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Protein composition and techno-functional properties of transgenic wheat with reduced α-gliadin content obtained by RNA interference

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Summary

An RNA interference approach was applied to silence α-gliadin genes in hexaploid wheat. A 313 bp fragment from the 5' coding region of an α-gliadin sequence was cloned into the "hairpin" (hpRNAi) constructs pGliaRNAiSpacer and transformed into winter wheat *cv. Florida* by biolistic transformation. The resulting transgenic lines were then analysed for their α-gliadin content by an extraction/RP-HPLC method. Distinct differences of the α-gliadin content ranging from unaltered to strongly reduced were found. Kernels of two lines with strongly reduced α-gliadin content were mixed, milled to white flour, analysed for quantitative protein composition and used for functional testing in comparison with the wild type flour. Protein analysis revealed that the lack of α-gliadins was compensated by an increase of albumins/globulins, ω-gliadins, γ-gliadins and HMW glutenin subunits. Dough resistance and extensibility of wild type and transgenic lines were similar, whereas gluten resistance of the transgenic increased drastically and extensibility decreased slightly. 2D gel electrophoresis showed that the transgenic wheat #6/6 lacked at least 20 storage proteins, but other distinct proteins were more abundant compared with the wild type. Major reductions in the α-gliadins do not make discernable differences in micro-scale measurements of flour functionality and have only a slightly detrimental effect in micro-baking tests.

Introduction

The unique dough properties and the baking quality of wheat are mainly determined by the structure and quantity of the storage (gluten) proteins. They are present in the endosperm as complex polymorphous mixture; two-dimensional gel electrophoresis distinguishes several hundred different components. The gluten proteins can be divided into two main fractions according to their solubility in aqueous alcohols: the soluble gliadins (GLIA) and the insoluble glutenins (GLUT) (Osborne, 1907). GLIA are mainly monomeric proteins and can be classified according to their amino acid sequences into α-, γ-, and ω- type (Wieser, 2007), disulphide bonds are either absent or present in intrachain cross links. GLUT comprise polymeric proteins linked by interchain disulphide bonds and consist of high-molecular-weight glutenin subunits (HMW-GS) and low-molecular-weight glutenin subunits (LMW-GS) (Payne et al., 1981; Shewry et al., 1986; Shewry and Tatham, 1990). Some gliadins are found in the glutenin fraction because they contain an odd number of cysteine residues and can thus form inter-chain disulphide bonds to glutenin subunits.

α-Gliadins are the major subgroup of gluten proteins and can be divided into five homologous classes on the basis of small differences in amino acid sequences. These differences in sequence are basically due to base substitutions, duplications or deletions within, or very close to direct repeats (Okita et al., 1985; Payne et al., 1982; Payne et al., 1984;).

Up to date it is not trivial to successfully transform hexaploid wheat by using RNAi. Formerly, it was rarely possible to eliminate specific cereal seed storage proteins using antisense or RNAi approaches (Fire et al., 1998), and to analyse changes in the amount of different seed storage proteins and as a consequence also changes in amino acid composition (Gil-Humanes et al., 2008; Huang et al., 2004; Lange et al., 2007; Maruta et al., 2001; Segal et al., 2003; Wu and Messing, 2010; Yue et al., 2008). In the mean time, three scientific approaches were published showing successful down regulation of single- (γ-gliadins reduced; Gil-Humanes et al., 2008; Piston et al., 2011) to multiple gliadin multigene families (α-, γ- and ω-gliadins reduced; Gil-Humanes et al., 2010) by RNAi. The aim of the present study was to decrease the expression of α-gliadins only, which represents the major protein subgroup in wheat, via RNAi and to analyse the resulting lines for protein composition and technological properties.

Materials and methods

Choice of the α-gliadin target nucleotide sequence and cloning into the vector pGliaRNAiSpacer

The basis for a successful RNAi approach to eliminate all α-gliadins in wheat was the identification of a nucleotide sequence within all known α-gliadin sequences, which share nearly 100% sequence homology to each other. Therefore, an alignment of 29 α-gliadin mRNA- or genomic sequences deposited in the GenBank Data Base (NCBI) was set up (GenBank Acc. No. AJ133602, AJ133603, AJ133604, AJ133605, AJ133606, AJ133607, AJ133608, AJ133609, AJ133610, AJ133611, AJ133612, K02068, K03074, K03075, M10092, M11073, M11074, M11075, M11076, U08287, U50984, U51303, U51304, U51306, U51307, X00627, X01130, X02540, X17361). The sequence homology is nearly identical for the different genes, especially in the first 248 bases (from ATG), the sequence homology is in the range of 90-100% identical. In addition there are single base substitutions, gaps (position 249-290) and insertions. For the construction of the RNAi transformation vectors we selected a region of 313 bp, starting 5 nucleotides after the translational starting point. To amplify a sequence from a wheat caryopsis cDNA library the Gli-forward primer (Glia for) 5'-CCTTTCTCATCCTTGCCCTCCT-3' and the Gli reverse primer (Glia rev) 5'-TACTGTGGTTGCGGTTGTGGA-3' were used. The amplified sequence was: 5`CCTTTCTCATCCTTGCCC TCCTTGCTATCGTGGCGACCACCGCCACAACTGCAGTT AGAGTTCCAGTGCCACAATTGCAGCCACAAAATCCATC TCAGCAACAGCCACAAGAGCAAGTTCCATTGGTACAAC ACCAACAATTTCTAGGGCAGCAACAACCATTTCCACCA CAACAACCATATCCACAGCCGCAACCATTTCCATCACA ACAACCATATCTGCAACTGCAACCATTTCCGCAGCCGC AACTACCATATTCACAGCCACAACCATTTCGACCACAA CAACCATATCCACAACCGCAACCACAGTA 3'.

The PCR amplification was then performed in a 100 µL reaction volume using the following substances: 1 x PCR-buffer (Invitrogen, Karlsruhe, Germany), 2 U Taq DNA-polymerase (Invitrogen, Karlsruhe, Germany), 0.2 mmol/L each dNTPs, 1.5 mmol/L $MgCl_2$,

1 µmol/L each Glia for and Glia rev primers, 1.1 x 10^9 pfu wheat caryopsis cDNA library *T. aestivum* cv. 'Florida' (provided by M. Block, University Hamburg, unpublished). The PCR cycle were 95°C, 4 min; [95°C 1 min; 60°C 30 sec; 72°C 1 min] x 28 cycles; 72°C 10 min. The amplified PCR fragment had 100% sequence identity to the α-gliadin gene with the accession number EU018293.
For the stable transformation of wheat the fragment was cloned into the hpRNAi constructs pGliaRNAiSpacer, which was designed to produce single stranded RNA in sense and antisense orientation (Fig. 1a). The cloning vector contained a spacer sequence of about 1002 bp, which derived from the β-*glucuronidase* gene (*uidA* or *gus*). The ubiquitin1 promoter from maize drove the expression of the construct (CHRISTENSEN et al., 1992). The cloning of the transformation vectors was kindly provided by DNA-Cloning Service (Hamburg, Germany).

Plant transformation
For stable transformation experiments we used the hexaploid wheat *cv*. "Florida". This wheat variety is a wheat/ rye hybrid carrying the 1BL/1RS chromosome translocation, which is correlated with poor breadmaking quality. The green house growth conditions and the stable transformation procedure of wheat was described by BECKER et al., (1994) and BECKER and LÖRZ, (1996), with the following modifications. The explants were cultured on a modified MS-medium and contained macro and micronutrients, FeEDTA and 30 g/L sucrose. For bombardment, plasmid pGliaRNAiSpacer was used in combination with the plasmid pCaIneo (MÜLLER et al., 1996), which carried the selectable marker gene *nptII* with resistance against kanamycin under the control of the CaMV 35S promoter and the *AdhI* intron from maize. For the selection of transgenic calli and plants, 150 mg/L kanamycin was added to the culture medium. The immature embryos were directly bombarded after isolation with the Bio-Rad PDS 1000-He particle delivery system (BioRad, Munich, Germany).

