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Analysis of Storage and Structural Carbohydrates in Developing Wheat (*Triticum aestivum* L.) Grains Using Quantitative Analysis and Microscopy

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Supporting Information

ABSTRACT: In this paper, the content of all major carbohydrates and the spatial distribution of starch, arabinoxylan and β-glucan in developing wheat kernels (*Triticum aestivum* L. var. Homeros) from anthesis until maturity were studied. By combining information from microscopy and quantitative analysis, a comprehensive overview on the changes in storage and structural carbohydrates in developing grains was obtained. In the phase of cell division and expansion, grains were characterized by a rapid accumulation of water and high concentrations of the water-soluble carbohydrates fructan, sucrose, glucose and fructose. During the grain filling phase, starch, protein, β-glucan and arabinoxylan accumulated, while during grain maturation and desiccation, only a loss of moisture took place. The comprehensive approach of this study allowed finding correlations, which are discussed within the context of grain development. Particular attention was given to the transient presence of high fructan concentrations, which was associated with the most striking compositional changes during grain development.

KEYWORDS: *carbohydrates, dietary fiber, fructan, starch, arabinoxylan, β-glucan, grain development, Triticum aestivum L., microscopy*

INTRODUCTION

Knowledge on wheat grain composition is crucial for the evaluation of its nutritional value. Wheat is rich in digestible carbohydrates, mostly starch, and contains lower concentrations of dietary fibers such as arabinoxylan, β-glucan and fructan.[1] The balance between digestible and indigestible carbohydrates varies greatly during grain development. Fine-tuning of their synthesis, degradation or conversion offers opportunities for wheat nutritional quality improvement. Indeed, insight into the changes of grain composition in immature grains paves the way for enhancement of wheat nutritional value based on plant breeding or genetic engineering.

The development of wheat kernels has been studied intensively at morphological, biochemical and genetic levels.[2] Several phases can be distinguished during kernel development, which have been described in detail by Bechtel et al.[2] and more recently by Shewry et al.[3] In short, early development includes fertilization, syncytium formation, cellularization, endosperm cell differentiation and endosperm cell expansion. During the grain filling period, storage compounds are accumulated, and during the final maturation phase, programmed cell death, dormancy and desiccation take place.[3,4]

Grain development is characterized by an increase in grain size and weight and by major shifts in kernel composition. Indeed, mature wheat kernels are known to contain high concentrations of starch in contrast to immature kernels.[5] Fructans are abundantly present in immature wheat kernels in which fructan concentrations up to 30% have been reported,[5] whereas mature kernels contain between 0.7 and 2.9% fructan on a dry matter basis.[6] Apart from storage compounds, cell wall polysaccharides are the most important compounds of mature wheat kernels. Although the spatial distribution of arabinoxylan and β-glucan in developing wheat grains has been studied in detail using microscopy,[7,8] information on the ontogeny of cell wall polysaccharide contents is scarce. Beaugrand et al.[9] studied arabinoxylans only in the external layers from immature wheat kernels. Toole et al.[10] performed a semiquantitative analysis of β-glucan and arabinoxylans in developing grains. They treated immature wheat samples with lichenase [endo-1,3(4)-β-D-glucanase] and endoxylanase and analyzed the hydrolysis products by high performance anion exchange chromatography (HPAEC). The majority of the released glucooligosaccharides (GOS) and arabinoxylanoligosaccharides (AXOS) were identified on the HPAEC profiles. There was a steady increase in the total area of released and identified GOS, and hence β-glucan, per seed, whereas the total area of AXOS peaks, and hence arabinoxylan, per seed increased more rapidly during development.[10]

Although the above-mentioned studies contain useful information regarding the ontogeny of one or a few individual grain compounds, they do not provide an exhaustive overview of the changes in carbohydrate levels during wheat grain development. A more integrated approach was used by Shewry et al.,[3] who combined the results of multiple studies on the

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biochemical and transcriptional changes during grain development. They gave an in depth description of wheat grain development, but focused little on the quantitative changes of carbohydrate concentrations. Furthermore, fructans were not investigated despite their crucial role in grain development[11] and their potential prebiotic properties.

In the present study, the ontogeny of all major wheat kernel carbohydrates during kernel development was investigated simultaneously, both through quantification of the levels of the different compounds and through visualization of their spatial distribution with microscopy.

■ MATERIALS AND METHODS

Materials. All chemicals, solvents and reagents were purchased from Sigma-Aldrich (Bornem, Belgium) and were of analytical grade unless specified otherwise. A total starch assay kit and a mixed linkage β-glucan assay kit were purchased from Megazyme (Bray, Ireland) for the quantification of starch and mixed linkage β-glucan (further referred to as β-glucan), respectively. Wheat grain samples were taken from two plots (10×10 m^2) on a wheat field (*Triticum aestivum* L. var. Homeros, 2011) in Leefdaal (Belgium). Plants were marked and dated at anthesis, or the appearance of anthers on the ear. Four ears were taken from each plot at several time points during development and frozen immediately in liquid nitrogen. Grain kernels from the first and second plots were taken from the middle part of the frozen ear and equally distributed over three aliquots. The kernels were ground under liquid nitrogen to a wheat whole meal, lyophilized and used for the carbohydrate analysis. A second set of three grain aliquots was taken in the same way and used for the determination of kernel weight, moisture content and protein content.

Determination of Kernel Weight and Moisture Content. Fifteen to thirty kernels were weighed in triplicate, lyophilized, weighed again and ground to a wheat whole meal. A first part of the whole wheat meal was used to determine moisture content with an adapted version of AACC method 44-15.02.[12] The remainder was used to determine the crude protein content. The total moisture content was calculated as the sum of moisture lost during lyophilization and the moisture remaining in the wheat whole meal.

