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Exploring abiotic stress on asynchronous protein metabolism in single kernels of wheat studied by NMR spectroscopy and chemometrics

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Abstract

Extreme climate events are being recognized as important factors in the effects on crop growth and yield. Increased climatic variability leads to more frequent extreme conditions which may result in crops being exposed to more than one extreme event within a growing season. The aim of this study was to examine the implications of different drought treatments on the protein fractions in grains of winter wheat using ^{1}H nuclear magnetic resonance spectroscopy followed by chemometric analysis. *Triticum aestivum* L. cv. Vinjett was studied in a semi-field experiment and subjected to drought episodes either at terminal spikelet, during grain-filling or at both stages. Principal component trajectories of the total protein content and the protein fractions of flour as well as the ^{1}H NMR spectra of single wheat kernels, wheat flour, and wheat methanol extracts were analysed to elucidate the metabolic development during grain-filling. The results from both the ^{1}H NMR spectra of methanol extracts and the ^{1}H HR-MAS NMR of single kernels showed that a single drought event during the generative stage had as strong an influence on protein metabolism as two consecutive events of drought. By contrast, a drought event at the vegetative growth stage had little effect on the parameters investigated. For the first time, ^{1}H HR-MAS NMR spectra of grains taken during grain-filling were analysed by an advanced multiway model. In addition to the results from the chemical protein analysis and the ^{1}H HR-MAS NMR spectra of single kernels indicating that protein metabolism is influenced by multiple drought events, the ^{1}H NMR spectra of the methanol extracts of flour from mature grains revealed that the amount of fumaric acid is particularly sensitive to water deficits.

Key words: Chemometrics, drought, fumaric acid, grain-filling, HR-MAS NMR, PARAFAC, PCA trajectory, single kernel, wheat.

Introduction

Increased climatic variability leading to more frequent extreme conditions may result in crops being exposed to more than one extreme event in a single growing season. As with temperature, variability in drought can occur through variation in its timing, intensity, and duration (IPCC, 2007). Due to limited water resources, drought has become the single most limiting factor to crop production worldwide (Wollenweber *et al.*, 2003). In the last decade, severe

droughts leading to significant yield losses have become a major problem in parts of Europe. The overall prediction is that climate change will affect yield quantity (higher grain yield due to higher dry matter), but potentially at the expense of yield quality.

Besides starch, proteins are the most important components of wheat grains governing end-use quality. However, the quantity of protein per grain is mainly under environmental control. The deposition of the various protein fractions takes place asynchronously, which means that both the amount and concentration of these components vary throughout the maturity period of the grains (Martre *et al.*, 2003). One consequence of this is that conditions shortening grain-filling, such as episodes of high temperature and/or water deficits, will affect the balance of protein fractions (Jamieson *et al.*, 2001). With these considerations in mind, it is important both to understand the environmental constraints on crop quality and to predict how quality will be influenced by the interaction of multiple extreme climatic conditions (Raven *et al.*, 2004).

To date, there are only very few studies on the effect of environmental variability on the quantitative variation in crop protein composition (Martre *et al.*, 2003; Triboi *et al.*, 2003; Wollenweber *et al.*, 2003). The developmental stage of crops experiencing stress events will determine the degree of possible damage experienced by the plant. It has recently been shown that extreme heat events at the vegetative growth stage of double ridge does not affect subsequent growth and development of wheat exposed to heat stress at anthesis as well, while the development of fertile grains is affected by high temperature episodes (Martre *et al.*, 2003; Triboi *et al.*, 2003; Wollenweber *et al.*, 2003).

Nuclear magnetic resonance (NMR) spectroscopy is a powerful non-invasive analytical technique for measuring multiple parameters of plant tissue *in vivo* (Krishnan *et al.*, 2005). ^{1}H NMR can be used as a single analytical method to obtain information about the vast majority of metabolites in a plant system, since all proton-bearing compounds such as carbohydrates, amino acids, organic and fatty acids, and lipids can be simultaneously detected. However, a challenge in ^{1}H NMR spectroscopy of plants is the solid nature of plant tissue. In solids, anisotropic interactions like homo- and heteronuclear dipole-dipole couplings as well as chemical-shielding anisotropy induce significant line broadening in the NMR spectra. These effects may be eliminated in ^{1}H NMR spectra of soft materials (high mobility) by the use of high resolution magic angle spinning (HR-MAS) and spectral resolution similar to liquid-state NMR is obtained. ^{1}H HR-MAS NMR spectroscopy has already been used in metabonomic studies of animal and human tissue without the need of chemical extraction or further sample preparation (Garrod *et al.*, 2001; Lindon *et al.*, 2001; Griffin *et al.*, 2003). Recently, carbohydrate grain-filling of barley mutants has been studied by ^{1}H HR-MAS NMR (Seefeldt *et al.*, 2008).

The overwhelming information produced by spectroscopic screening of complex biological samples calls for multivariate data analysis such as chemometrics in order to extract systematic information. Such analysis requires a minimum of assumptions and the relationships may be visualized by intuitive illustrations by means of the graphic computer interface (Winning *et al.*, 2008).

In the field of metabolomics, the analysis of metabolic changes in time is a fundamental aspect of understanding the biochemical response of an organism to an external perturbation (Lindon *et al.*, 2001). Thus, the aim of the current study was to investigate the implications of one or more drought events on protein quality in wheat grains using information from chemical protein analysis, liquid state ^{1}H NMR of methanol extracts, and ^{1}H HR-MAS NMR of single kernels and flours followed by unsupervised exploratory chemometric data analysis.

