

Nitrogen topdressing timing modifies the gluten quality and grain hardness related protein levels as revealed by iTRAQ



Yingxin Zhong^{a,c}, Weiling Wang^a, Xin Huang^a, Mingming Liu^b, Kim Henrik Hebelstrup^c, Donglei Yang^b, Jian Cai^{a,*}, Xiao Wang^a, Qin Zhou^a, Weixing Cao^a, Tingbo Dai^a, Dong Jiang^{a,*}

^a National Technique Innovation Center for Regional Wheat Production/Key Laboratory of Crop Physiology and Ecology in Southern China, Ministry of Agriculture/National Engineering and Technology Center for Information Agriculture, Nanjing Agricultural University, PR China

^b National Laboratory of Plant Genetics and Germplasm Enhancement, Nanjing Agricultural University, PR China

^c Department of Molecular Biology and Genetics, Section of Crop Genetics and Biotechnology, Aarhus University, Denmark

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ABSTRACT

Nitrogen fertilization regimes significantly affect both grain quality and yield. Wheat plants were subjected to different application timing of topdressed nitrogen at the emergence of the top fifth (TL5), top third (TL3) and top first leaf (TL1), respectively. The iTRAQ (isobaric tag for relative and absolute quantitation) technology was adopted to obtain the complete proteome of wheat flour and to identify the differentially expressed proteins (DEPs) as regulated by nitrogen topdressing timing. Collectively, 591 proteins into 17 functional categories in flour of mature grains were identified. In comparison to TL3, 50 and 63 DEPs were identified in TL5 and TL1, respectively. Nine of the DEPs commonly dependent on nitrogen topdressing timing are the γ-gliadins or high-molecular-weight glutenin subunits. Additionally, delaying nitrogen topdressing modified the grain hardness and allergic protein content. The results suggested that altering nitrogen topdressing timing is a potential strategy for pursuing targeted processing quality of wheat flour.

Abbreviations: ADP-RF, ADP-ribosylation factor; ANOVA, analysis of variance; DAA, days after anthesis; DEP, differentially expressed protein; FDR, false discovery rate; GMP, glutenin macropolymers; GPC, grain protein content; GSP, grain softness protein; HMW-GS, high molecular weight glutenin subunits; iTRAQ, isobaric tag for relative and absolute quantitation; LMW-GS, low molecular weight glutenin subunits; LSD, least significant difference; N, nitrogen; PDI, protein disulfide isomerase; qRT-PCR, quantitative real-time PCR; SDS, sodium dodecyl sulfate

* Corresponding author.

** Co-corresponding author.

E-mail addresses: yingxinzhong@mbg.au.dk (Y. Zhong), 2015201012@njau.edu.cn (W. Wang), 2015101005@njau.edu.cn (X. Huang), 2014201021@njau.edu.cn (M. Liu), kim.hebelstrup@mbg.au.dk (K.H. Hebelstrup), dlyang@njau.edu.cn (D. Yang), caijian@njau.edu.cn (J. Cai), xiaowang@njau.edu.cn (X. Wang), qin Zhou@njau.edu.cn (Q. Zhou), caow@njau.edu.cn (W. Cao), tingbod@njau.edu.cn (T. Dai), jiangd@njau.edu.cn (D. Jiang).

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1. Introduction

Wheat is one of the most important staple crops in the world. As a primary food, wheat provides approximately 20% of the calories and protein for the humankind (<http://www.fao.org/faostat/zh/#data/FBS>). For daily diet, wheat is consumed mainly as flour, after being processed to bread, pasta, noodles and various other baked products.

The extensive use of wheat flour in diet products is due to its unique properties related to protein content and quality. Wheat flour protein is typically divided into storage proteins and metabolic proteins (Shewry, Halford, & Lafiandra, 2003). The storage proteins, which represent 80%–85% of the total proteins in wheat grain, can be further classified into the monomeric gliadins and the polymeric glutenins (Wrigley, 1996). Gliadins are stabilized by intramolecular disulfide bonds, whereas glutenins are polypeptides of the high- and low molecular weight subunits (HMW-GS and LMW-GS) that are stabilized by both intermolecular and intramolecular disulfide bonds (Shewry et al., 2003). Both content and composition of storage proteins in wheat flour are important for the processing quality (Goesaert et al., 2005).

In addition, the non-storage proteins involving in various biological processes during endosperm development also affect the breadmaking quality, the hardness of the wheat grain and regulation of starch biosynthesis in grain. For instance, protein disulfide isomerase (PDI) contributes indirectly to wheat quality by regulating formation of the glutenin polymers (Kimura, Higashino, Kitao, Masuda, & Urade, 2015). Grain hardness, which has significant impacts on milling and baking quality of wheat flour, is closely correlated with a 15 kDa endosperm specific protein named Grain Softness Protein (GSP) (Jolly, Rahman, Kortt, & Higgins, 1993). In addition, some proteins may also trigger allergies such as baker's asthma, which is recognized as one of the most common types of occupational asthma (Houba, Doekes, & Heederik, 1998). In this context, it is important to understand the fraction of metabolic proteins, although they are among the less abundant wheat grain proteins.

Wheat grain protein content (GPC) is determined by both genetic and environmental factors. Wheat genotypes for bread-baking produce 2% higher GPC than other wheat types when grown under the same conditions (Monaghan, Snape, Chojecki, & Kettlewell, 2001). However, N availability or rate could have much greater impact (up to 2-fold) on GPC than genetic factors (Kindred et al., 2008). Increasing total N dose is an effective practice to improve GPC in wheat. However, since unintended side effects such as lowered N use efficiency, increased risk of environmental pollution, as well as other ecological concerns derived from high N application (Foulkes et al., 2009), supplementary agronomic methods should be explored to reduce these concerns. The split of N fertilization and modification of topdressing timing are commonly recommended approaches in intensive wheat production for maintaining satisfying GPC without increasing N dose (Foulkes et al., 2009). Late application of N fertilizers at heading or anthesis is found to increase GPC of wheat (Xue, Schulte aufm Erley et al., 2016). In addition, as an important quality trait affecting processing functionality, nutritional value and immunogenic potential, protein composition is also strongly regulated by N application. It has been reported that delaying N topdressing timing increases content of the gluten proteins (Blandino, Vaccino, & Reyneri, 2015). However, information focusing on the effects of N topdressing timing on the overall protein fractions is rare so far.

Proteomics-based approaches are very powerful to understand the complexity of wheat protein compositions in response to genotypes, environmental factors and management practices (Colgrave et al., 2015; Debiton et al., 2011). During the past decade, proteomic approaches have advanced research on the wheat flour proteins. The first phase of proteomics research is primarily descriptive and involves the separation of individual flour proteins by two-dimensional gel electrophoresis (2-DE) coupled with their identification by mass spectrometry (MS) (Dupont, Vensel, Tanaka, Hurkman, & Altenbach, 2011;

Altenbach et al., 2011). However, the 2-DE and 2D-DIGE methods showed obvious limitations in identification rate, quantification accuracy, reproducibility, and difficulty in separation of hydrophobic proteins (Ge et al., 2013). In recent years, the so-called 'isobaric Tag for Relative and Absolute Quantitation (iTRAQ)' has been acknowledged as a powerful proteome analysis technique that allows to identify more proteins with better quantitation (Karp et al., 2010). Ma et al. (2014) performed the first iTRAQ-based quantitative proteome of developing wheat at different stages and identified 1146 non-redundant proteins. Yang, Dong, Zhao, and Gao (2016) focused on protein profiles during the early stage of wheat grain development (4DAA, 8DAA, 12DAA) and observed 1762 differential expressed proteins (DEPs) between different grain filling stages (Yang et al., 2016). This implies the high potential of the iTRAQ technology for profiling the proteome in wheat grains. However, the power of iTRAQ has not been evident in profiling proteins in wheat flour until now.

