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Transcript profiles of genes expressed in endosperm tissue are altered by high temperature during wheat grain development

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Abstract

Timing of transcript accumulation for genes involved in a variety of cellular processes was assessed by RT-PCR in endosperm from developing wheat grains grown under moderate (24/17 °C day/night) and high (37/28 °C day/night) temperature regimens. Under moderate temperatures, transcripts for proteins with storage functions were present at all five time points examined between 7 and 34 DPA, while transcripts for proteins involved in signal transduction, protein synthesis and metabolism were most abundant from 7 to 20 DPA. Transcripts for proteins that play roles in defense were present from 14 DPA, about the time that starch accumulation commenced, to 34 DPA. High temperatures advanced and compressed the timing of transcript accumulation during grain development. Comparisons of transcript profiles with the timing of key events in grain development identified genes whose transcripts were accumulated at equivalent stages under the two temperature regimens and might serve as markers for grain development. These comparisons also revealed a number of genes with transcript profiles that were shifted under high temperatures in a manner that was not consistent with developmental events. These genes may be involved in responses to high temperature that are distinct from effects on the timing of developmental processes.
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Keywords: *Triticum aestivum* L.; Endosperm development; Transcript profiles; High temperature

1. Introduction

Complex programs of gene expression within the developing wheat grain control the timing of biochemical and physiological processes, including cell division, water uptake, accumulation of starch and protein, maturation and desiccation. Environmental conditions during grain development influence these processes in unique ways, resulting in changes in grain yield and flour quality that are of considerable importance to growers and end-users. While genomic and proteomic studies will elucidate some of the basic molecular mechanisms involved in wheat grain development, using these approaches to understand the effects of environmental variables on these mechanisms will be challenging because changes in gene expression due to the environment must be distinguished from normal developmental changes. Temperature, in particular, influences rates of biochemical reactions and timing of developmental processes in the grain. The response to temperature depends on the wheat variety, the specific temperature, and the time in the developmental program that the temperature regimen is imposed (Dupont and Altenbach, 2003; Sofield et al., 1977; Tashiro and Wardlaw, 1990; Wardlaw and Moncur, 1995).

A recent study (Altenbach et al., 2003) in which the US wheat 'Butte 86' was exposed to either moderate or very high temperatures from anthesis to maturity demonstrated that high temperatures both advanced and compressed the progression of grain development and had dramatic effects on kernel properties. The time from anthesis to harvest maturity spanned 44 days under a 24/17 °C day/night

Abbreviations: DPA, days post-anthesis; ESTs, expressed sequence tags; HMW, high molecular weight; RT-PCR, real time-polymerase chain reaction.

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regimen but only 26 days under a 37/28 °C day/night regimen. Times to maximum water content and maximum dry weight were notably shorter under high temperature conditions and indications of apoptosis of endosperm tissue became evident earlier. The onset and cessation of starch and protein accumulation also occurred earlier under high temperatures. Average kernel weight was reduced nearly 60% under the 37/28 °C regimen and mature kernels contained higher proportions of the A type starch granules formed during early stages of grain development (Hurkman et al., 2003).

The accumulation of transcripts for the major gluten proteins and a variety of enzymes involved in starch biosynthesis also was examined in endosperm developing under the two temperature regimens. Transcripts for all classes of gluten proteins were present throughout much of grain development while transcripts for the starch biosynthetic enzymes were accumulated during early stages. Under moderate temperatures, gluten protein transcripts first appeared by 8–10 days post-anthesis (DPA) and disappeared between 36 and 38 DPA whereas these transcripts were detected by 5 DPA and disappeared between 20 and 22 DPA under the high temperature regimen (Altenbach et al., 2002). Transcripts for starch biosynthetic enzymes that were most abundant between 12 and 16 DPA under moderate temperatures were prevalent around 7 DPA under high temperatures (Hurkman et al., 2003).

The availability of tentative consensus sequences for genes expressed in developing endosperm and grains makes it possible to examine the timing of transcript accumulation for genes involved in other processes during grain development. These sequences have been assembled from more than 500,000 expressed sequence tags (ESTs) generated by wheat genome projects (Clarke et al., 2000; Ogihara et al., 2003; http://wheat.pw.usda.gov/NSF/). In this paper, we survey the accumulation profiles of transcripts in developing endosperm for a collection of 57 genes involved in metabolism, signal transduction, carbohydrate and storage protein synthesis or defense and identify transcripts that are present at various stages of grain development. While the timing of expression for a few of these genes was reported previously (Altenbach et al., 2002; Gautier et al., 1990, 1994; Grimwade et al., 1996; Hurkman et al., 2003; Monnet et al., 2001; Sanchez de la Hoz et al., 1994; Singh et al., 1993), these experiments are the first to compare the expression of genes involved in many different processes in wheat endosperm tissue. By examining patterns of transcript accumulation in endosperm under two controlled regimens, we assess the effects of high temperatures on the timing of gene expression during grain development and highlight the difficulties in examining gene expression in terms of developmental time rather than chronological time. These studies form the background for more detailed analyses of gene expression and protein accumulation using microarray analysis and proteomics.

2. Experimental

2.1. Growth of plants and collection of endosperm

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. The maximum and minimum temperatures were maintained for 5 and 11 h, respectively, separated by 4 h periods at 21 °C. Plants were watered with a dilute solution of Plantex 20-20-20 fertilizer (0.3 g/l) using an automatic drip irrigation system equipped with a fertilizer injector. Natural light was supplemented with 100 W high-pressure sodium lights to maintain a daylength of 16 h. Pots were rotated weekly to minimize positional effects in the greenhouse. At anthesis, heads were tagged and the date was noted. When the majority of heads had undergone anthesis, half of the plants were transferred to a second greenhouse equipped with similar irrigation and lighting systems. This greenhouse was maintained at a maximum daytime temperature of 37 °C and a minimum nighttime temperature of 28 °C, held for 4 and 11 h, respectively, and separated by 4 or 5 h periods at 30 °C. Environmental data was collected at 5 min intervals throughout the experiment using GrowLink v. 2.1 software (MicroGrow Greenhouse Systems, Inc., Temecula, CA). There were 17 pots per treatment, each containing 7 plants. Endosperm tissue from individual heads was harvested at 7, 14, 20, 28 and 34 DPA from plants grown under the 24/17 °C regimen and at 5, 8, 12, 16, and 20 DPA from plants subjected to the 37/28 °C regimen. Twenty heads were harvested from the 7 DPA time point from the 24/17 °C regimen and from the 5 and 8 DPA time points from the 37/28 °C regimen. Ten heads were harvested for all other time points. Endosperm was collected, frozen immediately in liquid nitrogen and stored at −80 °C. In this experiment, average kernel fresh weights at the 7, 14, 20, 28 and 34 DPA time points under the 24/17 °C regimen were 17.08, 48.13, 62.67, 79.52 and 87.75 mg, respectively. Average kernel fresh weights at the 5, 8, 12, 16, and 20 DPA time points under the 37/28 °C regimen were 18.98, 28.89, 33.84, 35.92 and 29.55 mg, respectively. The average weight for mature kernels was 58.04 mg for the 24/17 °C regimen and 20.00 mg for the 37/28 °C regimen.