Determination of Crude Protein Content. Crude protein content was determined using an adaptation of the AOAC Official Method 990.03.[13] An automated Dumas protein analysis system (EAS VarioMax N/CN, Elt, Gouda, The Netherlands) was used with 5.7 as the nitrogen to protein conversion factor. All concentrations were measured in triplicate, except for day 0 (anthesis), for which only one value was obtained.

Carbohydrate Analysis. Determination of starch, including malto-oligosaccharides, was performed with an adapted version of AACC method 76-13.01.[12] The amount of glucose released after α-amylase and amyloglucosidase hydrolysis was quantified with gas chromatography as described by Courtin et al.[14] To quantify arabinoxylans, an acidic treatment was applied to hydrolyze arabinoxylans and gas chromatography to quantify the released arabinose and xylose.[14] β-Glucan content was determined with the Megazyme mixed linkage β-glucan kit, as described in AACC method 32-23.[12] Samples were incubated with lichenase and β-glucosidase for complete β-glucan hydrolysis, and the released glucose was quantified with a spectrophotometer. Glucose, fructose and sucrose were extracted with water and measured with HPAEC while fructans were first hydrolyzed by a mild acid treatment before HPAEC analysis.[15] Prior to HPAEC analysis, wheat whole meal (50 mg) was heated first in ethanol (1.0 mL, 90 °C) until all added ethanol was evaporated to avoid enzymatic degradation. Carbohydrate concentrations were measured in triplicate except for starch at day 0 and 2 DAA (single value) and β-glucan at 5 DAA (in duplicate).

Microscopy. Grain kernels were fixed overnight with 0.10 M sodium potassium phosphate buffer pH 7.0 containing 3.0% formaldehyde and 1.0% glutaraldehyde, dehydrated in a series of ethanol solutions, infiltrated with the Leica Historesin Embedding Kit and fixed with the Leica Historesin mounting medium (Leica Microsystems, Bensheim, Germany) as described before.[16] After embedding, thin kernel sections (4 μm) were cut with a Leica RM2255 microtome equipped with a tungsten carbide edge knife (16 cm, profile d) and transferred onto microscope slides (Thermo Scientific Cel-line; SSG Braunschweig, Germany). For each time point in kernel development, at least four individual kernels were embedded. Several sections obtained from these kernels were stained and incubated in closed polystyrene foam containers. Finally, the stained sections were rinsed with water and dried at room temperature. A first set of grain sections was stained with Lugol's solution to visualize starch granules. Neutral red was used as counterstain. Grain sections were incubated for 2 h with 100 μL of a neutral red buffer solution (0.04% neutral red in 0.2 M mannitol−0.01 M HEPES, pH 7.8) and rinsed for 30 min in 0.2 M mannitol−0.01 M HEPES buffer solution as described by Gonzalez et al.[17] Next, sections were colored with Lugol's solution (0.33% I_2 and 0.68% KI) for 4 min, rinsed with water and dried at room temperature. A second set of grain sections was used to visualize arabinoxylans as described by Dornez et al.[18] In short, sections were incubated for 1.0 h with 100 μL of a 1 μM Alexa Fluor 488-labeled catalytically inactive *Bacillus subtilis* xylanase solution, prepared in sodium Hepes buffer (50 mM, pH 7.0), rinsed with water and dried at room temperature. To visualize β-glucan, sections were stained by incubation with 100 μL of calcofluor white (1 g/L) for 3 min, rinsed with water and dried at room temperature.[19,20] The stained grain sections were examined with a Nikon ECLIPSE 80i epifluorescence microscope (Nikon Inc., Melville, New York, USA) equipped with a Nikon Digital Sight DS-U2 camera and an Intenslight C-HGFI lamp (Nikon). NIS-Elements BR software (Nikon) was used to produce composited images of multiple individual images. Starch granules were examined with light microscopy, arabinoxylans with epifluorescence microscopy using a FITC filter cube (excitation 465−495 nm, dichroic mirror 505 nm long pass, emission 515−555 nm) and β-glucan with epifluorescence microscopy using a DAPI filter cube (excitation 325−375 nm, dichroic mirror 400 nm long pass, emission 435−485 nm). Multiple images at 40× magnification and 100 ms exposure time were combined to obtain images of complete grain kernels stained for arabinoxylan. Exposure times of 20 and 30 ms were used for arabinoxylan stained sections at 100× and 400× magnification, respectively. To visualize β-glucan in complete kernels of 5, 16, and 56 DAA, images at either 40× or 100× magnification with an exposure time of 4 ms, 10 ms and 30 ms, respectively, were combined. The same sections were examined at 100× magnification with exposure times of 8 ms, 10 ms and 1 ms, respectively, while an exposure time of 4 ms was used at 400× magnification. The annotations of Wilkinson et al.[21] were used for grain tissue identification.

Statistics. Statistical analysis was performed using SAS software 9.3 (SAS institute Inc., Cary, NC, USA). To find correlations between kernel components, the Spearman correlation coefficient (ρ) between the average component concentrations was calculated, and 0.01 was used as significance level. Spearman rank-order correlation is a nonparametric measure of association based on the ranks of the data values.[22]

■ RESULTS AND DISCUSSION

Phases of Grain Development. The development of grain dry matter and grain moisture content was studied from anthesis until 69 days after anthesis (DAA) when maturity was reached (Figure 1). Kernel moisture content was high during the first two weeks after anthesis (75.1 ± 0.2% moisture at anthesis and 71.7 ± 0.1% 14 DAA) and decreased continuously thereafter until a final moisture content of 10.4 ± 0.6% at maturity. Kernel dry weight increased steadily during the first two weeks after anthesis (0.8 ± 0.2 mg/kernel at anthesis and 11.3 ± 0.4 mg/kernel at 14 DAA). Subsequently, dry matter accumulated faster until 42 DAA (50.2 ± 3.6 mg/kernel), after which the deposition of dry matter ceased (51.4 ± 1.8 mg/kernel at maturity). These changes of grain moisture and dry