In the present study, we performed the first iTRAQ-based quantitative global proteome analysis on flour of one elite Chinese bread wheat cultivar Yangmai 16. Three N topdressing timing treatments were conducted according to the leaf ages. The proteome of flour obtained from the different set of treatments were compared. The objectives of this study were to: (1) obtain a complete proteome of wheat flour, (2) identify specific proteins modified by N topdressing timing, and (3) clarify the possible mechanism underlying the modified protein accumulation by N topdressing timing. The results demonstrate the N fertilization effects on flour protein composition, and provide clues for the regulation approaches for accumulation of specific flour proteins. The work also constructs a database for further studies on the roles of proteins in flour.

2. Materials and methods

2.1. Plant materials and growth conditions

The field experiments were conducted at the Tangquan Experimental Station of Nanjing Agricultural University, Nanjing (32°08' N and 118°51' E), Jiangsu Province, P. R. China in three growth seasons of 2013–2014, 2014–2015 and 2015–2016. The soil fertility and date of each nitrogen topdressing treatment, average temperature and precipitation in the growing seasons are shown in [Supplementary File 1](#). A locally released and widely planted winter wheat cultivar with medium grain protein content, Yangmai 16, was sown in October with a seeding rate of ca. 180 seedlings m⁻² and a row space of 25 cm. Half of the total nitrogen (120 kg ha⁻¹) in the form of urea was applied as basal nitrogen before sowing. Another half of the nitrogen was topdressed at either of three different leaf ages: 1) at the emergence of the top-fifth-leaf stage (TL5, after re-greenness, which was around Stage 17 in the Zadoks coding system (Zadoks, Chang, & Konzak, 1974), 2) the top-third-leaf stage (TL3, in accordance to the jointing stage or the Stage 31 in the Zadoks coding system, which is the recommended timing of nitrogen topdressing in practice), or 3) the top-first-leaf stage (the emergence of the flag leaf or the Stage 37 according to the Zadoks coding system, TL1) of the main stem. The agricultural practices were performed according to the local recommendations. The plot size was 3 m × 3.2 m. The treatments were arranged in a randomized complete block design with three replicates.

2.2. Sampling

Uniform spikes on the same day of flowering were tagged and were then sampled on the 7th, 13th, 19th, 25th and 31st days after anthesis (DAA). The collected spikes were immediately put into liquid nitrogen and then stored at –80 °C till qPCR analysis. Grains from the central spikelets of the tagged spikes were collected for analysis of genes expressions.

At maturity, heads from 4 m² were harvested and threshed to collect

grain samples. Spike numbers were determined by counting stems in 1 m^2 (4 rows $\times$ 1 m) in each plot. Grain number per spike was determined by counting the grains from 20 randomly selected spikes. The 1000-grain weight was determined using the weights of three sets of 1000 kernels from each plot. Grains were dried at room temperature and kept for two months before hardness measurement and milling.

2.3. Measurement of quality parameters

The mature grains were used for determining grain hardness by method of AACC 39-70A (AACC, 2004) using a Perten DA 7250 near infrared reflectance analyzer (Hamburg, Germany). Dried grains were ground into fine flour with a Buhler experimental mill (MLU-202, Buhler Equipment Engineering Wuxi Co., Ltd., China), followed by being sieved through a $150\text{-}\mu\text{m}$ screen. The protein fraction was separated and analyzed according to Luthe (1983). Four protein fractions were sequentially extracted from 0.5 g flour in 25 ml following solvents: albumin in 10 mM Tris-HCl, (pH 7.5); globulin in 1 M NaCl and 10 mM Tris-HCl (pH 7.5), gliadin in 70% (v/v) ethanol and 10 mM Tris-HCl (pH 7.5), and glutenin in 0.5% SDS, 1% β -mercaptoethanol and 10 mM Tris-HCl (pH 7.5) by stirring the flour for 2 h at room temperature. The contents of protein and protein fractions (albumin, globulin, glutenin and gliadin fraction) in flour were determined by the micro-Kjeldahl method of AACC 46-13.01 (AACC, 2004).

Glutenin macropolymers (GMP) were determined according to our previous report (Zhong et al., 2018). Briefly, 50 mg flour was suspended in 1 ml of SDS solution (1.5%) followed by being centrifuged at $15,500g$ at 20°C for 30 min. The sediment was washed twice with 1.5% SDS solution. The final sediment was dissolved in 2 ml NaOH (0.2%) for 30 min. Afterwards, 3 ml Biuret reagent was added to the solution to evaluate the nitrogen content for further calculation of GMP content. HMW-GS and LMW-GS were separated by SDS-PAGE and quantified according to our previous method (Zhong et al., 2018).

Contents of wet and dry gluten, and gluten index were determined following the AACC 38-12.02 procedure (AACC 2004) with the Glutomatic 2200, the Centrifuge 2015 and the Glutork 2020 (Perten instruments AB, Stockholm, Sweden). Starch content was quantified as described in AACC 38-12 procedure (AACC 2004).

2.4. Proteomic analysis using the iTRAQ technology

2.4.1. Protein extraction

500 mg flour was dissolved in 800 μl BPP extraction buffer (100 mM EDTA, 50 mM Borax, 1% PVPP, 1% Triton X-100, 2% β -mercaptoethanol, 100 mM Tris-HCl buffer (pH 8.0)), by shaking for 10 min at room temperature. After adding an equal volume of Tris-Phenol, the mixture was shaken for another 10 min, followed by centrifugation at $15,000g$ for 15 min. The supernatant was transferred to a new tube, to which equal volume of BPP extraction buffer was added, and then vortexed for 5 min. After being centrifuged at $15,000g$ for 15 min, the supernatant was collected and mixed with 5-time volume of saturated ammonium sulfate methanol solution, followed by incubation at -20°C overnight. Then the supernatant was discarded after being centrifuged at $15,000g$ for 15 min. The pellet was then fully washed with 1 ml cold methanol, followed by washing with 1 ml acetone for two times. After being dried at room temperature, 500 μl urea lysate was added to dissolve the protein pellet overnight.

Protein quantity was measured using the Bradford method (Kruger, 1994). Afterwards, 200 μg protein from each sample was incorporated into 5 μl dithiothreitol (DTT) and kept at 37°C for 1 h. After adding 20 μl 1 M indoleacetic acid (IAA), the solution was incubated for 1 h in darkness. The detergent, DTT and other low-molecular-weight components were removed using the UA buffer (8 M urea and 150 mM Tris-HCl, pH 8.0) combined with repeated ultrafiltration (30 kD Microcon; Millipore Corp., Billerica, MA, USA) (Wiśniewski, Zougman, Nagaraj, & Mann, 2009). Thereafter, 100 μl of 0.05 M iodoacetamide in UA buffer

was added to block the reduction of cysteine, followed by dark incubation for 20 min. The filter was washed three times with 100 μl 0.5 M Tetraethylammonium Bromide (TEAB). The mixture was finally subjected to overnight trypsin treatment, with a trypsin-to-protein ratio of 1:50 adjusted according to the protein quantification result.