2.2. RNA preparation and RT-PCR analysis

Endosperm tissue from 3 heads was combined and total RNA was isolated as described previously (Altenbach, 1998). Poly A RNA was prepared using an Oligotex kit (QIAGEN, Inc., Valencia, CA), according to the manufacturer's instructions. The amount of poly A-RNA was quantified by scanning on a spectrophotometer. Because insufficient quantities of mRNA were obtained at 20 DPA from the 37/28 °C regimen, two mRNA preparations were

Table 1
Selection of tentative consensus sequences and genes from TIGR *Triticum aestivum* Gene Index (TaGI) and NCBI Genbank, respectively, primers specific for each sequence, sizes of expected amplification products, representation of sequences in selected cDNA libraries and expression profiles in endosperm developing under a 24/17 °C regimen

Tentative consensus sequence or gene	ID	Forward primer	Reverse primer	Amplicon size (bp)[a]	#ESTs in #AQN[b]	#ESTs in 5468[c]	24/17 °C transcript profile[d]
Storage							
CM1 *alpha*-amylase/trypsin inhibitor	TC168914	CAGGTCCATATTGCTACGCC	ATATGCATGCCACACATCC	429	0	0	A
	TC168912			429	8	18	
CM16 *alpha*-amylase/trypsin inhibitor	TC147093	AAGCTGCCGTGACTATGTGG	CATGTATGGTCGCAACATGC	356	10	17	A
CM17 *alpha*-amylase/trypsin inhibitor	TC147299[e]	ACTACAATCTCCTCTTCACGGC	CATGTATGGTCGCAACATGC	465	5	11	A
	X59791[f]			465	N/A	N/A	
CM3 *alpha*-amylase/trypsin inhibitor	TC146656	TGGCTAGAATACCATGGCG	AGATCCACAGAGGCTGTTCC	517	27	32	A
CMx trypsin inhibitor	TC147979[e]	TCACATACGAGTCGCTTAACG	AACATCATCCTCTCGGATGC	458	4	0	E
	TC147978[e]			452	1	10	
	X75610[f]			458	N/A	N/A	
	X75609[f]			458	N/A	N/A	
	X75608[f]			458	N/A	N/A	
Globulin	TC169700	TATGGCAGTGAGACAGGAGG	AGAGCTCACACATTGCATCG	301*	0	0	A
	TC169699[e]			292*	1	1	
Grain softness protein-1a	TC146459	AATTGCGAGGAAGAGCAGC	CGGATCAATGTTGCACTGG	300	4	26	A
Grain softness protein-1b	TC170239	CGAGGAAGAGCATCCAAAGC	GATCAATGTTGCACTTGGAGG	293	1	7	A
HMW glutenin subunit 1Dy10	X12929[f]	AGCAGCTCCGAGATGTTAGC	TGGCCTGGATAATATGACCC	228	N/A	N/A	A
Ima1 monomeric *alpha*-amylase inhibitor	TC169519	AAGGAGCCATAACAGTGGTCC	GCAGATTCGCTTGACTAGACG	393	14	22	A
Puroindoline b	TC147582	GTGGTTCTCAACAATGTCCG	TTGAATACCTCACCTCGCC	262	4	15	A
Triticin	TC168319	CAGGTTGTCAATGACCATGG	GACTCTTCTTCGCCGTTAGG	338	3	5	A
Unknown protein 1[g]	TC169873	GAAGACAATGTTCATCCTCGC	AATGATAGCCTCTTCCACGC	319	5	6	E
Unknown protein 2[h]	TC168313	TGTCCAGTCACAGATGTGGC	TACCACCAACACAGGTAGCG	350	1	0	A
	TC168310			347	5	4	
WASI *alpha*-amylase/subtilisin inhibitor	TC85394[i]	ACGAGTACAAGCTCATGGCG	AGTGAGGACAGCACATCACC	261	3	0	A
	TC144618[e]			261	6	4	
Protein synthesis/folding/transport/turnover							
20S proteasome alpha subunit B 1	TC169834	GACTGCTGCTGTTATGCAGG	CATCAAGCTCCATATCTTCGG	218*	0	1	B
	TC169832[e]			218*	1	0	
26S proteasome AAA-ATPase subunit 2	TC169887	TGTTGGAGTTGCTGAATCAGC	GCACTTGGATAATGCCTTCG	354*	0	0	B
	TC169886			354*	0	2	
26S proteasome AAA-ATPase subunit 3	TC65933[i]	CCATCAAGTTGGTTCAGAAGC	TTGAGAGTCTCGGAATTGCC	324*	1	1	B
	AF475120[e,f]			328*	N/A	N/A	
ADP ribosylation factor	TC145165	GTTACGTGATGCTGTGCTGC	GGTGATTCAATCGTCCATCC	259*	1	1	A
	TC145173			259*	1	4	
Aspartic proteinase	TC67604[i]	TTCAGCCAAGAGTGCAAGG	CTCATCCGGTCATCTATGCC	586	1	5	B
	TC147809[e]			586	1	5	
Clp protease	TC166654	GGATCAAGAGCTTGGTGACC	CGGAACTTCTCTGTTACCTGG	193*	0	0	C
Cyclophilin	TC146664	AGTTCGTCCACAAGCACACC	CAGCAGACACAGGAAGATAACC	453*	1	2	B
	TC168882			437*	0	1	
	TC146524			441*	1	2	

(*continued on next page*)

Table 1 (*continued*)

