Environmental Conditions During Wheat Grain Development Alter Temporal Regulation of Major Gluten Protein Genes

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ABSTRACT

The accumulation of transcripts for the major gluten storage proteins was assessed during grain development in wheat plants (Triticum aestivum L. ‘Butte 86’) grown under seven controlled environments where temperature, water, and fertilizer conditions were varied after anthesis. Transcripts within the α-, γ- and ω-gliadin, low molecular weight glutenin subunit (LMW-GS), and high molecular weight glutenin subunit (HMW-GS) gene families followed similar patterns of accumulation throughout grain development under each environmental regimen. Under moderate daytime temperatures in plants that were well watered and fertilized, transcripts from all gene families were detectable by eight days postanthesis (8 DPA), were present at high levels until ∼34 DPA, and disappeared between 36 and 38 DPA. Under high temperature regimens, transcripts from all gene families appeared slightly earlier, but the time frame of accumulation was shorter. The presence or absence of postanthesis fertilizer did not alter the temporal regulation of the gluten genes under the moderate temperature regimen. However, under the high daytime temperature regimen, transcripts disappeared slightly earlier in kernels from plants that did not receive postanthesis fertilizer. Reverse transcriptase polymerase chain reaction (RT-PCR) using primer pairs for specific HMW-GS and LMW-GS transcripts revealed that individual genes within each family exhibited identical patterns of temporal regulation under different environmental conditions.

The gluten proteins, consisting of gliadins and glutenin subunits, are the major storage proteins accumulated in wheat (Triticum aestivum L.) endosperm and confer the elasticity and extensibility properties essential for the functionality of wheat flours (Shewry et al 1994, 1995). The gliadins, encompassing ≈40–50% of the total endosperm protein, are a polymorphic collection of proteins soluble in 70% alcohol that range in size from 30 to 60 kDa and can be separated into α, γ, and ω subgroups on the basis of size and sequence homology. The gliadins are encoded by large complex gene families (Anderson et al 1997, 2001; Hsia and Anderson 2001). For example, the α-gliadin gene family may contain as many as 150 genes (Anderson et al 1997). A second class of proteins, the glutenins, are relatively insoluble polymers that consist of high molecular weight glutenin subunits (HMW-GS) of ≈90 kDa and low molecular weight glutenin subunits (LMW-GS) of ≈30 kDa linked by interchain disulfide bonds.

There are six HMW-GS genes in most hexaploid wheat cultivars, only five of which are generally expressed, and as many as 30–40 LMW-GS genes (Cassidy et al 1998). In terms of flour functionality, the gliadins contribute to extensibility while the glutenins contribute to strength and elasticity. Genetic studies have demonstrated that breeding quality correlates with the presence or absence of specific allelic variants of HMW-GS (Payne 1987; Wieser and Zimmermann 2000) and to a lesser extent with specific LMW-GS (Gupta et al 1989). There is also a correlation between the amount of HMW-GS contained within the gluten polymers and the breadmaking quality of the flour (Field et al 1983).

Environmental conditions during wheat grain development influence the quality of flour (Peterson et al 1992, 1998; Graybosch et al 1995), creating challenges for commercial bakers who strive to produce uniform high-quality products. Changes in the amount of gluten transcripts or in the temporal regulation of gluten protein genes in response to environmental conditions could lead to alterations in flour quality. Ratios of the different classes of proteins or of specific proteins within each class could change and thus affect the formation of glutenin polymers. In this report, we examine the accumulation of transcripts for the major classes of gluten storage proteins throughout grain development in kernels from plants subjected to seven different environmental regimens between anthesis and harvest maturity. Our experiments address the effects of temperature and water availability under two different postanthesis fertilizer regimens on the regulation of genes encoding the α-, γ-, and ω-gliadins and the LMW-GS and HMW-GS in a U.S. spring wheat.

