measured by SDS-PAGE were significantly correlated with the corresponding data for average gliadin-related gene expression in both 2009 (r = 0.335, p = 0.013, n = 54, F-test) and 2010 (r = 0.372, p = 0.006, n = 54, F-test) (Figure 6A). In particular, the proportions of gliadin proteins and the gene expression were lowest at 100 kg N/ha, with the 200 and 350 kg N/ha samples having higher proportions of gliadin protein but more variable gliadin gene expression. Similarly, gliadin gene expression was positively correlated with the proportion of gliadin proteins measured by SE-HPLC (F4 fraction) (2009: r = 0.641, p = 0.004, n = 18, F-test; 2010: r = 0.768, p < 0.001, n = 18, F-test) (Figure 6B). No significant (p < 0.05, F-test) correlations were observed between glutenin subunit (HMW or LMW) gene expression and the proportions of the proteins determined by SDS-PAGE, but HMW subunit gene expression showed a negative correlation with the proportion of large glutenin polymers determined by SE-HPLC (Figure 6C) (2009: r = −0.387, p = 0.112, n = 18, F-test; 2010: r = −0.637, p = 0.004, n = 18, F-test), presumably because this smaller fraction, when expressed as a percentage of AT, is diluted by the increasing amounts of F4.

Table 4. Significance (F-Statistics and p-Values) of the Main Effects and Interactions between the Two Factors Cultivar and N Level for $\log_e$ Gene Expression Analysis

	source					
	HMW GS		LMW GS		gliadins	
	F	p-value	F	p-value	F	p-value
cultivar	19.88	<0.001	132.74	<0.001	27.80	<0.001
N level	45.66	<0.001	0.16	0.853	137.78	<0.001
cultivar·N level	1.48	0.163	0.93	0.507	2.24	0.022

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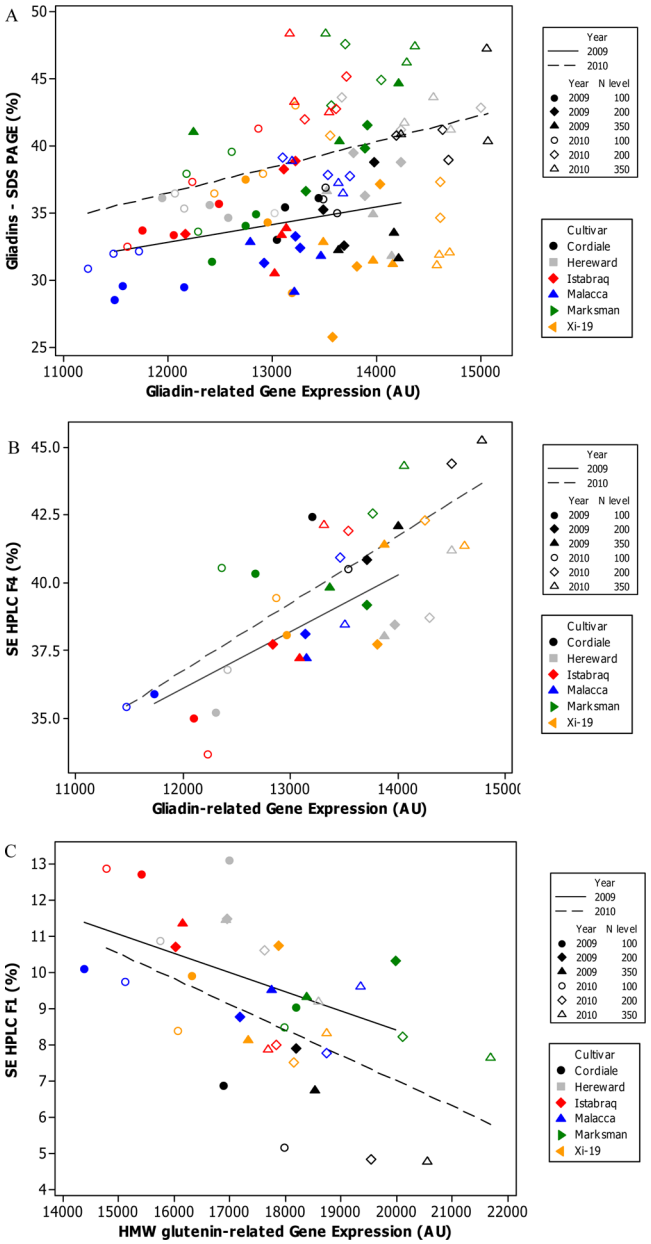

Figure 6. Correlations between gene expression values and protein contents determined by SDS-PAGE and SE-HPLC. (A) Gliadin gene expression and % gliadin proteins determined by SDS-PAGE (2009: $r = 0.335$, $p = 0.013$, $n = 54$; 2010: $r = 0.372$, $p = 0.006$, $n = 54$; for data on $\log_e$ scale). (B) Gliadin gene expression and monomeric gliadin proteins determined by SE-HPLC (F4 fraction) (2009: $r = 0.641$, $p = 0.004$, $n = 18$; 2010: $r = 0.768$, $p < 0.001$, $n = 18$; for data on $\log_e$ scale). (C) HMW subunit gene expression and large glutenin polymers (F1 fraction) measured by SE-HPLC (2009: $r = -0.387$, $p = 0.112$, $n = 18$; 2010: $r = -0.637$, $p = 0.004$, $n = 18$; for data on $\log_e$ scale). Data are for six cultivars grown at Rothamsted Research in 2009 and 2010 with three biological replicates, analyzed at 21 DPA for gene expression profiles and at maturity for protein composition.

