JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. XXXX, XXX, XXX−XXX

pubs.acs.org/JAFC

Gradients of Gluten Proteins and Free Amino Acids along the Longitudinal Axis of the Developing Caryopsis of Bread Wheat

Zhiqiang Shi,[†,‡] Yan Wang,[‡] Yongfang Wan,[‡] Kirsty Hassall,[§] Dong Jiang,[†] Peter R. Shewry,*[,‡] and Malcolm J. Hawkesford[‡]

[†]National Technology Innovation Center for Regional Wheat Production, Key Laboratory of Crop Physiology, and Ecology and Production in Southern China, Ministry of Agriculture, National Engineering and Technology Center for Information Agriculture, Nanjing Agricultural University, Nanjing 210095, P.R. China

[‡]Plant Sciences Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, U.K.

[§]Computational and Analytical Sciences Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, U.K.

Supporting Information

ABSTRACT: Gradients in the contents and compositions of gluten proteins and free amino acids and the expression levels of gluten protein genes in developing wheat caryopses were determined by dividing the caryopsis into three longitudinal sections, namely, proximal (En1), middle (En2), and distal (En3) to embryo. The total gluten protein content was lower in En1 than in En2 and En3, with decreasing proportions of HMW-GS, LMW GS, and α/β- and γ-gliadins and increasing proportions of ω-gliadins. These differences were associated with the abundances of gluten protein transcripts. Gradients in the proportions of the gluten protein polymers which affect dough processing quality also occurred, but not in total free amino acids. Microscopy showed that the lower gluten protein content in En1 may have resulted, at least in part, from the presence of modified cells in the dorsal part of En1, but the reasons for the differences in composition are not known.

KEYWORDS: *wheat grain, gluten proteins, free amino acids, longitudinal gradients*

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the most important cereal crop in temperate countries, providing a source of energy, protein, and other components for human nutrition and health. Most wheat is consumed after milling to produce white flour which is then processed into bread, other baked products, pasta, and noodles. The ability to process wheat into these foods is determined by the gluten proteins, which confer unique viscoelastic properties to dough.[1,2]

The wheat caryopsis is a single seeded fruit, in which the filial embryo and endosperm tissues are surrounded by maternal tissues (pericarp and testa). The embryo is located on the dorsal side of the proximal end of the wheat caryopsis and comprises a single storage cotyledon (the scutellum) and the embryonic axis (plumule, radicle and hypocotyl). The endosperm comprises two tissues, with starchy endosperm cells being surrounded by a single layer of aleurone cells. In the mature wheat the embryo accounts for about 3% of the grain dry weight, the aleurone about 6.5%, the outer layers (pericarp and testa) about 7−8%, and the starchy endosperm about 83−84%.[3] The starchy endosperm is the major storage tissue, comprising mainly starch and about 10% of protein, while the aleurone and embryo are richer in protein (about 23% and 34%, respectively).[3]

The starchy endosperm is often treated as a simple homogeneous tissue, with commercial flour millers aiming to recover all of the cells in the form of white flour. However, more detailed studies show differentiation into three types of cells.[4] The cells immediately below the aleurone, called peripheral or subaleurone cells, are about 60 μm in diameter, while beneath those are prismatic cells, which radiate in columns and are about 128−200 μm long and 40−60 μm wide.[5] Finally, the cells within the center of the cheeks of the grain are rounded with dimensions ranging from 72 to 144 to 69−120 μm.[5] The cells also differ in their compositions, with radial gradients between the outer and inner parts of transverse sections of the starchy endosperm having been reported for a range of components, including proteins, cell wall polysaccharides, starch, and lipids.[6−12] In particular, the content of gluten proteins decreases but the proportion of glutenin polymers increases from the outer to the inner layers of starchy endosperm.[8−10] By contrast, the starch content increases with changes in the distribution of starch granule types.[9,11] These gradients may also be reflected in differences in the compositions of flour streams produced by commercial roller milling and, hence, have implications for grain utilisation.[9]

The mechanisms which control the accumulation of proteins in the different cells and tissues of the grain are not known but appear to be both genetically programmed[13] and influenced by environmental factors.[10,14−16] The requirement for nutrients (mainly amino acids) varies during the development of the wheat caryopsis.[17−19] In the early stages, for about the first 10 days after fertilization, nutrients are required to support cell division and expansion, particularly of the starchy endosperm cells, to establish the basic structure of the grain.[20,21] After this

Received: May 1, 2019
Revised: July 5, 2019
Accepted: July 16, 2019
Published: July 16, 2019

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

stage the main nutrient requirement of the starchy endosperm is to support the synthesis and deposition of storage compounds, mainly starch[11] and protein.[6] By contrast, the growth of the embryo continues throughout grain development. Consequently, the embryo is a powerful sink for nutrients and may compete for these with the starchy endosperm cells.[22]

Pulse-chase analysis of ^{15}N glutamine fed to developing caryopses showed that amino acids enter the developing starchy endosperm through the transfer cells in the groove and are then transported radially to the subaleurone cells,[23] suggesting that protein accumulation is determined by sink activity and/or the activity of specific transporters. However, protein accumulation is also affected by nitrogen availability, and higher concentrations of proteins and larger-sized protein bodies being deposited in the outer layers of endosperm cells under high nitrogen levels.[15,24]

Although there is information on the differences in cell type and composition in radial sections of the grain, little is known about whether gradients also occur along the longitudinal axis of the starchy endosperm tissue. This study therefore compares three longitudinal sections (proximal, middle, and distal to embryo) of the developing starchy endosperm, determining the amount and composition of gluten proteins, gluten protein gene expression patterns, and the contents of free amino acids.

MATERIALS AND METHODS

Plant Material and Harvest. Five winter bread wheat cultivars (Crusoe, Gallant, Hereward, Skyfall, and Solstice) were grown at Rothamsted Research (Harpenden, U.K.) with three biological replicates at two nitrogen levels (100 and 350 kg/ha) in 2017, as part of the Wheat Genetic Improvement Network (WGIN) field trial. Ears were tagged at anthesis, and a total of 80−90 whole caryopses were harvested from five ears at 14, 21, and 28 days post-anthesis (DPA) and transversely cut (after removing the embryos) into three parts of equal length. These were defined as proximal (En1), middle (En2), and distal (En3) to the embryo (shown in Figure 1). The dissected tissues were freeze-dried and ground into fine powder using a 2010 Geno/Grinder (Metuchen, NJ, USA).

Total Nitrogen Determination. Total nitrogen concentration was determined based on the Dumas combustion method,[25] using a Leco combustion analyzer (Leco Corp., St. Paul, MN, USA).

Quantification of Gluten Protein Composition by SDS-PAGE. Gluten proteins were separated and quantified by SDS-PAGE as described before by Wan et al.[24] Ten milligram samples were extracted twice with 200 μL of extraction buffer [50% (v/v) aqueous propan-1-ol containing 2.5% (w/v) DTT] at 50 °C for 30 min. The combined supernatants were freeze-dried and then dissolved in 100 μL of gel loading buffer [100 mM Tris-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol, 2% (w/v) dithiothreitol (DTT), and 0.1% (w/v) bromophenol blue]. The extracts were denatured at 90 °C for 5 min and centrifuged for 15 min at 14100 rpm. Next, 8 μL of the supernatant was separated on precast 4−12% Bis-Tris Nu-PAGE gels (Invitrogen, Paisley, U.K.). The gels were stained overnight in staining solution [0.1% (w/v) Coomassie Brilliant Blue R250, 10% (v/v) trichloroacetic acid (TCA), 40% (v/v) methanol], distained for 12 h in 10% (w/v) TCA, and scanned with a HPG4010 scanner. The images from gray tiff files were processed with Phoretix 1D advanced software (Nonlinear Dynamics, Durham, NC, USA).