MATERIALS AND METHODS

Growth of Plant Material

The U.S. hard red spring wheat ‘Butte 86’ was grown in a climate-controlled greenhouse with a daytime maximum temperature of 24°C and a nighttime minimum temperature of 17°C until anthesis. Plants were well watered with a dilute solution of Plantex 20-20-20 fertilizer (0.6 g/L) before anthesis and natural light was supplemented with 100W high-pressure sodium lights to maintain a day length of 16 hr. Heads were tagged with the date of anthesis. From the day on which the majority of heads had undergone anthesis, the plants were placed under one of the environmental regimens shown in Table I. In one experiment, plants grown under the 24/17, 37/17, and 37/17°C plus drought regimens, with and without postanthesis fertilizer, were compared. There were six pots per treatment, each containing seven plants. Developing kernels from single heads were harvested at one-day intervals between 3 and 8 DPA and at two-day intervals from 10 DPA until maturity. In another experiment, plants grown under the 24/17 and 37/17°C regimes with and without postanthesis fertilizer were compared. There were nine pots per treatment and developing kernels from single heads were harvested at two-day intervals between 3 and 7 DPA and at three- to five-day intervals from 10 to 35 DPA. Two heads were sampled at each developmental stage. In two additional experiments, plants grown under the 24/17 and 37/28°C regimes with postanthesis fertilizer were compared. In the first additional experiment, there were six pots per treatment and heads were harvested at two-day intervals from 3 DPA until maturity. In the replicate experiment, 13 and 22 pots containing seven plants each were grown under the 24/17 and 37/28°C regimes, respectively, and heads were harvested at two-day intervals from 4 DPA until maturity. All tissue was frozen immediately in liquid nitrogen and stored at −80°C. RNA was isolated from kernels obtained from single heads as described previously (Altenbach 1998). The integrity of each RNA sample was examined by electrophoresis on 1.5% formaldehyde agarose gels.

Hybridization Analysis

After denaturation at 65°C for 15 min in 20 mM MOPS (4-morpholinepropanesulfonic acid), 5 mM NaOAc, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.0, containing 50% formamide

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and 6.5% formaldehyde, 50 or 100 ng of each total RNA sample was applied to a nylon membrane (Roche Molecular Biochemicals, Indianapolis, IN) using a slot blot manifold. RNA was cross-linked to the membrane (Stratalinker crosslinker, Stratagene, La Jolla, CA) and hybridized to digoxigenin-labeled probes in DIG Easy Hyb buffer (Roche Molecular Biochemicals) at 42°C overnight. After hybridization, the membrane was washed at 65°C in 0.1x SSC, 0.1% SDS. Detection of hybridized sequences was performed using a luminiscent detection kit (DIG, Roche Molecular Biochemicals) according to the manufacturer’s directions.

Digoxigenin-labeled probes were generated using the PCR synthesis kit (DIG Probe, Roche Molecular Biochemicals). For the α-gliadins, most of the coding sequence was amplified from genomic clone CNN10 (Genbank Accession #U51303) (Anderson et al 1997) using primers with the sequences CTCCTTGCTATCGTGCGG and AGTACCGAAGATGCCAAAATGG. For the γ-gliadins, a 755 bp fragment was amplified from genomic clone G1 (Genbank Accession #AF234647) (Anderson et al 2001) using primers with the sequences GCCAATATGGAAGTCGCC and CAACTACCCGCTGATCCC. The 1,030 bp probe used to detect α-gliadin transcripts resulted from the amplification of clone G3 (Genbank Accession #AF280605) (Hsia and Anderson 2001) with the primers GCTAGGCAGCTAAACCCTAGC and CCGATGCTTGTAAGACTACTCCC. For the LMW-GS, a 484 bp fragment spanning the