Materials and methods

Plant material

In a semi-field pot experiment, winter wheat (*Triticum aestivum* L. cv. Vinjett) was grown outdoors at the semi-field facility of the Faculty of Agricultural Sciences at Flakkebjerg (Slagelse, Denmark) in the growing season of 2005. Pots with both depth and diameter of 25 cm were equally filled with 4.2 kg 1:2:1 (by vol.) mixture of peat substrate, loamy soil, and sand. A dose of 5.25 g K_2SO_4, 3.5 g $(NH_4)_2SO_4$, 4.67 g NH_4NO_3, 1.9 g $CaSO_4$, 1.9 g $MgSO_4$, 0.4 g $MnSO_4$, 0.4 g $CuSO_4$, and 11.67 g $CaCO_3$ per pot was also mixed in the soil. Spring wheat was sown at a rate of 15 seeds per pot and then thinned to five seedlings per pot at three-leaf stage.
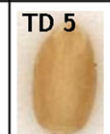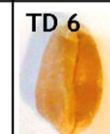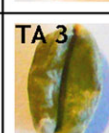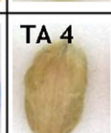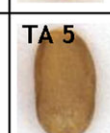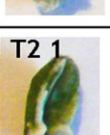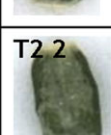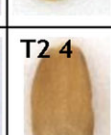
Water deficits were applied during two growth stages, namely terminal spikelet (end of spikelet initiation) and at anthesis by withholding irrigation. Spikes were harvested 10 d after anthesis (DAA), at four time points during the grain-filling period (17, 23, 31, and 43 DAA) and at harvest maturity (50 DAA), yielding six harvests in total (Fig. 1). The spikes were harvested and immediately frozen in liquid nitrogen and stored at –80 °C. Afterwards, the spikes were freeze-dried for 2 d.

The freeze-dried grains were milled (0.5 mm, Cyclotec 1093, Foss Tecator AB, Högenas, Sweden). The flour material was stored in sealed plastic bottles at 4 °C until analysis. A flour sample unit consisted of the seeds from two spikes. A total of 48 flour samples were analysed, covering a total of six harvests including two replicates of all four treatments (6×4×2).

See Fig. 1 for an overview of the experimental design. The four treatments constitute of the control treatment (CT) which has sufficient water supply throughout the period, the early drought treatment which is exposed to drought at double ridge (TD), the late drought treatment which is exposed to drought at anthesis (TA), and the fourth treatment which is exposed to drought at double ridge and at anthesis (T2).

Methanol extract preparation

Samples were prepared using a protocol performed by Baker *et al.* (2006) which was a modified form of the

Harvest 2005	8th July	15th July	21th July	29th July	10th August	17th August
Days after anthesis	10	17	23	31	43	50
Control: No drought	Contr 1	Contr 2	Contr 3	Contr 4	Contr 5	Contr 6
Early drought: Drought at double ridge	TD 1	TD 2	TD 3	TD 4	TD 5	TD 6
Late drought: Drought at anthesis	TA 1	TA 2	TA 3	TA 4	TA 5	TA 6
Two drought periods: Double ridge+ anthesis	T2 1	T2 2	T2 3	T2 4	T2 5	T2 6

Fig. 1. The experimental design of the four different drought treatments: control (CT), early drought (TD), late drought (TA), and double drought (T2) together with the six harvests.

method described by Ward *et al.* (2003). Replicate aliquots of white flour (30 mg) were weighed into 1.5 ml Eppendorf tubes. D_2O–CD_3OD (1 ml, 80:20) containing 0.05% (w/v) TSP-d4 was added to each sample. The contents of the tube were mixed thoroughly and heated at 50 °C in a water bath for 10 min. The samples were then spun down in a micro centrifuge for 5 min; 800 µl of the supernatant was transferred to an Eppendorf tube and kept at 90 °C in a water bath for 2 min. The high-temperature (90 °C) step was incorporated to ensure that enzyme activity had stopped. The samples were then stored at 4 °C for 45 min prior to recentrifugation for 5 min (still at 4 °C); 700 µl of the supernatant was transferred to a 5 mm (o.d.) NMR tube. The residual CD_2HOD multiplet in the region 3.36–3.32 ppm was excluded from all data sets.

^{1}H HR-MAS NMR

^{1}H HR-MAS NMR spectra were obtained using a Bruker AVANCE-400 (Bruker BioSpin, Rheinstetten, Germany) spectrometer, operating at a frequency of 400.13 MHz for protons equipped with a HR-MAS double channel probe using a 50 µl zirconia rotor (4.0 mm o.d.). Samples were prepared in the rotor using approximately 14 mg flour or one single kernel and 30 µl of D_2O (with 5.8 mM of TSP-d4 (per-deuterated 3-trimethylsilyl propionate sodium salt). Data were accumulated at 298 K employing a pulse sequence using presaturation of the water resonance during

the 2 s recycle period followed by a composite 90 degree pulse (Bax, 1985) with an acquisition time of 2.045 s, 256 scans, and a spectral width of 8012.82 Hz, resulting in 16 k complex data points. A spin-rate of 7 kHz was used for all experiments. All samples were individually tuned and matched and the corresponding spectra were automatically phased and baseline-corrected and referenced to TSP-d4 at 0.0 ppm. Prior to Fourier transformation, each FID was apodized by Lorentzian line broadening of 0.3 Hz and zero filled to 64 k points. Flour and whole kernels were analysed approximately 2 h after preparation. All spectra were normalized relative to the TSP-d4 signal. The spectral range from 0.45–8.70 ppm was chosen, resulting in 27 000 data points. Bruker Topspin 1.3 (Bruker BioSpin 2005) was used for acquisition and processing of NMR data.