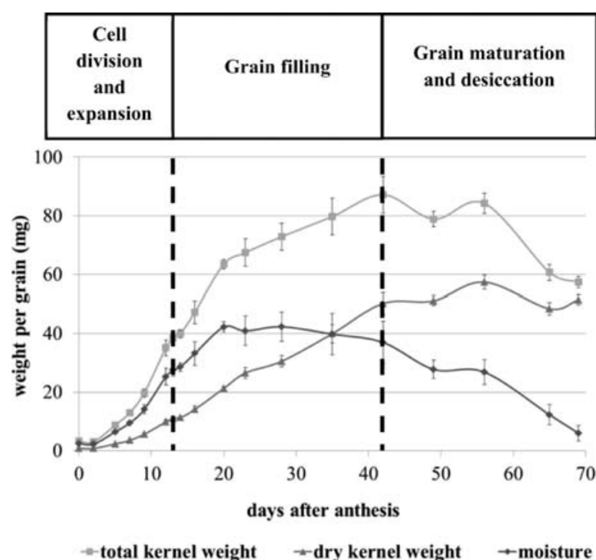

Figure 1. Change of kernel weight and moisture content. Total kernel weight (light gray square), dry kernel weight (dark gray triangle) and moisture content per kernel (black diamond) as a function of development time. Error bars are standard deviations on triplicate measurements. Vertical dashed lines separate the three consecutive phases of kernel development: the division and expansion phase, the grain filling phase and the maturation and desiccation phase.

matter content are in agreement with the general knowledge on grain development and were used to divide grain development into three consecutive phases as described earlier.[3,23,24] The first phase, which occupied the first two weeks after anthesis, is the phase of cell division and expansion,[3] also called the phase of grain enlargement.[24] During this initial phase, there is a rapid accumulation of water in the grain.[23] The second phase, called grain filling phase, is characterized by a rapid accumulation of dry matter and lasted until approximately 42 DAA in the present study. The final phase is the phase of grain maturation and desiccation (42 DAA until 69 DAA), in which the kernel dry matter content remained constant and the kernel moisture content decreased.

Protein. The crude protein content, which was calculated based on the total nitrogen content, decreased from 18.3% at anthesis to 11.8 ± 1.5% in the mature kernel (Figure 2A). This decrease is in line with the results of Jennings and Morton,[25] who furthermore demonstrated that the majority of the total nitrogen compounds in wheat kernels consisted of proteins except for kernels in the first phase of development. At 8 DAA, about 52% of the total nitrogen was present in proteins, while the remaining was mostly present in free amino acids. Yet, the importance of the latter fraction decreased rapidly later on during grain development.[25] In accordance with literature data,[24−26] proteins accumulated mainly during the grain filling stage (Figure S1 in the Supporting Information). There was a vast increase in the crude protein content per kernel during this phase (1.53 ± 0.06 mg/kernel at 14 DAA and 5.67 ± 0.55 mg/kernel at 42 DAA) but not during the final stage of grain maturation and desiccation (6.08 ± 0.85 mg/kernel at 69 DAA).

Glucose, Fructose and Sucrose. The appearance and disappearance of glucose, fructose and sucrose in the developing kernels is shown in Figure 2B (on a percentage basis) and in Figure S1 in the Supporting Information (per kernel basis). Glucose and fructose concentrations were almost identical and were the highest during the first phase of development with a maximum value of 3.2 ± 0.3% and 3.1 ± 0.3% for glucose and fructose, respectively, at 9 DAA. Glucose and fructose concentrations decreased during grain filling and in the mature grain only traces of these hexoses were detected (0.04 ± 0.01% glucose and 0.04 ± 0.02% fructose at 69 DAA) as reported before.[27] Sucrose concentrations were, similar to glucose and fructose concentrations, high during the phase of cell division and expansion, decreasing during grain filling and constantly low during grain maturation (Figure 2B). However, the highest sucrose concentrations were reached between 12 and 16 DAA (7.3 ± 0.7% and 6.3 ± 0.3% sucrose, respectively) when glucose and fructose concentrations were already declining. This discrepancy caused a shift in the monosaccharide (sum of glucose and fructose) to sucrose ratio. Indeed, the monosaccharide to sucrose ratio was high during the first two weeks after anthesis and reached a maximum at 7 DAA (1.5 ± 0.6). However, this ratio sharply decreased when grain filling commenced (0.41 ± 0.05 at 16 DAA) and remained low during grain filling (0.11 ± 0.06 at 42 DAA) and grain maturation (0.08 ± 0.03 at maturity). A decline in the monosaccharide to sucrose ratio was also seen in *Arabidopsis thaliana*,[28] *Vicia faba*[29] and *Brachypodium distachyon*[30] and correlated with the start of the deposition of storage compounds while a high monosaccharide/sucrose ratio correlated with cell division.

