

Transformation of the US bread wheat 'Butte 86' and silencing of omega-5 gliadin genes

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Complex groups of proteins determine the unique functional properties of wheat flour and are sometimes responsible for food intolerances and allergies in individuals that consume wheat products. Transgenic approaches can be used to explore the functions of different flour proteins, but are limited to the few wheat cultivars that can be transformed and also by the lack of detailed information about genes and proteins expressed in grain from those cultivars. The US bread wheat Butte 86 has been extensively characterized and a comprehensive proteome map was developed in which flour proteins were distinguished by mass spectrometry and associated with specific gene sequences. Here, this information has been used to design an RNA interference construct to silence the expression of genes encoding omega gliadins that trigger the food allergy wheat-dependent exercise-induced anaphylaxis (WDEIA). The construct was introduced into immature embryos from Butte 86 using biolistics and bialaphos-resistant plants were regenerated. Stable transformation and inheritance of the transgene were confirmed by PCR. Analysis of proteins in grain from transgenic plants demonstrated that the omega-5 gliadins were either absent or substantially reduced relative to non-transformed controls. The ability to genetically transform Butte 86 makes it possible to alter flour composition in a targeted manner in a commercial US wheat cultivar and should accelerate future research on flour quality and immunogenic potential.

Introduction

Wheat flour has played an important role in the human diet for thousands of years and is an essential ingredient used in a wide variety of breads, noodles and other food products because of the unique viscoelastic properties conferred by the gluten proteins. The gluten proteins comprise about 70% of the flour protein and consist of gliadins and glutenins, all of which have repetitive sequences with an abundance of glutamine and proline residues (reviewed in ref. 1 and 2). A single bread wheat cultivar may contain more than 100 genes encoding gluten proteins and there is considerable heterogeneity among the genes expressed in different wheat cultivars. The gliadins, separated into alpha, gamma and omega gliadin groups, are generally present as monomers and contribute extensibility to wheat flour dough. The glutenins contribute elasticity to dough and consist primarily of two types of proteins, the high molecular weight glutenin subunits (HMW-GS) and the low molecular weight glutenin subunits (LMW-GS), linked by disulfide bonds into some of the largest polymers found in nature.³ Some gliadins with an odd number of cysteine residues also may be incorporated into the glutenin polymer.⁴⁻⁶ Interactions among the different gluten proteins are important for flour quality but are complex and difficult to study, in part because of the many similar proteins that are involved.

In addition to conferring unique viscoelastic properties, certain proteins in wheat flour cause serious food intolerances or

allergies in sensitive individuals. Some gluten proteins, particularly those within the alpha gliadin group, have been identified as triggers of celiac disease (CD), an important food intolerance that affects nearly 1 out of 100 people in the US and other countries.⁷ Additionally, wheat flour is among the top eight causes of IgE-mediated food allergies. A number of flour proteins cause food allergies, including some types of omega gliadins as well as non-gluten proteins that perform a variety of different functions in the grain and account for minor proportions of the total flour protein (reviewed in ref. 8). Certain flour proteins also cause an occupational allergy referred to as baker's asthma that results from the inhalation of flour, primarily by millers and bakers who handle large quantities of flour.

In an attempt to better understand the molecular basis of flour quality, dissect the complex interactions among the gluten proteins, and define the roles of specific proteins in celiac disease and allergies, we have extensively characterized both genes and proteins expressed in developing grain from the US spring wheat cultivar Butte 86.⁹⁻¹⁵ Additionally, we have used this cultivar for detailed studies of the effects of high temperature and fertilizer on grain development.¹⁶⁻²¹ Most recently, we developed a two-dimensional electrophoresis (2-DE) map of flour proteins in which 230 protein spots representing more than 93% of the total wheat flour proteome were identified by tandem mass spectrometry (MS/MS) and associated with 157 specific gene sequences, many from Butte 86.²²

