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ARTICLE

Proteome Changes in Wheat Subjected to Different Nitrogen and Sulfur Fertilizations

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Controlling the quality of wheat for breadmaking is a major concern for the milling and baking industry. Wheat flour quality depends on both the genetic background and environmental factors during growth and storage. Amount and timing of application of fertilizer are factors that affect wheat quality. This study investigated the effect of different levels of nitrogen and sulfur on the tris-soluble and glutenin protein fractions by 2D-electrophoresis. Multivariate analysis was performed to study changes in the proteome pattern. In the tris-soluble fraction 20 proteins were changed in abundance due to S fertilization, whereas 16 proteins were changed in the glutenin protein fraction. It was found that induced sulfur deficiency during growth resulted in the most pronounced effect on protein composition. Understanding which proteins are affected by varying levels of fertilizers may help tailor specific traits in various wheat varieties.

KEYWORDS: Proteomics; sulfur fertilization; wheat; 2D-electrophoresis; gliadins; glutenins

INTRODUCTION

Wheat is one of the most important crops for global food supply and is processed into a range of products that require different qualities of the raw material. The gluten proteins are the most important determinants of the dough viscoelastic properties that are essential for processing and product quality. The large genetic variation in the composition of the gluten proteins causes a correspondingly large variation in dough properties. This is utilized in the breeding of wheat cultivars for specific end-use qualities such as bread, noodles, biscuits, and cakes. Gluten protein composition and viscoelastic properties are also affected by environmental factors, as reviewed in Dupont and Altenbach (*1*). This may cause unpredictable variations in wheat quality, which are unprofitable for the baking industry. In particular, the soil availability of sulfur (S) and nitrogen (N) may affect both the amount and the composition of gluten proteins. Shewry et al. (*2*) classified the wheat prolamins into S-poor (mainly ω-gliadins) and S-rich (mainly the γ- and α-gliadins and the LMW-GS) and HMW prolamins (HMW-GS). In low S soils, a shift in the prolamin composition toward lower proportions of the S-rich and a higher proportion of the S-poor prolamins has been reported (*3*, *4*). These compositional changes were associated with dough rheological changes, giving increased resistance to dough extension and decreased extensibility when S availability from the soil was low (see ref *4* for review). Wieser et al. (*5*) studied the changes in proportion of the different prolamin types caused by different S fertilizations. They found that decreased S content in the soil correlated with a considerable increase in the proportion of ω-gliadins, a moderate increase in the proportion of HMW-GS, and a decrease in the proportion of γ-gliadins and LMW-GS.

About 80% of the organic S in the plant is incorporated into proteins (*4*). Because only a portion of the organic S is redistributed to the grain from vegetative tissues during grain development (*6*), sufficient availability of S throughout the growth season is important to obtain stable baking quality and should be recommended. Split application of S was shown to be effective in securing optimal levels of grain S and predictable dough properties (*7*).

Further studies are needed to understand how N and S availability in the soil may affect the composition and buildup of the gluten proteins. Due to the complexity of flour protein composition, proteomics may be a useful tool in measuring the effects of variable growth conditions on the many individual prolamins and in understanding their implications for wheat quality parameters (*8*).

Although the enzymes in mature wheat grain have low activity and abundance, their presence can still have an impact on the quality of the wheat flour. Whereas the baking properties of wheat are mainly determined by the gluten protein fraction, the tris-soluble proteins may also be of interest. One of the allergens associated with baker's asthma is α-amylase inhibitors from wheat grains (*9*, *10*). Any observed change in abundance of these proteins according to applied N and S fertilization could therefore be of interest to the baking industry.

In an earlier study (*7*), we observed a significant effect of N and S fertilization on SDS sedimentation volume and on viscoelastic properties of the dough measured by Kieffer

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extensigraph. Therefore, samples from this earlier study were selected to further explore effects of N and S fertilization on quality using proteome analyses. The objective of this study is to study the effect of N and S fertilization on the tris-soluble proteins and gluten protein fractions using two-dimensional gel electrophoresis (2-DE) and mass spectrometry. The relationships between protein content, SDS sedimentation volume, and dough properties were analyzed as an effect of the N/S ratio in the wheat grain.

EXPERIMENTAL PROCEDURES

Sample Material. The wheat samples were obtained from an earlier experiment in which plants were grown in pots in a greenhouse with different N and S fertilization regimens and published in Flaete (*7*). The experiment is briefly described below. The experimental design consisted of four treatment factors: (1) N given at sowing (N_{tot}), (2) S given at sowing (S_1), (3) additional S given as split application during growth (S_2), and (4) the developmental stage when split application was added (GS). The design was a full factorial design with N_{tot}, S_1, and GS having two levels each and S_2 having three levels (**Figure 1**). N was applied at sowing and as a late split application during growth. The two levels of N_{tot} correspond to field applications of 150 + 50 kg ha^{-1} (level 1) and 220 + 80 kg ha^{-1} (level 2), which are considered to be moderate and high levels, respectively, in commercial production. For S_1, the levels used correspond to 10 kg ha^{-1} (level 1) and 30 kg ha^{-1} (level 2). These can be compared to low and high levels in commercial production. The levels of S_2 included zero (level 1). For level 2 and 3, the amount of S given was adjusted according to N_{tot}, and a lower amount was given combined with level 1 of N_{tot} (corresponding to 5 and 8 kg ha^{-1}, respectively, for level 2 in S_2) and a higher amount was given combined with level 2 of N_{tot} (corresponding to 10 and 16 kg ha^{-1}, respectively, for level 3 in S_2). Further details are given in Flæte (*7*) regarding application of the mineral fertilizers. The plant development stages for applying split applications were at stem elongation and early heading [decimal codes 31 and 49, respectively (*11*)]. Three pots of each treatment were grown. The experiment was repeated in the same greenhouse and at the same period during the year in 2002 and 2003, to obtain biological replicates. The mature seeds were harvested, and grains from the three parallel pots were pooled. The grain samples were milled to whole-meal by a Falling Number 3100 hammer mill using a 0.5 mm sift (Perten Instruments AB, Huddinge, Sweden) and kept at cool conditions (below 10 °C) prior to analyses.