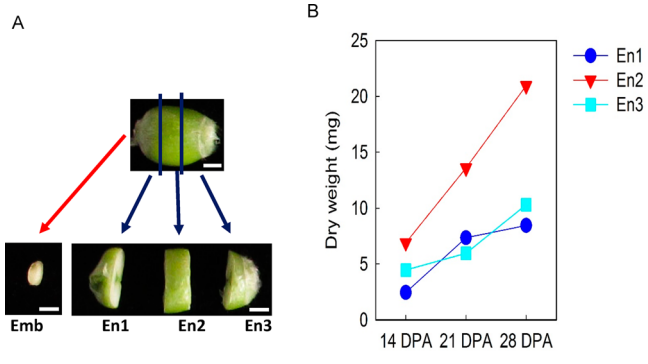

Figure 1. (A) Dissection of a developing caryopsis of cv. Hereward at 21 DPA, after removal of the embryo (Emb), into three longitudinal sections: En1 (proximal), En2 (middle), and En3 (distal). Scale bar: 2 mm. (B) Dry weight (mg) of the three longitudinal sections (En1, En2, and En3) during development at 14, 21, and 28 DPA.

Determination of Gluten Protein Polymers by SE-HPLC. The polymer size distribution of gluten proteins were determined by size-exclusion high-performance liquid chromatography (SE-HPLC). First, 16.5 mg samples were mixed with 1.5 mL of 0.5% (w/v) SDS in 0.05 M phosphate buffer (pH 6.9), sonicated for 45 s at amplitude 6 in a Soniprep instrument fitted with a 3 mm exponential microtip, and then centrifuged for 30 min at 10000 rpm. The supernatants were filtered through a 0.45 μm Durapore membrane filter and sealed in HPLC vials before analysis. SE-HPLC analysis was carried out using a Waters system operating with a Phenomenex column (300 mm × 7.8 mm, 5 μm) and a guard column (75 mm × 7.8 mm, 5 μm) at 35 °C. Protein polymers were separated with 50% (v/v) aqueous acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.7 mL/min for 25 min and detected at 214 nm. Chromatograms were analyzed as described by Chope et al.[26]

Analysis of Free Amino Acids. Free amino acids were extracted according to methods from Curtis et al.[27] First, 10 mg samples were suspended and extracted in 400 μL of 0.01 N HCl for 30 min at room temperature. After centrifugation at 10000 rpm for 15 min, the supernatants were filtered through a 0.22 μm poly(ether sulfone) filter before analysis. Amino acids were separated using a Waters Alliance 2795 HPLC system (Waters Corp., Milford, USA) coupled with a Waters 474 scanning fluorescence detector. First, 15 μL of sample was derivatized with the same volume of OPA (*o*-phthalaldehyde) solution [5.4 mg/mL OPA, 2% (v/v) 2-mercaptoethanol, 556 mM sodium borate (pH 9.2)], and the precolumn derivatization process was automatically completed with the autosample injector before separation. OPA solution was prepared 24 h before use. Amino acids were separated at 45 °C on a Phenomenex Kinetex column (50 mm × 4.6 mm, 2.6 μm) and a Security Guard column (2 mm × 4.6 mm, 2.6 μm) using a multistep gradient. Fluorescence was monitored at excitation and emission wavelengths of 340 and 450 nm, respectively. Chromatograms were analyzed with Millennium 32 software (Waters Corp., Milford, USA).

Eighteen free amino acids (comprising all protein amino acids except cysteine and proline) were quantified using standards, and the amounts were combined to give total free amino acids as mmol/kg dry weight. The proportions of the individual amino acids were expressed as a percentage of total free amino acids. The mean proportions of the individual amino acids from three biological replicates were log transformed, and the TBtools software (http://github.com/CJ-Chen/TBtools) was used to generate a heatmap,[28] with red representing the high proportion and blue the low proportion.

RNA Extraction and RT-qPCR. The En1, En2, and En3 parts of caryopses (after removal of embryos) were prepared from field-grown plants, immediately frozen in liquid nitrogen, and ground using a SPEX Sample Prep 6870 Freezer/Mill (Metuchen, NJ, USA). Total RNA extraction was performed based on the CTAB (cetyltrimethylammonium bromide) method as described previously.[24]

Total RNA was purified with a mini RNeasy RNA isolation kit (Qiagen) and treated RNase-free TURBO DNase (Ambion). A 2 μg aliquot of total RNA was used for reverse transcription with SuperScriptIII reverse transcriptase (Invitrogen) using anchored oligo$(dT)_{23}$ primers (Sigma-Aldrich). cDNA diluted to a 1:6 ratio was used for RT-qPCR in a 20 μL reaction with 1 × SYBR Green

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

PCR master mix (Invitrogen). RT-qPCR was carried out on an ABI 7500 (Applied Biosystems) thermocycler.

The transcript Ta.2526.1S1_at was used as an internal control gene as it showed the most stable expression in caryopses of Hereward during development between 6 and 42 DPA.[24,29,30] The primers designed for RT-qPCR are shown in Supplementary Table S1. PCR efficiency was calculated using the LinRegPCR software. Relative gene expression was calculated by 7500 sequence detection software version 1.4 (Applied Biosystems) in the formula ratio = $E.T.^{-Ct.T}/E.I^{-Ct.I}$, where E.T and Ct.T are the primer efficiency and Ct of the target gene, and E.I. and Ct.I are the primer efficiency and Ct of the internal control gene, respectively.

Microscopy. Endosperm sections from fresh grains (cv. Hereward) were cut into 2 mm transverse sections and immediately fixed in 4% (w/v) paraformaldehyde in 0.1 M Sorenson's phosphate buffer ($NaH_2PO_4{\cdot}2H_2O$ and $Na_2HPO_4{\cdot}12H_2O$, pH 7.0) with 2.5% (w/v) glutaraldehyde for light microscopy. After dehydration in increasing concentrations of ethanol, tissues were infiltrated with LR White Resin for several days at room temperature and polymerized at 55 °C. The resin-embedded grains were sectioned at 1 μm thickness using a Reichert–Jung Ultracut ultramicrotome. Protein bodies were stained with 1% (w/v) naphthol blue black in 7% (w/v) acetic acid, and the cell structure was stained by toluidine blue. Sections were mounted in DPX mountant and observed with a Zeiss Axiophot microscope. Images were acquired with a RetigaExi CCD digital camera (Qimaging, Surrey, BC, Canada) under bright-field optics and MetaMorph software version 7.5.5.9 (Molecular Devices, Sunnyvale, CA, USA)

Statistical Analysis. Data were analyzed using multistrata ANOVA accounting for the split-plot field design and nested sampling structure over time and sections. Where necessary, data were log transformed to satisfy homogeneity of variance. Unless otherwise stated, the least significant difference (LSD) values presented are the LSD associated with comparisons between sections within a fixed N level, development stage, and wheat varieties and are obtained from the lowest stratum of pairwise comparisons. Analyses were done using the GenStat (19th edition, VSN International Ltd., Hemel Hempstead, U.K.).