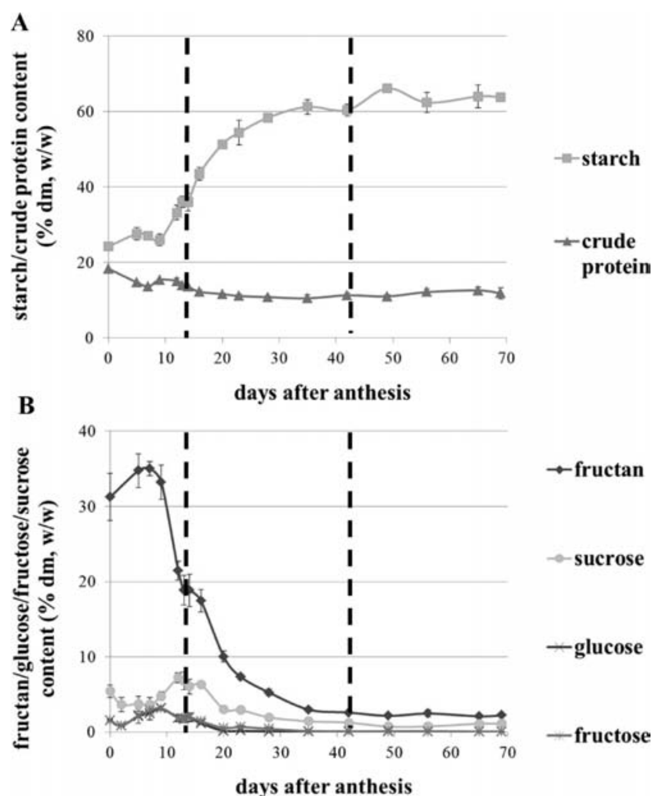

Figure 2. Crude protein, starch, fructan, glucose, fructose and sucrose concentrations in developing wheat kernels. Starch and crude protein concentrations as a percentage of kernel dry matter (A) and glucose, fructose, sucrose and fructan concentrations as a percentage of kernel dry matter (B) as a function of development time. Error bars are standard deviations on triplicate measurements. Vertical dashed lines separate the three consecutive phases of kernel development: the division and expansion phase, the grain filling phase and the maturation and desiccation phase.

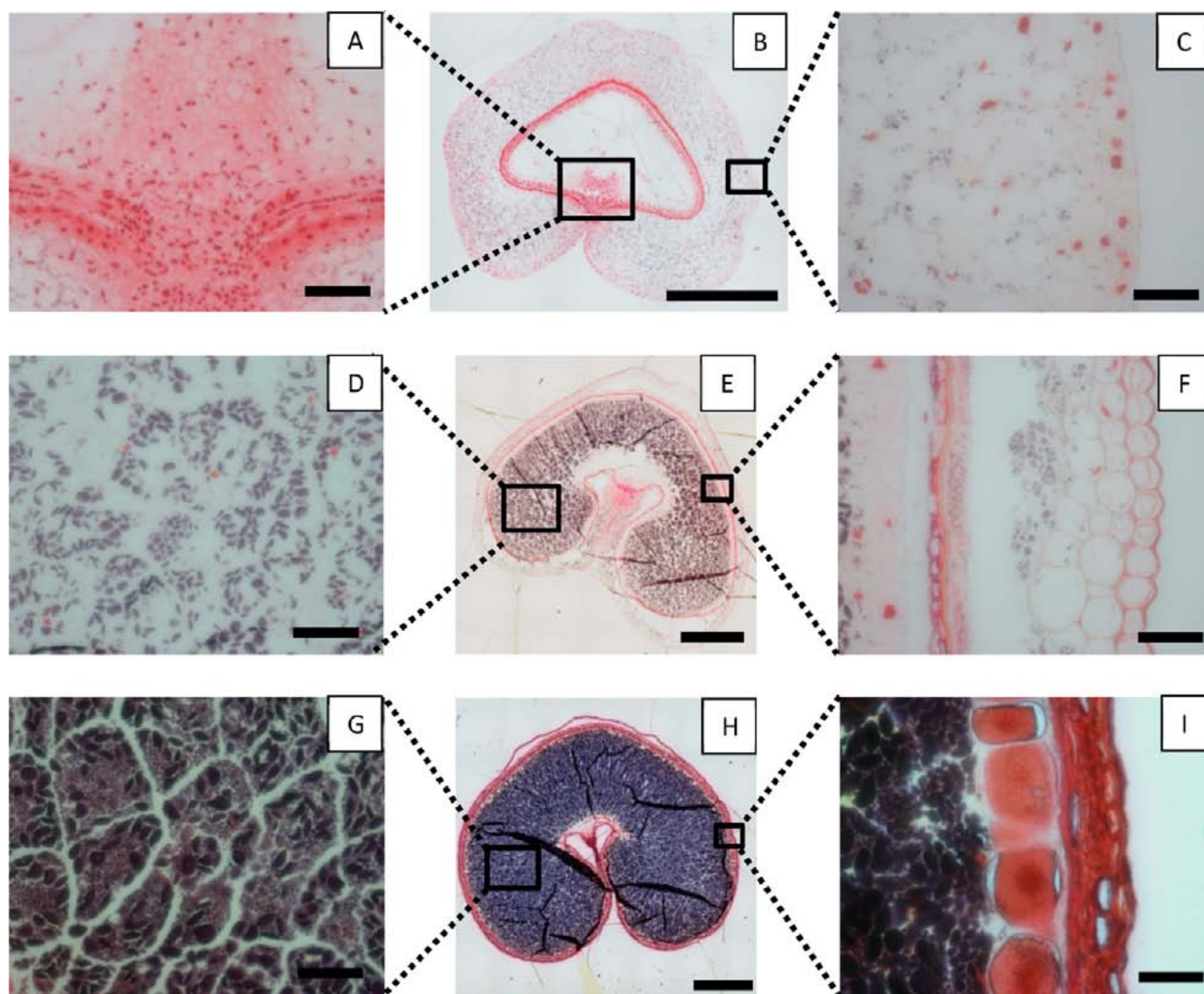

Figure 3. Light micrographs of grain sections stained with neutral red and Lugol's solution, 5 DAA (A, B and C), 16 DAA (D, E and F) and 69 DAA (G, H and I). Bars measure 1000 μm (B, E and H), 100 μm (A, D and G) or 50 μm (C, F and I). The large and small rectangles in images B, E and H indicate the zones in which images A, D and G and C, F and I, respectively, were taken.