In Flæte et al. (*7*), S_2 was shown to be the most influential factor for gluten quality. Due to this, we decided to analyze the glutenin fraction from samples where N_{tot} and S_1 were constant at high and low levels, respectively (**Figure 2A**). For the analysis of tris-soluble proteins we decided to use all four factors, but limiting S_2 to only low ($S_2 = 1$) and high ($S_2 = 3$) level. The samples were chosen on the basis of a fractional reduction of the original design, and the selected samples are shown in **Figure 2B**.

Quality Analyses. The samples were analyzed for N content by using a LECO CHN-1000 apparatus. S content was determined by ICP after digestion with aqua regia (300 mL of HCl and 100 mL of HNO$_3$ diluted with 1 L of H$_2$O). Breadmaking quality was analyzed using the SDS sedimentation test [AACC Approved Method 56-70, 1995 (*12*)], and the dough rheological properties were analyzed using the SMS/Kieffer Dough and Gluten Extensibility for the TA XT2i Texture Analyzer (Stable Micro Systems, Godalming, U.K.) (*13*).

Protein Extraction and 2D-Electrophoresis. The extraction procedure of Tatham et al. (*14*) for extracting glutenin subunits was used with some modifications. Fifty milligram of whole-meal flour was extracted by 70% ethanol and then by 50% propanol containing 1% acetic acid and 1% mercaptoethanol.
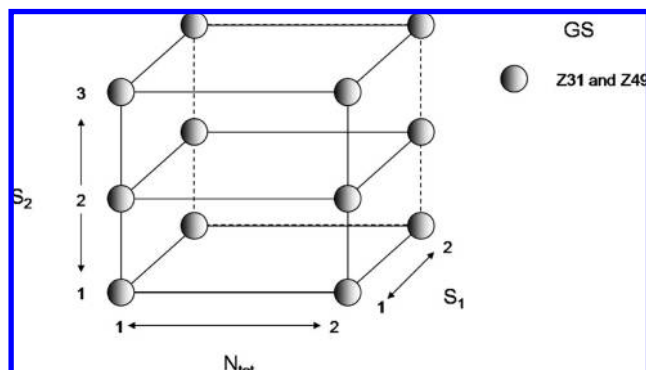

Figure 1. Design of the original experiment. Experimental factors were total nitrogen (N_{tot}), sulfur applied at sowing (S_1), amount of sulfur applied as split application during growth (S_2), and timing of application of fertilizer during growth (GS). The levels of each of the three first factors correspond to S_1, 10 and 30 kg ha^{-1}; N_{tot}, 150 + 50 and 220 + 80 kg ha^{-1}; and S_2, 0, 5/8, and 10/16 kg ha^{-1}. The two numbers for levels of S_2 refer to an adjustment of the applied sulfur according to whether the plants got a low or high amount of N_{tot}, respectively. Circles indicate treatment combinations used in the experiment. Black and white shaded circles show that the treatment combination included both levels of GS. The growth stages for late split application were stem elongation (Zadoks 31) and early heading (Zadoks 49).
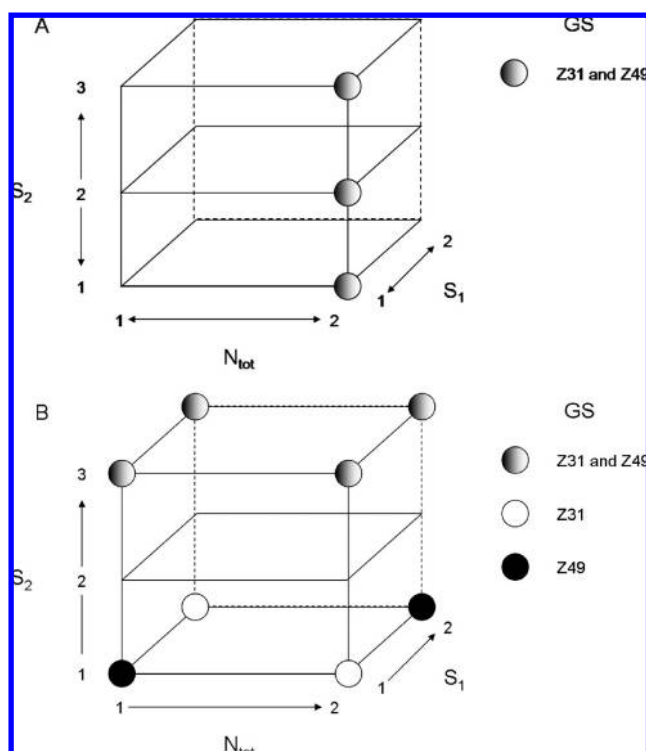

Figure 2. Design of the samples selected for the proteome studies of glutenins (**A**) and the tris-soluble proteins (**B**). Factors and levels are the same as in **Figure 1**. Circles indicate which of the treatment combinations were used in the reduced designs. Black and white shaded circles show that the treatment combination included both levels of growth stage (GS). Full white or black circles indicate that split application of fertilizer was given at only one of the two levels of GS (Z31 and Z49, respectively).

The pellet was resuspended in 50% propanol containing 1% acetic acid and 2% mercaptoethanol overnight and centrifuged, and the supernatant liquid was transferred to new tubes and kept at −70 °C until use. For extraction of tris-soluble proteins, 100 mg of wheat flour was mixed with 1 mL of 50 mM tris, pH 8.8, with shaking for 1 h at 4 °C. After centrifugation,

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the supernatant liquid was transferred to a new tube and kept at −70 °C until use.

Protein concentrations were measured with a commercial kit at 760 nm (*RC DC* Protein Assay, Bio-Rad) in a spectrophotometer (Ultrospec 3000, Pharmacia Biotech) using BSA as standard. We used 50 μg of tris-soluble proteins and 100 μg of glutenins for analytical gels and 1000 μg of protein for preparative gels. The proteins were mixed with rehydration buffer [8.5 M urea, 2 M thiourea, 40 mM tris, pH 8.8, 2% (v/v) NP-40, 2% (v/v) IPG 3−10 buffer, 25 mg/mL DTT, and a few crystals of bromophenolblue] and used for rehydration of 18 cm immobilized pH gradient (IPG) strips pH 4−9 (homemade) (tris-soluble fraction) and pH 6−11 (GE Healthcare Biosciences, Uppsala, Sweden) (glutenin fraction). Isoelectric focusing (IEF) was performed on an IPGphor apparatus (GE Healthcare) with a stepwise procedure at a total of 52 kVh. The IPG strips were equilibrated for 2 × 15 min in equilibration buffer (50 mM tris, pH 8.8, 6 M urea, 30% glycerol, 2% SDS) first with 60 mM DTT and second with 140 mM iodoacetamide. Proteins were separated on a 12.5% (tris-soluble proteins) or 10% (glutenins) acrylamide gel in the second dimension using the Ettan Dalt *twelve* apparatus (GE Healthcare). Silver staining of the gels was performed according to the method of Blum et al. (*15*). For preparative gels the staining was stopped with 5% acetic acid. Two 2-DE gels per sample were performed, using protein from two separate extractions (technical replicates). In addition, the two biological replicates of the experiment, grown in the two subsequent years, were analyzed.