2.4.2. The iTRAQ labeling and high-pH reversed-phase chromatography

100 μg protein peptides from each group were labeled using the eight plex iTRAQ reagents multiplex kit (ABI, Foster City, CA, USA) according to the manufacturer's instructions (isobaric tags 113, 114 and 115 for the treatment TL5, isobaric tags 116 and 117 for the treatment TL3 and isobaric tags 118, 119 and 121 for the treatment TL1. TL3 was taken as control). After labeling, the samples were dissolved in a mobile phase A (98% H_2O , pH 10). Then, the samples were loaded on a reverse-phase column (Luna C18, $4.6 \times 150\text{ mm}$; Phenomenex, Torrance, CA, USA) and eluted using a gradient chromatograph procedure comprised of 0–30 min from 5% to 35% B eluant solution (98% acetonitrile hydrate (ACN), pH 10), 30–32 min from 35% to 95% B, 32–37 min of 95% B, 37–39 min from 95% to 5% B and 39–45 min of 5% B at a flow rate of 0.7 ml min^{-1} . The eluates were auto-collected in each 1.5 min and then freeze-dried. All the collections were dissolved into 5 μl 0.5% FA respectively and finally combined into six pools and desalted on C18 Cartridges (Empore™ standard density SPE C18 Cartridges, bed I.D. 7 mm, 3 ml volume; Sigma, St. Louis, MO, USA).

2.4.3. LC-electrospray ionization-MS/MS analysis

10 μl of the reconstituted peptides was analyzed with the Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a nano high-performance liquid chromatography system (UltiMate 3000 LC Dionex; Thermo Fisher Scientific). The peptides were loaded on C18-reversed phase column ($3\text{ }\mu\text{m}$ -C18 resin, $0.1 \times 20\text{ mm}$) and separated on an analytical column ($1.9\text{ }\mu\text{m}$ C18 resin, $0.15 \times 120\text{ mm}$; Dr. Maisch GmbH, Ammerbuch, Germany) using a mobile phase A: 0.1% formic acid (FA)/ H_2O and B: 0.1% FA/ACN at a flow rate of 600 nl min^{-1} , using a 78 min gradient.

MS/MS data were acquired on an Orbitrap Fusion. In detail, all MS spectra were acquired over m/z 300–1800 in the Orbitrap. The automatic gain control was set to accumulate 1×10^6 ions, with a maximum injection time of 50 ms. Data-dependent tandem MS analysis was performed using a top-speed approach (cycle time of 3 s). The normalized collision energy was optimized at 32% for HCD. MS^2 spectra were acquired with a fixed first m/z of 100. The intensity threshold for fragmentation was set to 5×10^4 for orbitrap methods. A dynamic exclusion of 18 s was applied.

2.4.4. Sequence database search and data analysis

The raw mass data were processed for the peptide data analysis using Mascot 2.2 and Proteome Discoverer 1.4 (Thermo Fisher Scientific) with a false discovery rate ($\text{FDR} = \text{N}(\text{decoy})^2 / ((\text{N}(\text{decoy}) + \text{N}(\text{target}))) < 1\%$ and expected cutoff or ion score < 0.05 (with 95% confidence) for searching against the uni_Triticum_a_0920.fasta. The following protocol was used to identify the proteins: Peptide mass tolerance = $\pm 15\text{ ppm}$, MS/MS tolerance = 0.02 Da , enzyme = trypsin, missed cleavage = 2, fixed modification: iTRAQ8plex (K) and iTRAQ8plex (N-term), Carbamidomethyl (C), variable modification: oxidation (M), database pattern = decoy. The up-regulated or down-regulated proteins in three replicates with relative quantification p-values < 0.05 and 1.2 fold-changes were selected as being differentially expressed candidate proteins. The Gene Ontology (<http://www.geneontology.org>, GO) bioinformatics analysis was performed on the differentially expressed proteins to categorize the proteins according to the molecular functions, cellular components and biological processes. The allergenicity related proteins were evaluated by online database PREAL^W (<http://lilab.life.sjtu.edu.cn:8080/prealw/index.php>) and Allergome (<http://www.allergome.org/>).

2.5. The qRT-PCR analysis

Developing seeds from the central spikelets from five spikes were combined and ground into powder with liquid nitrogen. The total RNA was extracted by using the Total RNA Purification Kit (Genemark) according to the manufacturer's instructions. Approximately 500 ng purified mRNA was used to synthesize cDNA by using a HiScript II One Step RT-PCR Kit (Vazyme, China). The cDNA was used for quantitative real-time PCR (qRT-PCR). ADP-ribosylation factor (ADP-RF) was used as housekeeping gene. Details of the primers are listed in the [Supplementary File 2](#). All primers were gene specific as checked by electrophoresis and unique melting peak verification. The efficiencies of all primers ranged from 90% to 100% as determined by the standard curves using a series of cDNA dilutions at 13 DAA in TL3. The qPCR reactions included an initial incubation at 94 °C for 3 min, followed by 40 cycles at 94 °C for 20 s, 58 °C for 15 s, 72 °C for 20 s. The gene expression data were standardized according to the CFX96 Real-Time system (Bio-Rad). Relative gene expression was calculated using the comparative delta cycle threshold ($\Delta\Delta$ method) using ADP-RF as the reference gene ([Livak & Schmittgen, 2001](#)). Triple PCR reaction analysis and three biological replicates were performed for each gene. Raw data of relative gene expression is shown in [Supplementary File 3](#).

2.6. Statistics

All data were subjected to analysis of variance (ANOVA) using the SPSS (Statistical Product and Service Solutions) Version 17.0. The ANOVA mean comparisons were performed in terms of the least significant difference (LSD), at the significant level of $P < 0.05$.

3. Results and discussions

3.1. Yield and quality of grain and flour

The emergence of the top 3rd leaf on the main stem (TL3) is the recommended N topdressing timing in wheat production in China. Thus, TL3 was taken as the control in the present study. As expected, the maximum grain yield was observed in TL3 in all the three growing seasons from 2013 to 2016 ([Supplementary File 4 & Table 1](#)). Predating N topdressing (TL5) decreased 14.01%, 16.04% and 9.98% in grain yield in relation to TL3 in three seasons, respectively. Delaying N topdressing (TL1) also caused yield loss of 9.05%, 11.67% and 12.98% in the three growth seasons, respectively. The yield loss in TL5 was mainly due to the lowest grain number and grain mass, while in TL1 was related to the lowest spikes number and lowered grain number. The highest spikes number in TL5 and highest grain mass in TL1 seemed to partially compensate the yield loss. Pearson correlation analysis showed significantly positive relationship ($P < 0.01$) of grain yield between the three seasons ([Supplementary File 4](#)).