Molecular analyses
For Southern blot analyses, 25 µg of genomic DNA was cleaved with restriction enzymes in a total volume of 40 µL, separated on a 0.8% agarose gel and blotted on a Hybond NX nylon membrane (GE Healtcare, Munich, Germany). Hybridisation was done with DIG-labeled DNA probes (20-25 ng/mL hybridisation solution) at 42°C using DIG Easy Hyb solution (Roche, Mannheim, Germany). Chemiluminescence detection was done with CSPD substrate according to the manufacturer's prescriptions (Roche, Mannheim, Germany). The primer pair used for amplification of the *uidA* specific DNA-probe in a DIG-labeling reaction was GUS-Sp For (5'-CCGAAAGAACTGTACAGCGAAGA-3') and GUS-Sp Rev (5'-CGGTGATGATAATCGGCTGATGC-3').

Detection of RNAi transcripts in transgenic plants
The expression of the RNAi construct pGliaRNAiSpacer was studied by reverse transcription PCR. Total RNA was prepared from 14 days old seedling leaves with peqGOLD TriFast™ Trizol reagent (peqLab, Erlangen, Germany), and the first strand cDNA synthesis was performed according to the manufacturers instruction (RevertAid H Minus First Strand cDNA Synthesis Kit, Fermentas, St. Leon-Rot, Germany). The first strand DNA synthesis was started with 0.7 µg of total RNA and the use of 1U desoxyribonuclease I incubated for 1 h at 37°C. The first strand synthesis was then started by adding the GUS-Spacer reverse primer (GUS-Sp Rev) 5'-CGGTGATGATAATCGGCTGATGC-3'.
The PCR amplification mixture contained: 0.65 µg RNA from the first strand synthesis, 1 x PCR-buffer (Invitrogen, Karlsruhe, Germany), 2 U Taq DNA-polymerase (Invitrogen, Karlsruhe, Germany), 0.2 mM dNTPs each, 1.5 mmol/L $MgCl_2$, 1 µmol/L each GUS-Sp For and GUS-Sp Rev. The PCR amplification was performed in a 100 µL reaction volume. The PCR cycles were 95°C, 4 min; [95°C 1 min; 60°C 1 min; 72°C 1 min] x 28 cycles; 72°C 10 min. The size of the resulting amplification product was 396 bp.

Flour preparation and Osborne fractionation of proteins
Single kernels of the transgenic lines (T_0 and T_1 generations) were cut into halves by means of a razor blade and the halves without embryo were crushed in a mortar. Kernels of the transgenic lines #30/5 (39 g) and #11/10 (21 g) were mixed, milled into white flour (0.55 % ash content) by means of a laboratory mill (Quadrumat Junior Brabender, Duisburg, Germany) and sieved through a 0.2 mm mesh (Retsch, Haan, Darmstadt, Germany). White flour was produced in the same manner from a kernel mixture of the *nptII* gene expressing T_0 transgenic line #10 (42 g) and a sample of wild type cultivar Florida (52 g, Saatzucht Schweiger, Moosburg, Germany).
Protein fractionation was performed according to a modified Osborne procedure described by WIESER et al., (1998). Two separate extraction experiments were carried out for each flour sample.

RP-HPLC
Aliquots (0.5 mL) of the extracts were filtered through a 0.45 µm membrane and used for HPLC analysis under the following conditions (WIESER et al., 1998): instrument, solvent module 126 with a System Gold software (Beckman, Munich, Germany); column, Nucleosil 300 - 5 C_8, 4.6 x 240 mm (Macherey-Nagel, Dueren, Germany); temperature, 50°C; injection, 150 µL (ALGL), 50 µL (GLIA), or 200 µL (GLUT). In addition, 500 µL of 0.1% (v/v) trifluoroacetic acid (TFA) was injected into the sample loop (2 mL) before and after sample solution; elution system, (A) TFA (0.1%, v/v), (B) acetonitrile (99.9%, v/v), 0.1% (v/v) TFA; linear gradients, 0 min 20% B, 20 min 60% B (ALGL), 0 min 28% B, 30 min 56% B (GLIA), 0 min 28%, 90 min 56% (GLUT); flow rate, 1.0 mL/min; detection, UV absorbance at 210 nm.
For preparative purposes, the HPLC eluates of between 19 and 24 min (elution region of α-gliadins, Fig. 2A) of GLIA from line #6/15 were collected and freeze-dried.

Further protein analyses
The crude protein content (N x 5.7) of white flours was determined by the Dumas method (ICC method no. 167) using an FP-328 combustion instrument (Leco, Kirchheim, Germany). The analysis of N-terminal amino acid sequences of freeze-dried α-gliadin eluates was performed by automated Edman degradation using a protein sequencing system (Procise 492, Applied Biosystems, Weiterstadt, Germany).

Rheological tests
Rheological dough and gluten properties were characterised by methods on a micro-scale developed previously (KIEFFER et al., 1998). Due to the limited amount of flour the methods were down-scaled from 10 to 7 g flour. In preliminary experiments, tests with 10 g or 7 g of flour were compared using a flour mix of *nptII* transgenic line #10 and wild type flour. In the main experiments 7 g of flour was mixed with 0.14 g of NaCl in a micro-farinograph (Brabender, Duisburg, Germany), and distilled water was added to give a dough consistency of 550 Brabender units (BU) in the maximum of the curve. The dough was kneaded for 20 min at 20°C

and 60 rounds per minute and then divided into five strands. After a resting time of 40 min at 20°C and 90% humidity, maximum resistance to extension (RE) and extensibility (EX) of single strands were determined using a Texture Analyzer TA-XT2 (Stable Micro Systems, Godalming, Surrey, United Kingdom) equipped with the SMS/Kieffer Dough and Gluten Extensibility Rig (Kieffer et al., 1998). Afterwards, strands were combined and washed in a Glutomatic (Perten Instrument, Huddinge, Sweden) with distilled water for 10 min at 22°C and centrifuged (4000 x *g*, 10 min, 22°C). The resulting wet gluten was pressed into a polytetraflouroethylene mould (two strands) and, after a relaxation time of 30 min at 22°C, RE and EX were determined.

Protein extraction for 2-dimensional gel analysis

Wheat grain proteins for isoelectric focusing were prepared using a dithiothreitol-trichloroacetic acid- (DTT-TCA) acetone precipitation method adopted from Zörb et al., (2009).

Isoelectric focusing (IEF) and two-dimensional gel electrophoresis (2D GE)

Two-dimensional gel electrophoresis (2D GE) with some modifications was performed using protocols from Görg and Weiss, (1998). Commercially purchased IPG strips (18 cm, pH 3-10, linear, GE-Healthcare, Munich, Germany) were used. Trays were loaded with 300 µg protein diluted in 360 µL protein sample buffer. The strips were subsequently incubated for 15 h. Filter papers (Whatman) soaked with 3% (w/v) DTT were placed at the basic site of the strip to prevent renaturation of basic proteins. Finally, strips were covered with paraffin oil to prevent evaporation. IEF was carried out in a Multiphor apparatus (GE-Healthcare, Munich, Germany) according to the following conditions: 10 h rehydration; 2 h 100 V; 1 h 500 V; 2 h 1000 V; and 2 h 8000 V. System temperature was 20°C and current was set to 45 µA per strip.

To run the second dimension, strips were placed in equilibration buffer (50 mmol/L Tris-HCl, pH 8.8; 6 mmol/L urea; 30% (v/v) glycerol, 2% (w/v) SDS; 0.001% (w/v) bromophenol blue containing 1% (w/v) DTT) and gently agitated for 10 min. Strips were incubated again for 10 min in equilibration buffer containing 4% (w/v) iodoacetamide instead of DTT under gentle agitation and rinsed three times with SDS-PAGE running buffer (25 mmol/L Tris;
192 mmol/L glycine; 0.1% (w/v) SDS). SDS-PAGE was performed by using 12% (w/v) acrylamide 20 cm x 20 cm gels. Molecular weight standards from 10,000 to 150,000 were used (Sigma, Taufkirchen, Germany). 2D gels were fixed with 50% (v/v) ethanol and 12% (v/v) acetic acid. Subsequent staining was done with Coomassie R 250.

