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Mini Review

Molecular and biochemical impacts of environmental factors on wheat grain development and protein synthesis

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Abstract

Wheat grain yield and flour quality are strongly influenced by the effects of environment during grain fill. Environmental variables such as temperature, water and fertilizer influence the rate and duration of wheat grain development, protein accumulation and starch deposition in unique ways, and by different mechanisms. The effects of environment are superimposed on the intrinsic temporal patterns of gene expression during grain development. Integration of genomic and proteomic studies with developmental studies under controlled environmental conditions should make it possible to resolve complex patterns of gene expression during grain development, pinpoint key regulatory processes that are influenced by the environment, and reveal the molecular basis for environmental impacts on flour composition and quality.
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Keywords: Environment; Fertilizer; Gene expression; Genomics; Grain development; Protein synthesis; Proteomics; Temperature; Wheat

1. Introduction

Wheat is one of the primary grains consumed by humans and is grown around the world in diverse environments, from cool rain-fed to hot dry-land areas. It has long been recognized that productivity and quality vary considerably as a result of environmental conditions during grain fill (Bailey, 1925). Yield is a major concern for wheat growers, while millers and bakers cite variability in the functional properties of flour as one of their biggest problems. Despite years of research, there remain critical gaps in our understanding of factors controlling yield and quality. New information is needed on the molecular mechanisms underlying quality and the response of the developing grain to environmental stress.

Prior to anthesis, environment affects germination, photosynthesis, tiller formation, and inflorescence development, thereby impacting grain number (reviewed by Herzog (1986) and Egli (1998)). After anthesis, environmental conditions primarily affect kernel size and composition. This review summarizes current information on the effects of post-anthesis temperature, fertilizer and water on the temporal program of grain development and on the regulation of protein and starch accumulation in the starchy endosperm. Applications of proteomics and genomics for understanding the effects of environment on grain development are addressed. Where possible, recent reviews are cited. If information on wheat is not available, studies of barley, rice or maize are discussed.

2. Endosperm development

The wheat grain is a composite of different tissues, each with a unique temporal pattern of gene expression during grain fill. Technically, the mature grain is a caryopsis, with an outer testa closely appended to the seed (Hoseney, 2002). The seed includes the outer, maternal pericarp layer, the embryo, and the endosperm. The endosperm consists of an outer aleurone layer and inner columns of starchy endosperm cells. The embryo, aleurone, and pericarp plus testa are removed during milling, leaving the starchy endosperm as the principal contributor to white flour.

Abbreviations: ABA, abscisic acid; DPA, days post-anthesis; BPBF, barley prolamin binding factor; bZIP, basic leucine zipper; DOF, DNA binding with one finger; GA, gibberellic acid; HMW-GS, high molecular weight glutenin subunit; LMW-GS, low molecular weight glutenin subunit.

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Developing wheat is often described using the Feekes, Zadoks or Heun scales that assign numbers to visual landmarks that are useful for crop management purposes (Chang et al., 1974; Large, 1954). These subjective scales divide grain development into early, medium and late milk stages, and early, soft and hard dough stages. The temporal pattern of grain development also can be described in terms of transition points in the accumulation of total dry matter, starch, protein, and water, in order to pinpoint times in grain development when changes in gene expression and protein accumulation are likely to occur (Altenbach et al., 2003) (Fig. 1).

Endosperm development begins with fertilization of a diploid cell, followed by repeated division of the triploid nuclei, gradual formation of cell walls, and partitioning of the original vacuolated cell into a characteristic cellular pattern (Olsen, 2001; Olsen et al., 1999; Lohe and Chaudhury, 2002). Next is a period of cell expansion in which water content increases and starch and protein reserves accumulate. Endoreduplication of DNA within the nuclei continues during this phase and has been reported to influence final grain size in maize (Engelen-Eiges et al., 2000). The maximum amounts of starch and protein that accumulate in each grain depend on the number of endosperm cells, determined early in grain fill, and the final size of the cells, which is influenced by water uptake, cell-wall extensibility and rate and duration of grain fill (Egli, 1998). Cell-wall-loosening enzymes may play a role in determining the extent of cell enlargement (Chanda and Singh, 1998). In maize, endosperm expansion was also reported to be influenced by cell division in the peripheral layers (Vilhar et al., 2002).