Protein, protein component and wet gluten contents were quantified in flour of three growing seasons ([Supplementary File 4](#)). N topdressing at the emergence of the top 1st leaf (the flag leaf) (TL1) significantly increased protein content in comparison to TL3 ([Table 1 & Supplementary File 4 & 5](#)), especially for the contents of gliadin and glutenin. In the three growing seasons, gliadin content separately increased 27.27%, 22.22% and 28.95%, while glutenin content increased 13.89%, 13.16% and 11.68% in TL1 in relation to TL3. Reversely, predating topdressed N (TL5) caused reductions of contents of total protein, gliadin and glutenin in all three growing seasons. Content of wet gluten showed the same trend in response to nitrogen topdressing timing. In accordance with yield data, significant correlation ($P < 0.01$) was also observed in protein indicators between years.

Grain yield (GY) and grain protein concentration (GPC) is reported to be negatively correlated ([Simmonds, 1995](#)). Here, we observed a similar trend as exemplified by the high GPC with low GY in delaying N topdressing treatment (TL1) in relation to TL3 ([Table 1](#)). Previous

Table 1

Effects of nitrogen topdressing timing on grain yield and quality traits of wheat.

Traits	TL5	TL3	TL1
Spikes (10^4 ha^{-1})	365 ^a	344 ^b	329 ^c
Grain number	45.5 ^b	52.0 ^a	47.5 ^b
1000-grain mass (g)	44.5 ^c	46.1 ^b	48.9 ^a
Grain yield (kg ha^{-1})	6867 ^b	7628 ^a	6638 ^b
Grain hardness (dimensionless)	63.77 ^a	62.87 ^a	58.24 ^b
Protein content (%)	15.05 ^c	16.70 ^b	17.40 ^a
Albumin (%)	2.55 ^a	2.57 ^a	2.63 ^a
Globulin (%)	1.55 ^b	1.61 ^{ab}	1.69 ^a
Gliadin (%)	3.16 ^c	3.73 ^b	4.81 ^a
Glutenin (%)	3.66 ^c	3.94 ^b	4.4 ^a
Gliadin/Glutenin	0.86 ^c	0.95 ^b	1.09 ^a
GMP (%)	3.68 ^c	3.97 ^b	4.36 ^a
HMW-GS (%)	0.93 ^c	1.15 ^b	1.44 ^a
LMW-GS (%)	3.21 ^c	3.53 ^b	3.81 ^a
HMW/LMW-GS	0.29 ^c	0.33 ^b	0.38 ^a
Wet gluten (%)	36.0 ^c	40.9 ^b	42.8 ^a
Dry gluten (%)	13.2 ^c	14.3 ^b	15.3 ^a
Gluten index	0.80 ^a	0.78 ^a	0.75 ^a
Starch content (%)	66.4 ^a	66.9 ^a	66.1 ^a

Note: TL5, TL3 and TL1 indicate topdressing timing of nitrogen at emergence of the fifth, third and first leaf (flag leaf) from the top, respectively. All data were subjected to one-way analysis of variance (ANOVA) to determine the significant differences between treatments. Small letters in the same row indicate significant differences at $P < 0.05$. HMW-GS, high molecular weight glutenin subunits. LMW-GS, low molecular weight glutenin subunits. GMP, glutenin macropolymers.

studies also reported a negative relationship between protein and starch content under different N doses ([Kindred et al., 2008](#)). However, no significant change was observed here in terms of starch content in all the treatments ([Table 1](#)). Therefore, the lower yield in TL1 compared with TL3 could be related to the lowered spike numbers rather than reduction in starch content. This inferred that improving spike number can be a potential breeding trait to achieve both high GY and high GPC under delaying N topdressing conditions.

The contents of GMP, HMW-GS, LMW-GS and dry gluten showed close patterns to glutenin content in response to the N topdressing timing ([Table 1](#)). This result was consistent with previous report suggesting that topdressing nitrogen at the booting stage enhanced the percentages of gliadins and glutenins as well as HMW-GS ([Xue, Rossmann, Schuster, Koehler, & Mühling, 2016](#)). Though both wet and dry gluten contents were increased in TL1, no significant difference was observed between the N topdressing timing treatments in terms of gluten index. Moreover, N topdressing timing also affected grain hardness. Delaying N topdressing (TL1) decreased grain hardness 7.4% and 8.7% than TL3 and TL5, respectively.

3.2. Proteome profiling in wheat flour

Using the iTRAQ-based proteomic analysis, in total 1103 unique peptides, corresponding to 591 proteins were identified in flour of mature wheat grain ([Supplementary File 6](#)). The number was much higher than that of previous studies using the 2-DE technology, where 157 proteins ([Dupont et al., 2011](#)) and 114 proteins ([Altenbach et al., 2011](#)) were identified respectively. It was also much higher than previous studies using the LC-MS/MS or QTOF-LTQ technology where 151 and 127 proteins were identified, respectively ([Colgrave et al., 2015; Bromilow et al., 2017](#)). The gene ontology (GO) analysis further revealed that 493 proteins were associated with the GO categorization ([Supplementary File 7](#)). The proteins were categorized into 17 groups according to the putative molecular functions ([Fig. 1](#)). The main categories were defined by separate involvement in the amino acid metabolism (9.83%), oxidoreductase (9.40%), metal binding (8.97%), ATP binding (8.97%), and carbohydrate metabolism (8.69%). The remaining 36% proteins were related to categories including signal

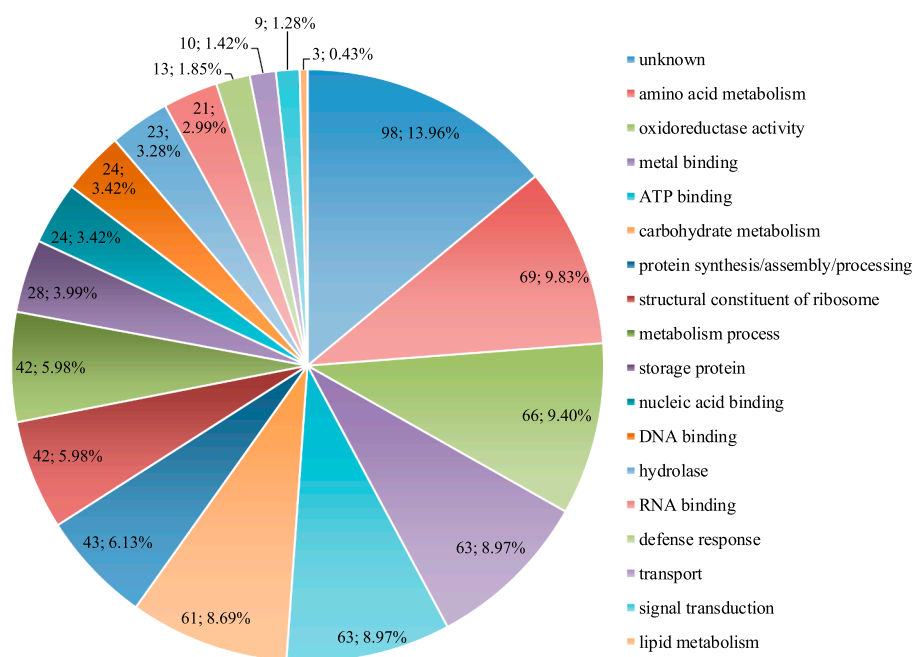


Fig. 1. Functional classifications of 591 proteins identified in wheat flour. Note: The number in the chart indicated the number of proteins in the corresponding category.

transduction, nucleic acid binding, structural constituents of ribosomes, lipid metabolism, transportation, various metabolic processes, protein synthesis/assembly/processing, RNA binding, storage protein, DNA binding, hydrolase and pathogen defense responses (Fig. 1).