DISCUSSION

A combination of transcriptomics and protein analysis was used to identify differences between varieties in terms of their ability to partition N into gluten proteins in response to increased N inputs. This showed a consistent and robust inverse relationship between wheat grain yield and grain %N, which is consistent with previous studies.[3,14] Although grain protein deviation (GPD) was not consistent between the two years, Marksman, Hereward, and Cordiale all tended to show positive GPD. However, both 2010 and 2011 were drier than average, particularly during the spring, which was reflected by a negative impact on N responses (on yield and/or grain N).

The relationships between genetically determined differences in gluten protein composition and grain processing properties are well established.[4,5] The most consistent effect of increased N observed in the present study was increased synthesis and accumulation of gliadins, whether determined as % gluten protein subunits by SDS-PAGE, as monomeric gluten proteins by SE-HPLC, or by gene transcript profiling. Previous studies have shown that increases in grain N result in increases in the proportions of the monomeric gliadins,[15−21] leading to increased dough extensibility. However, Pechanek et al. (1997)[22] showed that the effect of N on grain protein composition was not consistent, but varied between varieties. Our results support this conclusion, as clear statistically significant differences were observed between the six cultivars that were studied.

Less is known about the effects of nutrition on the glutenin fraction, either on the proportions of the individual subunits or on the size distribution of the glutenin polymers. Thus, both increases[23] and decreases[22] in the proportions of HMW subunits have been reported in response to N application, while Panozzo and Eagles (2000)[17] and Zhu and Khan (2001)[19] showed differential effects of N on glutenin polymers and processing properties in cultivars with different HMW subunit alleles. Our results showed that increasing grain N resulted in decreases in the % LMW GS but little effect on the % HMW GS in all cultivars, but that the absolute values and temporal patterns during development varied between the cultivars. Although these did not differ consistently between the five breadmaking cultivars and the single feed quality cultivar (Istabraq), the latter did have the lowest proportion of high molecular weight glutenin polymers (%F1 by SE-HPLC), which are known to be positively correlated with high dough strength.[24,25]

It has previously been shown that glutenin polymers increase significantly in size during the desiccation phase of seed development,[20,26,27] and the results from our study support this. Differential effects on this process could provide a mechanism for effects of the environment during the later stages of grain maturation on grain quality, but would not explain effects of the environment at earlier stages of grain development. In the present study a similar increase in gluten polymers was observed in all six wheat cultivars, with no significant difference between Hereward (which is favored by millers and bakers because it is more stable in quality between seasons), Istabraq (the feed cultivar), and the other four breadmaking wheat cultivars. Environmental effects on grain protein composition and quality that are not related to N content have also been reported by Kolster et al. (1991).[28]

In order to ensure relevance to commercial wheat production and utilization, we focused on field-grown samples of six cultivars of wheat, using material from combinations of five sites and three years (totaling 11 different environments) and three biological replicates. All cultivars showed similar trends in the relationship between N application and protein composition, but statistically significant differences were observed between the cultivars in terms of their varying trends in composition over development time. Although our data suggest that genetic differences in N utilization exist between cultivars,

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it is clear that larger scale studies are required, with more cultivar × site × year × N combinations, in order to identify and exploit genotypes that make more efficient use of applied N, in terms of efficiency of translocation to the grain and incorporation into quality-related proteins.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1: SDS-PAGE analysis of reduced total gluten protein fractions from the six cultivars showing the HMW glutenin subunits expressed in the six cultivars. Table S1: Log_e-transformed raw gene expression values for the mean of all HMW GS-related genes for six wheat cultivars grown at one site (Rothamsted Research) only. Table S2: Log_e-transformed raw gene expression values for the mean of all LMW GS-related genes for six wheat cultivars grown at one site (Rothamsted Research) only. Table S3: Log_e-transformed raw gene expression values for the mean of all gliadin-related genes for six wheat cultivars grown at one site (Rothamsted Research) only. Supporting information for Figure 5: Statistical analysis of gene expression data on log_e scale. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

cDNA, complementary DNA; DPA, days post-anthesis; GPD, grain protein deviation; HMW GS, high molecular weight glutenin subunits; LMW GS, low molecular weight glutenin subunits; LSD, least significant difference; PCO, principal coordinates analysis; REML, residual maximum likelihood; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE-HPLC, size exclusion high-performance liquid chromatography

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