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<th>Growth Regimens for Butte 86 Plants During Grain Development</th>
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<tr>
<td>Regimen</td>
<td>Temperature (°C)a</td>
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<tr>
<td>Moderate daytime temperatures with fertilizer</td>
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<tr>
<td>High daytime temperatures plus drought without fertilizer</td>
<td>37/17</td>
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a Day/night maximum; 24/17 and 37/17°C regimen temperatures maintained for 5 (day) and 11 hr (night), separated by intermediate 4-hr periods at either 21 or 26.7°C, respectively. 37/28°C regimen temperatures maintained for 4 (day) and 11 hr (night), separated by intermediate 4- or 5-hr periods at 30°C.
b Pots weighed daily and adjusted to 6 kg (80% of soil capacity) or 2.5 kg (10% of soil capacity).
c 0.6 g/L of Plantex 20-20-20.
repetitive region was amplified from clone F23 (Genbank Accession #U86027) (Cassidy et al 1998) using primers F2 and R10 as described in Altenbach (1998). For the HMW-GS, a 1,765 bp fragment was amplified from a clone encoding the Dy10 subunit (GenBank Accession #X12929) (Anderson et al 1989) using primers with the sequences TATGAGCAAACTGTGGTGCC and AACTGTGACACGCATCACG. All probes were tested by hybridization to cloned gene sequences representative of each gene family. A small amount of cross-hybridization was detected between the α-gliadin probe and a γ-gliadin gene and between the γ-gliadin probe and two different α-gliadin genes. All other probes hybridized only to the expected sequences. For use in evaluating the amount of total RNA loaded on each slot blot, a 242 bp probe for the 18S rRNA was generated by amplifying cDNA made from total kernel RNA with the primers TTCATACAGGTGCTGCATGG and AGACGACTTCG

RT-PCR Analysis Using Gene-Specific Primers

Primers specific for LMW-GS genes were described previously (Altenbach 1998). Primers with the following sequences were used to amplify the 5' regions of individual HMW-GS genes: Dx5: GCGGTAGTCCTCTTGTGG and TGCGGACAAGTTACACTTGG; Bx7: AGCAACTCCGAGACGTAGTAGACC; Ax2*: AGCGGTTGGTCTTTTTGCC and CTTTGTTGAGTGGTGTTGCC; Dy10: AGCGTCCGAGATGTTAGC and TGGCCTGGATAATATGACCC; and By9: AGCAGCTCCGAGATGTTTAGC and CTTGAGAAGACCTAGGCTTAGG. RT-PCR of RNA from developing grains of ‘Cheyenne’ at 15 DPA yielded single amplification products with each primer pair that were confirmed to be the desired HMW-GS sequences by digestion with specific restriction endonucleases (not shown).

RT-PCR was performed according to the basic protocols accompanying the reagents and enzymes (Applied Biosystems, Foster City, CA). Total RNA was reverse-transcribed in a reaction containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 5 mM MgCl2, 1 mM of each dNTP, 2.5 mM random hexamers, 1 unit/µL RNase inhibitor, and 2.5 units/µL MuLV reverse transcriptase in a final volume of 20 µL. The sample was incubated at room temperature for 10 min, followed by either 15 min (HMW-GS) or 60 min (LMW-GS) at 42°C, 5 min at 99°C, and 5 min at 5°C in a Perkin Elmer Cetus DNA Thermal Cycler 480. Amplifications were performed in 100-µL reaction volumes containing 20 µL of the reverse transcription mix, 2.5 units of AmpliTaq DNA polymerase, and 20 pmol of each oligonucleotide primer. The concentrations of Tris-Cl, pH 8.3, and

Fig. 2. Effect of high daytime and high nighttime temperatures on the accumulation of gluten protein transcripts in developing kernels supplied with postanthesis fertilizer. Equal amounts of total RNA from developing kernels were hybridized to α-gliadin (A), γ-gliadin (B), ω-gliadin (C), LMW-GS (D), HMW-GS (E), or 18S rRNA (F) probes. In each panel, lanes 1 and 2 contain RNA from plants grown under 24/17 and 37/28°C regimens, respectively. Ages of developing kernels are indicated on the left in days postanthesis. Lane 3 shows hybridization of each probe to increasing amounts of kernel RNA or to 100 ng of leaf RNA.