RESULTS

Gradients along the longitudinal axis of the grain were determined by removing the embryos from developing caryopses and then dividing the caryopsis into three equal sections, i.e., proximal (adjacent to the embryo) (En1), middle (En2), and distal (En3) to the embryo (Figure 1A). The analyses were also carried out on developing caryopses grown at two levels of nitrogen fertilization, i.e., 100 kg/ha which is typical of low input production systems used in many countries and 350 kg/ha which is higher than the levels used for commercial production. Results are reported here for the cultivar Hereward, a U.K. winter wheat which was commercially grown from 1989 to about 2010. Supporting data for four other U.K. commercial cultivars (Crusoe, Gallant, Skyfall, and Solstice) are presented in supplementary figures and are discussed where appropriate.

The dry weights of each section increased rapidly during development, with En2 accounting for 51–53% of whole caryopses (no embryo) dry weight and En1 and En3 each accounting for about 21–27% of the dry weight at 21–28 DPA. However, the relative dry weight of En1 was lower at 14 DPA (18%) (Figure 1B).

Total Nitrogen and Gluten Proteins. Total nitrogen (expressed as percent per dry wt) decreased slightly between En1 and En3 at 14 DPA, but a clear increase from En1 to En3 occurred at 21 and 28 DPA (panels a–c of Figure 2B) ($p < 0.05$; F test). The grain nitrogen contents were higher at 350 kg N/ha, by about 1.5-fold at 21 and 28 DPA, but decreased during development ($p < 0.05$; F test), probably due to dilution with starch which accumulates during grain filling to account for about 70% of the total grain dry weight.

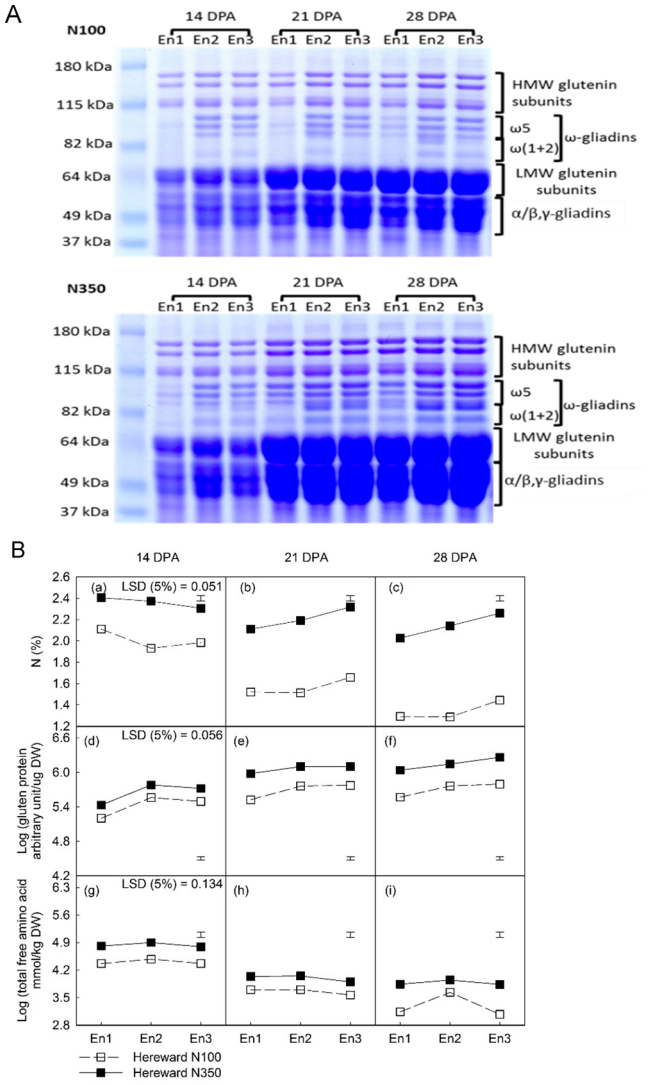

Figure 2. Analysis of proteins and amino acids in the three longitudinal sections (En1, En2, and En3) of cv. Hereward at 14, 21, and 28 DPA grown under low (N 100 kg/ha) and high (N 350 kg/ha) nitrogen. (A) Stained SDS-PAGE separations of gluten proteins. (B) Quantitative differences in fractions: (a–c) total nitrogen (% dry weight); (d–f) total gluten protein contents measured by quantitative scanning of SDS-PAGE separations; and (g–i) total free amino acid concentrations determined by HPLC. Data represent means of three replicate biological samples. LSD bar is shown for comparisons between En1, En2, and En3 sections. Significant differences were observed between N levels, between stages, and between fractions ($p < 0.001$, F test).

Total gluten protein fractions of cultivar Hereward (Figure 2A) and the other four cultivars (Supplementary Figure S1) were extracted and separated by SDS-PAGE. Quantitative scanning of the stained gels allowed the contents of gluten proteins to be determined as an arbitrary unit per microgram of dry weight (panels d–f of Figure 2B). Increases in the contents occurred in all sections between 14 and 28 DPA, particularly in En1 between 14 and 21 DPA ($p < 0.05$; F test). The contents were also higher at 350 kg N/ha ($p < 0.05$; F

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX–XXX

test). Similar patterns were observed for the four other cultivars (Supplementary Figure S1A) with Hereward having the highest protein content.

Free Amino Acids. To determine whether the observed longitudinal gradients in protein content were related to substrate availability, the contents of free amino acids (except cysteine and proline) were determined in the three sections of Hereward (panels g–i of Figure 2B). No consistent differences between the contents of free amino acids in the three sections were observed (LSD, F test), although the amount was higher in En2 at 28 DPA at 100 kg/ha. However, the contents in all sections were higher at 14 DPA than at 21 and 28 DPA, and in the N 350 kg/ha treatments in sections and all stages. Similar results were also observed for the cultivars Skyfall and Solstice (Supplementary Figure S1B).

The proportions of the individual free amino acids (expressed as the percent of total amino acids) are shown as a heatmap in Supplementary Figure S2A. Some differences in the proportions of some individual amino acids were observed between the three grain sections; notably there was a lower proportion of glutamine in the middle part (En2) compared with the proximal and the distal parts (En1 and En3) from 14 to 28 DPA (panels a–c of Supplementary Figure S2B) ($p < 0.05$; F test), and a lower proportion of asparagine in the proximal portion (En1) compared with the other sections (En2 and En3) at 14 DPA (Supplementary Figure S2C) ($p < 0.05$; F test).

Gluten Protein Composition. SDS-PAGE separates gluten proteins into four groups of bands, corresponding to high molecular weight subunits of glutenin (HMW-GS), ω-gliadins, mainly low molecular weight subunits of glutenin (LMW-GS), and mainly α/β and γ-gliadins (Figure 2A) (as discussed by Godfrey et al.[31]). Clear differences were observed between the sections and stages of development ($p < 0.05$; F test), particularly in the proportions of ω-gliadins. Quantitative gel scanning was therefore carried out to quantify the proportions of the four groups of bands (Figure 3 and Supplementary Figure S4). This showed that the proportion of ω-gliadins at 14 DPA increased from En1 to En3, with corresponding decreases in the proportions of the other groups. Similar increases in the proportions of ω-gliadins from En1 to En3 also occurred at 21 and 28 DPA, with decreases particularly in the proportion of LMW subunits.