3.3. Identification of the differentially expressed proteins (DEPs) between N topdressing timing treatments

In total, 97 DEPs were identified in response to N topdressing timing, of which 50 DEPs were found between TL5 and TL3, and 63 DEPs were found in TL1 vs TL3, while 16 DEPs were commonly identified in the two comparisons (Fig. 2). Of the 50 DEPs in TL5 vs TL3, 20 were up-regulated while 30 were down-regulated. The up-regulated proteins are mainly those that participate in DNA binding (5 DEPs). Almost half of the down-regulated proteins (14 DEPs) in TL5 vs TL3 are storage proteins, indicating that predating N topdressing negatively affected storage protein accumulation. In addition, three down-regulated proteins in TL5 vs TL3 are related to amino acid metabolism, and another three are pathogen defense response related. The proteins related to DNA binding, oxido-reduction, metal binding, ATP binding were up-regulated in TL5 as compared with TL3.

Of the 63 DEPs in TL1 vs TL3, 38 were up-regulated while 25 were down-regulated. In the up-regulated proteins, in total 11 DEPs were those related to protein synthesis-, assembly- or processing, while the second large category is storage protein (9 DEPs), indicating delaying N topdressing favored storage protein accumulation in wheat grain. In addition, of the 25 down-regulated proteins, the main categories belonged to ATP binding related proteins (5 DEPs) and C metabolism related proteins (5 DEPs), followed by oxido-reduction proteins (3 DEPs). Of the 16 co-differentially expressed proteins, 10 were up-regulated by TL1 while reversely by TL5, and 5 were up-regulated while 1 was down-regulated by both TL1 and TL5 in relation to TL3. However, no protein was observed to be up-regulated in TL5 vs TL3 while down-regulated in TL1 vs TL3.

Since our research interest here is focused on flour quality, we only analyzed the storage proteins, grain hardness related proteins and allergenicity related proteins in detail in the present study.

3.3.1. Storage proteins

Here, we only listed 78 DEPs with annotated functions in Tables 2 and 3 by removing those DEPs not annotated and those that scored less than 44.1 (with 95% confidence). Of the 78 DEPs, 14 were identified as storage proteins, which were negatively affected by predating N topdressing (TL5) as compared with TL3, including six gamma-gliadin proteins, and four HMW-GS (Table 2). Furthermore, one ω -gliadin and two proteins belonging to the 11S globulin types (W4ZXS7 and W5AKY9) were also lower in TL5. Delaying N topdressing up-regulated all the DEPs classified as storage proteins as compared with TL3. Here, the four gliadins increased in a range from 1.22 to 2.82 folds in TL1 than TL3. In addition, three HMW-GS and one LMW-GS were also up-regulated by TL1 (Table 3). Nine co-DEPs belonging to storage proteins were higher in TL1 while they were depressed in TL5 in relation to TL3 (Tables 2 & 3), indicating that delaying N topdressing improved accumulation of these storage proteins.

Contents of both gliadins and glutenins significantly increased with delaying N topdressing (Tables 2 & 3), which was in agreement with previous report (Xue, Rossmann et al., 2016). In addition, the gliadins seemed to be more sensitive to delaying N topdressing, resulting in an increased ratio of gliadin to glutenin under delayed N topdressing (Table 1). This suggests that though the content of total gluten protein increased, the synchronous increment of gliadin to glutenin ratio will increase the extensibility while reduce the strength of dough, which could negatively affect quality of bread-making (Goesaert et al., 2005). The iTRAQ analysis further revealed that one γ -gliadin (Q94G97) was highly responsive to delaying N topdressing (increased by 2.82-fold), inferring that this could be a candidate protein for breeders to reduce the side effect of delaying N topdressing on gliadin accumulation by silencing the corresponding gene (AF234643).

HMW-GS accounts for about 25% of the total glutenin proteins, and plays a major role for dough rheological properties by contributing more to dough strength than the LMW-GS (Dhaka & Khatkar, 2015). We observed that HMW-GS content was more affected by N topdressing timing than LMW-GS (Tables 2 & 3). The content of HMW-GS strongly increased, while the content of LMW-GS moderately increased with delayed N topdressing timing, resulting in a higher ratio of HMW-GS to LMW-GS (Table 1). Since HMW-GS plays an essential function for the