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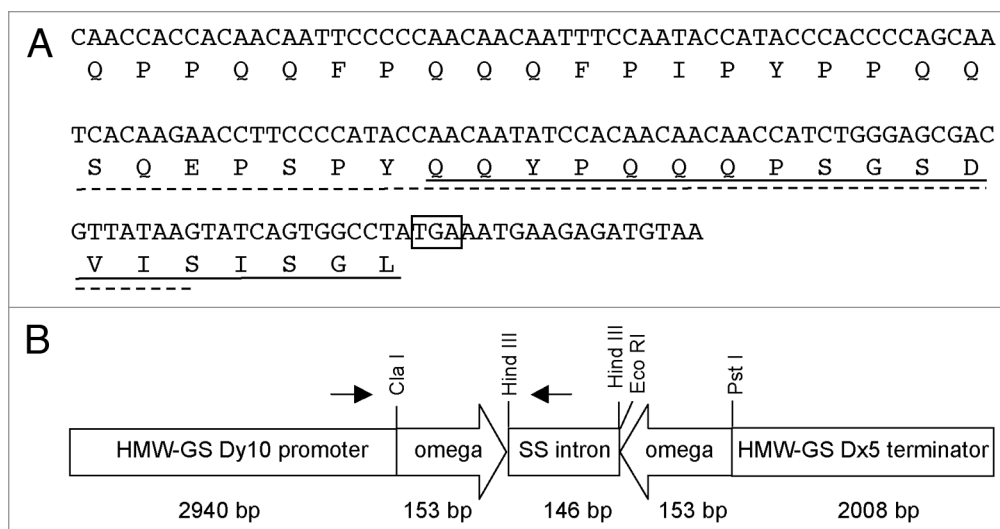


Figure 1. Omega gliadin RNAi construct. (A) Sequences of the 153 bp target region used in the RNAi construct and the portion of the protein encoded by the target region. In the DNA sequence, the stop codon is enclosed in a box. Chymotryptic and thermolytic peptides identified by MS/MS in a proteomic analysis of Butte 86 flour that correspond to portions of the target region are indicated with solid and dashed lines, respectively. (B) Diagram of the construct used for silencing omega-5 gliadin genes. SS denotes starch synthase intron used as a spacer between inverted copies of the 153 bp target region. Restriction sites used in construction of the transgene are indicated. Solid arrows show the positions of primers used to detect the transgene in transgenic plants.

Results

Selection of the RNAi target sequence. Of the 3,649 ESTs from Butte 86 developing grain that are catalogued in NCBI, only five contain sequences for omega-5 gliadins.⁹ None of these include the 5' portion of the coding region. Because the goal of the experiment was to inhibit the expression of all omega-5 gliadins in Butte 86, a target sequence was selected that matched at least a portion of all five Butte 86 ESTs. The 153 bp sequence shown in **Figure 1A** corresponds to the 3' end of the omega-5 gliadin coding region and is a perfect match with three ESTs, BQ806730, BQ806362 and BQ805583. It also is a perfect match with a 60 bp portion of BQ806240 and 53 and 55 bp regions of BQ805830. Proteomic data further suggest

We have now developed genetic transformation methods for Butte 86 and have transformed this cultivar with a construct designed to silence the expression of genes encoding a subset of omega gliadins that trigger a serious food allergy. Wheat-dependent exercise-induced anaphylaxis (WDEIA) occurs in sensitive individuals when the ingestion of wheat is followed by intense physical exercise. The omega-5 gliadins, encoded by genes on the short arm of chromosome 1B, have been implicated as major allergens in WDEIA.^{23,24} The omega-5 gliadins have N-terminal sequences beginning SRLL, contain about 70% glutamine and proline, and consist almost entirely of repetitive sequences with FPQQQ and QQIPQQ as the predominant motifs. These motifs also are found within several dominant IgE-binding epitopes identified using sera from WDEIA patients.²⁵⁻²⁷ Dupont et al.²⁸ found three distinct omega-5 gliadins in Butte 86 by SDS-PAGE that resolved into multiple spots in 2-DE. The omega-5 proteins differ from the omega-1,2 gliadins, encoded on chromosomes 1A and 1D, in N-terminal sequences and molecular weights as well as in the sequences of the repetitive motifs.^{9,28-30} Although 15 to 18 genes for omega gliadins are present in most bread wheat cultivars,³¹ there are very few DNA sequences for omega gliadins in the NCBI nr database, presumably because their repetitive sequences are difficult to clone. Additionally, it has not been possible to assemble complete omega gliadin coding regions from expressed sequence tags (ESTs) from any single wheat cultivar.³² In the current work, we relied on incomplete EST data as well as proteomic data from Butte 86 to design an RNA interference (RNAi) construct that effectively targets the omega-5 gliadins in this cultivar. We report on the first successful transformation of Butte 86 with this construct.

that this region would be a good sequence for use in an RNAi construct. Six protein spots in a 2-DE map of Butte 86 flour protein were identified as omega-5 gliadins in Dupont et al.²² Three were major spots with slightly different molecular weights (spots 60, 71, 74) and three were minor spots that differed from each major spot by isoelectric point and are likely charge trains (spots 63, 69, 73). The chymotryptic peptide QQYPQQPPSGSDVIS ISGL corresponding to the C-terminal region of the protein was obtained by MS/MS for two major spots (spots 71, 74) and one minor spot (spot 63), representing all three size classes of proteins. In addition, the thermolytic peptide SQEPSPYQQYPQ QQPSGSDVIS was obtained for spot 74. Both peptides are encoded by the 153 bp target sequence (**Fig. 1A**).