Fructans. Fructans were the major carbohydrates at the start of kernel development, and after 7 DAA, fructans formed 35.0 ± 1.0% of the grain dry matter (Figure 2B) corresponding to 1.2 ± 0.1 mg of fructan/kernel (Figure S1 in the Supporting Information). However, once starch concentrations started to increase at around 10 DAA, fructan concentrations rapidly decreased. This relative decrease of fructan concentrations was mainly caused by the accumulation of nonfructan compounds since only a limited net degradation of fructan took place during grain filling (2.5 ± 0.3 mg of fructan/kernel at 16 DAA and 1.3 ± 0.1 mg of fructan/kernel at 42 DAA). A similar decrease in fructan concentrations has been reported before for both developing *Triticum aestivum*[27] and *Triticum durum*[5] kernels.

Starch. Starch, including malto-oligosaccharides, was already present in kernels in the cell division and expansion phase (Figure 2A). In this phase, grains consisted mainly of maternal pericarp tissue and the developing endosperm constituted only a small part of the grain (Figure 3B). Small starch granules were visible in the pericarp but not in the developing endosperm (Figures 3C and 3A, respectively). The main accumulation of starch started at the end of the cell division and expansion phase and lasted until the end of grain filling (4.1 ± 0.3 mg of starch/kernel and 30.3 ± 2.5 mg of starch/kernel at 14 DAA and 42 DAA, respectively, Figure S1 in the Supporting Information). At the start of grain filling, cell nuclei were still visible and almost only large, lenticular shaped A-type starch granules were observed in the endosperm (Figure 3D). At 16 DAA, the pericarp had been degraded to a large extent (Figure 3F). Free starch granules remained at the inner side of the pericarp where cell walls were largely degraded. Starch accumulation ceased at 42 DAA, and starch concentrations remained constant during the last three weeks of grain development (32.8 ± 2.3 mg of starch/kernel at maturity) in accordance with earlier reported results.[25] In the endosperm of the mature kernel, both large and small starch granules were present while cell nuclei were crushed (Figures 3G and 3H). The microscopic observation that small starch granules were present in the pericarp but not in the endosperm of grains of 5 DAA confirms previous quantitative results. Chevalier and Lingle[31] found that starch concentrations in the pericarp reached a maximum at 6 DAA and decreased when starch accumulation in the endosperm started, around 9 DAA. Similarly, the main accumulation of starch started around 9 DAA in the present study. As reported before,[32] the synthesis of

A-type starch granules in the endosperm was initiated earlier than the synthesis of the smaller B- and C-type starch granules.

Arabinoxylan. Figure 4 shows that arabinoxylan concentrations rapidly increased at the end of the cell division and expansion phase with arabinoxylan concentrations of 3.0 ± 0.1% at 6 DAA and 6.5 ± 0.2% at 14 DAA. During grain filling, arabinoxylan concentrations remained roughly constant as both the total dry matter (Figure 1) and arabinoxylan content per kernel (0.73 ± 0.03 mg of arabinoxylan/kernel at 14 DAA and 3.52 ± 0.30 mg of arabinoxylan/kernel at 42 DAA, Figure S2 in the Supporting Information) increased continuously and in constant proportion. Finally, during the phase of maturation and desiccation, both the dry matter and arabinoxylan content per kernel remained constant. To study arabinoxylan spatial distribution in the developing kernel, grain sections were labeled with a fluorescent xylanase and examined with fluorescence microscopy (Figure 5). Almost no arabinoxylan staining was observed in kernels of 5 DAA, even at high exposure times (result not shown). In kernels of 12 DAA, a clear arabinoxylan staining was observed in the nucellar epidermis (Figure 5B and 5C) whereas in the endosperm only the cell walls in the ventral region of the grain were stained (Figure 5A). However, a few days later, at 16 DAA, all endosperm cell walls, including those of the starchy endosperm, gave a clear signal after xylanase labeling, while the nucellar epidermis was the most fluorescent part of the grain (result not shown). This is consistent with the earlier reported findings that arabinoxylan synthesis in the starchy endosperm is initiated at the beginning of the differentiation stage, around 12 DAA, when starch and protein accumulation start.[7] Besides, arabinoxylans were detected earlier in development in the transfer cells[33] and in the nucellar epidermis and the cross cells, while arabinoxylans were not seen in the maternal outer tissue of the pericarp.[7] The intense arabinoxylan staining of aleurone cell walls and of the interface between the nucellar epidermis and the aleurone cells during the grain filling stage (Figures 5D, 5E and 5F) and at maturity (Figures 5G, 5H and 5I) corresponds with the observations of Robert et al.[33]

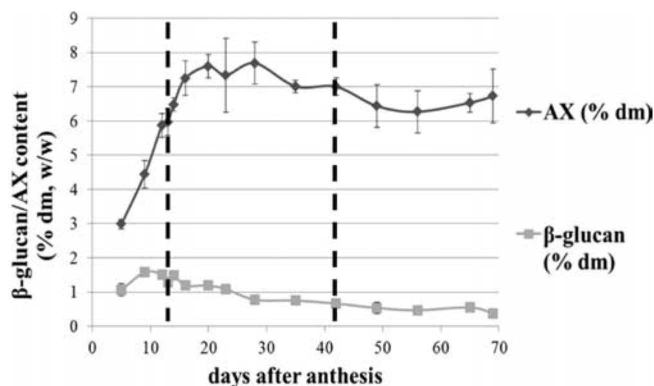

Figure 4. Arabinoxylans and β-glucan concentrations in developing wheat kernels. Arabinoxylan (black diamond) and β-glucan (light gray square) concentrations as a percentage of kernel dry matter as a function of development time. Error bars are standard deviations on triplicate measurements. Vertical dashed lines separate the three consecutive phases of kernel development: the division and expansion phase, the grain filling phase and the maturation and desiccation phase.