Liquid state ^{1}H NMR

The liquid state ^{1}H NMR spectra were obtained using a Bruker AVANCE-400 (Bruker BioSpin, Rheinstetten, Germany) spectrometer, operating at 400.13 MHz for protons using a broad band inverse detection probe head equipped with 5 mm (o.d.) NMR sample tubes. Data were accumulated at 298 K employing a pulse sequence using presaturation of the water resonance during the 2 s recycle period followed by a composite 90 degree pulse (Bax, 1985) with an acquisition time of 2.045 s, 256 scans, and a spectral width of 8012.82 Hz, resulting in 16 k complex data points.

All samples were individually tuned, matched, and shimmed. Prior to Fourier transformation, each FID was apodised by Lorentzian line broadening of 0.3 Hz and the corresponding spectra were automatically phased and baseline corrected and referenced to TSP-d4. In order to resolve the complex carbohydrate part of the spectra, a series of 1D and 2D experiments (^{13}C-HSQC, COSY, and TOCSY) were recorded at 18.8 T using a Bruker Avance 800 spectrometer operating at 799.92 MHz for protons and equipped with a 5 mm cryo probe.

Protein analysis

The protein content of flour (N×5.7) was determined by Kjeldahl analysis (Kjeldahl, 1883) in duplicate. The protein fractions (albumins, globulins, and gliadins) were extracted from flour in triplicate according to Osborne (Ghirardo *et al.*, 2005), and quantified according to Popov *et al.* (1975).

Chemometric analysis and software

Multivariate data analysis in the form of principal component analysis (PCA) (Hotelling, 1933) was applied to obtain systematic variations from the measured spectra. PCA is the primary tool for investigation of large bilinear data structures for the study of trends, groupings, and outliers. By means of PCA it is possible to find the main variation in a two-dimensional data set by creating new linear combinations, PCs, from the underlying latent structures in the raw data. To study the grain-filling process through time, PCA score trajectories are constructed from NMR data to identify changes in the biochemical profile. This procedure has previously been applied for analysis of dynamic change during bread baking (Engelsen *et al.*, 2001) and of metabolic time response to toxic lesion (Keun *et al.*, 2004).

In exploratory studies investigating changes over time, time series are naturally arranged as a three-way data set. The first dimension represents the treatment, the second dimension represents the harvest time, and the third dimension represents the metabolic profile measured by NMR (Castro and Manetti, 2007). Parallel factor analysis (PARAFAC) (Bro, 1997; Carrol and Chang, 1970; Harshman, 1970) can be considered as a multiway extension of PCA able to handle three-way data. The PARAFAC model is based on the decomposing of the data into trilinear components in a similar way to the bilinear components extracted in PCA. When such higher-order data are available, the so-called second-order data advantage gives unique solutions and, for example, the pure analyte spectra will be found in a mixture (Harshman, 1970). In this study, data were arranged in a three-way cube with single kernels with the four treatments in mode 1, the six harvest times in mode 2, and NMR spectra in mode 3 constituted by 27 000 data points, giving a data cube with dimensions 4×6× 27 000, and the PARAFAC model was calculated with non-negativity constraints and two factors. Data were mean-centred prior to the chemometric analysis.

The spectra were analysed using the chemometric software LatentiX 1.0 (www.latentix.com, Latent5, Copenhagen, Denmark), PLS Toolbox 4.11 (Eigenvector Research, Manson, Washington, USA), and MATLAB 2007a (The MathWorks, Inc., Natrick, Massachusetts, USA).

Results and discussion

Drought-induced changes in the accumulation of protein and the distribution in the main fractions, albumins, globulins, and gliadins were examined for the wheat kernels during grain-filling. Figure 2 shows the results from the Popov and Kjeldahl analysis demonstrating the asynchronous protein metabolism during grain-filling and the influence of the drought exposure.

A common feature in all the drought treatments and in the control is the rapid increase in the gliadin fraction from 10 DAA to 17 DAA accompanied by reductions in the albumin and globulin fractions, an observation that is in accordance with the function of gliadins in the further development of the kernel (Shewry and Halford, 2002).

The time development of the albumin fraction in the control wheat (CT) deviates from the wheat exposed to drought, with the TA treatment most affected. The time development of the gliadin fractions was less influenced by the different drought treatments. The globulins showed a marked response to drought. The globulin level of the CT wheat decreased from 10 DAA to 23 DAA after which the level increased throughout the rest of the period of growth. However, when wheat suffered from drought, whether early or late (TA and TD), the globulins show the same 'pattern' from 10 DAA to 43 DAA, but decrease, in fact, from 43 DAA to 50 DAA. Surprisingly, after experiencing two drought periods, the globulin fraction showed the same development profile throughout grain-filling as the control group which had sufficient water, except that the increase had already started at 17 DAA. A one-factor analysis of variance (ANOVA) model on the total protein content showed significant difference between treatments of all six harvests. However, a Fisher's least significant difference test showed that CT and T2 are not different at harvest times of 17, 23, and 50 DAA and that no significant difference is observed between CT and TD at harvest times of 31 and 43 DAA. This simple analysis indicates that the late drought treatment induces the most significant changes in the total protein content compared with the control. The asynchronous development of the protein fractions is underlined by an almost complete lack of correlation between the protein fractions during development. However, the albumin and the gliadin fractions in CT wheat during development were correlated with a correlation coefficient (R^2) of 0.88, but the correlation deteriorated significantly when exposed to drought.