Data analyses

All gels were scanned with an image scanner (HP Scan-Jet 4890, USA; 300 dpi and 16 bits per pixel). A computer-assisted 2D GE analysis was performed using Delta 2D 3.4 software (Decodon GmbH, Greifswald, Germany). Statistical calculations were based on three biological replications and two technical replicates within each biological replication (average 2D gels) of wild type and the α-gliadin-reduced transgenic lines, respectively. All 2D GE images were analysed by applying statistics at five levels: In order to analyse the accuracy of the analyses, (i) three technical 2-D gel-replications from each treatment were used to create an average 2-DE gel. The spot volumes were normalized to the total protein on each gel. The standard error of the mean of each spot volume was calculated (ii) using the three average mean value 2D GE from each treatment replicates. A Students t-test was performed (iii) to evaluate the reproducibility of the spots over the 2D GE gel replicates. Spots were disregarded, when the confidence value was above p = 0.05. A spot filter was applied using an intensity limit of 0.02 (*iv*). By this method, weak spots as well as any artificial speckles were eliminated in all 2-DE gels. For the detection of protein spots that proved to be different between wild type and transgenic line, a final filter (*v*) was introduced; resulting proteins differed at least by 50% between wild type and RNAi line (fold change). Individual protein spots from 2D gels were identified using MALDI-TOF MS as described by Zörb et al., (2009).

Description

Cloning, wheat transformation and molecular analysis

A 313 bp PCR amplicon from the 5' coding region of a α-gliadin gene was cloned in sense and antisense orientation into the vector pGliaRNAiSpacer (Fig. 1a). The hexaploid bread wheat *Triticum aestivum* cv. Florida was transformed in co-transformation experiments with the two vectors pGiaRNAiSpacer and pCaIneo. From biolistic transformation experiments, it was possible to regenerate more than 111 independent transgenic wheat lines. The integration of the pGliaRNAiSpacer vector was confirmed by Southern blot analysis using a DIG-labeled *uidA(gus)* gene probe. The expected hybridisation signal of 3.6 kb for the whole *SfiI* fragment (cuts twice in the construct; Fig. 1a) was detectable in lines T_0-plants #30, #42, #44 and #55, and was absent in the line #53. The number of integrated gene copies, determined in *BamH*I (cuts once in the construct, Fig. 1a) digested DNA was between one and up to 20 (Fig. 1b). To determine the co-transformation frequency, the banding pattern of *BamH*I restricted plant DNA was compared with each other. Only plants with different banding pattern were counted as independent transgenic lines. The estimated co-transformation frequency was calculated by number of T_0 plants survived kanamycin spraying divided by number of plants containing a full length the pGliaRNAiSpacer construct (3.6 kb) and was 64%.

Expression and inheritance of pGliaRNAiSpacer constructs in transgenic wheat

The functionality of the constructs was checked by RT-PCR analysis in all plants. In order to check the expression of the pGliaRNAiSpacer construct in the transgenics, leaf RNA was isolated, treated with DNAse and first strand cDNA was synthesised. Figure 1c shows an example for 9 primary transformants. In 7 out of 9 T_0 transgenic lines (#2, #4, #48, #51, #53, #55, #59) we were able to detect the expected amplification product of 396 bp (Fig. 1c). The specificity of the 396 bp fragment was demonstrated by Southern blot using a DIG-labeled *uidA* probe (Fig. 1c). Strong hybridisation signals are detectable in plants #2, #4, #48, #51, #53, #55 und #59. Results from Southern blot analysis confirmed that the additional band, with a size of about 600 bp is an unspecific amplification product.

All primary transformants were self-fertile and the seed set and seed morphology was comparable to wild type plants. The stable integration and expression of the RNAi construct, and the transmission of the phenotype were successful determined in the following generation in self-pollinated plants (data not shown).

Protein composition

The RP-HPLC profiles of ALGL from the wild type and transgenic lines were almost identical (not shown). In contrast, the RP-HPLC profiles of GLIA were, in parts, strongly different; the chromatograms of four examples are shown in Fig. 2a. In all cases the profiles of ω-gliadins (elution time 16 - 19 min) and γ-gliadins (24 - 32 min) were unchanged, whereas those of α-gliadins (19 - 24

Fig. 1a

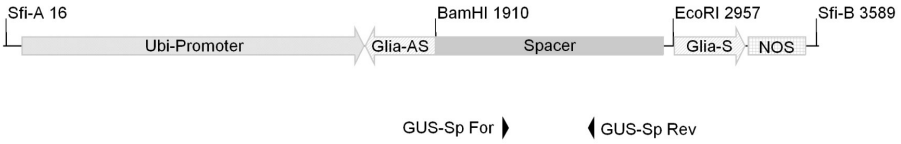


Fig. 1b

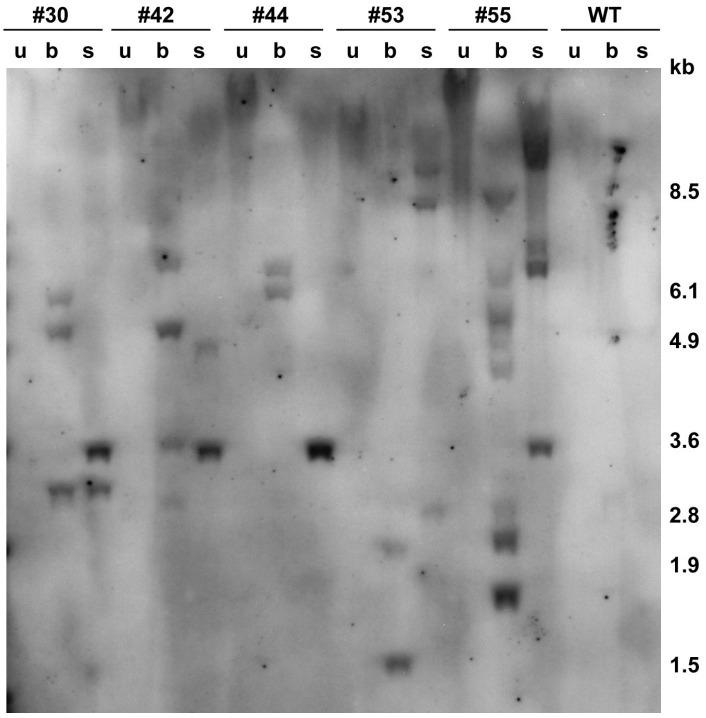


Fig. 1c

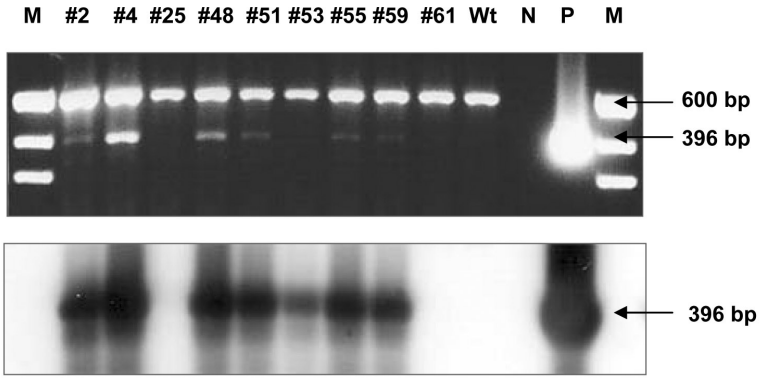


Fig. 1: Transformation construct and molecular analysis of transgenic plants

a Schematic representation of pGliaRNAiSpacer vector.