Image Analysis and Statistics. All gels were scanned using an office scanner with 8-bit color depth and a resolution of 240 dpi. The images were aligned with Z3 (Compugen Inc.), and spots were detected and quantified with ImageMaster 2D Platinum v5.0 (GE Healthcare). After the automatic spot detection and alignment, manual editing was performed to correct for any misalignments. The spot volumes were normalized by dividing by the total spot volume for all detected spots on the gel. The data table contained some missing values that were caused by a failure to match spots from the reference gel to the other gels. As discussed in Grove et al. (*16*), the missing values could be due to problems with the spot detection or spot matching, technical problems with the gels, or proteins being absent in the sample. Because of this and because the methods we used for variable selection could not handle missing values, we omitted protein variables with missing values from the analysis of tris-soluble proteins. For the glutenin protein data set, we included the protein variables with at most two missing values and used the *k* nearest-neighbor method (*17*) to estimate the missing values. The combined proteome data were analyzed by partial least-squares regression (PLSR). PLSR is a multivariate data analysis technique designed to investigate the relationship between two sets of multivariate data matrices. The technique compresses information from the data matrices by making a new set of variables called principal components (PC), which are linear combinations of the original variables. Each PC shows common variation explained by several of the original variables, and the PCs are ranked according to the amount of variation explained. The PCs can be visualized in a 2D plot where the samples are plotted according to the new coordinates from the transformed data set (score plot) and a corresponding plot of variables (loading plot). This gives an overview of the main structure of the data and the relationship between different proteins. The PLSR was performed using the protein volumes as regressor variables (X) and the indicator values for the design as response variables (Y). All protein spot volumes were log-transformed prior to the multivariate analysis. The Unscrambler v9.1 (CAMO ASA, Oslo, Norway) and Matlab v7.0.4 (The MathWorks Inc., Natick, MA) were used for data analysis. Cross-model validation (CMV) (*18*) was used to identify protein spots that were significantly changed in abundance between samples. CMV is based on PLS regression with jack-knife estimates and *t*-tests. In addition to the normal jack-knife procedure, the CMV

Table 1. Effects of N and S Treatments on Protein Concentration, SDS Sedimentation Volume, and Dough Properties Measured by Kieffer Ekstensigraph (Standard Errors of the Means Are Given)

N and S fertilization treatment	N/S ratio	protein, %	SDS[a], mL	R_{max},[b] g	ext,[c] mm
$N_{tot} = 1$, $S_2 = 0$	16.6	12.8	60	10.21	57.21
$N_{tot} = 2$, $S_2 = 0$	20.7	15.1	60	14.25	60.51
$N_{tot} = 2$, $S_2 = 1$	16.6	15.3	72	14.84	79.11
$N_{tot} = 2$, $S_2 = 2$	15.7	14.8	73	13.30	81.28
MSE	0.63	0.204	1.85	1.24	4.62

[a] SDS sedimentation volume. [b] Kieffer exstensigraph, maximum resistance. [c] Kieffer exstensigraph extensibility, distance.

uses an additional validation step, leaving out one subset of samples before the standard model building and cross-validation on the remaining samples. Changes in protein spot volumes were reported as significant if they were significant in at least 75% of the external cross-validation routines. For the CMV and PLSR analysis of the tris-soluble proteins, we included the four two-factor interactions involving S_2 in addition to the main factors (N_1, S_1, S_2, GS, and year). The two-factor interactions were represented as new design variables with high level, where both factors had the same level (either high−high or low−low) and low level where the factors had opposite levels (high−low or low−high).

Protein Identification. The protein spots were picked from preparative gels and washed twice for 15 min with 150 μL of 50% acetonitrile with shaking at room temperature. The liquid was removed, and the gel pieces were dried in a Speed-Vac centrifuge (ThermoSavant). After the addition of 150 μL of 10 mM DTT, the tubes were incubated at 56 °C for 45 min. After cooling to room temperature, the liquid was replaced with 150 μL of 55 mM iodoacetamide, and the samples were incubated for 30 min in darkness at room temperature. The gel pieces were washed with 150 μL of 50% acetonitrile and dried. Thirty microliters of trypsin solution (2 ng/μL in 50 mM $NH_4HCO_3^-$) was added to each tube, which were then set on ice for 30 min followed by 37 °C overnight (∼18 h). Then the supernatant liquid was transferred to a clean tube and 3 μL of formic acid was added. The peptide samples were washed and desalted using either OMIX microcolumns (Varian) or GELoader tips with a small disk of C_{18} material (*19*). Peptide samples were analyzed by mass spectrometry (MS) using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) in a Bruker Ultraflex MALDI-TOF/TOF (Bruker Daltronics). Database searches were performed using the Mascot search engine (www.matrixscience.com) and the NCBInr database, with all cysteines having a fixed modification with carbamidomethyl and all methionines having a possible oxidation modification. We used a mass tolerance of 100 ppm for the peptide fragment search and the same mass tolerance for the parent ions in the MS/MS search. The settings for mass tolerance for the fragment ions in the MS/MS search varied from 0.5 to 3 Da between searches. In those cases when a high mass tolerance was needed, the error distribution was checked to see if there was any systematic trend. If no valid systematic trend could be found, the results would be disregarded for the identification. Positive identification was concluded from the total combined score from the peptide mass fingerprint and MS/MS spectra from selected peptides. The MS/MS spectra were acquired if the peptide mass fingerprint did not yield any significant results or if there were some ambiguity in the results.