A search of the NCBI nr database revealed only nine accessions from *T. aestivum* with similarity to the 153 bp target sequence (**Table 1**). Two were gene sequences, four were cDNAs and three were pseudogenes. The Butte 86 target sequence was a perfect match with AB181300, a complete omega-5 gliadin sequence amplified from genomic DNA from the cultivar Norin 61. It also was a perfect match with 110 and 39 bp regions of cDNAs amplified from the cultivar Prinqual, FM212917 and FM212918, respectively. Both encode proteins very similar to omega-5 gliadins but are referred to as D-type LMW-GSs because they contain single cysteine residues near their C-termini. The Butte 86 target sequence was a perfect match with a 90 bp region from M11336, a cDNA clone from the cultivar Cheyenne that is a hybrid of omega-5 gliadin and LMW-GS sequences. Two accessions that encode proteins similar to omega-1,2 gliadins, AF280605 and FM212916, also were identified in the search, but they had only a 20 bp region that was a perfect match with the Butte 86 target

sequence. Pseudogenes from Norin 61, CN16-63 and Cheyenne also matched portions of the 153 bp target. A search of the NCBI EST database with the 153 bp target sequence returned eight Butte 86 ESTs that correspond to omega-1,2 gliadins in addition to the five ESTs for omega-5 gliadins. These were BQ839049, BQ838934, BQ807007, BQ806801, BQ806762, BQ806896, BQ804424 and BQ804207. The longest perfect match in these sequences to the 153 bp target was 20 bp.

The 153 bp omega-5 gliadin target sequence was inserted in sense and antisense directions on either side of a 146 bp intron from a wheat starch synthase gene and placed under the regulatory control of the HMW-GS Dyl0 promoter and HMW-GS Dx5 terminator so that transcription of the sequence would produce a hairpin RNA in developing endosperm tissue (Fig. 1B).

Transformation of Butte 86 and identification of transgenic plants. Five separate transformation experiments were conducted using a total of ~ 3,000 embryos obtained from Butte 86 developing grain collected between late May and early October. Initial experiments indicated that there was a very narrow window during which immature embryos from Butte 86 were able to form regenerable callus. Prior to 12 days post-anthesis (DPA), most embryos were very small and did not form callus. Beyond 12 DPA, embryos produced callus but had an increasing propensity to sprout. About 2,000 of the explants bombarded in the five experiments were embryos with attached scutella. The first three experiments included an additional 1,000 explants in which the embryo axes were either excised from the scutella or destroyed using a forceps. In the absence of embryo axes, sprouting was minimized and callus tissue proliferated. However, the resulting calli generally failed to form shoots when later placed on regeneration media. As a result, subsequent experiments utilized only explants containing both embryo axes and scutella.

Optimal levels of bialaphos for selection of transformed tissue in Butte 86 were determined empirically. In the first two experiments, 2.0 mg/L was included in both regeneration and rooting media. In subsequent experiments, calli were first placed on regeneration media containing 1.5 mg/L bialaphos for two weeks to increase the numbers of shoots produced, then transferred to regeneration and rooting media containing 2.0 mg/L bialaphos for the remaining selection. The five experiments yielded 82 plantlets with green shoots and sturdy roots that were transferred to soil. Of these, 54 were found to contain the transgene by PCR analysis using primers from the HMW-GS Dyl0 promoter and the starch synthase intron that flank the sense copy of the omega gliadin target sequence (Fig. 1B). These plants resulted from at least 45 independent transformation events. All transgenic plants had appearances that were indistinguishable from the non-transformed controls. PCR analysis further demonstrated that the transgene was transmitted to progeny. As an example, Figure 2 shows amplification products from DNA from T₁ plants from each of three independent lines. The expected 339 bp amplification product was obtained in 10 of 12 T₁ plants for SA-8-35b, 11 of 12 for SA-8-55b and 7 of 12 for SA-8-33c.