Tentative consensus sequence or gene	ID	Forward primer	Reverse primer	Amplicon size (bp)[a]	#ESTs in #AQN[b]	#ESTs in 5468[c]	24/17 °C transcript profile[d]
Elongation factor 1-alpha	TC145093	CCAACCTTGACTGGTACAAGG	ACGTTCTTGACGTTGAAGCC	294*	0	0	B
	TC145075		CCACATGAAGAGAATATCGGC	292*	0	1	
	TC145015			292*	13	3	
	TC145024			292*	1	4	
Endopeptidase	TC147628	CATCAAGCAGCAGTACGAGG		226	2	1	C
Protein disulfide isomerase	TC147747	TCATCCTCGTCGAGTTCTACG	GAATAGCCTAACCAATGGCC	500*	2	6	B
	TC148236			500*	4	4	
	TC144738			500*	0	4	
Sec61p	TC169339	AACTGCACCATCAAGTATGGC	ACGGAAGATGACAGGTGACG	631*	1	2	D
Transcription/RNA stability							
Glycine-rich RNA binding protein	TC166498[j]	CCACTCAGTCCTCGGTTCC	GTTGACGGTGATGTTGCG	438**	0	0	C
	TC166451			316*	7	1	
	TC166271[j]			316*	0	0	
	TC166434			314*	0	2	
	TC166539			308*	0	0	
Poly A binding protein 1	TC147521	ATGGTATGTTGGAAGAGCGC	ATTGGAACTGGCTGTGACG	819*	1	0	C
Poly A binding protein 2	TC143603	TGCCATGGATGATATTGGC	CACGCATTATCTTGCAAGAGG	590*	2	0	D
Prolamin binding factor	TC176019	TGGCAACACCAAGTTCTGC	CCACCATTGTTGTCATCACC	783	1	0	D
Signal transduction							
14-3-3 protein 1	TC166588	AACAGACACTGAAGATGGCG	CACAGCGTCAGGTTATCACG	728*	1	2	C
	TC166340[e]			727*	0	2	
14-3-3 protein 2	TC167346[j]	TGGTGAGCTCACTGTTGAGG	CCAGAGAGTCAAGTTGTCACG	602*	0	0	C
	TC167199			592*	1	3	
	TC167120[e]			592*	0	0	
14-3-3 protein (TaWIN1) 3	TC147806	GGCTTACAAGAATGTCATCGG	ATTCCTCGCCTAAGCTGTCC	497*	1	2	C
Metabolism							
Beta amylase	TC65947[i]	TGCAACAAGGCAACTATGTCC	CATCCAAGATCTTGTCACCG	822	9	7	E
	TC168650[e]			822	15	17	
Enolase	TC143002	GATGTGTGCTGCTCAGATGG	TTGTGGTACACTTCAACGCC	518*	0	0	C
	TC143544			518*	1	1	
	TC143094			518*	2	0	
Formate dehydrogenase 1	TC169343	GGCATCACTACATTGTCACCG	AACCTCAGCCACTGTCAAGC	227*	1	2	B
Formate dehydrogenase 2	TC169346[e]	CCAATGGACATCACTACATCG	TTACTTCCGGTGACCTCTGC	244	0	2	B
Pyruvate orthophosphate dikinase	TC170385	AAGAACTCAGCCTGCTCTGC	GCTGAGGATGATGTCCTTGC	356*	0	0	C
UDP-glucose pyrophosphorylase	TC143679	ACTTCTTGCCACTTCCAAGC	CTACAAGCCTCTTGATCGCC	411*	7	4	B
Starch synthase I	TC152276	CCATTCCAGAGCTCATGAGG	AGCTGTAACACGGACAGAGAGG	819	0	2	D
	TC173990			818	0	0	
Defense/oxidative stress							
Alpha purothionin	TC169310	TTGGTTCTGGAACAGGTGC	TGACCATGTAGTCACACACGG	253	3	29	E
Chitinase 1	TC143017	GATCACCAACATCATCAACGG	AAGCATCCATACGAGATGCC	286	3	0	E
	TC143032			286	3	0	
Chitinase 2	TC82949[i]	CTACTGCTTCAAGCAGGAACG	TAGCAGTCAAGGTTGTCGCC	462	2	0	E
	TC143017[e]			462	3	0	
	TC143692[e]			462	0	2	

Cu^{2+}–Zn^{2+} superoxide dismutase	TC168464	ATGGCTGCATGTCAACTGG	CTCTTGCTCAGCTCATGTCC	244*	2	2	C
	TC168469			244*	1	0	
Glyoxalase	TC166279	TATGCTTCGTGTTGGTGACC	ACCTGAGCATATGCATTGCC	207*	0	0	A
	TC166217			207*	1	3	
	TC166310[e,j]			207*	0	0	
Mn^{2+} superoxide dismutase	TC149927	GACCAACATCTGGAAGGTGG	GACAGGCACTGTTCTATGCG	226*	2	1	B
Peroxidase	TC171272	TCTTCACCAACGACATCACC	GCTTACAAGCTAGCCAAGCC	251	0	4	E
Serpin 1	TC169964	CCATTCATGTCCAGCATGG	ATGTAATGGCTGAGGCAACC	624	7	13	E
Serpin 2	TC171518	GCAGTGTGCAAGTACAAGGC	TCCAGGAAGTCTGGTTCAGC	446	8	0	E
	TC171068			446	4	7	
	TC169964[e]			449	7	13	
Serpin 3	TC148700	GTTCCTGGAGCAGCATATCC	CGATGAATAGAACCACACCG	368	2	1	E
Thionin V	TC170728	AGTACAGTGTTCCAGTGCGC	AACTGTCACAAGCAACACCG	208	1	2	E
Tritin	TC149724	GACAAGCTGACCAACGTCG	GCCTTCATCTCATTGCCG	242	1	4	F
Other							
Cell division cycle protein	TC168333	GTGGTCTGGAGAATGTCAAGC	TATGATGTCTGGCCTGTTGG	449*	2	1	D
Cellulase 1	TC170730	ACCTCAACGACCTCATCTCC	CATGTACTTGGCGTAGGTGG	299	2	1	D
Cellulase 2	TC170731[e]	CTGTGTTCCTTGCCTTCTCC	AACTTGACGTTGTTGCCGC	235	0	0	D
Lipid transfer protein	X63669[f]	CTCAGGTAATGCTCATGGCC	AGTCGATGTTGAGACTGATGG	327	N/A	N/A	F
Translationally controlled tumor protein (TCTP)	TC145960[j]	TGATGTGGACATTGGAGCC	TGGAGAGTCGACTGACAACC	431	0	0	A
	TC145955			458*	0	0	
	TC145968			458*	0	0	
	TC145953			457*	1	1	
	TC145954			458*	1	4	
28S ribosomal RNA	AY049041[f]	TCACCTTGGAGACCTGATGC	AAGGTTCCATGTGAACGGC	368*	N/A	N/A	A
18S ribosomal RNA	K01229[f]	TTCATACAGGTGCTGCATGG	AGACGACTTCGGTTCACACG	242*	N/A	N/A	A

Numbers after the gene name distinguish gene family members or sequences with similar names. Identifications are from TaGI Version 8.0 unless indicated. Sequences are grouped by proposed functions of encoded proteins.