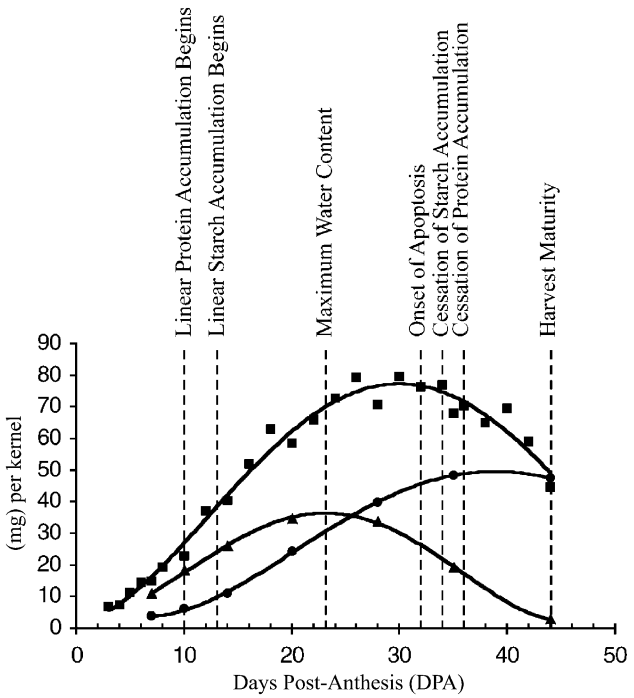

Fig. 1. The temporal pattern of grain development described by transition points in the accumulation of starch, protein and water and by the timing of the onset of apoptosis. ■, fresh weight; ●, dry weight; ▲, water content. Protein was measured by LECO nitrogen combustion analysis, starch was measured using a Megazyme total starch assay kit and the onset of apoptosis was identified by the analysis of genomic DNA. Plants were grown under a 24/17 °C day/night regimen. Data is from Altenbach et al. (2003).

Cell expansion and water accumulation stop before the cessation of dry matter accumulation, starch and protein replace cell water, and the kernel begins to desiccate (Berger, 1999; Lopes and Larkins, 1993; Rogers and Quatrano, 1983). Late in development, the formation of a waxy layer at the chalaza impedes input of sugars and amino acids into the grain (Sofield et al., 1977a; Cochrane et al., 1983), protein and starch deposition cease and the grain reaches maximum dry weight or physiological maturity (Fig. 1). At approximately this time the endosperm tissue undergoes a form of apoptosis, or programmed cell death, similar to that observed in other plant and animal tissues (Beltrano et al., 1994; Young and Gallie, 1999; Young and Gallie, 2000). Cell death, visualized by viability staining, progresses gradually throughout the endosperm tissue and is accompanied by internucleosomal fragmentation of genomic DNA. Only the aleurone cells remain viable. Finally, kernels desiccate rapidly, losing all but 10–15% of their water content, at which time they are ready for harvest. Several recent articles cover other aspects of seed development, including early stages of endosperm development (Olsen, 2001; McCarty, 1995; Finkelstein et al., 2002; Busk and Pagès, 1998; Sreenivasulu et al., 2001).

3. Effects of environment on the temporal pattern of wheat grain development

A considerable volume of literature addresses the effects of environment on the developing grain. Controlled growth experiments have made it possible to examine effects of individual variables, such as light intensity (Sofield et al., 1977b), temperature (Sofield et al., 1977b; Wardlaw et al., 1989; Tashiro and Wardlaw, 1990; Randall and Moss, 1990; Gibson and Paulsen, 1999; Guedira et al., 2002), water availability (Brooks et al., 1982; Kobata et al., 1992; Nicolas et al., 1984), fertilizer (Morris and Paulsen, 1985) and interacting effects of temperature, fertilizer and water (Altenbach et al., 2003; Yang et al., 2000; Daniel and Triboi, 2000) on the rate and duration of grain fill and on size and composition of the mature grain. To understand the molecular mechanisms that underlay the response to environment, it is valuable to determine the effects on the timing, duration and rate of specific developmental processes, although this is rarely done. Rates and durations sometimes are calculated in terms of heat units or degree-days rather than chronological days. The following discussion will refer to chronological days for ease of discussion.

Temperature impacts yield by altering the duration and rate of grain fill. The optimum temperature to achieve

maximum yields of wheat is generally considered to be between 15 and 20 °C. Temperatures in this range give the longest duration of grain fill and the greatest accumulation of starch per kernel. Such cool temperatures may be one of the factors responsible for the high wheat yields reported for Northern Europe. As average daily temperatures rise above 20 °C, the duration of grain fill is shortened (Wiegand and Cuellar, 1981). Temperature may also impact the rate of grain fill, depending on the wheat variety (Sofield et al., 1977b; Tashiro and Wardlaw, 1990; Wardlaw and Moncur, 1995). For some varieties, the rate of grain fill increases with temperature up to about 30 °C, presumably reflecting increased enzyme activity and metabolic processes. Increased rates may compensate for the effect of decreased duration on grain weight. For other varieties, rates of grain fill remain constant and kernel weight decreases. For example, Guedira and Paulsen (2002) observed a significant decrease in kernel weights because the duration, but not rate, of grain fill was reduced when roots, shoots, or whole plants were subjected to 30 °C instead of 15 °C during grain fill.