(nos: *Nopaline synthase* gene terminator; Glia-AS: 313 bp Gliadin anti-sense, Glia-S: 313 bp Gliadin sense; Spacer: 1002 bp *uidA*-gene spacer sequence (position 811 to1812 from ATG), Ubi-promoter: maize Ubiquitin promoter, GUS-Sp For and GUS-Sp Rev: forward and reverse GUS-Spacer primer binding sites; used for PCR amplification and Dig probe labeling. The vector backbone is pBlueSfi-BA.

b Southern blot analysis from T_0 plants transformed with GliaRNAiSpacer construct.

25 µg genomic DNA from the T_0 transgenic wheat plants #30, #42, #44, #53, #55 and a wild type plant were digested with *BamH*I to determine the number of integrated gene copies, with *Sfi*I to cut out the whole transgenic sequence (3.6 kb) and also uncut to show the genomic integration. After transfer onto a Hybond NX membrane, hybridisation was carried out using Dig-labeled *uidA* gene fragment. WT: wild type; u: uncut: b: *BamH*I-digested; s: *Sfi*I-digested.

c Detection of GliaRNAiSpacer transcripts with RT-PCR.

top: For reverse transcription, 0.65 µg total-RNA from T_0 plants #2, #4, #25, #48, #51, #53, #55, #59, #61, a wild type "Florida" sample (Wt) and a negative control without RNA (N) and the GUS-Spacer-Rev primer were used. cDNA were used as template for PCR-amplification using the GUS For- and GUS Rev primers. The expected size of the amplified fragment is 396 bp. M: Marker, N: negative Control without RNA; P: plasmid DNA pGliaRNAiSpacer.

bottom: Southern hybridisation of the same gel as shown in Fig. 1c using a Dig-labeled *uidA* probe (see Material and methods).

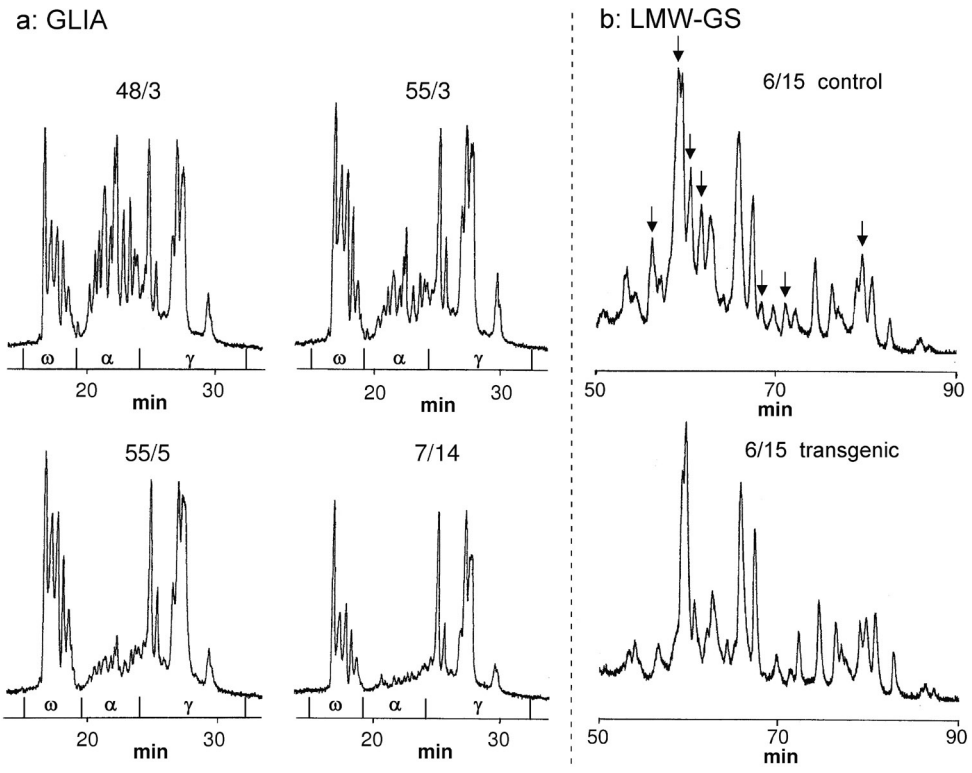


Fig. 2: RP-HPLC profiles of the GLIA fraction (a) and LMW-GS (b) from transgenic and wild type lines.
a RP-HPLC of the GLIA fraction from transgenic T_1 lines #48/3 (group 1), #55/3 (group 2), #55/5 (group 3) and #7/14 (group 4).
b RP-HPLC profiles of the LMW-GS in wild type and transgenic T_1 line #6/15. Changes are marked with arrows.

min) varied remarkably. Some lines such as #48/3 (T1-progeny from line#48)did not show any difference to the wild type and other lines had moderately (e.g. #55/3, T1-progeny from #55) or even strongly reduced (e.g. #55/5, T1-progeny from #55) α-gliadin peaks (Fig. 2a). Numerous lines such as T1-line #7/14 revealed chromatograms with only traces of peaks in the α-gliadin region.

The relative amounts of total GLIA and α-gliadins were determined via the absorbance units (AU) at 210 nm derived from the area of peaks, which has been shown to be highly correlated with protein quantity (WIESER et al., 1998). According to the results, the transgenic lines could be divided into four groups. The first group including 7 lines corresponded to the wild type with α-gliadin proportions of 30 - 36% based on total GLIA content. The second group (17 lines) was characterised by moderately reduced α-gliadin proportions of 24 - 30% and the third group (43 lines) by strongly reduced proportions of 18 - 24%. Twenty-four lines represented the fourth group with the lowest α-gliadin proportions of 12 - 18%. It should be mentioned that oligomeric proteins, the so-called ethanol-soluble glutenins (HUEBNER and BIETZ, 1993) are co-eluted with α-gliadins. This ethanol-soluble oligomeric fraction is composed by α- and γ-gliadins and LMW-GS bound by interchain disulfide bonds, and amounts to ca. 20% of total gliadins (KIEFFER et al., 1998).

To clarify whether α-gliadins were present in group 4 lines, the HPLC eluates in the region of α-gliadins of the wild type and #6/15 (12.3% absorbance area of total gliadins) were collected by means of several HPLC runs, freeze-dried and subjected to N-terminal amino acid sequence analysis to determine the first six positions of amino acids. As expected, the N-terminal sequence of α-gliadins (VRVPVP[a]) of the wild type was dominating accompanied by sequences of γ-gliadins (NIQVDP); sequences of LMW-GS could not be detected. In the case of transgenic line #6/15 (T1-progeny from line#6), the γ-gliadin sequence was clearly in evidence, whereas sequences of α-gliadins were not found. In contrast to the wild type sequences of LMW-GS (METSHI) were detectable because due to the lack of α-gliadins much less material was present in this fraction so that minor signals from LMW-GS were detectable during sequencing. In conclusion, the elution region of α-gliadins with low proportions related to total GLIA (e.g. 12.3%) did not contain any detectable α-gliadins and contained only oligomeric proteins consisting of γ-gliadins and LMW-GS. This showed that the applied RNAi technique allows eliminating α-gliadins completely.

According to GLUT, the HPLC profile of HMW-GS was unchanged, when wild type and transgenic lines from group 1 were compared (not shown). In contrast the profiles of LMW-GS (Fig. 2b) were different due to missing or reduced peaks in the chromatograms of transgenic lines of groups 2 - 4 (e.g. #6/15). This alteration of the chromatographic profile in the LMW-GS range could on the one hand be caused by off-target effects of the RNAi approach. On the other hand LEW et al., (1992) reported that GLUT usually contains α-gliadins bound by interchain disulfide bonds. Flour, which is deficient from α-gliadins could, therefore, lack α-gliadins in the glutenin fraction. However, this would need additional analysis for confirmation. Tab. 1 gives an overview about the α-gliadin reduction, transgene copy number and total protein content of all plants used for protein analysis. There is not a clear correlation between transgene copy number and degree of α-gliadin knock down.