[a] Expected from RT-PCR of endosperm mRNA. * indicates that primers also amplified leaf total RNA. ** indicates that product of this size was not detected from endosperm.

[b] AQN is a cDNA library from 'Butte 86' developing grains. 3627 ESTs from this library are included in TaGI Version 8.0.

[c] 5468 is a cDNA library from 'Cheyenne' developing endosperm. 4150 ESTs from this library are included in TaGI Version 8.0.

[d] Determined by RT-PCR. (A) present throughout endosperm development; (B) decreasing during endosperm development; (C) present early in endosperm development; (D) present during early to mid endosperm development; (E) present from mid to late endosperm development; (F) present during late endosperm development.

[e] Primers have 1 bp mismatch with tentative consensus sequence from TaGI Version 8.0.

[f] From Genbank.

[g] Contig matched to avenin using BlastX with probability 2×10^{-15}.

[h] Contigs matched to avenin using BlastX with probabilities of 4×10^{-14} and 2×10^{-11}, respectively.

[i] From TaGI Version 6.0.

[j] Contigs do not contain any ESTs from endosperm or grain cDNA libraries.

combined for this time point. The integrity of each RNA sample was assessed by Northern blot analysis using a digoxigenin-labeled probe specific for a ω-gliadin gene (Altenbach et al., 2002). Residual ribosomal RNA in the poly A sample was detected by RT-PCR using primers specific for the 28S and 18S rRNA genes (Table 1).

RT-PCR was performed according to the basic protocols accompanying the reagents and enzymes supplied by Applied Biosystems (Foster City, CA). Poly-A RNA (10 ng) or total RNA (100 ng) was reverse-transcribed in a reaction containing 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$, 1 mM of each dNTP, 2.5 μM random hexamers, 1 unit per μl RNase inhibitor, and 2.5 units per μl MuLV reverse transcriptase in a final volume of 20 μl. The sample was incubated at room temperature for 10 min, followed by 60 min 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 AmpliTaq DNA polymerase, and 20 pmol of each oligonucleotide primer. Oligonucleotide primers were 18–22 bases in length and were synthesized by QIAGEN Operon (Alameda, CA). The concentrations of Tris–HCl, pH 8.3 and KCl in the final reaction were 10 and 50 mM, respectively. Amplifications were carried out at 95 °C for 90 s, followed by 25 cycles of 95 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min. A final extension was carried out at 72 °C for 7 min and the samples were incubated at 4 °C until analysis. Amplification of each mRNA sample without prior reverse transcription confirmed the absence of contaminating DNA. Aliquots of RT-PCR products were analyzed in 1.5% agarose gels in TBE buffer following standard procedures.

3. Results

3.1. Selection of gene and primer sequences

DNA sequences expressed in developing wheat grains, endosperm or spikes were selected from The Institute for Genomic Research (TIGR) *T. aestivum* Gene Index (TaGI) (http://www.tigr.org/tdb/tgi/tagi/) or from the National Center for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/). Oligonucleotide primers specific for each DNA sequence were synthesized. Table 1 shows the selection of genes, sequences of primers corresponding to each gene and the identification numbers of tentative consensus sequences or gene sequences that match each set of primers. In addition to the 28S and 18S ribosomal RNA genes and HMW glutenin subunit 1Dy10 gene that serve as controls, the collection includes genes encoding proteins that serve primarily a storage function in the endosperm (triticin, globulin). Also included are genes encoding small cysteine-rich storage proteins that may have dual functions, such as *alpha*-amylase/trypsin inhibitors, implicated in defense (Gutierrez et al., 1990), and grain softness proteins and puroindoline b, suggested to play a role in kernel hardness (Morris, 2002). Two gene sequences that exhibit high-probability matches with avenin, an oat storage protein, by BLASTX searching also were selected. ESTs corresponding to many of these sequences are well represented in cDNA libraries from developing grains, endosperm or spikes in TaGI. The numbers of ESTs corresponding to each sequence in two representative cDNA libraries are indicated in Table 1. Library #AQN (referred to as cDNA library 11095 in NCBI) contains sequences expressed in developing grains from *T. aestivum* 'Butte 86' grown under 6 different environmental regimens and includes sequences expressed very early (3 DPA) as well as very late (44 DPA) in grain development. cDNA library 5468 (referred to as cDNA library 5468 in NCBI) contains sequences expressed specifically in endosperm tissue from 5–30 DPA developing grains of *T. aestivum* 'Cheyenne'.

A number of the selected sequences encode proteins that play various roles in protein synthesis (elongation factor 1-alpha), protein folding (protein disulfide isomerase and cyclophilin), protein transport (ADP ribosylation factor and sec61p), or protein processing and turnover (aspartic proteinase, Clp protease, endopeptidase, proteosome subunits). Several genes encode proteins involved in transcription and mRNA stability (prolamin binding factor, poly-A binding protein and glycine-rich RNA binding protein). Genes that play roles in signal transduction also were selected. These encode 14-3-3-like proteins that play diverse roles in many biological processes and may be involved in fine-tuning metabolism in response to changes in the environment or internal demands. The 14-3-3 protein called TaWIN1 (*T. aestivum* WPK4-interacting factor 1) is believed to be involved in nitrogen assimilation (Ikeda et al., 2000). Other genes encode enzymes involved in various metabolic pathways, including carbohydrate metabolism (UDP-glucose pyrophosphorylase, starch synthase I, *beta*-amylase), glycolysis (enolase), pyruvate metabolism (pyruvate orthophosphate dikinase), and formate metabolism (formate dehydrogenase). A number of genes encode proteins that probably play roles in plant defense and/or oxidative stress (chitinase, serpin, the ribosome-inactivating protein tritin, alpha purothionin, thionin V, glyoxalase, peroxidase and superoxide dismutase). Of these, ESTs for alpha purothionin and serpin 1 and 2 also are well represented in cDNA libraries from developing grains and/or endosperm (Table 1). Genes encoding a non-specific lipid transfer protein, cell division cycle protein, two cellulases and a protein similar to a translationally controlled tumor protein (TCTP) from human also were examined.