RESULTS

Effect of N and S Fertilization on Quality Parameters. The different N and S fertilization treatments greatly affected the N and S concentrations of the grain as well as the SDS sedimentation volume and dough properties (*7*). An overview of the quality variations, which made the basis for the selection of samples for this study, is shown in **Table 1**. In this
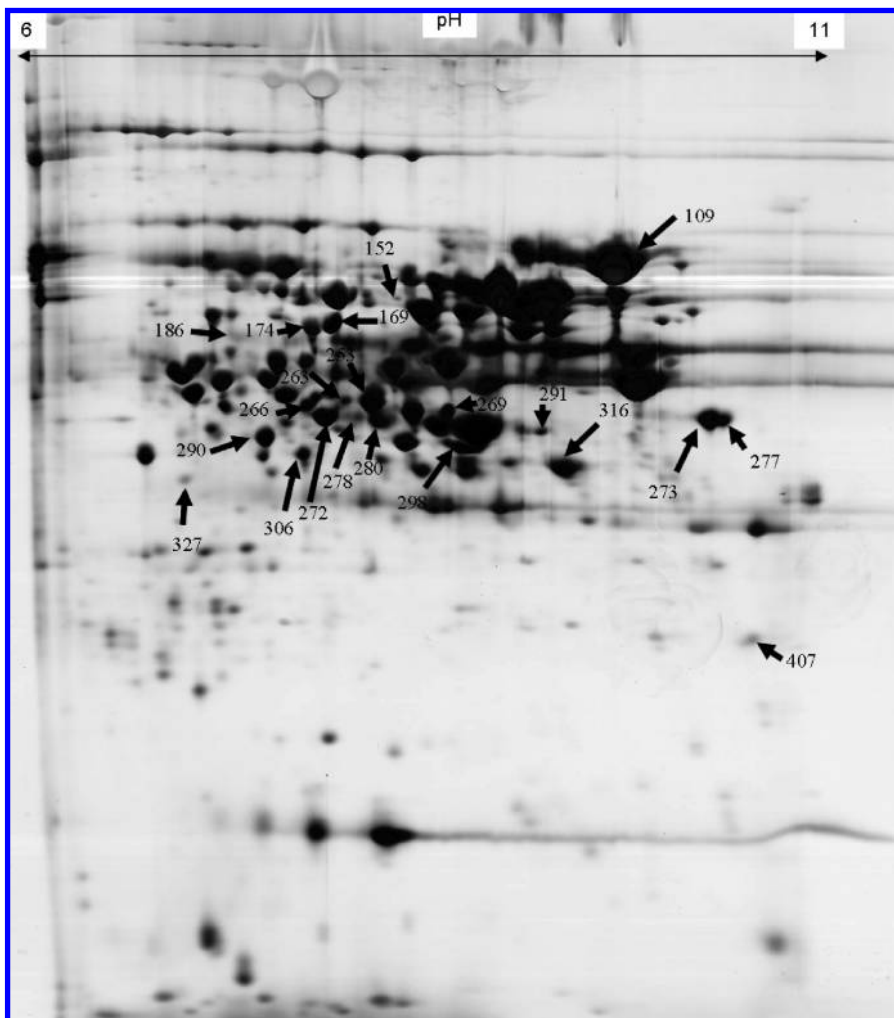
Figure 3. Representative image of a silver-stained 2-DE gel of wheat glutenin proteins. A total of 100 μg of protein was separated by IPG 6–11 in the first dimension and by 10% SDS-PAGE in the second dimension. Marked spot numbers refer to protein spots that showed a significant change correlated to the application levels of S fertilization.

table, four treatments are compared: (1) low N and no S in split application ($N_{tot} = 1$, $S_2 = 1$), (2) high N and no S in split application ($N_{tot} = 2$, $S_2 = 1$), (3) high N and medium amount S in split application ($N_{tot} = 2$, $S_2 = 2$), and (4) high N and high S in split application ($N_{tot} = 2$, $S_2 = 3$). The results showed strong effects of increasing N fertilization without giving extra S during growth, which resulted in highly increased protein concentration and elevated N/S ratio, but no increase in SDS sedimentation volume. The dough properties showed a substantial increase in dough resistance when N was increased, but no increase in extensibility. The high N/S ratio for this treatment clearly documented S starvation in the grain. According to Randall et al. (*20*), N/S ratios at 17 and higher indicate S deficiency. By adding extra S as spilt application during growth, the SDS sedimentation increased substantially. Also, changes in dough properties were documented, as the split applications of S significantly increased the dough extensibility. No significant differences were found between the different amounts of S given as split application (level $S_2 = 2$ compared to level $S_2 = 3$, both with high N_{tot}). This indicates that the lowest rate of split application provided enough S to secure a stable N/S value and to avoid variations in dough quality.

Proteome Analyses of the Glutenin Fraction. In this experiment we decided to focus on the glutenins and used an

extraction procedure developed to obtain high purity of glutenins. The protein extracts obtained should thus contain HMW and LMW glutenin subunits and only trace amounts of gliadins.