Fig. 3. Effect of temperature and drought on accumulation of gluten protein transcripts in developing kernels from plants that did not receive postanthesis fertilizer. Equal amounts of total RNA from developing kernels were hybridized to α-gliadin (A), γ-gliadin (B), ω-gliadin (C), LMW-GS (D), HMW-GS (E), or 18S rRNA (F) probes. In each panel, lanes 1, 2, and 3 contain RNA from plants grown under 24/17, 37/17, and 37/17°C plus drought regimens, respectively. Ages of developing kernels are indicated on the left in days postanthesis. Horizontal strip at bottom of panels shows hybridization of each probe to increasing amounts of kernel RNA or to 100 ng of leaf RNA.
KCl in the final reaction were adjusted to 10 and 50 mM, respectively. Amplifications of the LMW-GS and HMW-GS Bx7, Ax2*, and By9 genes were made at 95°C for 90 sec, followed by 25 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min. A final extension was made at 72°C for 7 min, and the samples were incubated at 4°C until analysis. The HMW-GS Dx5 and Dy10 genes were amplified under the same conditions, except that the annealing step was conducted at 60 instead of 56°C. Amplification of each RNA sample without prior reverse transcription confirmed the absence of contaminating DNA. Aliquots of RT-PCR products were analyzed in 1.5 or 2% agarose gels in TBE buffer following standard procedures.

RESULTS

Effect of High Temperatures and Drought on Accumulation of Gluten Protein Transcripts

In kernels from plants grown under the moderate daytime temperature regimen with postanthesis fertilizer, transcripts from each of the major storage protein gene families were apparent by 8 DPA and exhibited similar patterns of accumulation (Fig. 1A-E, lane 1). Transcripts increased in amount until ≈18 DPA, remained at reasonably constant levels until 34 DPA, and declined dramatically by 38 DPA. Sampling of RNA at two-day intervals late in grain development revealed that the levels of all transcripts tapered off at 36 DPA (Fig. 1G-K, lane 1). Under high daytime temperatures and high temperature regimen with postanthesis fertilizer, transcripts from each gene family were apparent slightly earlier, by 7 DPA, under the high daytime temperature regimen and were present only until 30 DPA (Fig. 1A-E, G-K, lane 2). When plants were subjected to drought conditions in addition to the high daytime temperatures, transcripts were detected even earlier, by 6 DPA, but were present only until 26 DPA (Fig. 1A-E, G-K, lane 3). Although there was some variation in amount of transcripts between samples, maximum levels of transcripts in kernels from plants subjected to moderate temperatures, high daytime temperatures, and high daytime temperatures plus drought were similar overall. The transcript accumulation profile was compressed even more dramatically when plants were subjected to high daytime as well as high nighttime temperatures during grain development. Transcripts appeared by 5 DPA, but were accumulated only until 20 DPA (Fig. 2A-E, lane 2). Again, transcripts within all gene families appeared and disappeared at the same time and levels of transcripts did not vary substantially with increased temperatures.

The timing of gluten protein transcript accumulation was similar when plants were grown under the moderate temperature regimen with or without postanthesis fertilizer (Fig. 3A-E, lane 1). However, under the high daytime temperature regimen, transcript levels declined somewhat earlier when postanthesis fertilizer was omitted, after 26 DPA (Fig. 3A-E, lane 2) rather than 30 DPA (Fig. 1A-E, lane 2). The earlier decline in transcripts was also noted in a replicate experiment in which RNA was sampled at less frequent intervals (Fig. 4). When high daytime temperatures were combined with drought, the profile of transcript accumulation in the absence of fertilizer was similar to that observed with fertilizer and transcripts appeared by 6 DPA and declined after 26 DPA (Fig. 3A-E, lane 3).