The ω-gliadins of wheat are classified into $\omega(1 + 2)$ and $\omega5$ based on previously reported studies with specific antibodies,[24] with 2 and 3 bands, respectively, present in Hereward (Figure 2A). Wheat cultivars differ in their number and proportions of these two types of ω-gliadin, with Skyfall and Solstice having similar compositions to Hereward (two $\omega(1 + 2)$ bands and three $\omega5$ bands) and to Crusoe and Gallant having two $\omega(1 + 2)$ bands but only one $\omega5$ band (Supplementary Figure S3). Nevertheless, all five cultivars showed similar increases in the proportions of ω-gliadins between En1 and En2 (Supplementary Figure S4C) with the increased proportion of ω-gliadins from En1 to En3 being statistically significant ($p < 0.05$; F test). Similarly, although the five cultivars differed in the proportions of other groups of gluten proteins, they showed broadly similar changes to Hereward from En1 to En3 and during development (Supplementary Figure S4).

Comparison of the two nitrogen treatments showed that the proportions of HMW-GS and ω-gliadins were higher at 350 kg N/ha and that the proportions of LMW-GS and other gliadins lower (Figure 3 and Supplementary Figure S4). The changes in

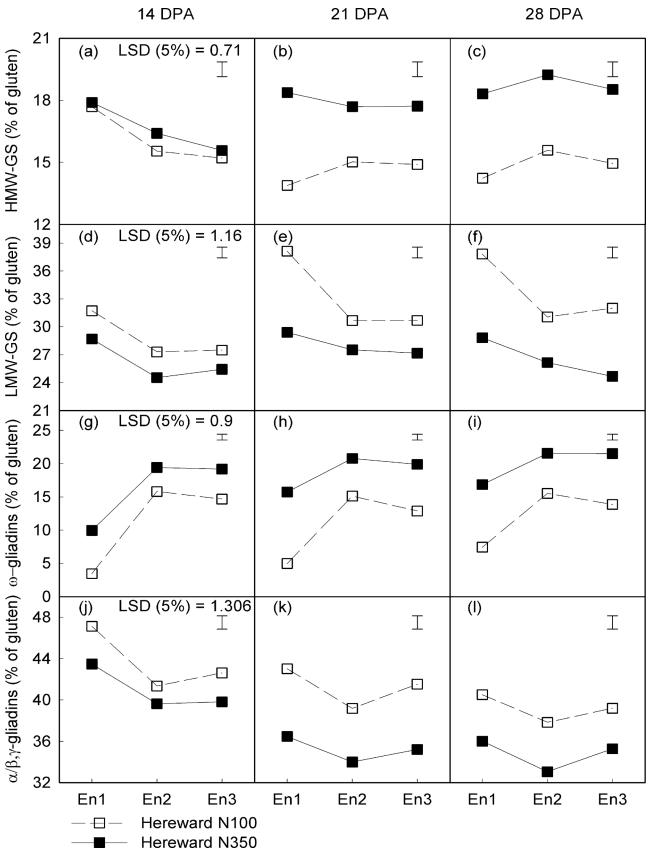

Figure 3. Proportions of gluten protein types (determined by quantitative scanning of SDS-PAGE separations as shown in Figure 2A) in the three longitudinal sections (En1, En2, and En3) of cv. Hereward at 14, 21, and 28 DPA grown under low (N 100 kg/ha) and high (N 350 kg/ha) nitrogen: (a–c) HMW-GS; (d–f) LMW-GS; (g–i) ω-gliadins; and (j–l) α/β and γ-gliadins. Data represent means of three replicate biological replicate samples. LSD bar is shown for comparisons between En1, En2, and En3 fractions. Significant differences were observed between N levels, between stages, and between fractions ($p < 0.001$, F test).

patterns were consistent between the five genotypes, at the two N levels and at the three developmental stages, in agreement with previous studies.[26,31] High nitrogen application reduced the gradients in ω-gliadins from En1 to En2 between 14 to 21 DPA, by increasing the content of ω-gliadins in En1. However, low nitrogen application increased the gradient from En1 to En2 for LMW-GS between 14 to 21 DPA.

Gluten Protein Polymers. The HMW-GS are important components of the high molecular mass glutenin polymers which strongly affect the functional properties of the grain.[32,33] The gradients in gluten protein composition would therefore be expected to result in similar gradients in the proportion of these polymers.

Total protein fractions were therefore extracted from the En1, En2, and En3 sections of developing caryopses and separated by SE-HPLC to determine the molecular size distribution of gluten proteins. This separates the proteins into five fractions corresponding to large glutenin polymers (enriched in HMW-GS) (F1), small glutenin polymers (enriched in LMW-GS) (F2), gliadin monomers (mainly ω-gliadins) (F3), gliadin monomers (mainly α/β, γ-gliadins) (F4), and nongluten protein (albumins and globulins) (F5).[31] High dough strength, and hence good breadmaking quality, of

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX–XXX

wheat flour is associated with a high proportion of the peak F1 and high ratios of F1/F2 (HMW/LMW polymers) and (F1 + F2)/(F3 + F4) (glutenin/gliadin).[10,26,31]

The %F1 and %F2 decreased from En1 to En3 and the %F4 increased in Hereward, while the %F3 decreased from En1 to En2 and then increased again in En3 (Figure 4A). Nitrogen fertilization resulted in decreased proportions of F1 and F2 and increased proportions of F3 and F4 during grain development ($p < 0.05$; F test). The glutenin/gliadin ratio (F1 + F2)/(F3 + F4) decreased from En1 to En3 and from 14 to 28 DPA and was higher at 100 kg N/ha than at 350 kgN/ha. However, the ratio of HMW/LMW polymers (F1/F2) was higher in En3 than in En1 and En2 and much lower at 28 DPA ($p < 0.05$; F test) (Figure 4B). These changes indicate that the proportion of glutenin polymers decreased during development and from En1 to En3, but the proportion of large polymers within the glutenin fraction increased.

Comparison with Skyfall and Solstice showed that Hereward had the lowest and Skyfall had the highest proportions of glutenin polymers (%F1 and %F2) ($p < 0.05$; F test) (Figure 4A and Supplementary Figure S5A). Nevertheless, all showed similar trends with %F1 and %F2 decreasing and %F3 and %F4 increasing between En1 and En3 and %F1 and %F3 decreasing and %F4 increasing between 14 and 28 DPA ($p < 0.05$; F test) (Figure 4A and Supplementary Figure S5A).