Protein analysis of grain from transgenic plants. Total proteins were extracted from individual grains from T₀ plants that

Table 1. *T. aestivum* sequences in NCBI nr database with similarity to 153 bp target sequence from Butte 86¹

Accession #	Cultivar	Type of Sequence	Longest Perfect Match (bp)
AB181300	Norin 61	genomic	153
FM212917 ²	Prinqual	cDNA	110
M11336 ³	Cheyenne	cDNA	90
FM212918 ²	Prinqual	cDNA	39
AF280605 ⁴	Cheyenne	genomic	20
FM212916 ^{2,4}	Prinqual	cDNA	20
AB181301	Norin 61	pseudogene	96
EF116277	CN16-63	pseudogene	27
AF280606	Cheyenne	pseudogene	27

¹determined by blastn of NCBI nr database limited to *Triticum aestivum*, word size 20, no filters or masks, database last searched on 3/18/11. ²encoded protein contains a cysteine residue and is referred to as a D-type LMW-GS. ³clone is a hybrid of omega-5 gliadin and LMW-GS sequences. ⁴sequence of encoded protein is similar to omega-1,2 gliadin.

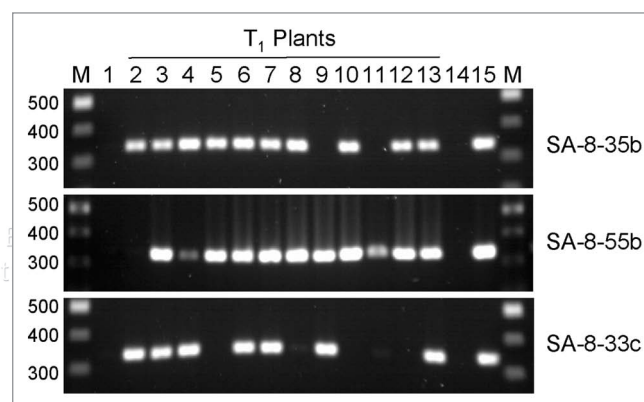


Figure 2. PCR analysis of leaf DNA from T₁ plants using the primers 4F73/4R73. In each part, amplification products were from: 1, non-transformed plant; 2-13, T₁ plants; 14, no template control; 15, non-transformed plant +5 pg Omega-RNAi-13. Sizes of DNA standards shown in lanes labeled M are indicated in bp on the left of each part.

contained the transgene. Previously, Dupont et al.²⁸ reported that the omega-5 gliadins from Butte 86 resolved into three bands with apparent MWs between 60,000 and 64,000 by SDS-PAGE. Figure 3 compares protein profiles from five grains obtained from each of six independently transformed plants with those from a non-transformed control. In grains from all six of the transgenic plants, the three protein bands just above the 60,000 dalton marker were either absent or substantially reduced in at least some of the samples relative to the non-transformed control. Suppression was observed in all five of the grains analyzed from SA-8-35b, SA-8-45a and SA-8-55b, in four of five from SA-8-32a, three of five from SA-8-33c and two of five from SA-8-18d. Examples of complete suppression (#1 from SA-8-18d) and partial suppression (#4 from SA-8-18d) were observed. The lack of silencing observed in some samples (#2, 3 and 5 from SA-8-18d; #5 from SA-8-32a; #4 and 5 from SA-8-33c) is likely due to the segregation of the RNAi transgene in the T₁ grain. Changes in