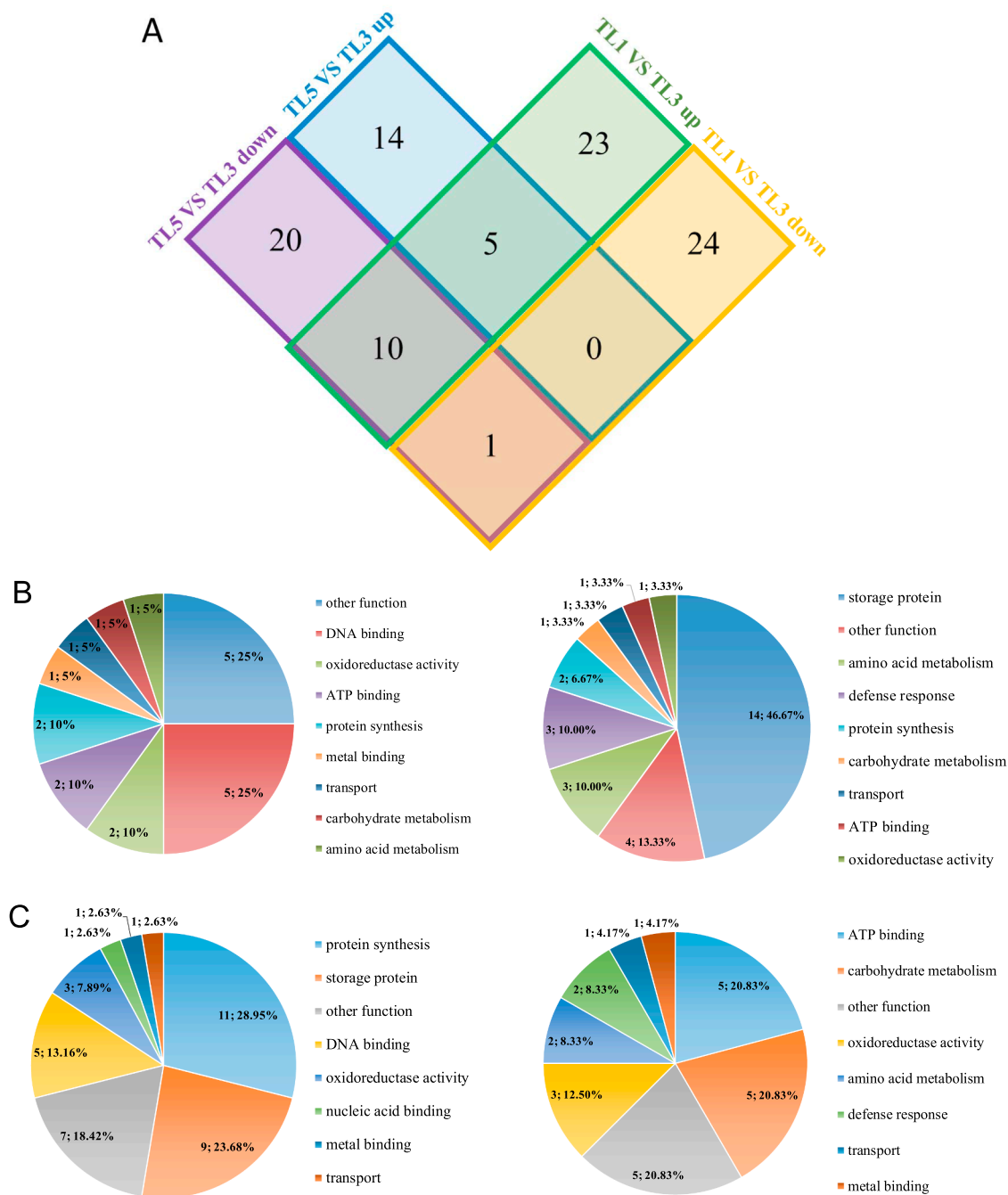


Fig. 2. Distribution (A) and functional classification (B & C) of 110 DEPs (differentially expressed proteins) in TL5 vs TL3 and TL1 vs TL3 identified by iTRAQ. Note: TL5, TL3 and TL1 indicate topdressing timing of nitrogen at emergence of the fifth, third and first leaf (flag leaf) from the top, respectively. A. purple, blue, green and yellow rectangle represents TL5 vs TL3 down-regulation, TL5 vs TL3 up-regulation, TL1 vs TL3 up-regulation and TL1 vs TL3 down-regulation, respectively. B. functional classification of up-regulation DEPs in TL5 vs TL3 (left) and TL1 vs TL3 (right). C. functional classification of down-regulation DEPs in TL5 vs TL3 (left) and TL1 vs TL3 (right). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

formation of the backbone of the glutenin network, a high ratio of HMW-GS to LMW-GS will benefit dough strength and stability (Goesaert et al., 2005). This may at least partially explain the unaffected gluten index with modified protein fractions observed when altering N topdressing timing (Table 1).

Further, the iTRAQ analysis identified three DEPs (one LEA protein (in TL5 vs TL3) and two PDI proteins (in TL1 vs TL3)), which could further help understand the regulatory mechanism of N topdressing timing on quality of the storage proteins (Tables 2 & 3). It has been reported that the LEA (late embryogenesis abundant) proteins are

effective in suppressing protein aggregation during grain desiccation (Goyal, Walton, & Tunnacliffe, 2005). Since the gluten proteins synthesis is active during grain desiccation (28 DAA to mature) (Shewry et al., 2012), LEA could negatively affect formation of storage protein by depressing protein aggregation. Here, the up-regulated LEA protein (A0A024CK72) in TL5 could at least partially explain the low content of gluten proteins than TL3. In addition, two PDI proteins (*F8THZ6* and *A0A024FR39*) were found to be up-regulated 1.30-fold to contribute to the higher glutenin content in TL1 than TL3, since PDIs function in the folding of storage proteins and play a fundamental role in the

Table 2
Differentially expressed proteins in TL5 vs TL3.

Accession	Description	Score	Cov.	MW [kDa]	Fold change (TL5/TL3)
<i>Amino acid metabolism</i>					
Q43665	Uncharacterized protein	86.91	13.48	9.5	0.65
P32112	Adenosylhomocysteinase	50.34	5.77	53.4	0.83
<i>ATP binding</i>					
A0A0C4BKH5	Uncharacterized protein	88.41	6.52	46.9	1.33
<i>Carbohydrate metabolism</i>					
Q5UHH6	0.19 dimeric alpha-amylase inhibitor	460.21	38.71	13.2	0.63
O82073	Sucrose synthase	77.9	3.59	92.5	1.29
<i>Defense response</i>					
J7K291	Alpha purothionin	53.88	27.03	11.9	0.66
W5G7R8	Glutathione peroxidase	57.18	13.09	20.9	0.76
<i>DNA binding</i>					
W5GER8	Histone H2A	53.21	6.55	17.7	1.31
W4ZU32	Uncharacterized protein	49.37	17.74	6.8	1.42
W5H573	Histone H2B	77.68	27.59	15.6	1.56
Q43213	Protein H2A.5	47.72	16.55	15.4	1.72
W5GES9	Histone H2B	73.8	33.33	15.5	1.93
<i>Metal binding</i>					
P04464	Calmodulin	93.52	26.17	16.8	1.54
<i>Oxidoreductase activity</i>					
A0A0F6QT98	Aldehyde dehydrogenase 7B1	44.72	6.78	54.4	1.27
<i>Protein synthesis/assembly/processing</i>					
W5H8V6	Uncharacterized protein	48.78	11.67	12.1	0.66
W5H631	60S ribosomal protein L27	45.85	9.49	15.5	2.33
<i>Storage protein</i>					
B6UKM6	Gamma-gliadin	216.24	8.50	27.9	0.54
Q7X9J9	Progesterone binding protein	44.45	7.46	23	0.55
R9XU99	Gamma-gliadin	288.1	6.80	35	0.59
I7KM78	Gamma-gliadin	151.41	8.87	38	0.63
Q8GV12	HMW glutenin subunit Dx2	84.93	6.10	17.6	0.66
F2X322	Gamma-gliadin	47.29	2.74	33	0.67
A0A060N0S6	Omega-gliadin	47.94	2.46	42.7	0.72
Q94G97	Gamma-gliadin	89.70	25.08	28.9	0.73
Q45R38	HMW glutenin subunit Bx7	112.42	5.52	88.8	0.76
P08488	HMW glutenin subunit Dy12	120.39	8.80	69.6	0.78
W5AKY9	Uncharacterized protein	408.26	17.5	64.9	0.81
W4ZXS7	Uncharacterized protein	443.18	19.91	51.2	0.82
Q03871	HMW glutenin subunit By9	102.44	7.66	75.7	0.83
<i>Other</i>					
A0A0A7AA38	Grain softness protein	54.67	10.74	16.3	0.67
Q8S4P7	Thaumatococcus-like protein	45.42	10.22	23.6	0.77
A0A0A7AA82	Grain softness protein	110.75	24.16	16.4	0.83
A0A024CK72	LEA protein	136.24	39.44	18.7	1.21
Q41585	Type V Thionin	52.89	7.69	13.6	1.28

Note: TL5 and TL3 indicate topdressing timing of nitrogen at emergence of the fifth and third from the top, respectively. TL3 was used as the control. Accession, predicted protein in UniprotKB database. Score, total ion score of peptide spectrum matches searched against Mascot. Cov., average percentage of assigned peptides to the predicted protein. MW, molecular weight. HMW, high molecular weight. LMW, low molecular weight. LEA, late embryogenesis abundant.

aggregation of glutenin macropolymers during dough formation (Kimura et al., 2015). These results were in agreement with a previous study showing that up-regulated PDIs were found in the best quality samples (Victorio et al., 2018).