β-Glucan. Despite an initial increase, β-glucan concentrations generally decreased slowly throughout development with 1.58 ± 0.12% as the highest β-glucan concentration at 9 DAA and 0.38 ± 0.03% as the lowest concentration at maturity (Figure 4). The total β-glucan content per kernel increased continuously during the first and second phases of development (0.02 ± 0.005 mg of β-glucan/kernel at 5 DAA and 0.33 ± 0.04 mg of β-glucan/kernel at 42 DAA, Figure S2 in the Supporting Information). Nevertheless, in contrast to the results of Toole et al.,[10] the β-glucan content per kernel slightly decreased during the last phase of development (0.19 ± 0.02 mg of β-glucan/kernel at 69 DAA). However, interpretation of the results of Toole et al.[10] is quite difficult as not all peaks generated from lichenase hydrolysis were identified and no standards were used to quantify the released sugars. Besides, Philippe et al.,[7] when interpreting the results of a microscopic analysis of β-glucan in developing wheat kernels, also suggested that β-glucan might be broken down during the stage of grain maturation.

Calcofluor was used to visualize β-glucan deposition.[20] At 5 DAA, calcofluor clearly stained the cell walls of the maternal pericarp (Figures 6A, 6B and 6C). This is in agreement with the earlier reported findings that (1−3)(1−4)-β-glucans are present in the maternal pericarp from the earliest stages of grain development before they appear in the developing endosperm. Indeed, Philippe et al.[7] demonstrated that the developing endosperm temporarily contained (1−3)-β-glucan during the cellularization stage and not (1−3)(1−4)-β-glucan. When grain filling started (Figures 6D and 6E), the endosperm cell walls were calcofluor labeled, mainly those of cells near the crease region. Coincidentally, the nucellar epidermis gave a clear signal (Figure 6F), similar to the situation with arabinoxylan labeling. However, in the mature kernels (Figure 6I), only a weak signal was observed in the nucellar epidermis and likewise the signal in the outer pericarp had almost disappeared. In the endosperm, the thick aleurone cell walls were the most prominently stained (Figures 6G, 6H and 6I) as reported before.[34,35]

Correlations between Kernel Constituents. Figure 7 shows the dry matter composition of the developing kernels from anthesis until maturity. The most striking compositional shift in kernel dry matter is the transient presence of high fructan concentrations. Fructans were the major carbohydrates in developing kernels the first 9 DAA, and this role was taken over by starch thereafter. This inverse relationship between starch and fructans is reflected by their negative Spearman correlation coefficient ($\rho = -0.97$, $n = 17$). Similar to fructan concentrations, the concentrations of the simple sugars glucose, fructose and sucrose were high during the first phase of development but lower thereafter. Hence, the sum of all small water-soluble carbohydrates (SWSC: glucose, fructose, sucrose and fructans) was also negatively correlated with starch concentrations ($\rho = -0.98$, $n = 17$). In addition, the initial phase of development was characterized by a high relative kernel moisture content that correlated positively with SWSC concentrations on the one hand ($\rho = 0.93$, $n = 17$) and with fructan concentrations on the other hand ($\rho = 0.95$, $n = 17$) throughout kernel development. Fructan accumulation was confined to the first phase of development, the phase of cell division and expansion, which is in agreement with the findings of Schnyder et al.,[11] who studied fructan metabolism in different tissues of developing wheat grains. They observed that fructan synthesis was limited to the period of rapid spatial growth of individual grain tissues and that it coincided with the deposition of water in these tissues. Fructan accumulation has been reported before in the growth zones of other cereal