In the glutenin protein extracts we observed 350 ± 74 (mean ± standard deviation) protein spots after separation by 2-DE. A representative gel image is shown in **Figure 3**. The HMW glutenins were observed as negatively stained spots on top of the gels and were excluded from further analysis. Because silver staining has problems quantifying both low- and high-abundant proteins at the same time, we decided to aim for visualizing as many proteins as possible. Due to this, the most abundant protein spots appeared saturated on the scanned images, making the quantification on those protein spots difficult. They were still included in the analysis because even though the spots were saturated, there was a possibility of observing differences due to different sizes of the spots between the gels. A total of 73 protein spots matched to a protein spot across all gels and were analyzed by PLSR to look for the variation in the samples connected to the levels of S_2 (**Figure 4**). The score plot from PLSR shows how the samples grouped according to this factor, and the distance between each pair of samples indicates the similarity of the proteome from those samples. The effect of missing values on the PLSR was tested by including protein variables with up to 50% missing values in the analysis. This did not
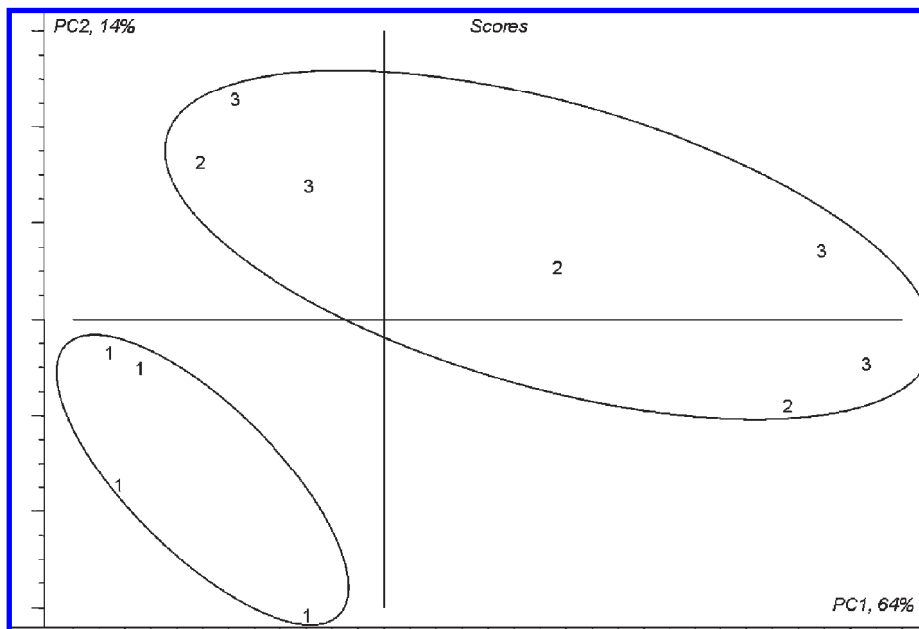
Figure 4. Partial least-squares regression of 73 protein spot volumes from the glutenin fraction of wheat endosperm. The numbers refer to the levels of added S at split application as described in **Figure 1**. The first two principal components showing the main variation in the samples according to the proteome data are shown. Only proteins without missing values were included in the analysis.
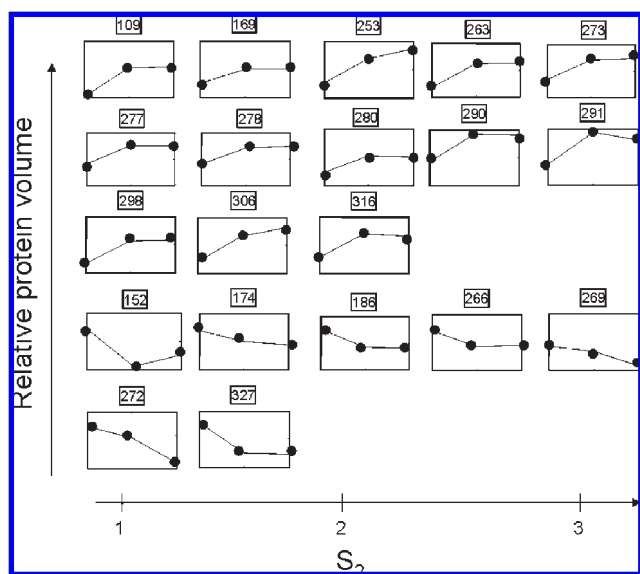

Figure 5. Expression profiles of the averaged spot volumes for the significantly changed proteins on the glutenin 2-DE gels at the three levels of late S application. The spot numbers are the same as in **Figure 3**, and the identified proteins are presented in **Table 2**.

change the results (not shown). The samples were separated into two groups by the PLSR analysis (marked by circles). One group contained the samples of no S as split application ($S_2 = 1$), and the other group contained the samples from the pots fertilized with medium ($S_2 = 2$) and high ($S_2 = 3$) levels of split S application.

The CMV analysis indicated that 20 protein spots were significantly changed in abundance depending on the levels of S_2. Thirteen of these proteins were positively correlated to S_2, whereas seven were negatively correlated (**Figure 5**). Of the 13 proteins positively correlated to S_2, 4 proteins were identified as γ-gliadins (169, 290, 298, and 316), 3 belonged to the LMW-GS group (109, 273, and 277), and one protein spot (306) was identified as a homologue to an avenin-like

b precursor from *Aegilops tauschii* (**Table 2**). Two proteins showed sequence homology to wheat gliadins but gave no significant hits in the database. The remaining proteins were not identified due to low protein amounts or difficulties with the protein extraction and the acquisition of suitable MS spectra. One reason for this can be the unique amino acid composition in wheat storage proteins, having a low number of lysine and arginine residues and, consequently, few cleavage sites for trypsin. This may result in few and large tryptic peptides, which make protein identification and MS/MS analyses challenging.

Proteome Analyses of Tris-soluble Proteins. In the analysis of the tris-soluble protein fraction we observed 1029 ± 145 (mean $\pm$ standard deviation) protein spots on each 2-DE gel (**Figure 6**). A total of 101 protein spots were matched across all gels and were analyzed by CMV. To find those protein spots showing a significant change between the two levels of S_2, all of the main factors and the two-factor interactions that included S_2 were used. We found 16 protein spots that were related to the levels of S_2. Four proteins (1764, 1817, 1855, and 1949) were negatively correlated to S_2, whereas the rest were positively correlated. Ten of the 12 proteins with a positive correlation were identified as defense proteins (α-amylase inhibitors, heat shock proteins, and others) (**Table 3**).

The PLSR analysis of the tris-soluble proteins was performed using only the significant proteins and the design variables to see if we could observe a correlation to the design. The first component separated the samples into two groups based on level of S_2 (**Figure 7A**), whereas the second component was influenced by the difference in year for samples with high level of S_2. The PLSR loading plot (**Figure 7B**) shows the correlation between the design variables and the protein spots. S_2 is located in the outer ring in the first component, which reflects that this design variable has the most influence on the variation in the protein spots. The two next most influential design variables are the yearly variation and the interaction between S_2 and year. The rest of the design variables, being located close to the middle of the plot, have little or no influence on these protein spots.