A mixture containing equal amounts of mRNA from 7, 14, 20, 28 and 34 DPA endosperm from plants grown under the 24/17 °C regimen was reverse-transcribed and amplified with each primer pair. Sizes of amplification products determined by gel electrophoresis corresponded to those

predicted for each primer pair (Table 1). Total RNA from leaf tissue also was reverse-transcribed and amplified with each primer pair. Primers that yielded amplification products from leaf RNA as well as endosperm RNA are indicated in Table 1.

3.2. Transcript accumulation profiles in endosperm under a 24/17 °C regimen

Transcript accumulation for each of the selected sequences was examined by RT-PCR at five time points during endosperm development under the 24/17 °C regimen. The earliest time point was at 7 DPA, when the starchy endosperm could be separated without difficulty from the pericarp, testa and embryo. Subsequent time points were at 6–8 day intervals with the latest time point at 34 DPA. Based on previous experiments (Altenbach et al., 2003), 7 DPA is shortly before the time that kernels begin to accumulate starch and protein and 34 DPA is shortly after the time that kernels reach maximum dry weight. The genes were categorized into 6 different groups based on expression profiles (Fig. 1, Table 1). Transcripts for the first group of genes were present at all time points from 7 until 34 DPA. Examples are shown in Fig. 1A and include the HMW glutenin subunit 1Dy10, triticin, Ima1 monomeric *alpha*-amylase inhibitor, grain softness protein-1b, and CM1 and CM3 *alpha*-amylase/trypsin inhibitors. Also included in this group were several other *alpha*-amylase inhibitors, globulin, grain softness protein-1a, puroindoline b, the avenin-like unknown protein 2, ADP ribosylation factor, glyoxalase and TCTP (Table 1). Since the RT-PCR methods used in these studies were end-point analyses in which saturation levels may have been attained, the detection of equivalent amounts of amplification products at all time points does not necessarily indicate similar transcript levels. Transcripts for a second group of genes also were present at all time points, however, the amounts of amplification products obtained from equivalent amounts of mRNA clearly were reduced at later time points. This group is typified by formate dehydrogenase 1, UDP-glucose pyrophosphorylase, elongation factor 1-alpha, cyclophilin, 20S proteosome subunit 1, and Mn^{2+} superoxide dismutase (Fig. 1B), but also included protein disulfide isomerase, formate dehydrogenase 2, 26S proteosome subunits 2 and 3, and aspartic

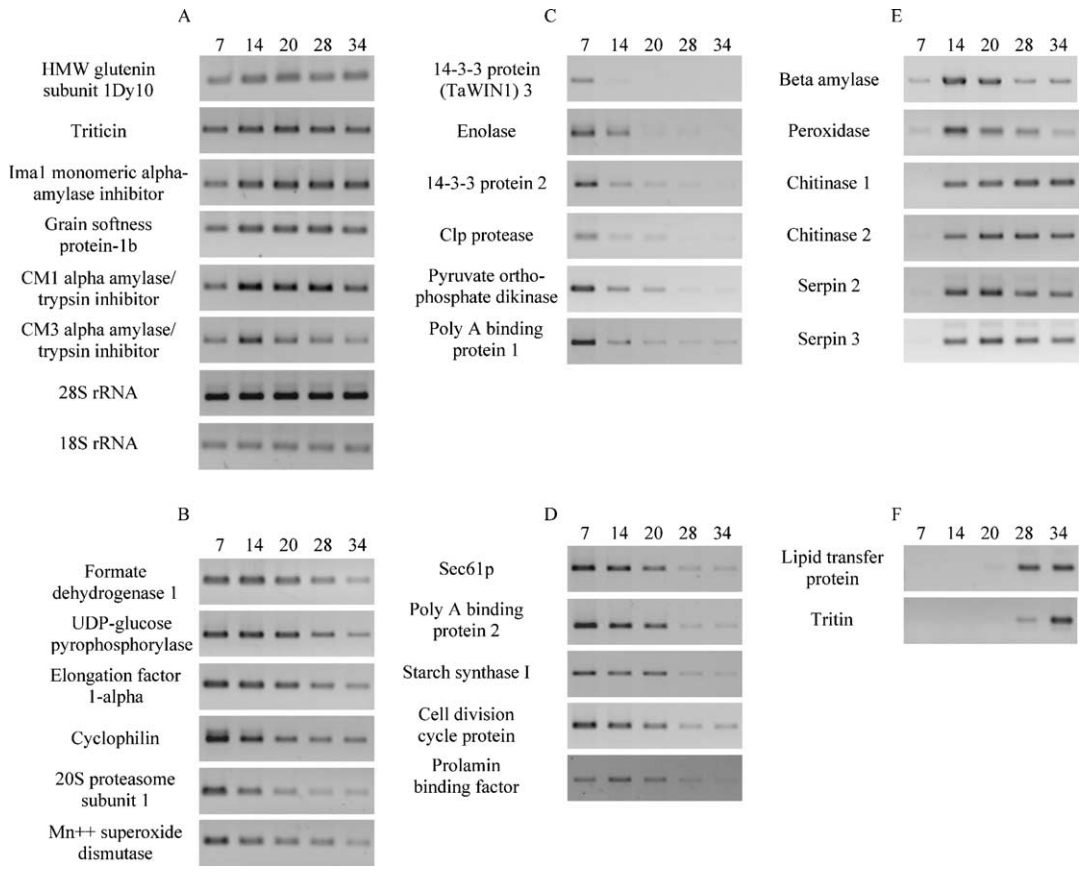

Fig. 1. Transcript accumulation profiles for genes expressed in developing wheat endosperm under a 24/17 °C regimen determined by RT-PCR using gene-specific primers. Examples of transcripts (A) present at all time points surveyed during endosperm development; (B) decreasing at later time points; (C) present at early time points; (D) present at early to intermediate time points; (E) present from intermediate to late time points; (F) present only at late time points. Genes correspond to sequences in Table 1. The age of endosperm tissue is shown at the top of each panel in DPA. Images of ethidium bromide stained gels were reversed for clarity. Amplification products obtained with primers specific for 28S and 18S rRNA are included in panel A.

proteinase (Table 1). Transcripts in a third group were accumulated at early time points. This group included the 14-3-3 protein TaWIN1 3, detected only at 7 DPA, enolase, detected at 7 and 14 DPA, and 14-3-3 protein 2, clp protease, pyruvate orthophosphate dikinase and poly A binding protein 1, predominant at 7 DPA but also detected at 14 and 20 DPA (Fig. 1C). Also included in this group were endopeptidase, glycine-rich RNA binding protein, 14-3-3 protein 1 and Cu^{2+}–Zn^{2+} superoxide dismutase, whose transcripts were detected at 7 and 14 DPA (Table 1). Fig. 1D shows transcripts that were accumulated at early and intermediate time points. Transcripts for sec61p, poly A binding protein 2, starch synthase I, cell division cycle protein and prolamin binding factor were most predominant at 7, 14, and 20 DPA. Cellulase 1 and 2 also had similar expression profiles (Table 1). Transcripts in the fifth group were accumulated at 14 DPA and all later time points. This group included *beta*-amylase, peroxidase, chitinase 1 and 2, and serpin 2 and 3 (Fig. 1E) as well as CMx trypsin inhibitor, the avenin-like unknown protein 1, alpha purothionin, serpin 1, and thionin V (Table 1). Small amounts of amplification products also were detected at 7 DPA for *beta*-amylase, peroxidase and alpha purothionin. The last group contained two genes, lipid transfer protein and tritin, whose transcripts were accumulated only at the 28 and 34 DPA time points (Fig. 1F).