3.3.2. Grain hardness related proteins

Grain hardness is an important trait for wheat quality. Normally, soft grain is preferred during milling since the hard seed is more difficult to crush and produces a coarser powder composed of angular aggregates of starch and protein, which are often the contents of a single or aggregated group of cells (Greer, Hinton, Jones, & Kent, 1951). Interestingly, we observed that N topdressing timing also affected grain hardness. Delaying N topdressing (TL1) decreased grain hardness 7.4% and 8.7% than TL3 and TL5, respectively (Table 1). Altman, McCuiston, and Kronstad (1983) also observed a decreased grain hardness by foliar N fertilization at around flowering and suggested that grain hardness negatively correlated to protein content. Reversely, Luo,

Branlard, Griffin, and McNeil (2000) observed positive relationship between grain hardness and protein content, since hardness and protein content simultaneously increased under N fertilizer. Moreover, Yamazaki and Donelson (1983) showed that there was no relationship between grain hardness and protein content. Regardless of the controversial views on the relationship between grain hardness and protein, there is consensus that the grain softness protein (GSP) correlates with grain hardness (Jolly et al., 1993). Here, the modification of GSP levels was demonstrated by iTRAQ, where two DEPs of GSP were identified (Tables 2 & 3). We found that GSP (A0A0A7AA38) increased when delaying N topdressing, with 2-fold and 3-fold contents in TL1 in relation to TL3 and TL5, respectively. This result was consistent with the lowered grain hardness in TL1 (Table 1).

3.3.3. Flour allergenicity related proteins

As an important part of human diet, flour has a drawback of triggering food allergies, which affects 6%–8% of children and 1%–3% of

Table 3
Differentially expressed proteins in TL1 vs TL3.

Accession	Description	Score	Cov.	MW [kDa]	Fold change (TL1/TL3)
<i>Amino acid metabolism</i>					
A0A096UU21	Uncharacterized protein	46.91	2.78	31.8	0.82
<i>ATP binding</i>					
P12783	Phosphoglycerate kinase, cytosolic	387.23	25.44	42.1	0.67
W5E8B9	Uncharacterized protein	49.48	2.48	99.8	0.73
<i>Carbohydrate metabolism</i>					
W5EK10	Beta-amylase	120.19	12.00	61.1	0.55
P17314	Alpha-amylase/trypsin inhibitor CM3	50.25	17.86	18.2	0.74
M4Q9V0	Enolase	148.59	15.92	48.1	0.80
A4ZIW9	Monomeric alpha-amylase inhibitor	130.42	35.54	13.1	0.81
Q5UHH6	0.19 dimeric alpha-amylase inhibitor	460.21	38.71	13.2	0.82
<i>Defense response</i>					
P32032	Alpha-2-purothionin	143.70	18.38	14.5	0.59
Q9S6Y2	Alpha purothionin	236.71	20.00	9.5	0.78
<i>DNA binding</i>					
A0A096UJW0	Uncharacterized protein	67.89	31.53	12.6	0.78
W4ZU32	Uncharacterized protein	49.37	17.74	6.8	1.46
W5H573	Histone H2B	77.68	27.59	15.6	1.51
W511S2	Histone H2A	166.21	11.98	17.6	1.57
W5GES9	Histone H2B	73.80	33.33	15.5	1.60
<i>Hydrolase</i>					
W5DZW0	Uncharacterized protein	47.39	5.46	37.6	0.78
<i>Nucleic acid binding</i>					
W5ECF2	Uncharacterized protein	45.77	12.12	7.3	1.23
<i>Oxidoreductase activity</i>					
W5ED28	Uncharacterized protein	53.37	2.42	51.4	0.67
P80602	2-Cys peroxiredoxin BAS1, chloroplastic	48.16	10.95	23.3	0.79
W5CMA3	Uncharacterized protein	46.76	1.90	56.6	0.81
W5FXC4	Uncharacterized protein	47.79	6.38	19.7	2.36
<i>Protein synthesis/assembly/processing</i>					
A0A024FR39	Protein disulfide-isomerase	747.64	34.43	53.8	1.24
W5A0K2	Uncharacterized protein	46.17	6.72	13.8	1.28
W5DI26	Uncharacterized protein	63.71	11.76	9.5	1.28
F8THZ6	Protein disulfide-isomerase	715.32	32.42	56.6	1.32
W5GBI3	Uncharacterized protein	49.15	4.83	16.4	1.36
Q5XUU9	Cytoplasmatic ribosomal protein S13	45.20	6.62	17.1	1.38
W5GVR2	Uncharacterized protein	46.34	15.98	24.5	1.56
W5H631	60S ribosomal protein L27	45.85	9.49	15.5	2.33
<i>Storage protein</i>					
Q7X9J9	Progesterone binding protein	44.45	7.46	23.0	1.21
I7KM78	Gamma-gliadin	151.41	8.87	38.0	1.22
D3UAL6	LMW glutenin subunit B3-1	99.08	9.21	36.3	1.23
Q03871	HMW glutenin subunit By9	102.44	7.66	75.7	1.24
W4ZXS7	Uncharacterized protein	443.18	19.91	51.2	1.25
Q8GV12	HMW glutenin subunit Dx2	84.93	6.10	17.6	1.26
Q45R38	HMW glutenin subunit Bx7	112.42	5.52	88.8	1.29
B6UKM6	Gamma-gliadin	216.24	8.50	27.9	1.36
A0A060N0S6	Omega-gliadin	47.94	2.46	42.7	1.55
Q94G97	Gamma-gliadin	89.70	25.08	28.9	2.82
<i>Transport</i>					
P46274	Mitochondrial outer membrane porin	46.36	10.91	28.9	0.83
W5F027	Uncharacterized protein	48.86	4.87	71.7	1.33
<i>Other</i>					
B7U6L4	Globulin 3	248.30	7.31	66.3	0.68
Q41585	Type V Thionin	52.89	7.69	13.6	1.37
A0A0A7AA38	Grain softness protein	54.67	10.74	16.3	1.98

Note: TL3 and TL1 indicate topdressing timing of nitrogen at emergence of the third and first from the top, respectively. TL3 was used as the control. Accession, predicted protein in UniprotKB database. Score, total ion score of peptide spectrum matches searched against Mascot. Cov., average percentage of assigned peptides to the predicted protein. MW, molecular weight. HMW, high molecular weight. LMW, low molecular weight.

adults (Östblom, Lilja, Pershagen, Van Hage, & Wickman, 2008). Among the major allergens identified in the water-/salt-soluble fractions (metabolic proteins) of wheat flour, the α -amylase inhibitors are active allergenic proteins that constitute a health risk for workers in the baking industry and causes atopic dermatitis, gastrointestinal symptoms or wheat-dependent exercise-induced anaphylaxis (Houba et al.,

1998). In this study, we detected allergenicity related proteins among DEPs involved in metabolism pathway by online database PREAL^W (<http://lilab.life.sjtu.edu.cn:8080/prealw/index.php>) and Allergome (<http://www.allergome.org/>), including the α -purothionin, 0.19 dimeric α -amylase inhibitor, thaumatin-like protein, and monomeric α -amylase inhibitor. The contents of both proteins belonging to the group

of α -amylase inhibitors were significantly lower in TL1 than those in TL3 (Table 3). Similarly, the contents of both α -purothionins in TL1 were 0.59- and 0.78-fold those of TL3. The results indicated the potential implement of delaying N topdressing to reduce the content of allergenicity related proteins.