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Table 2. Identified Proteins from the Glutenin Fraction

ID	GI	name	exptl pI	matched peptides[a]/sequence coverage/score	theor pI/M_r
109	GI 17425184	LMW glutenin subunit, group 3 type II (wheat)	9.68	6 (3)/20%/(276)	8.51/38K
169	GI 15148385	γ-gliadin (wheat)	7.86	2 (1)/8%/(80)	8.48/29K
273	GI 1857652	LMW glutenin storage protein group 8 type IV (wheat)	10.3	5 (2)/25%/(150)	9.03/34K
277	GI 1857652	LMW glutenin storage protein group 8 type IV (wheat)	10.4	12 (2)/32%/(122)	9.03/34K
290	GI 4836789	γ-gliadin (wheat)	7.43	3 (2)/12%/(68)	6.92/34K
298	GI 121103	γ-gliadin B precursor (wheat)	8.68	3 (1)/12%/(82)	8.48/33K
306	GI 89143132	Putative avenin-like b prec. (*Aegilops tauschii*)	7.68	10 (2)/45%/(169)	7.83/33K
316	GI 15148391	γ-gliadin (wheat)	9.32	1 (1)/6%/(50)	8.64/33K

[a] Numbers in parentheses indicate how many peptides were analyzed by MS-MS sequencing.
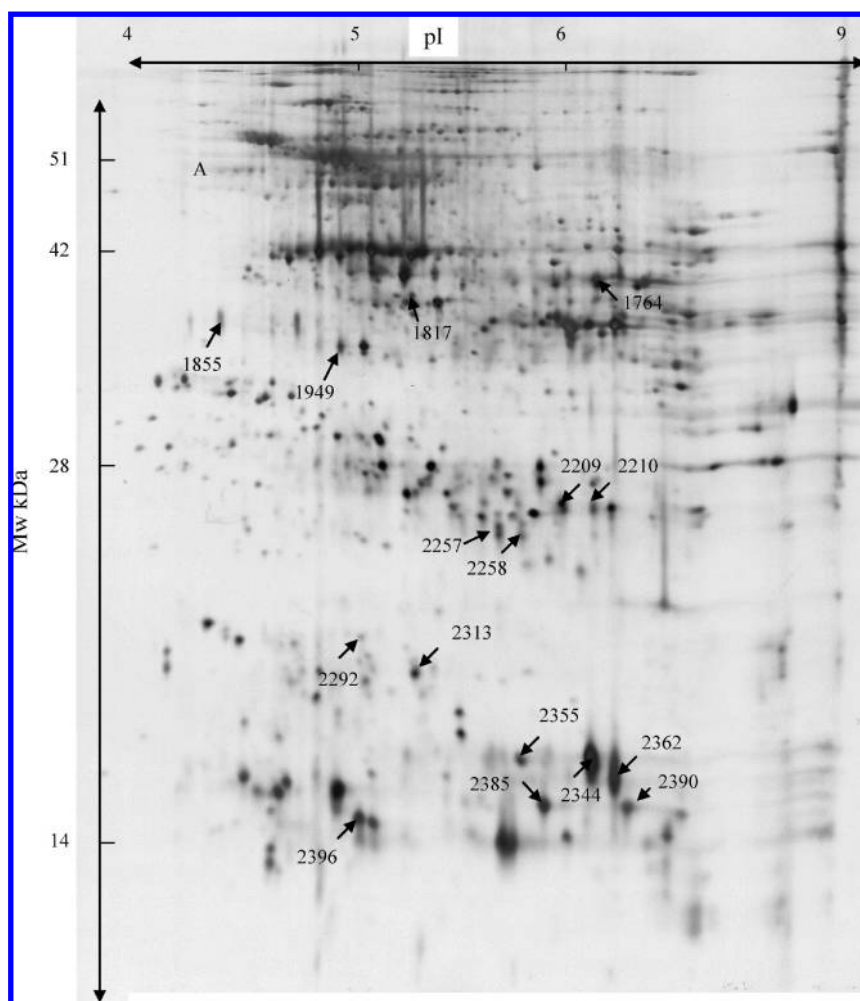

Figure 6. Representative image of a silver-stained 2-DE gel of tris-soluble wheat grain proteins. A total of 50 μg of protein was separated by IPG 4−9 in the first dimension and by 12.5% SDS-PAGE in the second dimension. Marked spot numbers refer to protein spots that showed a significant change correlated to the application levels of late S fertilization.

The clustering and positioning of the protein spots separate the proteins into three groups (**Figure 7B**). Two consist of amylase inhibitors and enzymes and are located close to S_2, indicating a high positive correlation to this design variable. The groups are separated along the second component, indicating a difference in response between the two sampled years. The third group is located at the opposite end of the plot, indicating a high negative correlation to S_2. It contains one enzyme (spot 1949) that was identified as a homologue to isoflavone reductase in maize.

DISCUSSION

We analyzed the effect of N and S fertilization on the tris-soluble and gluten protein fractions using 2-DE and mass spectrometry. Furthermore, we analyzed the relationship between protein content, SDS sedimentation volume, and dough properties as an effect of N/S ratio of the grain. The wheat grain samples were selected from a fertilization experiment that produced samples with large variation in N and S concentrations, as well as in breadmaking quality (*7*). Overall, the results showed that fertilization regimens of high N without giving extra S may induce an S deficiency in the grains that highly influence quality. Adding more N when the plant was starved on S increased the total amount of protein, but the rheological properties were highly changed. This is in accordance with earlier findings (*3*−*5*, *7*). However, adding extra S as a split application at stem elongation or heading of the plants alleviated the discrepancy between N and S at sowing.

Table 3. Identified Proteins from the Tris-soluble fFraction

ID	GI	name	exptl pI/M_r	matched peptides[a]/sequence coverage	theor pI/M_r	change
2390	gi 123955	α-amylase inhibitor, CM1 (wheat)	6.2/14K	6/42%	7.49/16K	+
2385	gi 100833	α-amylase inhibitor, CM2 (wheat)	5.9/14K	5 (1)/53%	6.86/16K	+
2362	gi 66841026	α-amylase inhibitor, 0.19 (wheat)	6.1/15K	6 (3)/70%	6.86/13K	+
2355	gi 100834	α-amylase inhibitor, CM3 (durum wheat)	5.8/16K	9/69%	7.44/19K	+
2344	gi 100834	α-amylase inhibitor, CM3 (durum wheat)	5.9/16K	8/57%	7.44/19K	+
2258	gi 1621627	manganese superoxide dismutase (wheat)	5.8/26K	10 (3)/53%	7.90/25K	+
2210	gi 34539782	Cys-peroxiredoxine (wheat)	6.1/27K	5 (1)/22%	6.08/24K	+
2209	gi 30793446	27K protein (wheat)	6.0/27K	6 (1)/27%	6.06/23K	+
1949	gi 1708421	isoflavone reductase homologue (IRL; maize)	5.1/39K	2 (2 + 2)/	5.69/33K	−
2313	gi 123545	heat shock protein (wheat)	5.4/19K	5 (4)/37%	5.83/17K	+
2396	gi 123966	α-amylase inhibitor 0.28 (CIII) (wheat	5.1/14K	7/52%	6.18/14K	+