In order to investigate changes in the protein profile of kernels affected by the different drought treatments, principal component trajectories have been calculated from the chemical data. These are presented in Fig. 3. Compared with the trajectory of CT, drought treatment apparently alters the trajectory pattern. The trajectories of the four
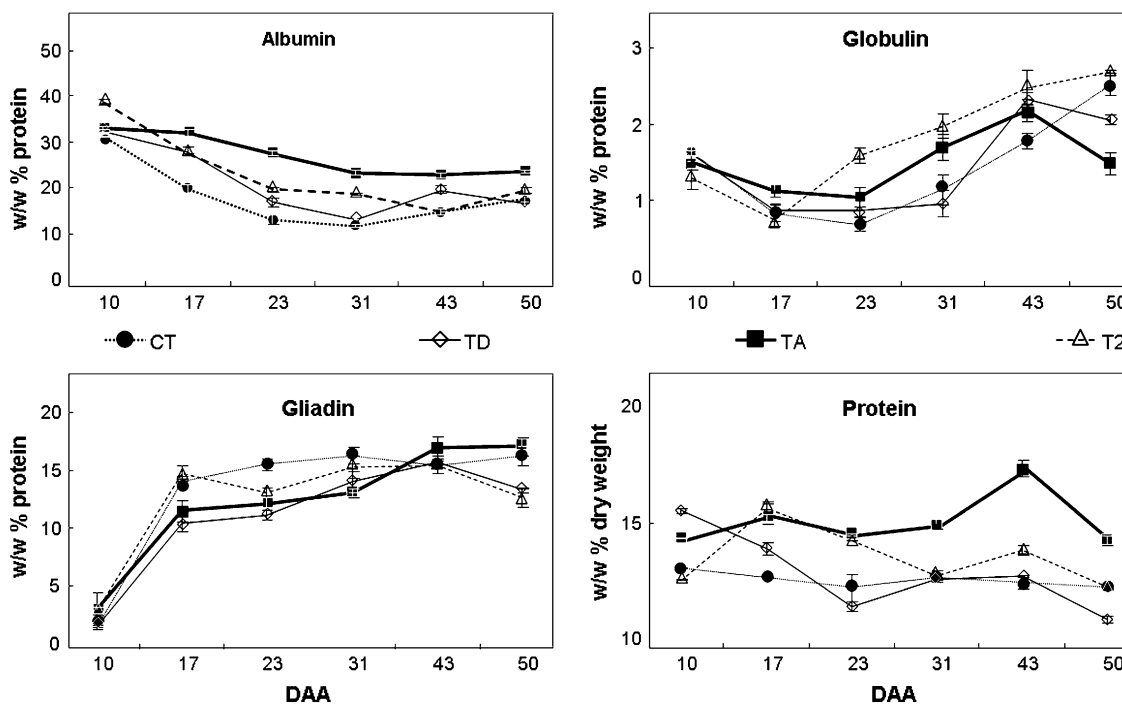
Fig. 2. Raw data from Popov and Kjeldahl analysis showing asynchronous protein metabolism in winter wheat as a function of DAA of the control group (CT), early drought (TD), late drought (TA), and twice drought (T2). Protein concentrations are measured in % dry weight kernel in duplicate. The albumin, gliadin, and globulin fractions are w/w % protein in triplicate: Error bars represent the deviation between replicates.
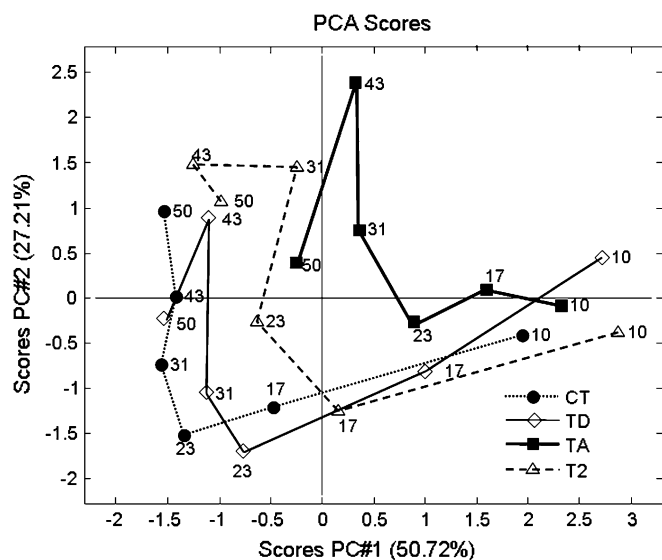

Fig. 3. PCA score plot of albumin, globulin, gliadin, and protein content during grain-filling showing different trajectories according to the drought treatment. PC1 and PC2 explain 51% and 27% of the variation, respectively.

treatments develop similarly progressing from 10-23 DAA along PC1, after which a bend along PC2 indicates that 23 DAA is a turning point for the protein synthesis of the CT, TD, and TA, whereas T2 deviates from this pattern by an increase in PC2 from 17–23 DAA. Moreover, a decrease in PC2 is observed from 43–50 DAA for the drought

treatment, but not for CT. An evaluation of the time development along PC1 shows that TD resembles CT the most and TA represents the most deviating treatment compared to CT. Altogether, the PCA revealed that late drought (TA) differs most from the control (CT) trajectory, indicating that the strongest effect on total protein content and the distribution in the protein fractions is after late drought. The PCA loadings (not shown) indicate that PC1 primarily concerns contributions from albumins and glia-dins which are negatively correlated. PC2 primarily concerns contributions from globulins and total protein content (both positively correlated). In conclusion, the treatment which most resembles the control regarding protein development is the TD treatment, whereas the TA treatment was the most deviating.