3.3. Transcript accumulation profiles in endosperm produced under a 37/28 °C regimen

Similar analyses were done using endosperm mRNA from plants subjected to a 37/28 °C regimen during grain development. Five time points were selected for analysis. The earliest time point was at 5 DPA. Subsequent time points were at 3–4 day intervals until 20 DPA. Based on previous experiments (Altenbach et al., 2003), the 5 DPA time point is shortly before kernels begin to accumulate starch and protein and the 20 DPA time point is shortly after kernels reach maximum dry weight. The genes could be separated into five groups based on expression profiles under the 37/28 °C regimen. Transcripts that were present at all five time points included HMW glutenin subunit 1Dy10, triticin, Ima1 monomeric *alpha*-amylase inhibitor, and the grain softness protein-1b (Fig. 2A). Grain softness protein-1a, puroindoline b, globulin, thionin V and ADP ribosylation factor also had similar accumulation profiles. Transcripts in a second group decreased in amount at later time points. This group included many of the same

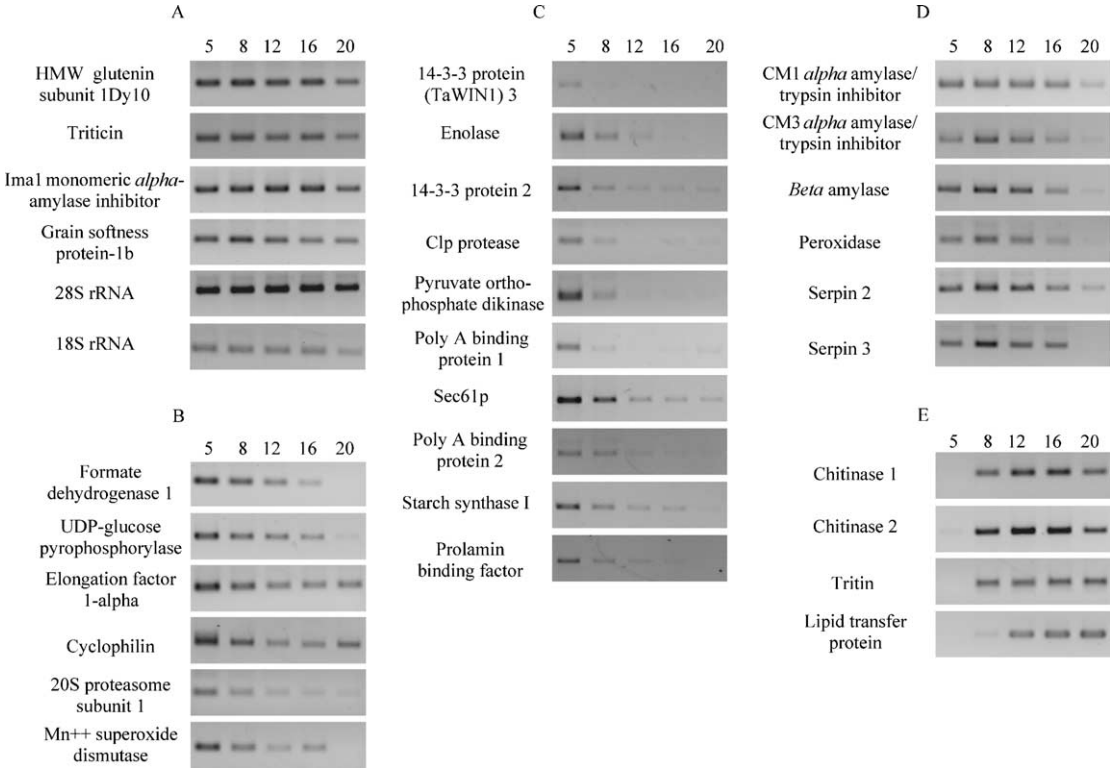

Fig. 2. Transcript accumulation profiles for genes expressed in developing wheat endosperm under a 37/28 °C regimen determined by RT-PCR using gene-specific primers. Examples of transcripts (A) present at all time points surveyed during endosperm development; (B) decreasing at later time points; (C) present at early time points; (D) present at the four earliest time points; (E) present from intermediate to late time points. Genes correspond to sequences in Table 1. The age of endosperm tissue is shown at the top of each panel in DPA. Images of ethidium bromide stained gels were reversed for clarity. Amplification products obtained with primers specific for 28S and 18S rRNA are included in panel A.

transcripts that decreased under the 24/17 °C regimen; formate dehydrogenase 1, UDP-glucose pyrophosphorylase, elongation factor 1-alpha, cyclophilin, 20S proteasome subunit 1 and Mn^{2+} superoxide dismutase (Fig. 2B) as well as formate dehydrogenase 2 and protein disulfide isomerase. However, transcripts for several of these genes either were not detected or barely detectable at the 20 DPA time point. Transcripts for a third group of genes were present only at early time points. Transcripts predominant at 5 DPA or 5 and 8 DPA included 14-3-3 protein TaWIN1 3, enolase, 14-3-3 protein 2, clp protease, pyruvate orthophosphate dikinase, poly A binding protein 1, sec61p, poly A binding protein 2, starch synthase I, and prolamin binding factor (Fig. 2C). Transcripts for genes in the fourth group were predominant at the 5, 8, 12, and 16 DPA time points. This group included the CM1 and CM3 *alpha*-amylase/trypsin inhibitors, *beta*-amylase, peroxidase and serpin 2 and 3 (Fig. 2D). Transcripts for these genes either were not detected or detected at low levels at 20 DPA. Finally, transcripts for the last group of genes were detected only at middle and late time points. Chitinase 1 and 2 and tritin transcripts were accumulated at 8, 12, 16, and 20 DPA, while lipid transfer protein transcripts were present at 12, 16 and 20 DPA.