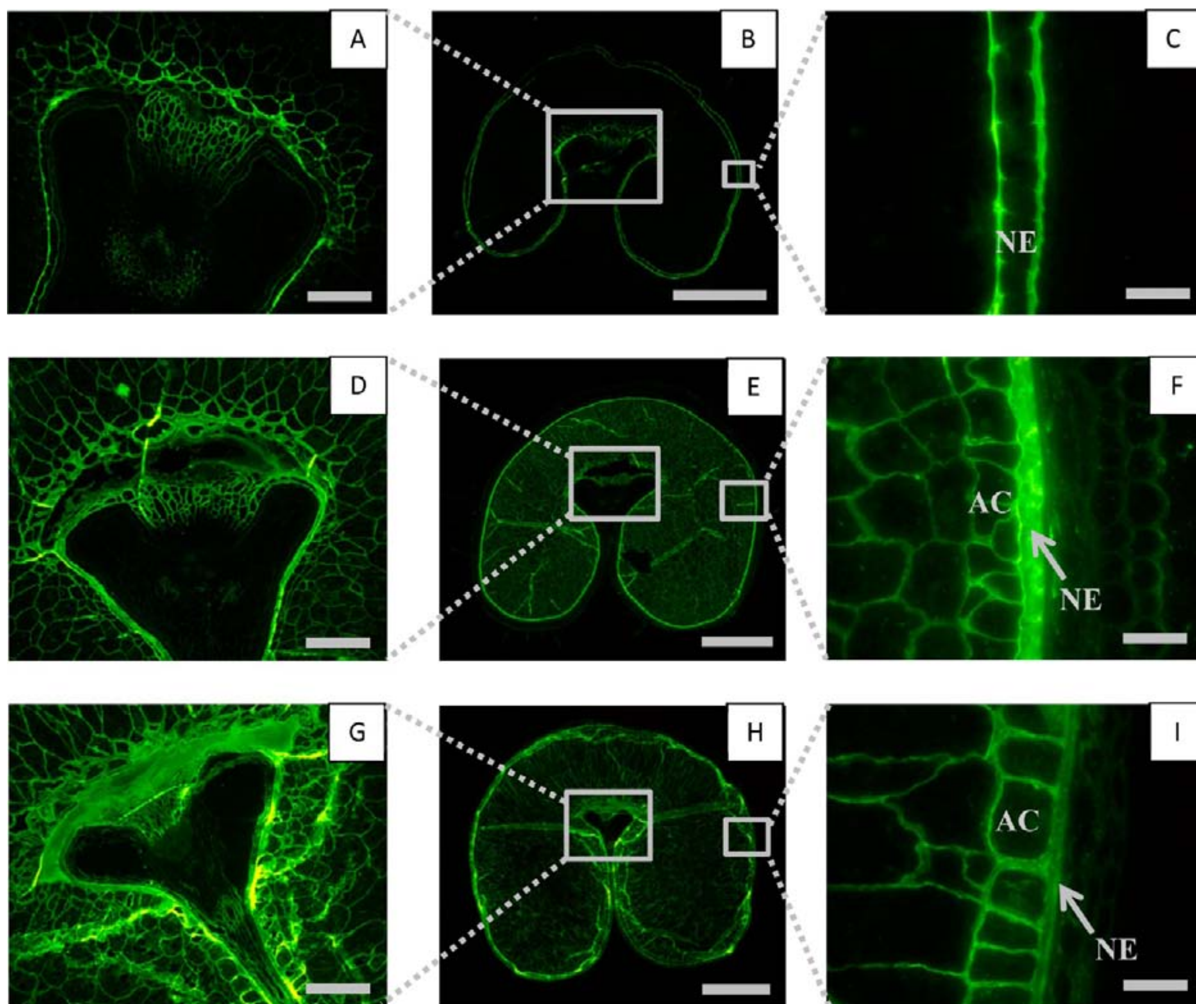

Figure 5. Fluorescence micrographs of grain sections stained with inactive fluorescent labeled xylanase, 12 DAA (A, B and C), 28 DAA (D, E and F) and 57 DAA (G, H and I). Bars measure 1000 μm (B, E and H), 200 μm (A, D and G) or 50 μm (C, F and I). Nucellar epidermis (NE) and aleurone cells (AC) are indicated. The large and small rectangles in images B, E and H indicate the zones in which images A, D and G and C, F and I, respectively, were taken.

tissues[36,37] and may serve to regulate osmotic pressure[38] or to sequester the surplus of imported sucrose. In the latter case, fructans buffer the transfer of photosynthesis products and help preserving a downhill sucrose gradient.[39,40] Indeed, soluble carbohydrates, mostly sucrose,[38] enter the developing grain via the tissues at the base of the crease, move through the endosperm cavity and finally move radially across the endosperm in an outward direction. Along this transport pathway, a downhill gradient of both sucrose and fructan from the endosperm cavity to the endosperm has been observed in kernels of 20 DAA.[41,42] Furthermore, in the pericarp tissue, fructan synthesis may function as a mechanism to prevent sucrose induced downregulation of photosynthesis. The cross cells and, to a lesser extent, the tube cells are photosynthetic during the first three weeks of kernel development,[43] and so there is a need to maintain sucrose concentrations below the threshold for the downregulation of photosynthesis.[44] Finally, it cannot be excluded that fructans play a role in scavenging reactive oxygen species (ROS) in developing wheat grains. Tissue parts with increased cell division and expansion typically show increased ROS levels,[45] and a role for fructans as ROS scavengers was recently proposed.[46] Considered together, fructans may act as ROS scavengers in developing wheat grains, but this remains a subject of further investigation. In conclusion, fructans presumably serve several functions in wheat grain development that are not mutually exclusive.

To find correlations between storage compounds, their average amounts per kernel were compared. As expected, the absolute amount of starch per kernel correlated strongly with the amount of crude protein per kernel (ρ = 0.98, n = 17) as both storage compounds are mainly accumulated during grain filling. In addition, both storage compounds correlated positively with the average arabinoxylan amount per kernel (ρ = 0.98, n = 17 for both crude protein and starch). However, the correlations between the amount of β-glucan per kernel and the amount of crude protein per kernel on the one hand (ρ = 0.77, n = 15) and the amount of starch per kernel on the other hand (ρ = 0.78, n = 15) were less strong. Indeed, arabinoxylans are almost exclusively synthesized during the grain filling phase, similar to proteins and starch. β-Glucan synthesis on the contrary was already ongoing when grain filling started, and the amount of β-glucan even decreased during grain maturation and desiccation. β-Glucan synthesis prior to arabinoxylan synthesis has been reported before.[7,10,47] β-Glucans were therefore suggested to be the structural elements of the early cell walls of growing cells[47] whereas arabinoxylans might