Flours of the wild type (FLOw) and the mixed transgenic T_1 lines #30/5 and #11/10 (FLOt) were compared by a detailed determination of protein quantities (Tab. 2). FLOw was a mixture of non-transgenic flour (cv. Florida) and transgenic flour from line #10, which contained and expressed only the kanamycin resistant gene. The crude protein contents (N x 5.7) were 9.96 (± 0.05) and 9.97 (± 0.27) %, respectively. This analogy was favorable for the comparison of protein fractions and types and, in particular, of baking quality. For

[a] One-letter-code for amino acids

Tab. 1: Alpha gliadin content, transgene copy number and total protein content in transgenic and wild type wheat plants used for protein analysis.

Line	Construct	Transgene copy number	RP-HPLC α-gliadins (%)	Total protein content (%)
Wild type			**39-43**	**12.4**
#6/6	**pGliaRNAiSpacer**	**4**	**16.4**	**10.9**
#6/15	**pGliaRNAiSpacer**	**4**	**15.7**	**11.7**
#7/14	**pGliaRNAiSpacer**	**11**	**17.8**	**10.5**
#11/10	**pGliaRNAiSpacer**	**4**	**18.3**	**12.4**
#30/5	**pGliaRNAiSpacer**	**3**	**16.5**	**11.5**
#48/3	**pGliaRNAiSpacer**	**8-10**	**39.6**	**12.3**
#55/3	**pGliaRNAiSpacer**	**9**	**25.7**	**12.1**
#55/5	**pGliaRNAiSpacer**	**9**	**18.2**	**11.8**

the characterisation of the quantitative protein compositions of the so-called Osborne fractions (ALGL, GLIA, GLUT) were analysed by means of a combined extraction/HPLC procedure (Wieser et al., 1998). Additional to the relative amounts (AU/mg flour) of Osborne fractions, those of single GLIA types (ω-, α-, γ-gliadins) and GLUT types (glutenin-bound ωb-gliadins, HMW- and LMW-GS) were determined (Tab. 2). In consistency with the crude protein content, the sum of all extracted proteins of FLOw and FLOt was nearly similar (1436 *vs*. 1407 AU/mg). In contrast, the amounts of Osborne fractions differed considerably: GLIA of FLOt decreased significantly (-16%), whereas ALGL (+34%) increased strongly and GLUT (+6%) increased moderately as compared to FLOw. The amount of total gluten proteins was lowered from 1193 to 1082 AU (-9%). The proportion of GLIA according to total extractable proteins was reduced from 57.0% to 48.7% and, accordingly those of ALGL (16.9 → 23.1%) and GLUT (26.1 → 28.2%) increased. The ratio of GLIA to GLUT, which is especially important for rheological dough and gluten properties (Wieser and Kieffer, 2001; Wrigley et al.,

Tab. 2: Gluten protein types, rheological dough and gluten properties in wild type (FLOw) and transgenic wheat flours from a mixture of T_1-lines #30/5 and #11/10 (FLOt)[a]. **a:** Relative amounts (AU/mg) and proportions (% according to total extractable proteins) of Osborne fractions and gluten protein; **b:** Rheological dough and gluten properties and bread volume of wild type (FLOw) and transgenic (FLOt) flours from T_1-lines #30/5 and #11/10[a]

a) Protein	FLOw		FLOt		Δ %[a]
	AU[a]	%	AU[a]	%	
ALGL[a]	243 ± 1	16.9	325 ± 4	23.1	+34***
GLIA[a]	818 ± 18	57.0	686 ± 1	48.7	-16***
ω	133 ± 1	9.3	162 ± 1	11.5	+23***
α	375 ± 11	26.1	151 ± 1	10.7	-63***
γ	310 ± 8	21.6	373 ± 1	26.5	+20***
GLUT[a]	375 ± 6	26.1	396 ± 1	28.2	+6***
ωb	12 ± 3	0.8	13 ± 0	0.9	+8[ns]
HMW	130 ± 1	9.1	151 ± 0	10.8	+16***
LMW	233 ± 1	16.2	232 ± 1	16.5	±0[ns]
GLUTEN[a]	1193 ± 13	83.1	1082 ± 3	76.9	-9***
Σ[a]	1436 ± 14	= 100.0	1407 ± 1	= 100.0	-2***
GLIA/GLUT	2.2 : 1	-	1.7 : 1	-	

[a] AU = HPLC absorbance units per mg flour (mean value of duplicate determinations ± standard deviation); ALGL = albumins/globulins, GLIA = gliadins, GLUT = glutenin subunits, ωb = glutenin-bound ω-gliadins, GLUTEN = gliadins plus glutenins, Σ = sum of extractable proteins, Δ% = percentual changes in FLOt as compared to FLOw (one-sample location T-test, - statistical significance: ***= p <0.001, ns = not significant, p >0.05)

b) Dough	RE[a] (N)			EX[a] (cm)		
	a[b]	b[b]	x	a[b]	b[b]	x
FLOw	0.109	0.125	0.117	9.20	10.20	9.70
FLOt	0.105	0.129	0.117	9.60	9.90	9.75
Δ%[a]	-3.7	+3.2	±0[ns]	+4.3	-2.9	+0.5[ns]
Gluten	**RE[a] (N)**			**EX[a] (cm)**		
	a[c]	b[c]	x	a[c]	b[c]	x
FLOw	0.910	0.727	0.819	7.90	8.40	8.15
FLOt	1.897	1.667	1.782	7.60	6.20	6.90
Δ%[a]	+109	+129	+118***	-4	-26	-15***
Bread	**BV[a] (ml)**					
	a	b	x			
FLOw	32.6	34.6	33.60			
FLOt	28.9	30.6	29.75			
Δ%[a]	-11.3	-11.6	-11.5***			

[a] RE = maximum resistance to extension, EX = extensibility, BV = bread volume, x = mean value, Δ% = percental changes of FLOt as compared to FLOw, (statistical significance: *** = p <0.001, ns = not significant, p >0.05)

[b] Mean value for 5 strands from one dough (7 g of flour)

[c] Mean value for 2 strands from one dough (7 g of flour)

2006) decreased from 2.2 : 1 to 1.7 : 1.
With respect to single gluten protein types, the amount and proportion of α-gliadins, the target for the applied RNA interference, were mostly affected; AU/mg was reduced from 375 (FLOw) to 151 (FLOt) (-63%) and the proportion according to total extractable proteins from 26.1 to 10.7%. With respect to total GLIA, the proportion of α-gliadins from FLOt amounted to 22%; this corresponds to the third group of the transgenic lines (see above). Considering the fact that oligomeric proteins containing disulfide-linked γ-gliadins and LMW-GS were eluted in the region of α-gliadins the reduction of α-gliadins was probably much higher than 63%. The loss of α-gliadins was compensated by the increase of ω-gliadins (23%), γ-gliadins (20%), HMW-GS (16%) and glutenin-bound ω-gliadins (8%). In contrast, the relative amounts of LMW-GS appeared to be not affected (±0). Obviously, the loss of α-gliadins present in the elution region of LMW-GS (Fig. 2b) was compensated by the increase of LMW-GS.

Dough properties

The small amount (31 g) of FLOt flour was the most limiting factor for rheological tests. Two measurements (7 g of flour each) on two subsequent days were performed for both rheological and baking tests. The results summarised in Tab. 2b demonstrated that FLOw and FLOt did not differ significantly in dough rheology. In contrast, FLOt gluten showed a much higher RE (+118%) and a lower EX (-15%) than FLOw. RE of FLOt gluten was enhanced in an extent not observed among numerous wheat cultivars previously studied (Kieffer et al., 1998). Because gluten consists almost entirely of GLIA and GLUT, the ratio (Tab. 2a), which has been shown to be highly correlated with gluten strength (Wieser and Kieffer, 2001), might be responsible for the difference between FLOw and FLOt. Dough, however, contains all flour constituents and the increased amount of ALGL in FLOt (+34%) might compensate for the influence of the ratio GLIA/GLUT resulting in similar rheological dough properties of FLOw and FLOt.