In addition to the Popov and Kjeldahl protein analysis, wheat single kernels and wheat flour were analysed by ^{1}H HR-MAS NMR. In Fig. 4, the spectra of flour and whole kernels from the control group at 10 DAA (Fig. 4a) and 50 DAA (Fig. 4b) are displayed. In the region 0–3 ppm, contributions arise from protons of the aliphatic side-chains of the amino acids and protons from the saturated parts of the lipids. The α- and β-protons from amino acids resonate in the 3–5.5 ppm region, overlapping with the HOD signal and the carbohydrates. The signals observed at 6.7–8.5 ppm arise from aromatic protons. The most obvious difference between the early and late harvests is that the spectra of the early harvest are dominated by signals from small carbohy-drates and free amino acids and the spectra of the late harvest are dominated by signals from lipids.
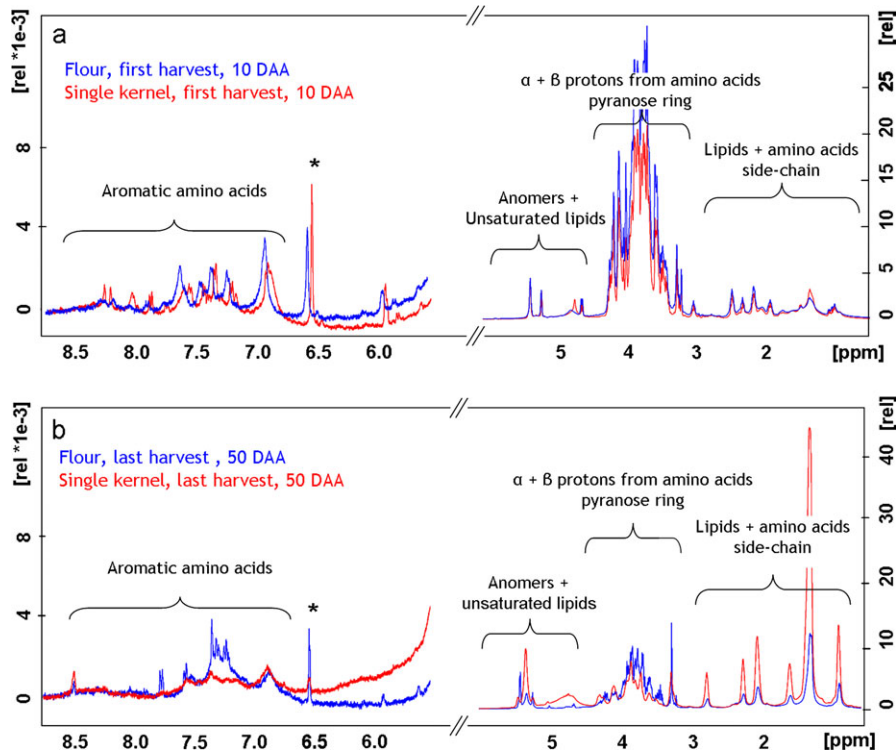
Fig. 4. ^{1}H HR-MAS NMR spectra of wheat flour and single kernels of the control group from the first harvest, 10 DAA (a) and the last harvest, 50 DAA (b), respectively. * Indicates fumaric acid. The region 5.5–9.0 ppm is vertically scaled by factor 20.

The NMR spectra of flour are better resolved in the 3–5.5 ppm region compared with the spectra of single kernels due to increased water accessibility of the molecules in the flour. In accordance with the continuous biosynthesis, amino acids, small carbohydrates etc. were observed in samples obtained at 10 DAA. Larger molecules (e.g. proteins or starch) are characterized by slower tumbling or a rigid structure which will increase the line width of the corresponding ^{1}H resonances. Indeed, they can even be broadened beyond detection in the liquid-state ^{1}H NMR spectrum. Therefore, the absence of resonances from the smaller molecules in the later stages of grain-filling implies that these are incorporated into larger molecules such as proteins, lipoproteins, glycolipids, or polysaccharides. The aromatic region is of particular interest with regard to proteins, since specific signals from aromatic amino acids (Phe, Trp, and Tyr) as well as His are located in this area without interfering signals from lipids and carbohydrates. Because it was possible to obtain information of the intact matrix of the wheat seed using single kernels, the ^{1}H HR-MAS NMR measurements of single kernels are preferred in this work, compared to the results obtained in a destroyed sample matrix as flour. Due to low signal intensity of the aromatic region, full spectra will be analysed.

The overall differences in lipid, carbohydrate, and protein accumulation pattern are all combined in the PCA kernel development trajectory score plot (Fig. 5) which is based on the average NMR measurements of two single kernels. The PCA of the scaled ^{1}H HR-MAS NMR spectra of the single kernels confirms that periods of drought change the development of the wheat kernel through grain-filling. Compared to the trajectory of the protein data in Fig. 3, the NMR spectra of the single kernels are more variable at the first harvest. This is probably due to physical differences (size of kernels, spike, and position of the spike etc.) between the chosen single seeds. Apart from this, the ^{1}H HR-MAS NMR spectra include information about carbohydrates, lipids, and proteins, whereas only proteins are included in Fig. 3. The PCA loadings (not shown) indicate that PC1 primarily describes aliphatic compounds, whereas PC2 primarily describes aromatic signals. The four trajectories have very different shapes according to treatment. However, the trajectory shape which most resembles the shape of the control is, in fact, the T2 trajectory which shows a parallel trajectory, although furthest from CT. The TD and the TA treatments follow different patterns in the score plot, with the TA treatment being the most deviating. The similarity of trajectories from T2 and CT indicates that treatment of wheat with two periods of drought does not significantly alter the final result. Compared to effects observed for TD and TA subjected to only one period of drought, this indicates that two drought periods induce better defence against drought compared to kernels treated with a single drought period. Apparently, the early drought treatment induces some drought immunity compared to drought later in the growth period. However, it was not possible to point to one compound expressing the drought treatment using the ^{1}H HR-MAS NMR single kernel spectra.
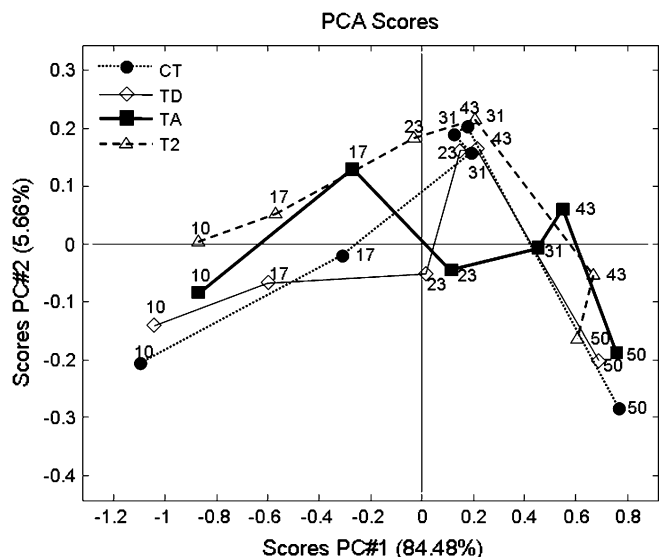
Fig. 5. PCA trajectory score plots obtained from single kernel ¹H HR-MAS NMR full spectra of the four different drought treatments (control (CT), early drought (TD), late drought (TA) and double drought (T2)). Spectra were block-scaled corresponding to the scaling used in Fig. 4. PC1 and PC2 explain 85% and 6% of the total NMR variation, respectively.