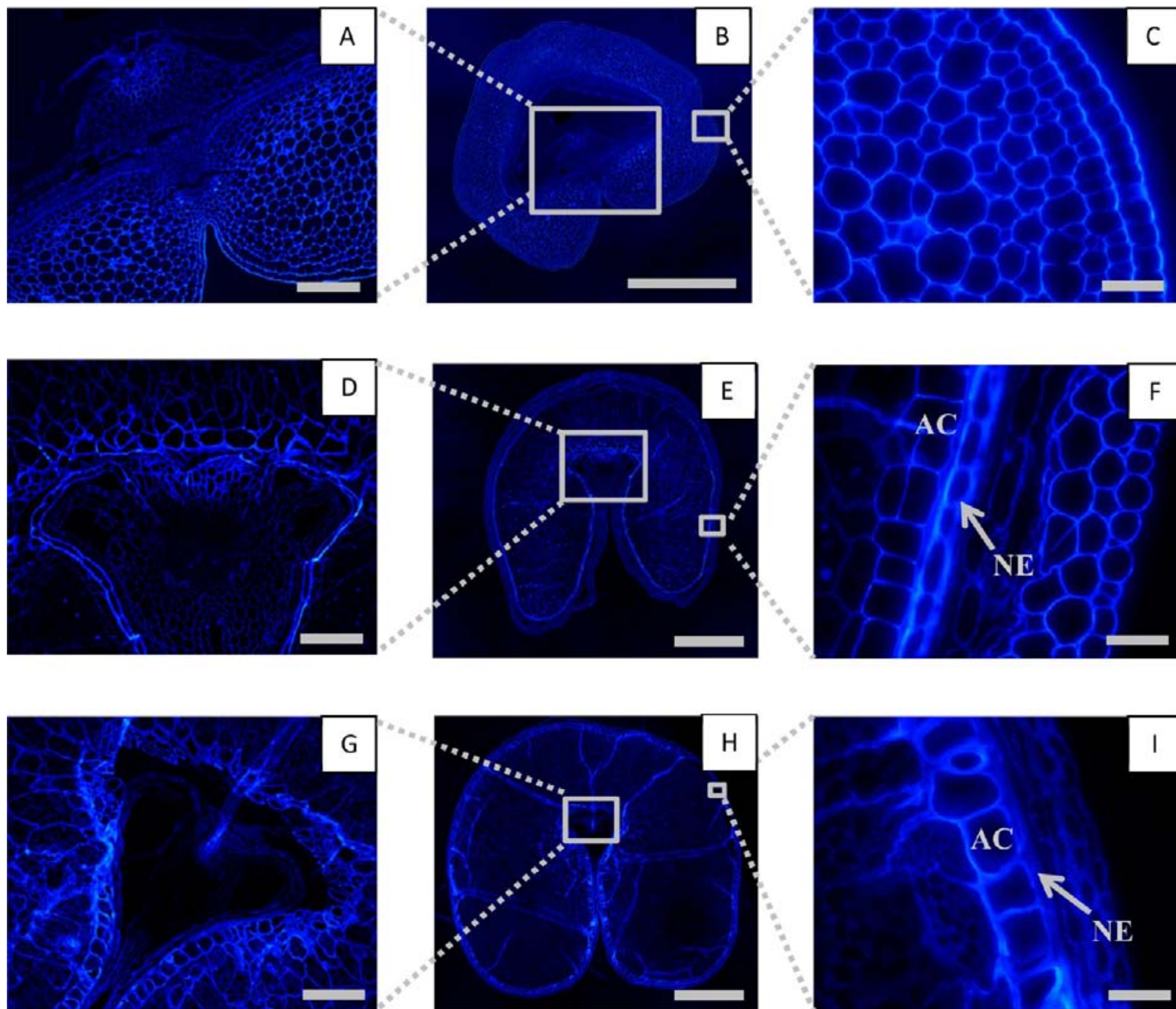

Figure 6. Fluorescence micrographs of grain sections stained with calcofluor, 5 DAA (A, B and C), 16 DAA (D, E and F) and 57 DAA (G, H and I). Bars measure 1000 μm (B, E and H), 200 μm (A, D and G) or 50 μm (C, F and I). Nucellar epidermis (NE) and aleurone cells (AC) are indicated. The large and small rectangles in images B, E and H indicate the zones in which images A, D and G and C, F and I, respectively, were taken.

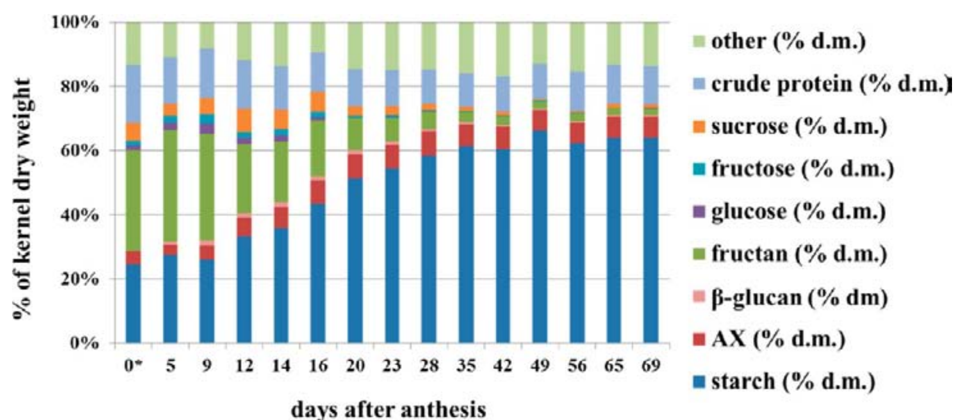

Figure 7. Changes in kernel dry matter composition in developing wheat kernels. The kernel crude protein, sucrose, fructose, glucose, fructan, β-glucan, arabinoxylan and starch content were measured in triplicate except for day 0 (*). Crude protein, arabinoxylan and starch content in kernels of day 0 were based on single measurements whereas β-glucan was not determined.

strengthen the walls of cells that have reached or almost reached their final size.[7]

In conclusion, the development of wheat grains could be divided into three consecutive phases based on the changes of grain dry matter and moisture content. During the first phase, which lasted the first two weeks, cell division and expansion took place. Grains consisted mainly of maternal pericarp containing β-glucan but no arabinoxylan and were rich in SWSC like fructan, glucose, fructose and sucrose. The accumulation of these SWSC correlated with rapid growth-

related water uptake. However, once grain filling started, SWSC were consumed while large amounts of starch, protein and arabinoxylans were deposited simultaneously. Both arabinoxylan and β-glucan were present in the entire endosperm but were more prominent in the ventral region of the endosperm. Grain filling ended when the grain attained maximal grain dry weight, around 42 DAA. During the last phase of grain maturation and desiccation, grain water content decreased strongly but no major shifts occurred in the composition of grain dry matter.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figure S1 showing the starch, crude protein, fructan, glucose, fructose and sucrose content per kernel as a function of development time. Figure S2 showing the arabinoxylan and β-glucan content per kernel as a function of development time. Figure S3 showing the changes of all analyzed components as a function of development time on a per kernel basis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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