[a] Numbers in parentheses indicate how many peptides were analyzed by MS-MS sequencing.
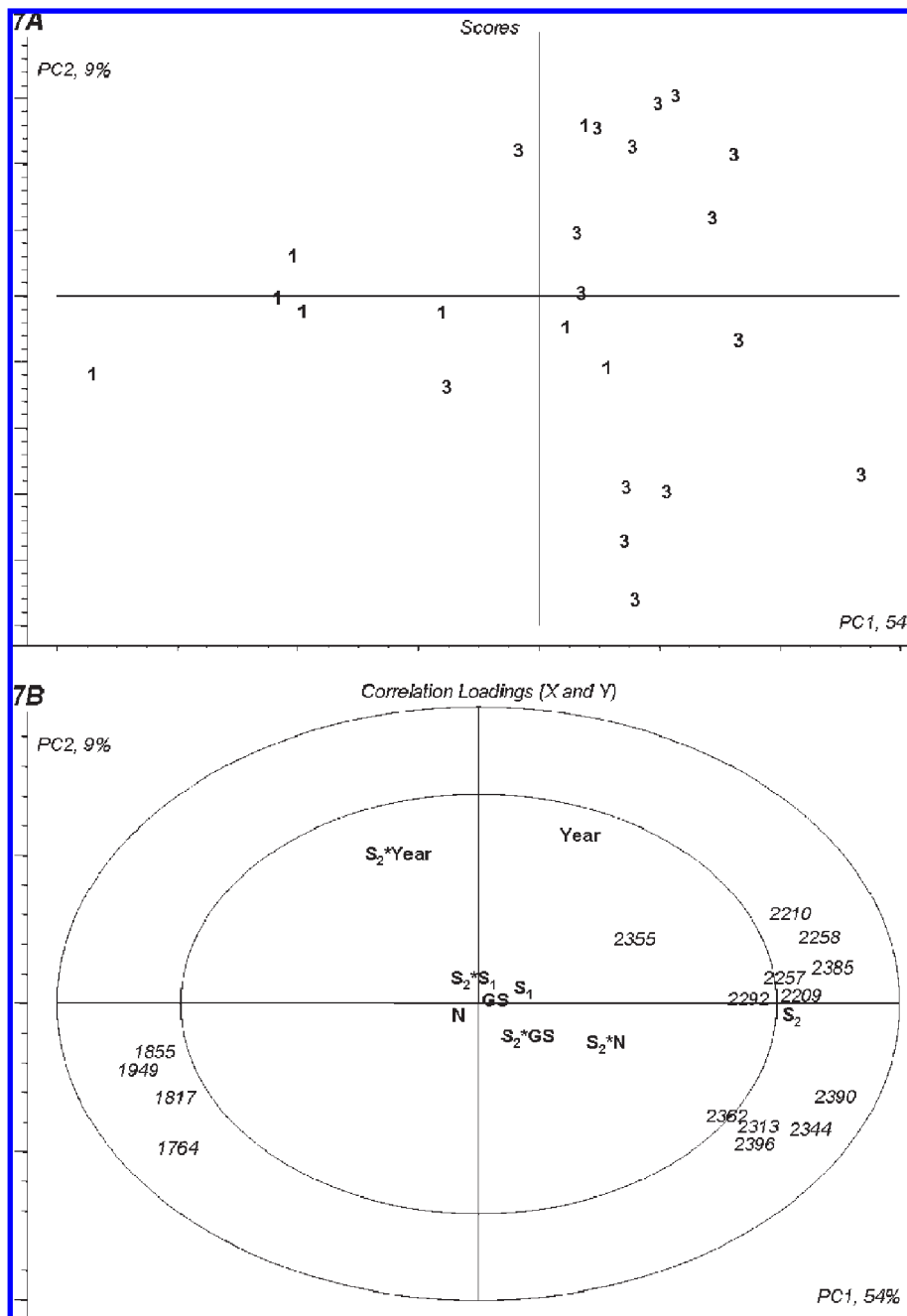

Figure 7. Multivariate analyses by PLSR of those protein spot volumes from the tris-soluble protein fraction found to be significantly correlated to late S application. The score plot (**A**) shows the grouping of samples according to the similarities in the expression profiles in the tris-soluble proteins. The loading plot (**B**) shows the grouping of treatments (in bold) and proteins (in italics) for the same analysis.

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We observed a difference in the effect on the glutenins for samples receiving S during growth ($S_2 = 2$ and 3) compared to samples receiving no S during growth ($S_2 = 1$). However, the lack of any clear difference between samples receiving medium levels of S_2 and samples receiving high levels of S_2 indicates that S stopped being a limiting factor above a certain amount. Six of the identified protein spots were identified as LMW glutenin subunits or γ-gliadins that increased in intensity when S_2 was applied. LMW glutenin subunits and γ-gliadins are among the sulfur-rich prolamins that are expected to increase in proportion with increasing S availability, as reported by Wieser et al. (5). Generally, few protein spots of the glutenin extracts responded to the S_2 treatment. One reason for this can be that many of the abundant protein spots were saturated on the gels and thus were not analyzed. However, the multivariate analysis showed an overall pattern of change in the proteome as response to S_2 when all proteins were viewed simultaneously.

In the analysis of the tris-soluble proteins we compared samples from plants given different amounts of N and S at sowing with samples from plants given late applications of S during growth. The results showed S_2 to be the most influential on the variation in abundance of tris-soluble proteins. The multivariate analysis showed that the proteins being positively correlated to S_2 were split in two groups depending on experiment year, demonstrating a biological variation that may be observed in different growth experiments. Among the tris-soluble proteins that decreased when the plants received a low level of S_2 are six isoforms of α-amylase inhibitor. These inhibitors are believed to be important for the defense of the seed against insects and microbial pests that are producing exogenous α-amylases to digest starch. These inhibitors are primarily located in the endosperm, and they show a rapid increase during maturation (21). Because this gene family has also been correlated to baker's asthma (10), further study of these proteins could be of interest. In addition, a superoxide dismutase protein decreased when the plants received low levels of S_2. This protein is part of an enzyme family that has been shown to be involved in cold and drought stress responses (22). These results indicate that a reduction in the available S may affect the defense mechanisms of the plants due to lower amounts of amylase inhibitors and other stress proteins. Isoflavone reductase increased when the plants received no S_2. This protein has been linked to S availability and is reported to be induced by S starvation in maize (23).