In order to investigate the measured ¹H HR-MAS NMR spectra further and to utilize the fact that the time dimension allows the application of a trilinear model, PARAFAC was applied on the ¹H HR-MAS NMR spectra of the single kernels (Fig. 6). The sample scores (Fig. 6a) of the four drought treatments were measured in duplicate showing the uncertainty of the replicates. The score plot reveals how the different drought treatments are clustered with the TD and CT placed close to each other and the TA and T2 placed together. The conclusion from this plot is that the ¹H HR-MAS NMR spectra of wheat treated with late drought, again, is mostly affected compared to the CT ¹H HR-MAS NMR spectra, whereas the TD treatment resembles the NMR profile of the control treatment. However, the result is not in total agreement with the results shown in Fig. 3 and Fig. 5. This is due to the modelling, since the three-way technique expresses only one concentration score value from each treatment and not a trajectory of the grain-filling pattern. The PARAFAC time loading (Fig. 6b) shows the time development of the two extracted components. The first time loading indicates how the NMR loading for Factor 1 increases during grain-filling. The Factor 1 NMR loading (Fig. 6c) resembles the profile of the fat fraction with characteristic signals from fatty acid methyl groups (0.9 ppm), methylene protons (1.3 ppm), β-carboxylic protons (1.6 ppm), α-methylic protons (2.0), α-carboxylic protons (2.3 ppm), aliphatic protons in conjugated chains (2.7 ppm), and protons on unsaturated carbons (5.3 ppm). The second time loading indicates how the NMR loading for Factor 2 decreases during grain-filling. The Factor 2 NMR loading (Fig. 6d) shows signals from protons in carbohydrates anomers and pyranose. The

small insert show the aromatic area which show small differences between Factor 1 and Factor 2. These have only a little influence on the model. These observations are consistent with observations from the ¹H HR-MAS NMR spectra of the early and late harvests in Fig. 4.

In order to enhance the resolution of the spectra in a relative simple manner, methanol extracts were prepared from wheat flour. During extraction of flour with a water–methanol mixture, the water-soluble proteins (e.g. albumins) and some alcohol-soluble proteins will be extracted. The extract contains no lipids or starch, only small saccharides and some water-soluble lipoproteins. The proteins and enzymes denature in the heating step and there is no hydrolysis of proteins to amino acids. Small peptides and amino acids can be naturally present in the flour from the onset (Shewry *et al.*, 1984).