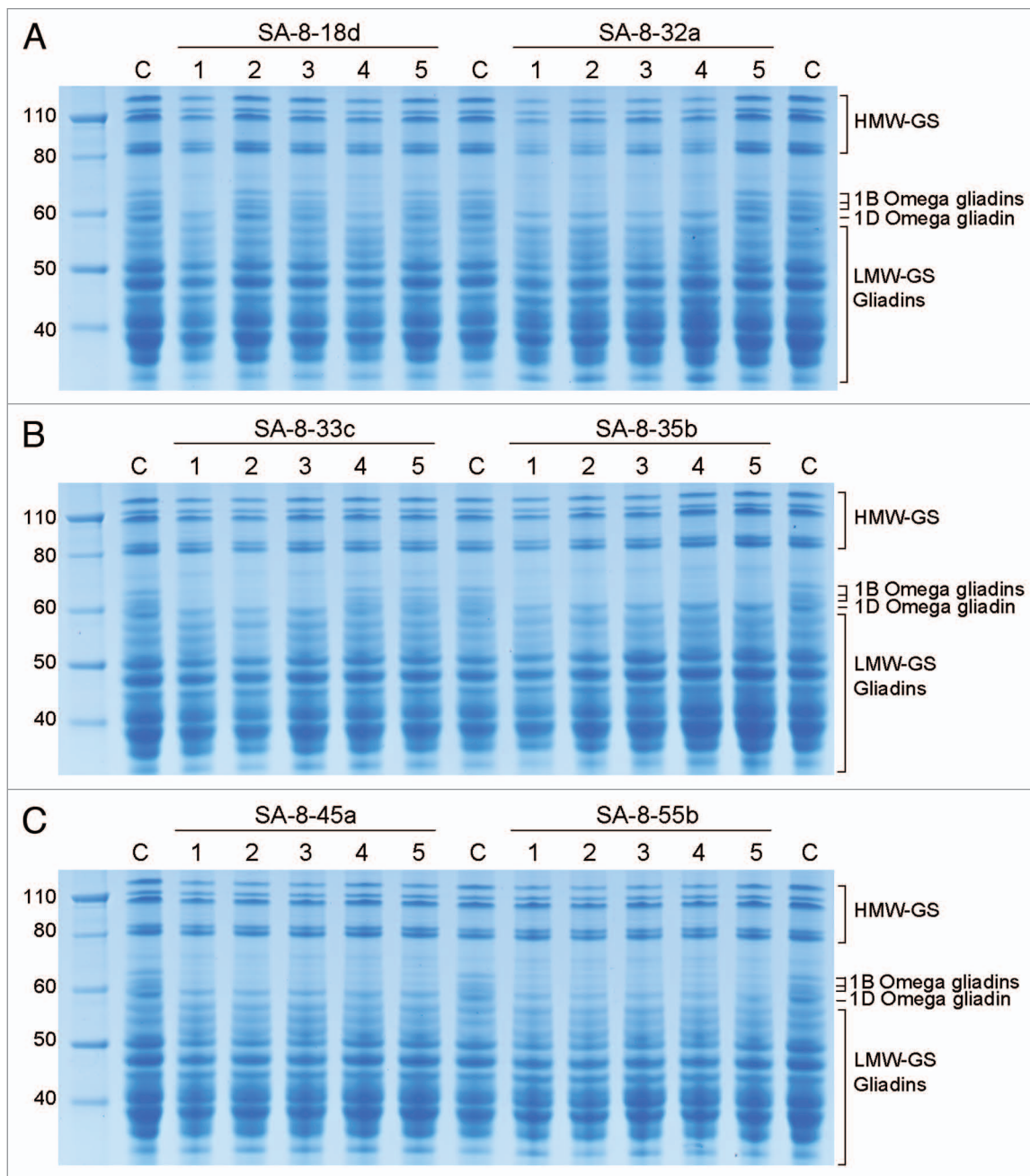


Figure 3. SDS-PAGE of total protein from individual grains from transgenic plants. In each part, protein from grain from non-transgenic control plants are indicated by C and proteins isolated from five individual T_1 grains of transgenic plants are labeled 1–5. The sizes of protein standards shown in the first lane of each part are indicated on the left in kDa. The positions of major gluten protein types are shown on the right. Positions of the omega-5 and omega-1,2 gliadins, labeled 1B and 1D omega gliadins, respectively, were determined by Dupont et al.²⁸

the levels of other protein types, including the omega-1,2 gliadin encoded by the 1D chromosome, were not obvious in any of the samples from transgenic plants.

To better visualize the omega-5 gliadins and demonstrate that the silencing occurs in subsequent generations, total gliadins were extracted preferentially from individual T_2 grains using 50% propanol as described by Dupont et al.²⁸ A small amount of HMW-GS and LMW-GS were also extracted by this method. With the removal of most of the LMW-GS, there are five distinct

bands in the region of the gel around the 60,000 dalton marker that are well separated from the bulk of the gliadins (Fig. 4). The three upper bands that were identified as omega-5 gliadins in Dupont et al.²⁸ are absent from all three samples derived from the SA-8-18d line and from two of the three samples derived from the SA-8-31a line, confirming the specificity of the silencing in the second generation. The lack of silencing in sample #3 from the SA-8-32a indicates that the transgene is still segregating in T_2 grain from this line.