3.4. Transcriptional expression of DEP encoding genes

For some of the important DEPs, we performed qPCR analysis to quantify the expressions of 15 representative DEPs encoding genes at 7, 13, 19, 25 and 31 days after anthesis (DAA). The change folds of the corresponding DEPs in flour are shown in Fig. 3. The qPCR analysis revealed that the transcriptional expression patterns of all the storage protein (HMW/LMW-GS, gliadins) encoding genes were well consistent with changes in the expressions of the corresponding proteins (Fig. 3). *HMW-Bx7*, *HMW-Bx9* and *LMW-B3* showed the highest expression at 25 DAA, which were later than the gliadin proteins that had their highest expression at 19 DAA. This is in agreement with previous observations suggesting a short delay of HMW-GS transcription compared with that of gliadin (Shewry et al., 2012). Here, the genes for ω -gliadin and γ -gliadin were highly expressed along with grain filling with a sharp initiation at 13 DAA. Delayed nitrogen topdressing up-regulated the expression of *HMW-Bx7*, *HMW-Bx9* and *LMW-B3* at 25 DAA in comparison with TL3, which was in accordance with the modified glutenin proteins (Table 1).

In addition, the genes encoding the β -amylase, 0.19 dimeric α -amylase inhibitor and alpha purothionin were expressed at their highest from 19 DAA to 25 DAA in TL3, which agreed with the highest protein expressions in TL3 flour. The significant differences were observed between N topdressing timing treatments in terms of expression level of GSP at 19 DAA and GSP content in mature grain flour. The genes encoding PDI and LEA, had a higher expression level in TL1 at 13 DAA and TL5 at 31 DAA, respectively, which were in accordance with the PDI and LEA content in the flour.

However, three genes encoding starch synthase, sucrose synthase and type V thionin showed opposed expression patterns with their final protein contents. Previous work demonstrated that the mRNA level and protein abundance can be inconsistent due to translational or post-translational regulation (Tada & Kashimura, 2009). Therefore, the poor consistency in starch synthase, sucrose synthase and type V thionin encoding genes between their transcriptional and translational levels could be explained by translational or post-translational regulation, which needed further evidences.

4. Conclusions

By modifying timing of topdressed N fertilizer in reference to the leaf ages, the present results found that delaying N topdressing improved GPC without increasing N dose, indicating a potential strategy to maintain quality of bread-making under low input of total nitrogen.

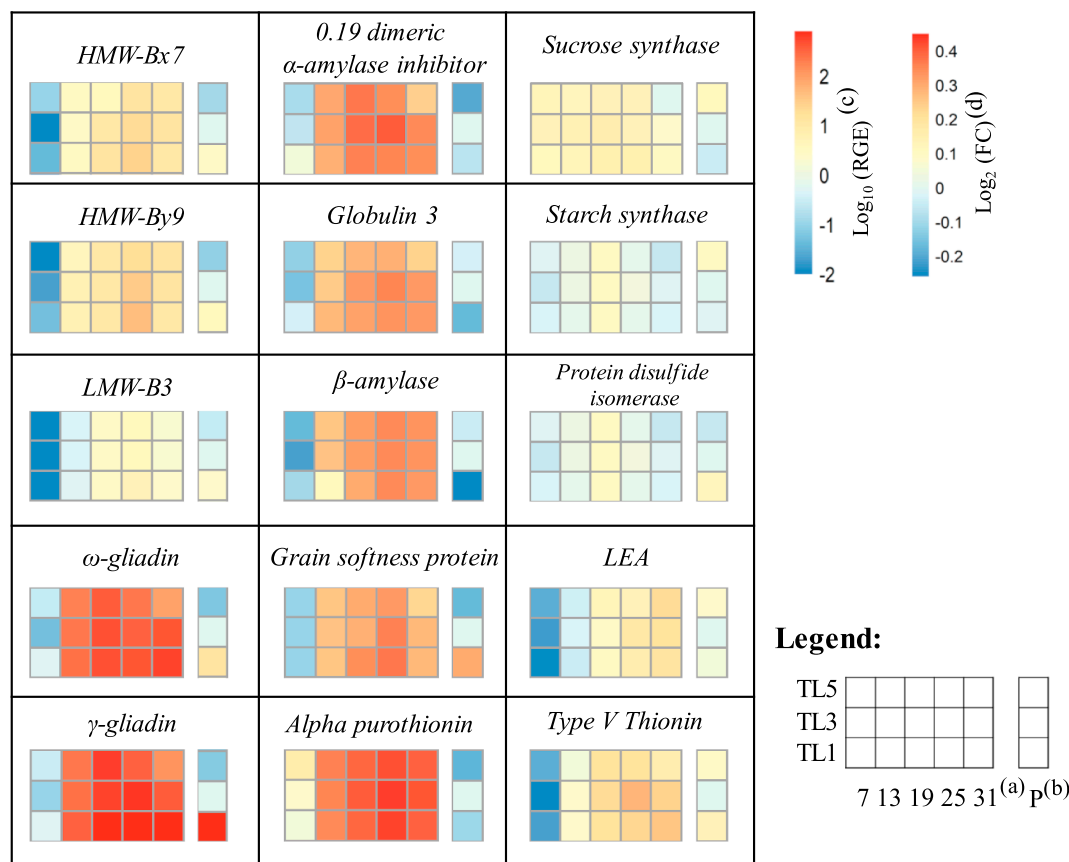


Fig. 3. Comparison of mRNA expression patterns and protein content of 15 representative DEPs (differentially expressed proteins) by qPCR and iTRAQ. Note: TL5, TL3 and TL1 indicate topdressing timing of nitrogen at emergence of the fifth, third and first leaf (flag leaf) from the top, respectively. (a) The numbers represent the corresponding day after anthesis. (b) P, protein content quantified by iTRAQ. (c) The scale for normalized relative gene expression during grain filling stage. REG, relative gene expression. (d) The scale for normalized fold change of protein content in TL5 or TL1 in comparison to TL3. FC, fold change. HMW, high molecular weight. LMW, low molecular weight. LEA, late embryogenesis abundant.

Furthermore, a proteomic study on flour using the iTRAQ technology confirmed that delaying N topdressing up-regulated the accumulation of gluten-related proteins, particularly the gliadins and HMW-GS. Delaying N topdressing down-regulated the synthesis of the globulin 3 and α -amylase inhibitors, to potentially reduce the risk of the allergic disease. Delaying N topdressing also up-regulated GSP, which indicated a modification of grain hardness. Furthermore, 591 proteins were identified in flour of a common wheat cultivar, of which 98 proteins have not been annotated to a biological function and 227 proteins of which the functions were predicted by domains. In the future, it will be of interest to clarify the function of the non-annotated proteins and introduce the differentially expressed proteins to breeding procedures and management practices targeting satisfying quality of end-products of wheat. In summary, the use of iTRAQ for identification and quantification of the global protein profile of wheat flour highlights the perspectives of nitrogen topdressing timing for grain quality modification.

Declarations of interest conflicts

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2018.10.071>.

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