3.4. Comparison of transcript accumulation under different temperature regimens

Fig. 3 compares the profiles of transcript accumulation for representative genes under two temperature regimens with the timing of transition points in grain development determined in a previous study where developing grains were evaluated at 2-day intervals (Altenbach et al., 2003). For many genes, indicated with open squares in Fig. 3, the timing of transcript accumulation relative to these transition points is consistent with the shortened developmental program under high temperature conditions. Transcripts detected primarily at 7 DPA under moderate temperatures, such as those encoding the 14-3-3 protein TaWIN1 3 (Figs. 1C and 3), were detected at 5 DPA, but not 8 DPA, under the high temperature regimen (Figs. 2C and 3). Under both temperature regimens, this time point is shortly before kernels begin to accumulate protein and starch. Transcripts that were predominant either at 7 and 14 DPA or at 7, 14, and 20 DPA under moderate temperatures (Figs. 1C and D) were accumulated at 5 and 8 DPA under high temperatures (Fig. 2C). Under both temperature regimens, these transcripts were most abundant at time points that were before kernels reached maximum water contents. Poly-A binding

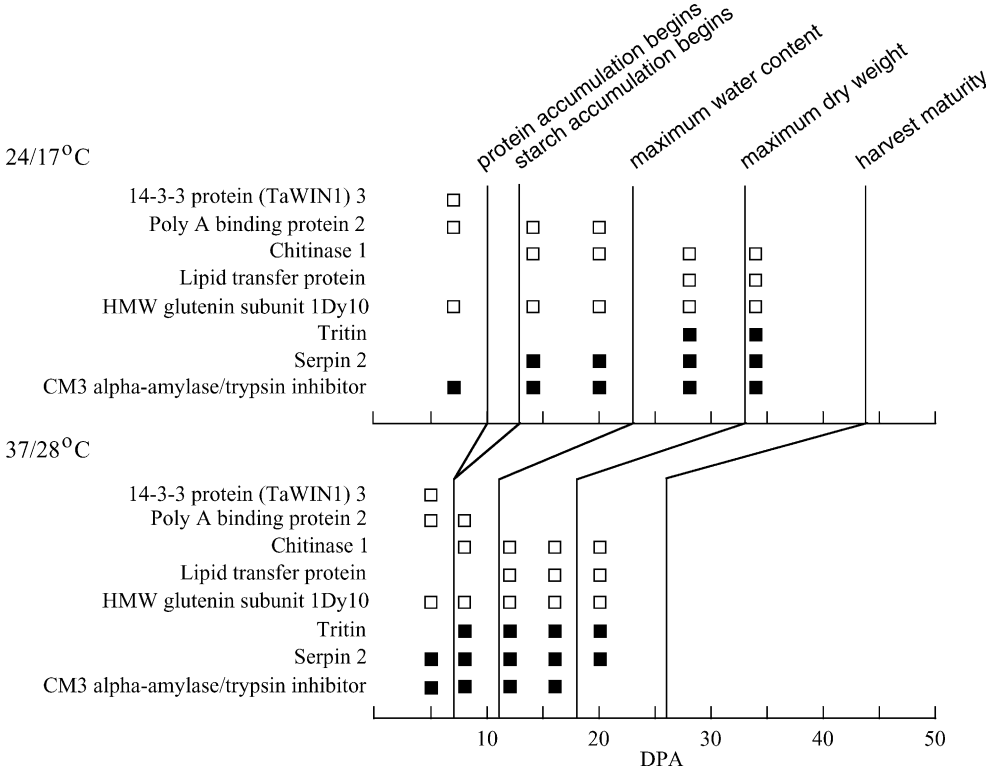

Fig. 3. Comparison of the timing of accumulation of transcripts for select genes with transition points in grain development under either a 24/17 or 37/28 °C regimen. Transition points indicated on the top of the figure were taken from Altenbach et al. (2003). The times in DPA that representative transcripts were detected by RT-PCR are indicated with squares. Open squares denote transcript profiles that changed with high temperature in a manner consistent with developmental events. Filled squares denote transcript profiles that were shifted under high temperatures in a manner not consistent with transition points in grain development.

protein 2 is an example of a gene that falls into this group (Fig. 3). The appearance of transcripts for two chitinase genes coincided with the previously determined time for the onset of starch accumulation under both temperature regimens (Altenbach et al., 2003). These transcripts were first detected at 14 DPA under moderate temperatures (Figs. 1E and 3) but at 8 DPA under the high temperature regimen (Figs. 2E and 3). Finally, transcripts for a non-specific lipid transfer protein were detected after kernels reached their maximum water contents, at 28 and 34 DPA under moderate temperatures (Figs. 1F and 3), and at 12, 16 and 20 DPA under high temperatures (Figs. 2E and 3). Transcript profiles for the HMW glutenin subunit 1Dy10 are shown for comparison. These transcripts were detected at all time points in this experiment, from shortly before protein accumulation was detected in the kernels to beyond the time that kernels achieved maximum dry weights. Previous work suggests that profiles of transcript accumulation for other glutenin subunits and gliadin storage proteins would be identical (Altenbach et al., 2002).

Transcript profiles for a number of other genes, some of which are indicated with filled squares in Fig. 3, were shifted under high temperatures in a manner that was not consistent with developmental events. For example, transcripts for tritin were predominant at 34 DPA under the 24/17 °C regimen, shortly after the kernels had achieved their maximum dry weights and the endosperm had started to undergo apoptosis, although a small amount of transcript was detected at 28 DPA (Figs. 1F and 3). Transcript profiles were shifted dramatically by high temperature and transcripts were apparent by 8 DPA, just as starch and protein were beginning to accumulate and before the kernels reached maximum water contents (Figs. 2E and 3). Transcripts for several serpin genes, *beta*-amylase and peroxidase were accumulated from 14 DPA, about the time that starch accumulation begins, through 34 DPA under moderate temperatures (Figs. 1E and 3). However, transcripts for all of these genes were detected by 5 DPA under the high temperature regimen, before the onset of protein and starch accumulation (Figs. 2D and 3). Finally, transcripts for a number of genes such as the CM *alpha*-amylase/trypsin inhibitors (Figs. 1A and 3) were present at the 34 DPA time point under the moderate temperature regimen, after kernels reached maximum dry weight. Transcripts for these same genes trailed off between 16 and 20 DPA under the 37/28 °C regimen, as kernels approached this same developmental milestone (Figs. 2D and 3).