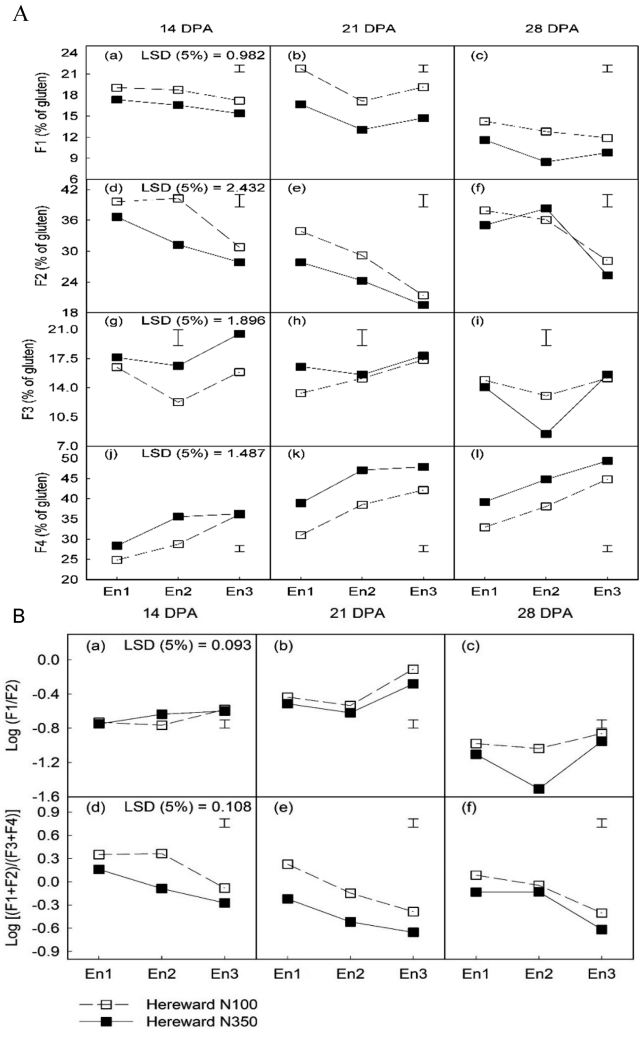

Figure 4. Proportions and ratios of gluten protein fractions separated by SE-HPLC in the three longitudinal sections (En1, En2, and En3) of cv. Hereward at 14, 21, and 28 DPA grown under low (N 100 kg/ha) and high (N 350 kg/ha) nitrogen. (A) Proportions of fractions expressed as a percent of total gluten proteins (F1 + F2 + F3 + F4): F1, large glutenin polymers (enriched in HMW-GS); F2, small glutenin polymers (enriched in LMW-GS); F3, gliadin monomers (mainly ω-gliadins); and F4, gliadin monomers (mainly α/β, γ-gliadins). (a–c) %F1; (d–f) %F2; (g–i) %F3; (j–l) % F4. (B) Ratio of peak areas: (a–c) ratio of HMW polymers to LMW polymers (F1/F2); (d–f) ratio of polymers to monomers (F1 + F2)/(F3 + F4). Data represent means of three biological three replicate samples. LSD bar is shown for comparisons between En1, En2, and En3 fractions. Significant differences were observed between N levels, between stages, and between fractions ($p < 0.001$, F test).

Gene Expression. RT-qPCR was used to determine the expression levels of genes encoding the major types of gluten protein in the En1, En2, and En3 sections of developing caryopses of Hereward (Figure 5). There was a significant ($p < 0.01$; F test) interaction between the caryopsis section, N application, and stage.

The levels of transcripts for all groups of gluten proteins increased from En1 to En2 and En3 at 14 DPA, with little difference between the two levels of nitrogen fertilization. In general, the expression levels of transcripts were highest at 28 DPA and with 350 kg N/ha. However, clear differences were observed at 21 DPA, with transcripts for ω(1 + 2)-gliadins, ω5-gliadins, and γ-gliadins increasing between En1 and En2 at 100 kg N/ha but not at 350 kg N/ha.

Microscopy. In order to relate the gradients to differences in grain structure, transverse sections of En1 and En2 from cultivar Hereward grown at 100 kg N/ha were prepared at 14 DPA (Figure 6 and Supplementary Figure S6) and 21 DPA (Supplementary Figure S7) and stained with either naphthol blue black (which is specific for protein) (Figure 6) or toluidine blue (which stains nuclei and other cell structures in addition to protein) (Supplementary Figures S6 and S7). Figure 6 shows clear differences between the abundance of protein deposits in the dorsal regions of the En1 and En2 sections. In En1, this region is adjacent to the embryo (which was removed before biochemical analysis) and comprises mainly prismatic cells with few or no protein bodies (Figure 6A). By contrast, the cells in the same region of En2 have numerous protein bodies including aggregates (Figure 6D). However, there are no obvious differences between the protein deposits in the lobes of the grain, with deposits in both the subaleurone and central cells (Figure 6C and 6F). Similar differences were observed at 21 DPA and when stained with toluidine blue, with the cells in the dorsal region of En1 being distorted by the expanding embryo (Supplementary Figure S7).

DISCUSSION

The presence of radial protein gradients in the starchy endosperm of wheat is well-established, based on immunolabeling,[7,8,23] analysis of pearling fractions,[8−10] and microdissection.[6] These studies have shown that the outermost subaleurone cells of the lobes of the grain are rich in gluten proteins, in particular ω-gliadins, α-gliadins, and LMW subunits of glutenin, while the inner central cells have low protein contents but are enriched in γ-gliadins and HMW subunits of glutenin. These gradients develop during the middle and late stages of grain filling and are increased by nitrogen application, elevated temperature post-anthesis,[15] and

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

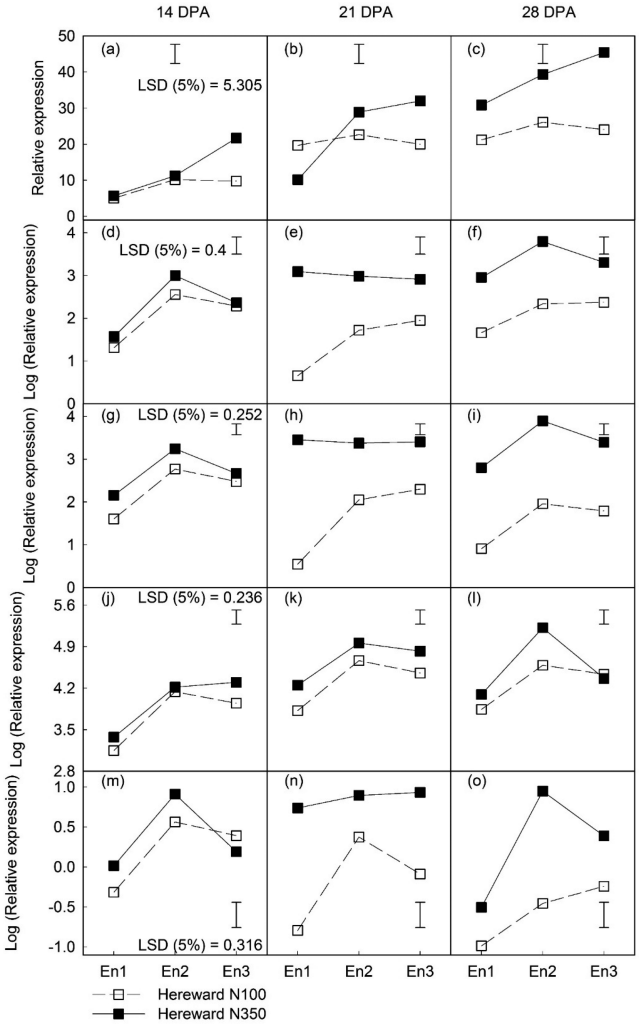

Figure 5. Relative expression levels of gluten protein genes in the three longitudinal sections (En1, En2, and En3) of cv. Hereward at 14, 21, and 28 DPA grown under low (N 100 kg/ha) and high (N 350 kg/ha) nitrogen: (a–c) HMW-GS subunit, (d–f) ω2-gliadins, (g–i) ω5-gliadine, (j–l) α/β-gliadins, and (m–o) γ-gliadins. Data represent means of three biological replicate samples. LSD bar is shown for comparisons between En1, En2, and En3 sections. Significant differences were observed between N levels, between stages, and between fractions ($p < 0.001$, F test).