Proteomics

Fig. 3a shows the image of a wild type (*cv*. Florida) grain proteome. In 2D gels, GLIA corresponds to protein spots in the range of 30,000 - 50,000 and a pI of 6 - 9, (Fig. 3c). The 2D gel image in Fig. 3b exhibits the proteome of the transgenic line #6/6 (T_1 progeny from transgenic line #6). Apparently, the transgenic line shows a clear reduction of the intensities of the α-gliadin spots in this range (Fig. 3c). In comparison to the wild type concentrations of 20 α-gliadin spots were decreased by a factor of -2 to -14.4. Additionally, two spots (No. 2336 and 2304, Fig. 3c) were decreased by a factor of -2 in the transgenic line and were identified as HMW-GS (HMW1 Ax1) and heat shock protein HSP 101 (Fig. 3c, Tab. 3a) by MALDI-TOF MS analysis and a subsequent database search (data not shown). The 2D images clearly indicated that the elimination of α-gliadins was actually successful and resulted in a decrease of 22 spots. Interestingly, a compensatory effect of storage protein in the transgenic line was detected (Fig. 3d). The abundance of 41 proteins was increased by a factor of 2 to 7. (with exception of protein no. 3870 wich was changed by a factor of +17.45) (Tab. 3). These proteins were not restricted to a certain pH range or molecular weight but spread all over the 2D gel image (Fig. 3d). Some of these up-regulated proteins were identified as further storage proteins by MALDI-TOF MS such as an HMW-GS, serpin (No. 2824, 2932), and few LMW-GS (No. 3051, 3121) or an avenin-like precursor (No. 3260). Others were enzymes like GAP-DH (No. 2941), and an ATP binding kinase (No. 3314) or a β-amylase (No. 2656) (Tab. 3). Enzymes of the starch biosynthesis seemed to be less affected. Some further protein spots could not be identified.

Discussion

The lack of already identified natural mutants makes it difficult to analyse the function of genes in hexaploid bread wheat. Concerning functional gene analysis and the identification of important genes for technological- and agronomical parameters, wheat is clearly lagging behind other major food crops such as rice and maize. The RNAi approach presented here, therefore, has a high impact for scientific and applied aspects especially for polyploid wheat. Since recent years, the RNAi approach has successfully been used to down-regulate transiently or stably expressed genes in wheat, which is a good hind to get knowledge about their function and regulation (Regina et al., 2006; Travella et al., 2006; Yue et al., 2008).
In our current work, it was possible to dramatically decrease α-gliadin proteins in some of the transgenic lines. This opens the opportunity to further understanding of a part of the gene regulation during seed storage protein expression. Moreover, for practical use it is now possible to understand the function of α-gliadins in dough formation and bread making. The work demonstrates that the RNAi technique is a powerful method to down-regulate whole gene families in wheat.

According to the results from RP-HPLC analysis the independent transgenic wheat lines were classified into four groups with respect to the degree of the decrease of α-gliadins. Transgenic copy number can explain one possible explanation for the variation in the degree of silencing and/or positional effects of particular DNA insertion events as reported previously (Gil-Humanes et al., 2008; Regina et al., 2006; Travella et al., 2006). Nevertheless, the variation in the degree of silencing observed in the transcript lines showed both a partial to complete reduction. These results are in agreement with other reports for cereals or for several dicotyledonous species (Chuang et al., 2000; Levin et al., 2000; Schweizer et al., 2000; Waterhouse et al., 1998).
Surprisingly, in our transgenic wheat plants the total protein content was not changed in comparison to the wild type (data not shown). The loss of α-gliadins was compensated by the increase of other protein types. Changes in the protein composition were reported previously in barley, wheat and rice (Gil-Humanes et al., 2008; Lange et al., 2007; Maruta et al., 2001).
Changes of single storage protein composition in transgenic and wild type flour was determined by RP-HPLC analysis. In transgenic wheat lines the lack of α-gliadins is not completely compensated by higher amounts of the remaining gliadins (mainly γ- and ω-gliadins) as it was reported by Piston et al. (2011) in transgenic wheat with reduced γ-gliadin content. They observed a compensation of reduced γ-gliadin content by an increase of α- and ω-gliadins The total amount of gliadins in transgenic- and wild type plants was not significantly altered. The total gliadin content of our transgenic wheat is significantly reduced (-16%) which is in accordance by the work from Gil-Humanes et al. (2010). An increase of total glutenin content in gliadin knock down wheat was measured by RP-HPLC analysis in all three experimental approaches (Piston et al., 2011; Gil-Humanes et al., 2010).
Due to the strong reduction of α-gliadins and the lower ratio of GLIA to GLUT, the rheological properties of gluten from the transgenic flour FLOt and the wild type flour FLOw differed strongly. In contrast, the rheological properties of the doughs were almost identical. Obviously, the increased content of ALGL in the FLOt dough compensated for the role of GLIA in the FLOw dough so that comparable dough properties were obtained. As gluten does not contain ALGL the altered GLIA to GLUT ratio of the two flours

Tab. 3: Compilation of proteins changed by RNAi knock-out of α-gliadin in wheat grain. **a:** down-regulated spots; **b**: up-regulated spots. Spot numbers according to Fig. 3 c, d; fold changes of spot volume of knock out wheat (#6/6) and wild type (cv. "Florida"). MW, molecular weight; pI, experimental pH of spots in the 2D gel. Statistical calculations were based on three biological replications and two technical replicates within each biological replication (average 2D gels). The spot volumes were normalized to the total protein on each gel.

3a down-regulated

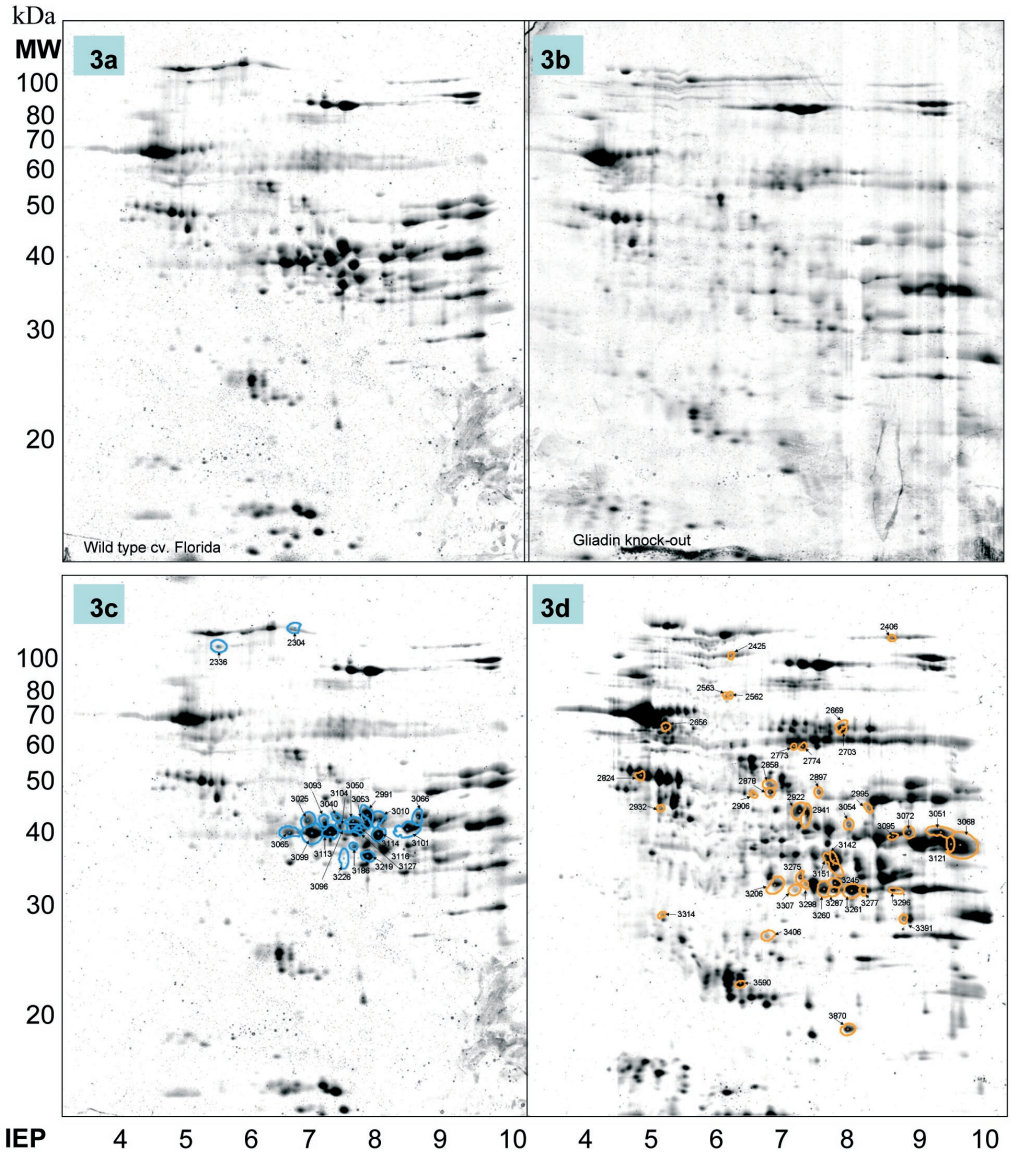
Spot No.	fold changes (#6/6/Florida)	MW / pI	Identification	down-regulated Spot No.	fold changes (#6/6/Florida)	MW / pI	Identification
3127	-14.36	40 / 7.7	α-gliadin	3010	-3.06	42 / 8.0	α-gliadin
3096	-7.78	41 / 7.1	α-gliadin	3093	-2.76	41 / 7.2	α-gliadin
2991	-6.02	43 / 7.7	α-gliadin	3040	-2.61	42 / 7.3	α-gliadin
3114	-4.81	39 / 8.0	α-gliadin	3219	-2.52	36 / 7.3	α-gliadin
3116	-4.50	41 / 7.7	α-gliadin	2304	-2.48	150 / 6.8	HSP101 (gi4558484)
3053	-4.38	42 / 7.6	α-gliadin	3099	-2.40	40 / 7.1	α-gliadin
3101	-4.04	40 / 8.4	α-gliadin	3066	-2.40	43 / 8.6	α-gliadin
3025	-3.42	42 / 7.0	α-gliadin	3050	-2.24	41 / 7.6	α-gliadin
3104	-3.41	41 / 7.5	α-gliadin	3226	-2.14	36 / 7.5	α-gliadin
3065	-3.39	40 / 6.7	α-gliadin	3186	-2.08	37 / 7.6	α-gliadin
3113	-3.30	40 / 7.3	α-gliadin	2336	-2.03	110 / 5.6	HMW1 Ax1 (gi21743)