Discussion

This work is the first report of the genetic transformation of the bread wheat Butte 86, a commercial US cultivar in which the genes and proteins expressed in developing grain have been extensively characterized. We have utilized molecular information from Butte 86 to silence a subclass of omega gliadin genes that encode proteins known to cause the food allergy WDEIA, significantly reducing the levels of the omega-5 gliadins in grain from transgenic plants. Omega-1,2 gliadins that share 20 bp of identical sequence with the omega-5 target sequence were not suppressed, demonstrating the specificity of the silencing mechanism. However, more detailed analysis of the proteins by quantitative 2-DE is needed to verify this finding.

As a general rule, RNA interference constructs must contain an uninterrupted stretch of at least 21 nucleotides identical to the target gene to trigger gene silencing.³³ In the current study, the design of the RNA interference construct was complicated by the limited number of omega gliadin sequences in NCBI and by the lack of complete coding sequences for omega gliadins from Butte 86. The highly repetitive nature of omega gliadin coding regions presented additional challenges. The 153 bp target sequence that was selected for use in the construct encodes the C-terminal end of the omega-5 gliadin that is the least repetitive portion of the protein. The target sequence matched at least 39 bp of continuous sequence from all omega-5 gliadins from *T. aestivum* in NCBI and at least 53 bp of continuous sequence from all five of the Butte 86 ESTs for omega-5 gliadins, but only 20 bp from NCBI accessions or Butte 86 ESTs that correspond to omega-1,2 gliadins. DNA sequence comparisons suggested that other portions of the omega-5 gliadin coding region also would have been suitable target sequences for silencing omega-5 gliadins without silencing other gluten proteins. However, these sequences were not used in the construct because they were highly repetitive and likely to present cloning difficulties. It is somewhat surprising that the omega-5 gliadin coding region did not have more than 20 bp in common with coding sequences for other gluten proteins given that all gluten proteins contain high levels of glutamine and proline and that the omega gliadins, in particular, contain around 70% glutamine and proline. However, the arrangement of these residues in repetitive motifs is sufficiently different in the omega-5 gliadins from those in the omega-1,2 gliadins and other gluten proteins. It is also interesting that the 3' untranslated regions of omega-5 gliadin ESTs BQ805583 and BQ806362 had 23 bp and 35 bp regions of perfect identity, respectively, with Butte 86 ESTs for omega-1,2 gliadins and thus were not suitable target sequences for RNA interference constructs.

The elimination or reduction of omega-5 gliadins in wheat flour should reduce risks involved in eating wheat-based products for individuals with WDEIA. However, first it will be essential to determine whether the elimination of these proteins impacts flour quality. It also will be interesting to determine whether the flour proteome compensates for the loss of the omega-5 gliadins when plants are grown under different fertilizer regimens. The Butte 86 omega-5 gliadins exhibit a large response to the application of post-anthesis fertilizer at both the transcript level⁹ and the

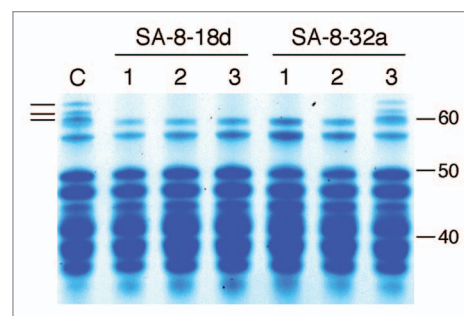


Figure 4. SDS-PAGE of a 50% propanol extract of gliadins from individual T₂ grains from transgenic lines. Gliadins from grain from a non-transgenic control plant are indicated by C. Gliadins isolated from individual T₂ grains of transgenic lines are labeled 1–3. The positions of protein standards are shown on the right in kDa. Positions of the omega-5 gliadins are indicated on the left.

protein level^{19,20} and encompass as much as 5% of total protein in flour.²² Further studies will be aimed at examining the effects of the transgene on the flour proteome, evaluating the role of the omega-5 gliadins in flour quality, and assessing the allergenic potential of flour from transgenic plants.