drought.[14] The mechanisms that result in the radial gradient are not clear yet, but it is known that the protein-rich subaleurone cells have a different origin to the central cells of the lobes, being derived from tangential divisions in the outer layer of cells which differentiate to form the mature aleurone.[34,35] Hence, this origin may be reflected in a different pattern of gluten protein gene expression, as proposed by Tosi et al.[8] A recent study[23] in which ^{15}N glutamine was fed to developing ears and followed in the developing endosperm using NanoSIMS showed that amino acid precursors are transported radially across the developing endosperm from the transfer cells in the groove to the outer layers of cells. Hence, it is likely that expression of gluten protein genes in the subaleurone cells provides a strong sink which drives this transport. In addition to proteins, gradients in other components also occur across the mature wheat grain, including arabinoxylan and β-glucan, lipids, and starch (reviewed by Tosi et al.).[9]

Due to the asymmetric nature of the mature wheat grain, with a pronounced ventral groove, it is not possible to study longitudinal gradients using single sections. One approach is to integrate data from serial sections, as described for cell wall polymers in developing wheat grain using MALDI-MS imaging[35] and hordein accumulation in developing barley grain.[36] However, this approach is expensive and time-consuming and does not provide sufficient material for biochemical analyses. We have therefore taken a simpler approach, by dividing the developing caryopsis into just three equal parts corresponding to region adjacent to the embryo (En1), the central part (En2), and the distal part (En3).

This has shown clear gradients in total nitrogen content and the content and composition of gluten proteins. The content of total gluten proteins was lower in En1 than in En2 and En3, with distinct gradients in composition of gluten proteins. These were steep decreases in the proportions of HMW-GS, LMW-GS, and α/β, γ-gliadins and increases in the proportion of ω-gliadins. These gradients were more pronounced at 14 DPA and were less pronounced at high nitrogen, which contrasts with the radial gradients.[10,15,24] The lower total contents of gluten proteins in En1 may relate to the presence of the embryo, which is associated with the differentiation of the cells surrounding this region to provide nutrients rather than store protein (Figure 6).[22,37−39] However, this does not account for low proportion of ω-gliadins in this region, as they have no known function except storage, which is shared with the other gluten proteins.

The expression levels of gluten protein genes are strongly upregulated by higher nitrogen application,[30,40−42] and we have shown that effects of nitrogen on the content and distribution of ω-gliadins are associated with differences in transcript profiles.[24] Our results are consistent with these studies, showing that the spatial and temporal changes in the proportions of gluten proteins during development are associated with differences in transcript abundances.

The proportions of HMW-GS and high molecular weight polymers (%F1), the ratios of high molecular weight to low molecular weight polymers (F1/F2), and the ratios of glutenin to gliadin (F1 + F2)/(F3 + F4) are strongly correlated with gluten strength and good bread making performance (reviewed by Shewry et al.).[33] All of these indicators of quality increase from the outer to inner endosperm, indicating the white flour from the central endosperm should have a lower total protein content but higher quality than the protein-rich flour from the outer starchy endosperm.[10] By contrast, in the current study the %HMW-GS, %F1, and (F1 + F2)/(F3 + F4) decreased from En1 to En3 with no consistent effects on F1/F2 (Figures 3 and 4).

Free amino acids are the major substrates delivered into the endosperm for storage protein synthesis.[43] The concentration of free amino acid was higher at 14 DPA than at later stages and was increased by nitrogen at all stages. This is consistent with previous studies[44], with the higher amino acid concentration at 14 DPA being due to less dilution with starch and gluten proteins. However, there were no differences in the concentrations of free amino acids in the three sections of longitudinal grain, suggesting that substrate availability did not determine the differences in protein accumulation.

Our study therefore demonstrated the existence of longitudinal gradients in gluten protein content and composition along the longitudinal axis of the wheat grain, which are associated with differences in transcript abundances.

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

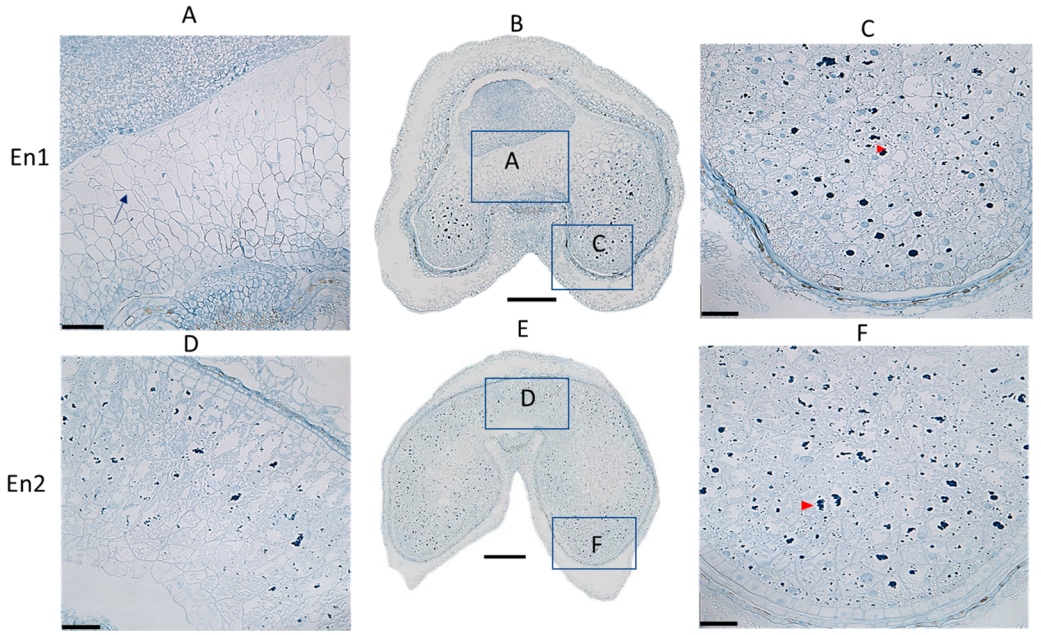

Figure 6. Transverse sections of the En1 (A–C) and En2 (D–F) regions of developing caryopses of wheat cv. Hereward at 14 DPA, stained for total protein using nephthol blue black. Scale bar: 250 μM in B and E; 100 μM in A, C, D, and F. Red arrow heads indicate protein bodies, and the black arrow indicates the prismatic cell.

The differences between the proximal (En1) part and the middle and central parts (En2 and En3) may be determined, at least in part, by the role of the prismatic cells in the dorsal part of En1 being modified to provide nutrients to the embryo instead of store protein. However, this does not provide an explanation for the differences in gluten protein composition and, in particular, the low proportion of ω-gliadins in this region.

The longitudinal gradients will almost certainly be reflected in differences in quality of flours produced from different parts of the grain, as discussed previously for radial gradients.[10] However, the exploitation of these differences by innovative processing is clearly a challenge.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.9b02728.