3b

up-regulated Spot No.	fold changes (#6/6/Florida)	MW / pI	Identification	up-regulated Spot No.	fold changes (#6/6/Florida)	MW / pI	Identification
2773	2.00	53 / 7.3	-	2932	2.46	40 / 5.3	Serpin (gi871551)
3277	2.02	28 / 8.3	-	2906	2.47	43 / 6.7	-
3095	2.02	36 / 8.7	-	3275	2.53	31 / 7.4	-
3072	2.02	37 / 9.0	-	2406	2.59	98 / 8.7	HMW Bx17(gi109452233)
3590	2.05	21 / 6.5	-	2425	2.63	84 / 6.4	-
3151	2.08	32 / 7.7	-	3261	2.65	28 / 8.2	-
3051	2.12	36 / 9.4	LMW sk2S (gi51870702)	2858	2.68	45 / 7.0	
3296	2.13	28 / 8.3	-	3287	2.72	28 / 7.8	-
3142	2.13	33 / 7.8	-	2824	2.99	47 / 5.1	Serpin (gi1885346)
3314	2.16	26 / 5.4	ATP bind. kin. (gi15229902)	2656	3.04	58 / 5.4	ß-amylase (gi32400764)
2922	2.16	40 / 7.5	-	2878	3.21	43 / 7.0	-
2562	2.18	67 / 6.4	-	3245	3.26	30 / 8.0	-
3406	2.19	24 / 6.9	27-K prot. (gi30793446)	3298	3.31	30 / 7.5	-
3054	2.26	37 / 8.2	-	2774	3.57	53 / 7.4	put. nucl. prot.(gi37537028)
2563	2.26	67 / 6.3	hyp. prot.(gi15226513)	2703	3.99	57 / 8.1	-
3260	2.31	29 / 7.7	avenin like pr. (gi89143122)	3307	5.26	28 / 7.3	-
3206	2.32	30 / 7.1	-	2995	5.46	40 / 8.4	-
2669	2.32	58 / 8.1	-	3068	6.27	34 / 9.6	-
3391	2.33	26 / 8.9	-	3121	6.94	34 / 9.5	LMW 4 type II (gi17425188)
2941	2.34	39 / 7.5	GAP DH (gi148508784)	3870	17.45	17 / 8.2	subtilisin IAAS (gi123975)
2897	2.46	43 / 7.7	-				

became effective and was, therefore, responsible for the differences of the rheological properties of the glutens.

The wheat α-gliadin elimination could also be observed by 2D electrophoresis. At least 22 spots of the storage proteins fraction were missing (Fig. 3). As detected by the proteome approach there was a compensation of the protein contents by increased expression of diverse other proteins. These proteins belong to other storage protein components such as γ-gliadins, HMW-GS, serpins or enzymes of the energy (ATPbinding kinase) and the nitrogen metabolism (GAP-DH). In total, the reduction of the 20 α-gliadins in the transgenic plants resulted in a decrease of 10.6% of the total spot volume in the 2D gel compared to the wild type. The 41 up-regulated protein spots compensated for the total spot volume by an increase of 7.6% compared to the wild type. In summary, the compensation effect in terms of storage protein production resulted in a loss of 3%. The up-regulation of some enzymes, which might have been needed for the alternative seed loading, could also have been affected. These enzymes could have been used the supplied resources of nitrogen and sulphur for increasing the expression of the alternative storage proteins. The α-gliadin deficient wheat could have used an alternative seed loading process, which finally resulted in the variation of the storage protein composition with an increased content of γ-gliadins, HMW-GS and other storage proteins. In a former study it was shown that α-gliadins were not changed by a different sulphur treatment of the plants. This anticipated that α-gliadins in contrast to γ-gliadins



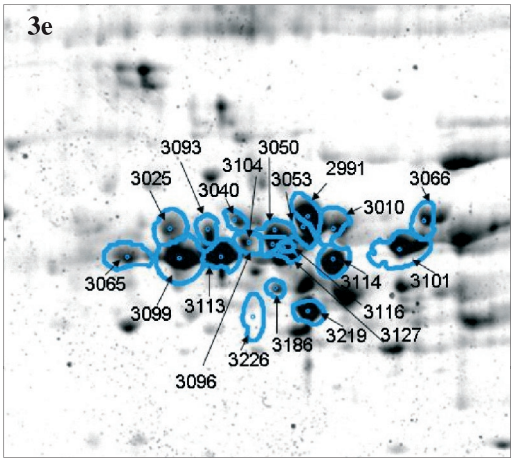


Fig. 3: Two dimensional gel images of wheat grain proteins from the wild type cultivar "Florida" (a, c) in comparison with those from T_1 seeds of transgenic line #6/6 with down-regulated α-gliadins (b, d). IEP, isoelectric point ranging from pH 3 to pH10.
c proteins which were down-regulated were marked with circles.
d proteins which were up-regulated in comparison to #6/6 and wild type "Florida" were marked with circles. Identification numbers were according to Tab. 3. For values of "fold changes" of up- and down-regulation of each spot refer to Tab. 3. (a, c and b, d represent biological replications).
e magnification of the central gel region shown in 3c.

were constitutively expressed under a changed sulphur availability of the plants (Zörb et al., 2009; Zörb et al., 2010). The α-gliadins may first and foremost function as sulphur storage. Because the plant would not germinate without having enough sulphur a compensation of the α-gliadins at the knockdown plants must have priority to maintain sulphur storage and, thus, maintain the ability to germinate.