3.5. Comparison of transcript profiles for closely related genes

During the course of these studies, the temporal regulation of different members of several gene families was examined. Coding sequences for the CM1, CM3, CM16 and CM17 *alpha*-amylase/trypsin inhibitors share between 58 and 85% identity while Ima1 and WAS1 sequences have less similarity to the other *alpha*-amylase inhibitors. All of these genes had similar transcript accumulation profiles under the 24/17 °C regimen (Fig. 1A, Table 1). Under the 37/28 °C regimen, transcripts for Ima1 and WAS1 were abundant at all time points (Fig. 2A) while transcripts for the CM proteins declined at 20 DPA (Fig. 2D). The timing of transcript accumulation for all of the serpin sequences was similar in kernels produced under the same environmental conditions, but the profiles differed between the 24/17 °C (Fig. 1E) and 37/28 °C regimens (Fig. 2D). The two consensus sequences for poly A binding proteins share 64% identity and exhibited different expression profiles. Under the 24/17 °C regimen, transcripts for poly A binding protein 1 were present at 7 DPA and declined rapidly (Fig. 1C) while transcripts for poly A binding protein 2 were present at 7, 14 and 20 DPA (Fig. 1D). Similarly, under the 37/28 °C regimen, transcripts for poly A binding protein 1 were detected only at 5 DPA while those for poly A binding protein 2 were detected at 5 and 8 DPA (Fig. 2C). Finally, transcripts for all three 14-3-3 protein sequences appeared early in grain development. However, transcripts for 14-3-3 protein TaWIN1 3 were detected only at 7 DPA under the 24/17 °C regimen (Fig. 1C) and at 5 DPA under the 37/28 °C regimen (Fig. 2C) while transcripts for 14-3-3 protein 1 and 14-3-3 protein 2 also were detected at later time points.

4. Discussion

By comparing the timing of transcript accumulation for a collection of 57 genes involved in a variety of different processes in endosperm from plants grown under a moderate temperature regimen, we provide a first glimpse at the transcriptional program of wheat grain development. In general, transcripts encoding proteins that play a storage function were accumulated throughout much of endosperm development while transcripts for genes involved in signal transduction, a variety of metabolic processes, and protein synthesis, transport and turnover were most predominant early in development, before kernels reached maximum water contents. Transcripts for a number of proteins involved in defense appeared about the time that starch began to accumulate and persisted throughout development. Transcripts for only two of the 57 genes, lipid transfer protein and tritin, were accumulated during the latter half of grain development, after kernels reached maximum water contents and as kernels accumulated the bulk of starch and protein.

It is somewhat surprising that we did not find more transcripts accumulated late in the developmental program. Endosperm tissue undergoes apoptosis late in grain development, a process that requires active gene expression. Although the key proteases and proteins involved in apoptosis have been well studied in animal systems, relatively little is known about the process in plants.

However, a recent survey of gene expression in *Arabidopsis* cell cultures undergoing apoptosis suggested that oxidative stress-related genes such as superoxide dismutase and cysteine proteases may serve as useful markers for plant apoptosis (Swidzinski et al., 2002). Accumulation profiles of two superoxide dismutase genes were examined in this study. However, neither gene was preferentially expressed late in grain development. A number of other genes, including ADP ribosylation factor, pyruvate orthophosphate dikinase and peroxidase, were expressed at later stages of seed development in *Arabidopsis* (Ruuska et al., 2002), but homologues for these genes exhibited different transcript accumulation profiles in developing wheat grains. Perhaps there are other members of these gene families that are expressed late in grain development.

Comparison of transcript profiles under the 24/17 and 37/28 °C regimens clearly showed that transcriptional programs were advanced and compressed by high temperatures. In addition, comparisons of transcript accumulation with physiological markers of grain development revealed those transcripts that were accumulated at equivalent stages of development under the two temperature regimens and thus might serve as markers of grain development. These included transcripts for many of the genes involved in signal transduction, RNA transcription and stability, and protein synthesis, transport and turnover, as well as chitinase, and a lipid transfer protein. Such markers establish the developmental equivalency of kernels produced under different environmental conditions and thus facilitate direct comparisons between the two treatments. This is particularly important in experiments where temperatures are outside of the range in which the degree-day concept is applicable.

The timing of accumulation for other transcripts was shifted under the high temperature regimen in a manner that was not consistent with key developmental events. In particular, transcripts for several putative defense proteins, such as peroxidase, serpin and tritin, were detected earlier in the developmental program under high temperatures. Likewise, a number of transcripts that were present after kernels reached maximum dry weight under moderate temperatures, such as the CM *alpha*-amylase/trypsin inhibitors and formate dehydrogenase declined as kernels approached this stage under high temperatures. It is possible that some of these discrepancies result from the frequency of time points selected for analysis. Alternately, these changes may represent responses to high temperature that are distinct from effects on the timing of developmental processes. While it might be expected that some processes would be disrupted in grains subjected to the very high temperatures used in this study, it is interesting that the main effect of high temperatures was on the timing of transcript accumulation.

More detailed analyses using microarrays will provide quantitative data on thousands of genes that play a role in wheat grain development. Microarray analysis already has been used to provide insight into transcriptional networks during seed development in *Arabidopsis* (Ruuska et al., 2002) and grain filling in rice (Zhu et al., 2003). Several studies also have demonstrated that major differences in metabolism sometimes occur with only minor changes at the transcript level. Indeed, comparisons of *Arabidopsis* seeds from a wild-type and mutant at three time points revealed that only 45 of 3500 surveyed transcripts varied more than 2-fold despite an 80% reduction in seed oil content. In maize, relatively few differences in gene expression were noted in the *sugary-1* mutant that is defective in starch synthesis, despite large differences in kernel phenotype (Hunter et al., 2002). In contrast, alterations in gene expression patterns were very pleiotrophic in a microarray analysis of opaque mutations in 18–22 DPA maize endosperm and included changes in expression of a number of genes associated with physiological stress (Hunter et al., 2002).

In wheat, high temperature conditions between anthesis and maturity clearly impact the composition of the grain. However, high temperature also alters the timing of developmental processes in the grain, making simple comparisons of gene expression between kernels of a similar age problematic. In our limited survey, we have described the effects of one high temperature regimen on the timing of transcriptional programs of a sampling of genes involved in many diverse processes. The data suggests that direct comparisons between developmentally equivalent time points may provide an indication of genes likely to be influenced by high temperatures. However, ultimately the expression of each gene must be examined within the context of grain development under each environmental regimen.

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