(Table S1) Primers for genes encoding gluten protein for RT-qPCR; (Figure S1) total gluten protein contents measured by quantitative scanning of SDS-PAGE separations of total protein fractions from four cultivars (Crusoe, Gallant, Skyfall, and Solstice) and total free amino acid concentrations determined by HPLC of two cultivars (Skyfall and Solstice); (Figure S2) Heatmap comparing proportions of individual free amino acid in sections of three cultivars (Hereward, Skyfall and Solstice) at 14, 21, and 28 DPA at 100 and 350 kg N/ha and proportion of glutamine and asparagine; (Figure S3) SDS-PAGE separations of total protein fractions from sections of developing caryopses of Crusoe, Gallant, Skyfall, and Solstice; (Figure S4) proportions of HMW-GS, LMW-GS, ω-gliadins, and α/β, γ-gliadins in sections of developing caryopses of four cultivars (Crusoe, Gallant, Skyfall, and Solstice) determined by gel scanning and expressed as a percentage of total gluten proteins at 14, 21, and 28 DPA; (Figure S5) proportions of gluten protein polymers and monomers determined by SE-HPLC in sections of developing caryopses of two cultivars (Skyfall and Solstice) grown at 100 and 350 kg N/ha; (Figure S6) microscopy of sections from En1 and En2 of cv. Hereward at 14 DPA and 100 kg N/ha stained with toluidine blue; and (Figure S7) microscopy of sections En1 and En2 of cv. Hereward at 21 DPA and 100 kg N/ha stained with toluidine blue (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: peter.shewry@rothamsted.ac.uk. Tel.: 0044-1582-763133.

ORCID

Peter R. Shewry: 0000-0001-6205-2517

Funding

Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the U.K. and the work forms part of the Designing Future Wheat strategic program (BB/P016855/1). The work was also supported by BBSRC Grant BB/N000854/1 and AHDB Grant AHDB RD-2170005 "Developing novel types of low protein wheat for breadmaking". S.-Z.Q. acknowledges funding by the China Scholarship Council.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the Defra-supported Wheat Genetic Improvement Network (CH1090) for providing samples from field trials.

■ ABBREVIATIONS USED

HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits; DPA, days post-

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX–XXX

anthesis; RT-qPCR, reverse transcription−quantitative polymerase chain reaction; cDNA, complementary DNA; LSD, least significant difference; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE-HPLC, size-exclusion high-performance liquid chromatography; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; CTAB, cetyltrimethylammonium bromide; TCA, trichloroacetic acid; OPA, *o*-phthalaldehyde; ANOVA, analysis of variance

REFERENCES

(1) Shewry, P. R.; Tatham, A. S. Disulphide bonds in wheat gluten proteins. *J. Cereal Sci.* **1997**, *25*, 207−227.

(2) Don, C.; Lichtendonk, W.; Plijter, J. J.; Hamer, R. J. Glutenin macropolymer: a gel formed by glutenin particles. *J. Cereal Sci.* **2003**, *37*, 1−7.

(3) Barron, C.; Surget, A.; Rouau, X. Relative amounts of tissues in mature wheat (*Triticum aestivum* L.) grain and their carbohydrate and phenolic acid composition. *J. Cereal Sci.* **2007**, *45*, 88−96.

(4) Evers, T.; Millar, S. Cereal Grain Structure and Development: Some Implications for Quality. *J. Cereal Sci.* **2002**, *36*, 261−284.

(5) Bradbury, D.; MacMasters, M.; Cull, I. M. Structure of the mature wheat kernel. III. Microscopic structure of the endosperm of hard red winter wheat. *Cereal Chem.* **1956**, *33*, 361−373.

(6) Ugalde, T. D.; Jenner, C. F. Substrate gradients and regional patterns of dry matter deposition within developing wheat endosperm. II. Amino acids and protein. *Aust. J. Plant Phys.* **1990**, *17*, 395−406.

(7) Tosi, P.; Parker, M.; Gritsch, C. S.; Carzaniga, R.; Martin, B.; Shewry, P. R. Trafficking of storage proteins in developing grain of wheat. *J. Exp. Bot.* **2009**, *60*, 979−991.

(8) Tosi, P.; Gritsch, C. S.; He, J. B.; Shewry, P. R. Distribution of gluten proteins in bread wheat (*Triticum aestivum*) grain. *Ann. Bot.* **2011**, *108*, 23−35.

(9) Tosi, P.; He, J. B.; Lovegrove, A.; Gonzáles-Thuillier, I.; Penson, S.; Shewry, P. R. Gradients in compositions in the starchy endosperm of wheat have implications for milling and processing. *Trends Food Sci. Technol.* **2018**, *82*, 1−7.

(10) He, J. B.; Penson, S.; Powers, S. J.; Hawes, C.; Shewry, P. R.; Tosi, P. Spatial patterns of gluten protein and polymer distribution in wheat grain. *J. Agric. Food Chem.* **2013**, *61*, 6207−6215.

(11) Ugalde, T. D.; Jenner, C. F. Substrate gradients and regional patterns of dry matter deposition within developing wheat endosperm. I. Carbohydrates. *Aust. J. Plant Phys.* **1990**, *17*, 377−394.

(12) Mills, E. N. C.; Parker, M. L.; Wellner, N.; Toole, G.; Feeney, K.; Shewry, P. R. Chemical imaging: the distribution of ions and molecules in developing and mature wheat grain. *J. Cereal Sci.* **2005**, *41*, 193−201.

(13) Drea, S.; Leader, D. J.; Arnold, B. C.; Shaw, P.; Dolan, L.; Doonan, J. H. Systematic spatial analysis of gene expression during wheat caryopsis development. *Plant Cell* **2005**, *17*, 2172−2185.

(14) Chen, X. Y.; Li, B.; Shao, S. S.; Wang, L. L.; Zhu, X. W.; Wang, W. J.; Xiong, F.; Yang, Y.; Yu, X. Accumulation characteristic of protein bodies in different regions of wheat endosperm under drought stress. *J. Integr. Agric.* **2016**, *15*, 2921−2930.

(15) Savill, G. P.; Michalski, A.; Powers, S. J.; Wan, Y. F.; Tosi, P.; Buchner, P.; Hawkesford, M. J. Temperature and nitrogen supply interact to determine protein distribution gradients in the wheat grain endosperm. *J. Exp. Bot.* **2018**, *69*, 3117−3126.

(16) Xue, Y. F.; Eagling, T.; He, J.; Zou, C. Q.; McGrath, S. P.; Shewry, P. R.; Zhao, F. J. Effects of nitrogen on the distribution and chemical speciation of iron and zinc in pearling fractions of wheat grain. *J. Agric. Food Chem.* **2014**, *62*, 4738−4746.

(17) Molino, I. M. M. D.; Rojo, B.; Martinez-Carrasco, R.; Pérez, P. Amino acid composition of wheat grain. 1: Changes during development. *J. Sci. Food Agric.* **1988**, *42*, 29−37.

(18) Lalonde, S.; Tegeder, M.; Throne-Holst, M.; Frommer, W. B.; Patrick, J. W. Phloem loading and unloading of sugars and amino acids. *Plant, Cell Environ.* **2003**, *26*, 37−56.