Accumulation of Transcripts Corresponding to Individual HMW-GS and LMW-GS Genes

The five HMW-GS genes expressed in Butte 86 encode the Dx5, Dy10, Bx7, By9, and Ax2* subunits. RT-PCR with primer pairs specific for each of these five genes was used to detect the presence of individual HMW-GS transcripts in RNA prepared from kernels early and late in grain development. Under the moderate temperature
regimen, transcripts corresponding to all five HMW-GS were barely detectable at 8 DPA but were clearly present by 10 DPA (Fig. 5A). Under high daytime temperatures in kernels from well-watered plants, the five primer pairs amplified fragments of the expected sizes at 7, 8, and 10 DPA (Fig. 5B). All five HMW-GS transcripts could be detected from six DPA in kernels from plants subjected to high daytime temperatures plus drought (Fig. 5C). A trace of Dy10 transcript detected in the 6 DPA sample from the heat-treated plants (Fig. 5B) may be due to the more efficient amplification of Dy10 sequences with the selected primers. Late in grain development, the transcript levels of all five HMW-GS genes declined after 34 DPA under moderate temperatures (Fig. 5D), 30 DPA under high daytime temperatures (Fig. 5E), and 26 DPA under high daytime temperatures plus drought (Fig. 5F).

RT-PCR also was used to examine the expression of a sampling of individual LMW-GS genes. The five primer pairs amplified LMW-GS sequences from 8 and 10 DPA kernels but not from 6 or 7 DPA kernels from plants grown under the moderate temperature regimen (Fig. 6A). The same LMW-GS sequences were evident by 7 DPA as well as 8 and 10 DPA in kernels from plants grown under high daytime temperatures (Fig. 6B) and at 6, 7, 8, and 10 DPA in plants subjected to high temperatures plus drought (Fig. 6C). Late in grain development, all five primer pairs amplified the expected bands from 26, 30, and 34 DPA kernels from plants grown under the moderate temperature regimen but not from 38 or 42 DPA kernels (Fig. 6D). When RNA from kernels produced under high daytime temperatures was used for RT-PCR, amplification products corresponding to the expected LMW-GS sequences resulted from 26 and 30 DPA samples, but not from 34 DPA samples (Fig. 6E). Amplification products were detected with all primer pairs from 22 and 26 DPA kernels but not from 30 DPA kernels when plants were subjected to high temperatures plus drought (Fig. 6F).

**Timing of Gluten Protein Transcript Accumulation Relative to Key Events in Wheat Grain Development**

In Fig. 7, the timing of gluten protein transcript accumulation is compared to the timing of key developmental events in kernels from plants grown under the same environmental regimens. Under all environmental conditions, gluten protein transcripts first appeared early in the developmental program as the kernels were taking up water and beginning to expand (Fig. 7A,B). The time in the developmental program when transcripts declined varied under different environmental conditions. When plants were well watered and fertilized, transcript accumulation paralleled total protein accumulation, and transcripts were abundant until several days after kernels reached maximum dry weight (Fig. 7A). However, maximum dry weight was achieved significantly earlier when plants were grown under high temperature rather than moderate temperature regimens. Under moderate temperatures in the absence of postanthesis fertilizer, transcripts also were present until several days after kernels achieved maximum dry weight, but protein accumulation ceased at least 10 days earlier (Fig. 7B). In comparison, under high daytime temperatures in the absence of postanthesis fertilizer, transcripts declined several days before kernels achieved maximum dry weight and transcript accumulation paralleled protein accumulation (Fig. 7B). When drought accompanied high daytime temperatures under either fertilizer regimen, gluten transcripts were present for about six days after the kernels achieved maximum dry weight, nearly until the time that kernels reached harvest maturity (Fig. 7A,B).