Figure 7 displays ¹H NMR spectra of the liquid methanol extracts from the last harvest (50 DAA), showing the final result of the differences between the four drought treatments. Several carbohydrates were indentified in the spectral region 3.5–5.5 ppm. The assignments were based on 2D experiments of the extracts, and include α-glucose (5.20, 3.50, 3.70, 3.39, and 3.82 ppm, H1–H5, respectively), β-glucose (4.61, 3.21, 3.45, and 3.38 ppm, H1–H4, respectively), maltose [α(1–4) 5.36, 3.55, 3.68, 3.40 ppm, H1–H4 and β 4.61, 3.25, 3.74, 3.61, 3.58 ppm, H1–H5, respectively, and α 5.20, 3.54, 3.96 ppm, H1–H3, respectively)], glucose-1-phosphate and glucose 1,6-diphosphate (5.41/5.40, 3.55/3.53, 3.74/3.74, 3.51/3.46, and 4.04/3.83 ppm, H1–H5, respectively), together with α-galactose (4.98, 3.81, 3.88, 3.81, and 3.98 ppm, H1–H5, respectively). In the aliphatic region (0–3.5 ppm), the signals from the side-chain of amino acids including aspartic acid [(2.64 ($H_β$), 2.80($H_{β'}$), and 3.87 ($H_α$) ppm, respectively) and alanine (1.48 ppm ($H_β$) and 3.75 ppm ($H_α$)], together with small acids such as malic acid [2.35 ($H_{β'}$), 2.66 ($H_β$), and 4.28 ($H_α$) ppm, respectively] arise. However, the most significant differences between the four treatments were observed in the region 7.1–7.8 ppm, covering the aromatic amino acids. In this region, the intensity increases in the order: T2< TA< TD< CT, indicating that the amount of water-soluble proteins is negatively affected by the drought treatment in this order. The opposite effect is observed for the sharp singlet at 6.51 ppm assigned to fumaric acid by confirmation by 2D NMR experiments. This assignment is in agreement with Ward *et al.* (2003) who measured methanol extracts of *Arabidopsis* and assigned the sharp singlet at 6.5 ppm to be fumaric acid, but without further augmentation (Ward *et al.*, 2003). This ordering of the drought treatments mentioned above is only observed for the last harvest, but the last harvest from the trajectory score plot of the ¹H HR-MAS NMR spectra of whole kernels (Fig. 5, 50 DAA) confirms that T2 is the treatment furthest from the CT and the TD treatment is closest. This agreement is not surprising since the results obtained from methanol extracts includes water-soluble proteins only, which is about the same protein fraction as the water-mobilized proteins included in the ¹H HR-MAS NMR spectra of whole single kernels.
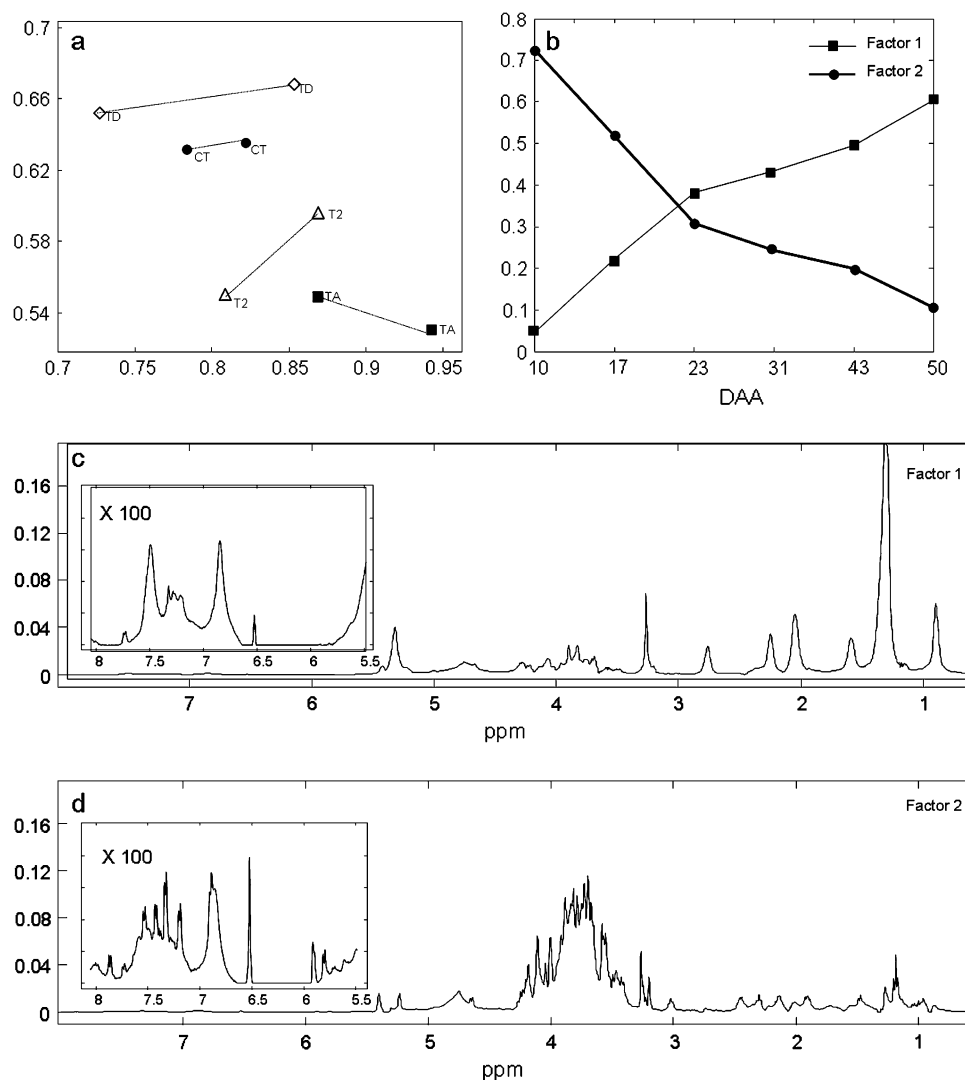
Fig. 6. The PARAFAC results: samples scores (a) of the four drought treatments: control (CT), early drought (TD), late drought (TA), and double drought (T2) together with PARAFAC time loading (b) PARAFAC NMR loading of Factor 1 (c) and Factor 2 (d).

Turning the focus on the spectral region (6.5–7.8 ppm) in Fig. 7, the aromatic ring protons show decreasing intensity with respect to severity of drought treatment, and they are highly (negatively) correlated (R^2=0.94) to the fumaric acid signal. This correlation between aromatic amino acids and a citrate cycle metabolite indicates that the effects of drought are almost equally severe on both.

The ^{1}H HR-MAS NMR spectra of single wheat kernels in Fig. 8 show the fumaric acid signal in mature wheat kernels (50 DAA) coloured by increasing drought intensity. As apparent from the figure, the fumaric signal is visible, but the signal does not show increasing intensity for increasing drought treatment as the spectra of the methanol extracts did (Fig. 7). This could be due to the fact that the size, the spike, and the position of the spike differs amongst kernels. These differences are not present when measuring flour or methanol extracts of flour as the intravariation in wheat kernels are averaged out. However, the ^{1}H HR-MAS NMR spectra of wheat flour did not show this interesting connection either. In wheatgrass metabolism, it was evident

that drought stress increased the extrusion from roots that contain higher concentration of fumaric and succinic acids (Henry *et al.*, 2007). Fumaric acid is involved in the citric acid cycle as succinate, is oxidized to fumarate by succinate dehydrogenase which is directly linked to the electron transport chain. The next step in the citric acid cycle is the hydration of fumarate to form L-malate, a step that needs water. We therefore speculate whether the lack of water during the growth of wheat, which apparently resulted in the accumulation of fumarate, has a negative effect on the citric acid cycle.