(19) Kapoor, A. C.; Heiner, R. E. Biochemical changes in developing wheat grains. Changes in nitrogen fractions, amino acids and nutritional quality. *J. Sci. Food Agric.* **1982**, *33*, 35−40.

(20) Olsen, O. A.; Linnestad, C.; Nichols, S. E. Developmental biology of the cereal endosperm. *Trends Plant Sci.* **1999**, *4*, 253−257.

(21) Sabelli, P. A.; Larkins, B. A. The development of endosperm in grasses. *Plant Physiol.* **2009**, *149*, 14−26.

(22) Smart, M. G.; O'brien, T. P. The development of the wheat embryo in relation to the neighbouring tissues. *Protoplasma* **1983**, *114* (1−2), 1−3.

(23) Moore, K. L.; Tosi, P.; Palmer, R.; Hawkesford, M. J.; Grovenor, C. R.; Shewry, P. R. The dynamics of protein body formation in developing wheat grain. *Plant Biotechnol. J.* **2016**, *14*, 1876−1882.

(24) Wan, Y. F.; Gritsch, C. S.; Hawkesford, M. J.; Shewry, P. R. Effects of nitrogen nutrition on the synthesis and deposition of the ω-gliadins of wheat. *Ann. Bot.* **2014**, *113*, 607−615.

(25) Dumas, J. B. A. Procedes de l'analyse organique. *Ann. Chim. Phys.* **1831**, *47*, 198−205.

(26) Chope, G. A.; Wan, Y. F.; Penson, S. P.; Bhandari, D. G.; Powers, S. J.; Shewry, P. R.; Hawkesford, M. J. Effects of genotype, season, and nitrogen nutrition on gene expression and protein accumulation in wheat grain. *J. Agric. Food Chem.* **2014**, *62*, 4399−4407.

(27) Curtis, T. Y.; Powers, S. J.; Wang, R. Y.; Halford, N. G. Effects of variety, year of cultivation and sulphur supply on the accumulation of free asparagine in the grain of commercial wheat varieties. *Food Chem.* **2018**, *239*, 304−313.

(28) Chen, C. J.; Chen, H.; He, Y. H.; Xia, R. TBtools, a Toolkit for Biologists integrating various biological data handling tools with a user-friendly interface. *BioRxiv* **2018**, 1.

(29) Wan, Y. F.; Poole, R. L.; Huttly, A. K.; Toscano-Underwood, C.; Feeney, K.; Welham, S.; Gooding, M. J.; Mills, C.; Edwards, K. J.; Shewry, P. R.; Mitchell, R. A. C. Transcriptome analysis of grain development in hexaploid wheat. *BMC Genomics* **2008**, *9*, 121.

(30) Wan, Y. F.; Shewry, P. R.; Hawkesford, M. J. A novel family of γ-gliadin genes are highly regulated by nitrogen supply in developing wheat grain. *J. Exp. Bot.* **2013**, *64*, 161−168.

(31) Godfrey, D.; Hawkesford, M. J.; Powers, S. J.; Millar, S.; Shewry, P. R. Effects of crop nutrition on wheat grain composition and end use quality. *J. Agric. Food Chem.* **2010**, *58*, 3012−3021.

(32) Payne, P. I.; Nightingale, M. A.; Krattiger, A. F.; Holt, L. M. The relationship between HMW glutenin subunit composition and the bread-making quality of British-grown wheat varieties. *J. Sci. Food Agric.* **1987**, *40*, 51−65.

(33) Shewry, P. R.; Halford, N. G.; Tatham, A. S.; Popineau, Y.; Lafiandra, D.; Belton, P. S. The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. *Adv. Food Nutr. Res.* **2003**, *45*, 219−302.

(34) Becraft, P. W. Cell fate specification in the cereal endosperm. *Semin. Cell Dev. Biol.* **2001**, *12*, 387−394.

(35) Fanuel, M.; Ropartz, D.; Guillon, F.; Saulnier, L.; Rogniaux, H. Distribution of cell wall hemicelluloses in the wheat grain endosperm: a 3D perspective. *Planta* **2018**, *248*, 1505−1513.

(36) Davies, J. T.; Shewry, P. R.; Harris, N. Spatial and temporal patterns of B hordein synthesis in developing barley (*Hordeum vulgare* L.) caryopses. *Cell Biol. Int.* **1993**, *17*, 195−203.

(37) Yu, X. R.; Yu, H.; Shao, S. S.; Zhang, J.; Zhou, L.; Zheng, Y. K.; Wang, Z.; Xiong, F. Structural development of conducting cell and its functions in wheat caryopsis. *Rev. Bras. Bot.* **2015**, *38*, 401−409.

(38) Xurun, Y.; Xinyu, C.; Liang, Z.; Jing, Z.; Heng, Y.; Shanshan, S.; Fei, X.; Zhong, W. Structural development of wheat nutrient transfer tissues and their relationships with filial tissues development. *Protoplasma* **2015**, *252*, 605−617.

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

(39) Zheng, Y. K.; Yang, J. C.; Wang, Z. Structure characteristics and function of wheat endosperm transport tissues. *Rev. Bras. Bot.* **2015**, *38*, 679−687.
(40) Altenbach, S. B.; Kothari, K. M. Omega gliadin genes expressed in *Triticum aestivum* cv. Butte 86: effects of post-anthesis fertilizer on transcript accumulation during grain development. *J. Cereal Sci.* **2007**, *46*, 169−177.
(41) Zheng, T.; Qi, P. F.; Cao, Y. L.; Han, Y. N.; Ma, H. L.; Guo, Z. R.; Wang, Y.; Qiao, Y. Y.; Hua, S. Y.; Yu, H. Y.; Wang, J. P.; Zhu, J.; Zhou, C. Y.; Zhang, Y. Z.; Chen, Q.; Wang, J. R.; Jiang, Q. T.; Yan, Z. H.; Lan, X. J.; Fan, G. Q.; Wei, Y. M.; Zheng, Y. L.; Kong, L. Mechanisms of wheat (*Triticum aestivum*) grain storage proteins in response to nitrogen application and its impacts on processing quality. *Sci. Rep.* **2018**, *8*, 11928.
(42) Yu, X. R.; Chen, X. Y.; Wang, L. L.; Yang, Y.; Zhu, X. W.; Shao, S. S.; Cui, W. X.; Xiong, F. Novel insights into the effect of nitrogen on storage protein biosynthesis and protein body development in wheat caryopsis. *J. Exp. Bot.* **2017**, *68*, 2259−2274.
(43) Fisher, D. B.; Macnicol, P. K. Amino acid composition along the transport pathway during grain filling in wheat. *Plant Physiol.* **1986**, *82*, 1019−1023.
(44) Howarth, J. R.; Parmar, S.; Jones, J.; Shepherd, C. E.; Corol, D.-I.; Galster, A. M.; Hawkins, N. D.; Miller, S. J.; Baker, J. M.; Verrier, P. J.; Ward, J. L.; Beale, M. H.; Barraclough, P. B.; Hawkesford, M. J. Co-ordinated expression of amino acid metabolism in response to N and S deficiency during wheat grain filling. *J. Exp. Bot.* **2008**, *59*, 3675−3689.

DOI: 10.1021/acs.jafc.9b02728
J. Agric. Food Chem. XXXX, XXX, XXX−XXX