The current work extends the range of US wheats that can be transformed. Although the first success in the genetic transformation of wheat was reported more than 18 years ago,³⁴ transformation methods remain genotype-dependent and have been limited to the few bread wheat cultivars that can be regenerated efficiently, the most common of which is the model spring wheat Bobwhite. Other US wheat cultivars that have been successfully transformed include the winter wheats Jagger³⁵ and Florida³⁶ and the spring wheats Hi-Line³⁷ and Anza.³⁸ While Bobwhite has poor flour quality and is not grown commercially in the US, it has been used in many transformation studies, some of which have addressed questions about flour quality. Some of the first studies examined the effects of expression or overexpression of different HMW-GS on flour quality.^{39–41} An RNA interference approach has also been used to silence a specific HMW-GS gene in Bobwhite.⁴² While these studies have shown that different HMW-GS can have distinct effects on dough mixing properties, ultimately it is desirable to introgress transgenes from Bobwhite into advanced cultivars by conventional breeding to truly assess the effects of the transgenes on flour quality.^{43,44} Only a few transgenic studies using Bobwhite have focused on gluten proteins from the more complex LMW-GS and gliadin families. Masci et al.⁴⁵ overexpressed a LMW-GS and Gil-Humanes et al.⁴⁶ used RNA interference to silence gamma gliadin genes. More recently, Gil-Humanes et al.⁴⁷ created an RNAi construct that consisted of conserved regions from alpha, gamma and omega gliadins and effectively silenced the bulk of gliadin genes in Bobwhite.

While the goals of some studies are to silence all members of a gene family, RNA interference also is a powerful method for silencing specific genes within a family.³³ Such approaches require knowledge of the complement of genes expressed in a cultivar so that target sequences that are unique to particular genes can be identified. It is interesting that most of the alpha and gamma gliadin gene sequences expressed in Butte 86 were not found

Table 2. Primers used for the construction and detection of the omega gliadin RNAi transgene

Primer	Sequence	Gene
4F50	TGT GTG ATC GAT CAA CCA CCA CAA CAA TTC	Omega gliadin
4R50	CTA TCT AAG CTT TTA CAT CTC TTC ATT TCA TAG G	Omega gliadin
4F30	GAG ACT GCA GCA ACC ACC ACA ACA ATT C	Omega gliadin
4R30	CAT GGA ATT CTT ACA TCT CTT CAT TTC ATA GG	Omega gliadin
4F39	GAG AAA GCT TGT AAG ATC AAG ATC AAG CAC GC	Starch synthase
4R39	CTA GAA GCT TGG CGC GCA ACA AGG	Starch synthase
4F73	TGT CCT GAA CCT TCA CCA CG	Dy10 HMW-GS
4R73	AGC TTG AAC TAG TAG GCG TGC	Starch synthase

among the hundreds of gliadin sequences already reported in NCBI.^{13,14} Thus, the ability to distinguish closely-related gluten protein gene sequences in Butte 86 combined with the capability to genetically transform this cultivar provides unique opportunities to use biotechnological approaches to test hypotheses about the roles of specific gluten proteins in gluten polymer formation and flour quality and to devise strategies to reduce the immunogenic potential of wheat flour for patients with celiac disease and food allergies.

Materials and Methods

Growth of plants, preparation of RNA and DNA. The US hard red spring wheat *Triticum aestivum* 'Butte 86' was grown in a climate-controlled greenhouse under a moderate temperature regimen with a daytime maximum temperature of 24°C and a nighttime minimum temperature of 17°C as described in Altenbach et al.¹⁷ except that Peters Professional water soluble 15-16-17 fertilizer (Scotts-Sierra Horticultural Products Company) was substituted for Plantex 20-20-20 fertilizer. Heads were tagged at anthesis. Total RNA was prepared from endosperm obtained from grains at 8, 16, 24 and 32 DPA and genomic DNA was prepared from young leaf tissue as described previously in reference 48. Equal amounts of RNA from each time point were pooled for use in RT-PCR.

Omega gliadin RNA interference construct. Three DNA fragments were amplified for use in the RNA interference construct. Two 177 bp fragments contained a 153 bp portion from the 3' end of the coding region of the omega-5 gliadin and were amplified from cDNA prepared from Butte 86 RNA using either primer pair 4F50/4R50 or 4F30/4R30 which differ in the restriction sites added to the 5' ends of the primers (Table 2). In addition, the fourth intron from the wheat starch synthase gene was amplified from Butte 86 genomic DNA using the primer pair 4F39/4R39 (Table 2). For cloning, the omega gliadin fragment amplified with 4F50/4R50 was digested with Cla I and Hind III and inserted into pBluescript SK⁺ that had been digested with the same enzymes, resulting in the plasmid, Omega-Trig-1. The starch synthase intron amplified with 4F39/4R39 was then digested with HindIII and inserted into the Hind III site of Omega-Trig-1, generating the plasmid Omega-Trig-1-SS. Finally, the omega gliadin fragment amplified with 4F30/4R30 was digested with EcoRI and PstI

and inserted into the Omega-Trig-1-SS plasmid that had been digested with EcoRI and PstI, resulting in the plasmid Omega-Trig1-SS-Trig2#5 containing the omega gliadin hairpin. The omega gliadin hairpin sequence was removed from pBluescript SK⁺ by digesting with HincII and Sma I and inserted into the Hpa I site of plasmid pJL10P5 between the HMW-GS Dy10 promoter and HMW-GS Dx5 terminator (pJL10P5 kindly provided by Dr. Ann Blechl, USDA-ARS, Albany, CA) (Fig. 1B). The final plasmid was named Omega-RNAi-13. At each step, junctions between the DNA fragments were verified by DNA sequencing. Omega-RNAi-13 will be made available upon request.