Conclusions

In this paper, the effects of water deficiency during grain-filling in wheat have been investigated with emphasis on the asynchronous protein synthesis monitored by ^{1}H NMR analysis of single kernels, flour, and methanol extracts. Protein fractions and methanol extraction were tested for
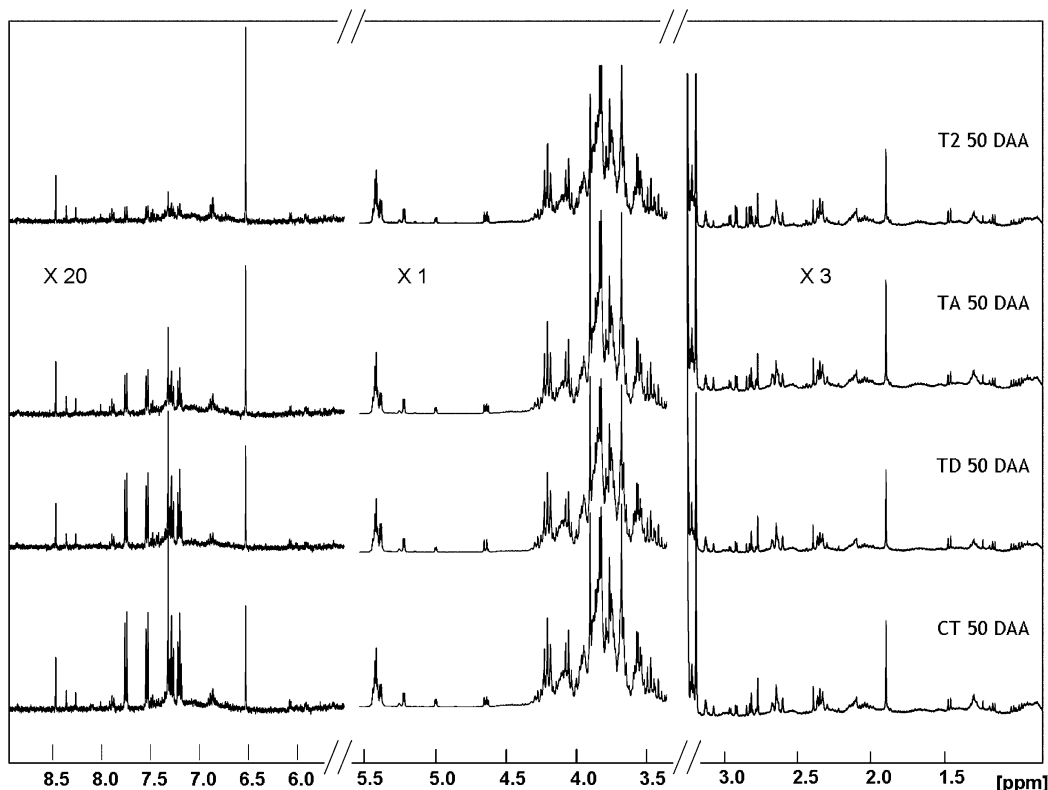
Fig. 7. Liquid-state ^{1}H NMR spectra of methanol extractions of wheat harvested at 50 DAA showing the differences between the four drought treatments (control (CT), early drought (TD), late drought (TA), and double drought (T2)).
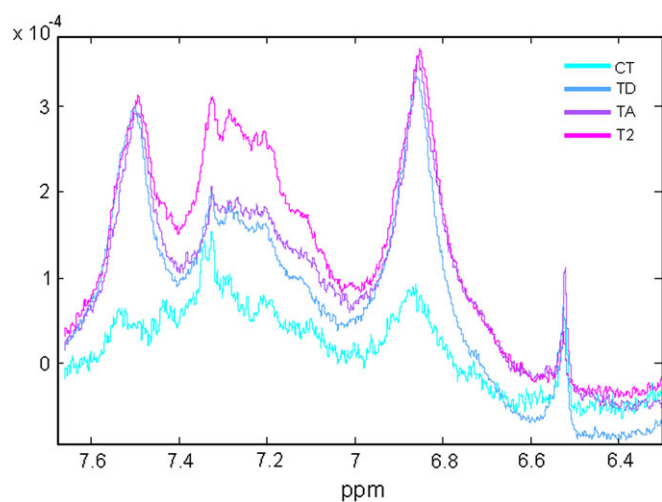
Fig. 8. ^{1}H HR-MAS NMR spectra of single wheat kernels from the last harvest coloured according to drought treatment.

their ability to differentiate between drought treatments, each method applying a different view on the protein depending on the solubility. Visualization of the data by PCA trajectories for the drought treatments yielded good contrast of the protein development during grain-filling. The results indicate that two periods of drought do not have as remarkable an influence as late drought. Apparently, some kind of resistance to drought is induced when

the wheat is drought-treated early in grain-filling. The ^{1}H NMR spectra of methanol extracts of wheat flour samples provided better spectral resolution and enabling assignment of a sharp singlet at 6.51 ppm to fumaric acid. This metabolite, which is also found in the single kernel ^{1}H HR-MAS NMR spectra, was found to be a potential marker for drought treatment in mature kernels.

The ^{1}H HR-MAS NMR spectra of single wheat kernels show considerable differences between early and late harvest. For the first time, data from grain-filling has been analysed by a complex multiway model and the result showed an excellent overview of the data, where the main variation in the data is expressed in two spectral profiles.

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