Among the tris-soluble enzymes we found a general decrease in defense proteins when sulfur availability was low. There were also identified changes in the composition of the glutenin fraction.

ABBREVIATIONS USED

LMW-GS, low molecular weight glutenin subunits; HMW-GS, high molecular weight glutenin subunits; PCA, principal component analysis; PLSR, partial least-squares regression; CMV, cross-model validation.

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LITERATURE CITED

(1) Dupont, F. M.; Altenbach, S. B. Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis. *J. Cereal Sci.* **2003**, *38* (2), 133–146.

(2) Shewry, P. R.; Tatham, A. S.; Forde, J.; Kreis, M.; Miflin, B. J. The classification and nomenclature of wheat gluten proteins—a reassessment. *J. Cereal Sci.* **1986**, *4* (2), 97–106.

(3) Wrigley, C. W.; Ducros, D. L.; Fullington, J. G.; Kasarda, D. D. Changes in polypeptide composition and grain quality due to sulfur deficiency in wheat. *J. Cereal Sci.* **1984**, *2* (1), 15–24.

(4) Zhao, F. J.; Hawkesford, M. J.; McGrath, S. P. Sulphur assimilation and effects on yield and quality of wheat. *J. Cereal Sci.* **1999**, *30* (1), 1–17.

(5) Wieser, H.; Gutser, R.; von Tucher, S. Influence of sulphur fertilisation on quantities and proportions of gluten protein types in wheat flour. *J. Cereal Sci.* **2004**, *40* (3), 239–244.

(6) Hocking, P. J. Dry-matter production, mineral nutrient concentrations, and nutrient distribution and redistribution in irrigated spring wheat. *J. Plant Nutr.* **1994**, *17* (8), 1289–1308.

(7) Flæte, N. E. S.; Hollung, K.; Ruud, L.; Sogn, T.; Faergestad, E. M.; Skarpeid, H. J.; Magnus, E. M.; Uhlen, A. K. Combined nitrogen and sulphur fertilisation and its effect on wheat quality and protein composition measured by SE-FPLC and proteomics. *J. Cereal Sci.* **2005**, *41* (3), 357–369.

(8) Skylas, D.; Van Dyk, D.; Wrigley, C. W. Proteomics of wheat grain. *J. Cereal Sci.* **2005**, *41* (2), 165–179.

(9) Sotkovsky, P.; Hubalek, M.; Hernychova, L.; Novak, P.; Havranova, M.; Setinova, I.; Kitanovicova, A.; Fuchs, M.; Stulik, J.; Tuckova, L. Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. *Proteomics* **2008**, *8* (8), 1677–1691.

(10) Sanchezmonge, R.; Gomez, L.; Barber, D.; Lopezotin, C.; Armentia, A.; Salcedo, G. Wheat and barley allergens associated with bakers asthma—glycosylated subunits of the α-amylase-inhibitor family have enhanced Ige-binding capacity. *Biochem. J.* **1992**, *281*, 401–405.

(11) Zadok, J. C.; Chang, T. T.; Konzak, F. C. A decimal code for growth stages of cereals. *Weed Res.* **1974**, *14*, 415–421.

(12) American Association of Cereal Chemists. *Approved Methods of the AACC*, 9th ed.; AACC: St. Paul, MN, 1995; pp 56−70.

(13) Kieffer, R.; Wieser, H.; Henderson, M. H.; Graveland, A. Correlations of the breadmaking performance of wheat flour with rheological measurements on a micro-scale. *J. Cereal Sci.* **1998**, *27* (1), 53–60.

(14) Tatham, A. S.; Gilbert, S. M.; Fido, R. J.; Shewry, P. R. Extraction, separation and purification of wheat gluten proteins and related proteins in barley, rye and oats. In *Celiac Disease—Methods and Protocols*; March, M. N., Ed.; Humana Press: Totowa, NJ, 2001; pp 55−74.

(15) Blum, H.; Beier, H.; Gross, H. J. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **1987**, *8*, 93–99.

(16) Grove, H.; Hollung, K.; Uhlen, A. K.; Martens, H.; Faergestad, E. M. Challenges related to analysis of protein spot volumes from two-dimensional gel electrophoresis as revealed by replicate gels. *J. Proteome Res.* **2006**, *5*, 3399–3410.

(17) Troyanskaya, O.; Cantor, M.; Sherlock, G.; Brown, P.; Hastie, T.; Tibshirani, R.; Botstein, D.; Altman, R. B. Missing value estimation methods for DNA microarrays. *Bioinformatics* **2001**, *17* (6), 520–525.

(18) Anderssen, E.; Dyrstad, K.; Westad, F.; Martens, H. Reducing over-optimism in variable selection by cross-model validation. *Chemom. Intell. Lab. Syst.* **2006**, *84* (1−2), 69–74.

(19) Rappsilber, J.; Ishihama, Y.; Mann, M. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. *Anal. Chem.* **2003**, *75*, 663–670.

(20) Randall, P. J.; Spencer, K.; Freney, J. R. Sulfur and nitrogen-fertilizer effects on wheat. 1. Concentrations of sulfur and nitrogen and the nitrogen to sulfur ratio in grain, in

relation to the yield response. *Aust. J. Agric. Res*. **1981**, *32* (2), 203–212.

(21) Pace, W.; Parlamenti, R.; Rab, A. U.; Silano, V.; Vittozzi, L. Protein α-amylase inhibitors from wheat-flour. *Cereal Chem*. **1978**, *55* (2), 244–254.

(22) Pearce, R. S. Molecular analysis of acclimation to cold. *Plant Growth Regul*. **1999**, *29* (1−2), 47–76.

(23) Petrucco, S.; Bolchi, A.; Foroni, C.; Percudani, R.; Rossi, G. L.; Ottonello, S. A maize gene encoding an NADPH binding enzyme highly homologous to isoflavone reductases is activated in response to sulfur starvation. *Plant Cell* **1996**, *8* (1), 69–80.