**DISCUSSION**

The accumulation of transcripts in each of the major gluten gene families was analyzed in detail in developing kernels from plants grown under different postanthesis environmental regimens.
Frequent sampling of RNA made it possible to pinpoint the appearance and disappearance of transcripts in each of the major gluten gene families within a one- to two-day time frame and thereby demonstrate the highly coordinated regulation of the α-, γ-, and α-gliadin and the HMW-GS and LMW-GS genes. RT-PCR further revealed that individual genes within the HMW-GS and LMW-GS gene families share the same pattern of temporal regulation. Reeves et al. (1986) previously demonstrated the coordinated regulation of genes within the α- and γ-gliadin families using hybridization analysis of RNA prepared from kernels at seven timepoints between 3 and 31 DPA, and Grimwade et al. (1996) found that transcripts from all gluten gene families were first detected during a six-day window between 5 and 11 DPA when RNA was examined at six timepoints between 5 and 25 DPA. RNA was not sampled late enough in grain development in either study to show that the decline in gluten transcripts was also synchronized among the different gene families. The striking similarity of transcript profiles for genes within different families suggests that the gluten protein genes are activated by a common signal early in grain development and respond to a common signal late in grain development.

The coordinated regulation of the gluten protein genes was not uncoupled by growth under high temperatures. However, temperature did exert a significant effect on the temporal regulation of the genes. Transcripts from all gluten gene families accumulated slightly earlier in development and disappeared several days earlier when temperatures increased, reflecting the acceleration and compression of the developmental program observed in the accumulation of water, dry weight, and protein in kernels from the same plants. Transcripts were detectable for a period of 28 days under the 24/17°C regimen, 23 days under the 37/17°C regimen, and 15 days under the 37/28°C regimen, when plants were supplied with postanthesis fertilizer.

Despite alterations in the temporal regulation of the gluten protein genes, there was little change in the amount of transcripts accumulated under the different temperature regimens. Perrotta et al. (1998) also reported that the levels of gluten protein transcripts did not change when developing grains from two durum and two bread wheat cultivars were subjected to high temperatures. Their study differed from the study presented here in that high temperature stress was induced either in the field by covering plants with plastic tunnels or in growth cabinets by increasing the daytime temperature gradually from 20 to 40°C over a 14-day period after anthesis. Nonetheless, neither study supports the hypothesis put forth by Blumenthal et al. (1990) that high temperatures affect the transcription of gliadin genes, thereby altering the gliadin to glutenin ratio and affecting dough properties. This hypothesis was based largely on the presence of putative heat shock consensus elements in the upstream regions of several α-gliadin genes. In fact, we did not find any evidence from steady-state RNA analyses to suggest that the complement of gliadins and glutenins synthesized at any stage during grain development might change with temperature, nor did we observe the preferential expression of specific glutenin subunit genes that might affect glutenin polymer formation.

The coordinate regulation of the gluten protein genes was not uncoupled by withholding fertilizer or by applying drought. However, the temporal control of the gluten protein genes was complicated by the application of additional stresses. The absence of postanthesis fertilizer did not influence the timing of gluten transcript accumulation under the 24/17°C regimen, but shortened the time that transcripts were present in plants grown under the 37/17°C regimen by several days. It is interesting that total protein accumulation ceased by ~26 DPA in plants grown in the absence of fertilizer under either temperature regimen. Because transcripts remained abundant until 36 DPA under the 24/17°C regimen, it is likely that translational regulation plays an important role under these conditions. This was not the case under the 37/17°C regimen where the accumulation of transcripts more closely reflected the pattern of protein accumulation. Addition of drought to the high daytime temperature regimen also resulted in earlier decline in gluten protein transcripts, although transcripts were present much later in the developmental program than in kernels from well watered plants.

Gliadins and glutenins encompass a majority of the grain protein and are important determinants of flour quality. Thus, identifying the factors that control expression of the gluten genes is central to unraveling the complex effects of the environment on the developing grain. However, the production of gluten proteins is only one of many molecular processes influenced by environmental conditions during grain development. High-throughput transcript profiling techniques such as those used recently with Arabidopsis (Girke et al. 2000) should make it possible to develop a comprehensive molecular picture of the key events and metabolic pathways in wheat grain development that are affected by changing environmental conditions. Knowing the precise times when the very abundant gluten protein transcripts appear and disappear under different environmental conditions will be helpful for interpreting transcript profiles for nonstorage protein genes and should make it easier to pinpoint transcriptional factors that control gluten protein synthesis.

**LITERATURE CITED**


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