Conclusion

The application of our RNAi approach on hexaploid wheat resulted in a partial or complete elimination of α-gliadin expression. In spite of the altered protein composition of the transgenic wheat the dough and bread making properties were comparable to those of the wild type showing that α-gliadins are not an essential protein group for baking quality.

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References

Becker, D., Brettschneider, R., Lörz, H., 1994: Fertile transgenic wheat from microprojectile bombardment of scuttelar tissue. Plant Journal 5, 299-307.

Becker, D., Lörz, H., 1996: Production of fertile transgenic wheat by microprojectile bombardment. Plant Tissue Culture Manual 12, 1-20.

Chuang, C.F., Meyerowitz, E.M., 2000: Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA 97, 4985-4990.

Christensen, A.H., Sharrock, R.A., Quail, P.H., 1992: Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Molecular Biology 18, 675-689.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998: Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 19, 806-811.

Gil-Humanes, J., Piston, F., Hernando Alvarez, J.B., Shewry, P.R., Barro, F., 2008: Silencing of γ-gliadins by RNA interference (RNAi) in bread wheat. J. Cereal Science 48, 565-568.

Gil-Humanes, J., Piston, F., Tollefsen, S., Sollid, L.M., Barro, F., 2010: Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. Proceedings of the National Academy of Sciences USA 107, 17023-17028.

Görg, A., Weiss, W., 1998: High-resolution two-dimensional electrophoresis of proteins using immobilized pH gradients. In: Celis, J. (ed.), Cell Biology. A Laboratory Handbook, 386-397. Academic Press NY, 1998.

Huang, S.S., Adams, W.R., Zhou, Q., Malloy, K.P., Voyles, D.A., Anthony, J., Kriz, A.L., Luethy, M.H., 2004: Improving nutritional quality of maize proteins by expressing sense and antisense zein genes. J. Agricultural and Food Chemistry 52, 1958-1964.

Huebner, F.R., Bietz, J.A., 1993: Improved chromatographic separation and characterization of ethanol-soluble wheat proteins. Cereal Chemistry 70, 506-511.

Kieffer, R., Wieser, H., Henderson, M.H., Graveland, A., 1998: Correlations of the breadmaking performance of wheat flour with rheological measurements on a microscale. J. Cereal Science 27, 53-60.

Lange, M., Vincze, E., Wieser, H., Schjoerring, J.K., Holm, P.B., 2007: Suppression of C-hordein synthesis in barley by antisense constructs results in a more balanced amino acid composition. J. Agricultural and Food Chemistry 25, 6074-6081.

Levin, J.Z., De Framond, A.J., Tuttle, A., Bauer, M.W., Heifetz, P.B., 2000: Methods of double-stranded RNA-mediated gene inactivation in *Arabidopsis* and their use to define an essential gene in methionine biosynthesis. Plant Molecular Biology 44, 759-775.

Lew, E.J.L., Kuzmicky, D.D., Kasarda, D.D., 1992: Characterization of low molecular weight glutenin subunits by reversed-phase high-performance liquid chromatography, sodium dodecyl sulfatepolyacrylamide gel electrophoresis, and N-terminal amino acid sequencing. Cereal Chemistry 69, 508-515.

Maruta, Y., Ueki, J., Saito, H., Nitta, N., Imaseki, H., 2001: Transgenic rice with reduced glutelin content by transformation with glutelin A antisense gene. Molecular Breeding 8, 273-284.

Müller, E., Lörz, H., Lüttcke, S., 1996: Variability of transgene expression in clonal cell lines of wheat. Plant Science 114, 71-82.

Okita, T.W., Chessbrough, V., Reeves, C.D., 1985: Evolution and heterogeneity of the α-/β-type sequences and γ-type gliadin DNA sequences. J. Biological Chemistry 260, 8203-8213.

Osborne, T.B., 1907: The Proteins of the Wheat Kernel. In: Carnegie Institution, Washington, DC, No. 84, 1-119.

Payne, P.I., Holt, L.M., Law, C.N., 1981: Structural and genetic studies on the high-molecular-weight subunits of wheat glutenins. Part I. Allelic variation in subunits amongst varieties of wheat (*Triticum aestivum*). Theoretical and Applied Genetics 60, 229-236.

Payne, P.I., Holt, L.M., Lawrence, G.J., Law, C.N., 1982: The genetics of gliadin and glutenin, the major storage proteins of the wheat endosperm. Qualitas Plantarum – Plant Foods for Human Nutrition 31, 229-241.

Payne, P.I., Holt, L.M., Jackson, E.A., Law, C.N., 1984: Wheat storage proteins: their genetics and their potential for manipulation by plant breeding. Philosophical Transaction of the Royal Society London 304, 359-371.

Piston, F., Gil-Humanes, J., Rodrí´guez-Quijano, M., Barro, F., 2011: Down-regulating c-gliadins in bread wheat leads to non-specific increases in other gluten proteins and has no major effect on dough gluten strength, PLoS ONE 6, e24754, 2011.

Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar-Hashemi, B., Li, Z., Rahman, S., Morell, M., 2006: High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. Proceedings of the National Academy of Sciences USA 103, 3546–3551.

Schweizer, P., Pokorny, J., Schulze-Lefert, P., Dudler, R., 2000: Double-stranded RNA interferes with gene function at the single-cell level in cereals. Plant Journal 24, 895-903.

Segal, G., Song, R., Messing, J., 2003: A new opaque variant of maize by a single dominant RNA-interference-inducing transgene. Genetics 165, 387-397.

Shewry, P.R., Tatham, A.S., Forde, J., Kreis, M., Miflin, B.J., 1986: The classification and nomenclature of wheat gluten proteins: a reassessment. J. Cereal Science 4, 97-106.

Shewry, P.R., Tatham, A.S., 1990: The prolamin storage proteins of cereal seeds: structure and evolution. Biochemical Journal 267, 1-12.

Travella, S., Klimm, T.E., Keller, B., 2006: RNA interference-based gene silencing as an efficient tool for functional genomics in hexaploid bread wheat. Plant Physiology 142, 6-20.

Waterhouse, P.M., Graham, M.W., Wang, M.B., 1998: Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proceedings of the National Academy of Sciences USA 95, 13959-13964.

Wieser, H., Antes, S., Seilmeiser, W., 1998: Quantitative determination of gluten protein types in wheat flour by reversed-phase high-performance liquid chromatography. Cereal Chemistry 75, 644-650.

Wieser, H., Kieffer, R., 2001: Correlations of the amount of gluten protein types to the technological properties of wheat flour determined on a micro-scale. J. Cereal Science 34, 19-27.

Wieser, H., 2007. Chemistry of gluten proteins. Food Microbiology 24, 115-119.

Wrigley, C.W., Bekes, F., Bushuk, W., 2006: Gluten: a balance of gliadin and glutenin. In: Wrigley, C., Bekes, F., Bushuk, W. (eds.), Gliadin and glutenin – the unique balance of wheat quality, 3-32. AACC International: St. Paul, Minnesota, USA.

Wu, Y., Messing, J., 2010: RNA interference-mediated change in protein body morphology and seed opacity through loss of different zein proteins. Plant Physiology 153, 337-347.

Yue, S.J., Li, H., Li, Y.W., Zhu, Y.F., Guo, J.K., Liu, Y.J., Chen, Y., Jia, X., 2008: Generation of transgenic wheat lines with altered expression levels of 1Dx5 high-molecular weight glutenin subunit by RNA interference. J. Cereal Science 47, 153-161.

Zörb, C., Steinfurth, D., Seling, S., Langenkämper, G., Köhler, P., Wieser, H., Lindauer, M.G., Mühling, K.H., 2009: Quantitative protein composition and baking quality of winter wheat as affected by late sulfur fertilization. Journal of Agricultural and Food Chemistry 57, 3877-3885.

Zörb, C., Grover, C., Steinfurth, D., Mühling, K.H., 2010: Quantitative proteome analysis of wheat gluten as influenced by N and S nutrition. Plant and Soil 327, 225-234.

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