Wheat transformation. Wheat transformation methods developed by Weeks et al. for the cultivar Bobwhite with modifications by Blechl and Anderson⁴⁰ and Okubara et al. were further adapted to Butte 86. In short, wheat heads were collected from Butte 86 plants at 12 DPA and kernels were removed, surface sterilized and dissected the same day. Immature embryos with attached scutella were removed aseptically from the kernels and placed embryo axis side down on MMS media containing 2 mg/L of 2,4 D. In some cases, embryo axes were removed or destroyed and only the scutella were cultured as described by Barcelo and Lazzari.⁵¹ Explants were cultured for six to eight days in the dark to allow the formation of callus. Four hours prior to bombardment, calli were transferred to media containing 0.5 M sucrose. Two plasmids, Omega-RNAi-13, containing the RNAi transgene and pAHC20,⁵² containing the selectable marker gene *bar* under the control of the maize *Ubi1* promoter, were co-bombarded into Butte 86 tissue at a 2:1 molar ratio using a Model PDS-1000/He Biolistic Particle Delivery System (BioRad) and 0.6 micron gold particles (BioRad). Gold particles were coated with 12.5 µg pACH-20 and 42 µg Omega-RNAi-13 as described by Weeks et al. Approximately 20 hours after bombardment, explants were returned to MMS media containing 2 mg/L of 2,4 D and cultured at room temperature in the dark for 3–4 w with one intermediate transfer to fresh media. The resulting calli were then transferred to MMS media containing 0.1 µg/ml 6-benzylaminopurine and either 1.5 or 2 mg/L bialaphos (Gold Biotechnology) in addition to 0.2 mg/L 2,4 D and incubated in the light to promote shoot formation. Calli were transferred to new media at 2 w intervals. Green shoots greater than ½ inch were transferred to culture tubes containing MMS media and 2 mg/L bialaphos. Plantlets with green shoots that formed

strong roots were transferred to soil and allowed to acclimate as described in Weeks et al. The plants were then transferred to the greenhouse.

Analysis of transgenic plants. Leaf DNA was prepared from transgenic plants and amplified with the primers 4F73/4R73 (Table 2 and Fig. 1B) using the REDExtract-N-Amp Plant PCR kit (Sigma Aldrich, Inc.,) according to the manufacturer's instructions. PCR reactions were carried out for 35 cycles with denaturation at 94°C, annealing at 65°C for 30 sec and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The size of the fragment amplified from Omega-RNAi-13 was 339 bp.

For extraction of total protein, individual mature wheat grains were crushed, transferred to 1.5 ml microfuge tubes containing 200 µl 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 8.5, 1% DTT and mixed for 20 sec using a Talboys Model 102 mixer at setting 10. An additional 200 µl of the extraction buffer was added to each sample and proteins were extracted at room temperature for 30 min. Samples were centrifuged for 10 min at 4°C in a TOMY MR-150 microfuge and 3 µl of each supernatant fraction diluted 1:4 in extraction buffer was analyzed by SDS-PAGE. For preferential extraction of gliadins, individual grains were ground in 200 µl 50% propanol as for the total protein extraction. An additional 200 µl 50% propanol was added to each sample and proteins were extracted for 30 min at 22°C with constant mixing in an Eppendorf

Thermomixer 5436 at a speed setting of 12. After centrifugation for 10 min at 4°C in a TOMY MR-150 microfuge, the resulting supernatant fractions were removed and completely dried in a Speed Vac (Savant). The samples were resuspended in 400 µl 2% SDS, 10% glycerol, 62.5 mM Tris-HCl, pH 8.5, 1% DTT at 22°C with constant mixing in an Eppendorf Thermomixer 5436 at a speed setting of 14 for 30 min. The resulting protein samples were analyzed on 4–12% NuPAGE Novex Bis-Tris gel in MES buffer (Invitrogen), according to the manufacturer's instructions.

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