In many regions of the world, including parts of the United States, Australia, India, and Mexico, wheat plants are exposed to episodes of very high temperatures that greatly reduce the duration of grain fill. Tashiro and Wardlaw (1989) observed that large decreases in duration were accompanied by decreases in rate when Australian varieties were grown under controlled conditions at day/night temperatures of 33/28 °C rather than 21/16 °C. In contrast, when a US wheat variety was grown under a moderate and two very high temperature regimens (24/17 °C, 37/17 °C and 37/28 °C day/night), high temperature had little impact on the rate of grain fill, although duration and mature kernel weights were greatly reduced (Altenbach et al., 2003). The period between anthesis and kernel maturity spanned 44, 35 and 26 days under the respective regimens (Fig. 2). This study also found that the timing of all stages of grain development was altered by the higher temperature regimens, as though the overall temporal program of grain development was accelerated and compressed. Starch and protein accumulation began earlier and the time to achieve maximum water content and maximum fresh and dry weight was shortened under the high temperature regimens. Discrete ladders of fragmented genomic DNA characteristic of apoptosis also appeared earlier when kernels were subjected to the high temperature regimens.

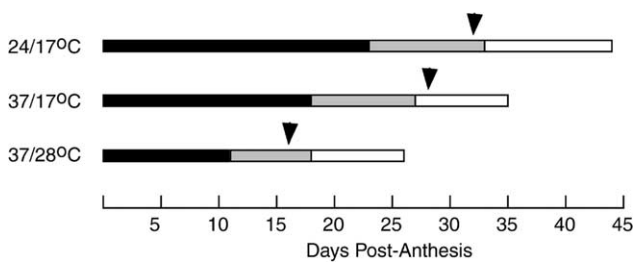

Fig. 2. Summary of grain development in plants grown under three temperature regimens between anthesis and maturity. The solid, hatched and open bars denote time to maximum kernel water content, maximum kernel dry weight and harvest maturity, respectively. The onset of apoptosis as determined by the fragmentation of genomic DNA is indicated by the solid triangles. Data is summarized from Altenbach et al. (2003).

Despite its importance, there have been relatively few controlled environment studies on the effects of drought on grain development, and it is not clear whether responses to water deficits vary with genotype, as was observed with temperature. Several studies demonstrated that drought decreased kernel size by shortening the duration, but not the rate of grain fill (Altenbach et al., 2003; Brooks et al., 1982; Kobata et al., 1992; Nicolas et al., 1984). The combination of high temperature and drought reduced the duration of grain fill more than either treatment alone (Altenbach et al., 2003; Nicolas et al., 1984). Addition of drought to a high temperature regimen compressed the temporal pattern of grain development, and maximum water content, fresh weight and dry weight were significantly less than with high temperature alone (Altenbach et al., 2003). Under some circumstances, however, drought may promote remobilization of nutrients from leaf and stem and increase the rate of grain fill (Yang et al., 2000).

It is difficult to generalize about effects of mineral nutrients on rate and duration of grain fill, flour composition, and flour quality from the multitude of field studies, which vary in timing and amount of fertilizer applied, wheat variety, soil type, temperature, and water availability. Controlled environment studies are essential to elucidate the underlying molecular basis for effects of fertilizer on grain fill but few studies have examined the effects of fertilizer on the pattern of grain development in wheat (Morris and Paulsen, 1985; Donovan, 1979). In one report, post-anthesis fertilizer had only minor effects on the temporal pattern of grain development, although it influenced duration of protein deposition and timing of leaf senescence (Altenbach et al., 2003).

4. Starch biosynthesis

Starch is a major determinant of yield, accounting for 65–75% of the grain dry weight and up to 80% of the endosperm dry weight (Rahman et al., 2000; Slattery et al., 2000). A series of enzymes synthesize the amylose and amylopectin chains that comprise starch (Rahman et al., 2000; Ball et al., 1998). Within the amyloplast, ADP-glucose pyrophosphorylase converts glucose 1-phosphate to ADP-glucose, which then is converted into amylose and amylopectin polymers by starch synthases and branching enzymes. The starch polymers form layered granules within the amyloplasts. Large type A granules are initiated about 4–7 days after anthesis (DPA), and smaller type B granules appear around 10–12 DPA (Bechtel et al., 1990; Parker, 1985; Buleon et al., 1998; Peng et al., 1999; Langeveld et al.,

2000). Many of the genes that encode enzymes required for starch biosynthesis have been sequenced (McCue et al., 2002; Murai et al., 1999; Nair et al., 1997; Vrinten and Nakamura, 2000; Li et al., 2000) and there are a few studies on transcriptional and post-transcriptional regulation of these genes in cereal grains (Morell et al., 1997; Sun et al., 1998). Some information is available on interactions between transcription factors and promoter binding sites of genes encoding starch biosynthetic enzymes in barley and maize endosperm (Zentella and Yamauchi, 2002; Kim and Guiltinan, 1999). Post-translational regulation of the starch biosynthetic enzymes has been shown to be important in chloroplasts and in potato tuber amyloplasts (Tiessen et al., 2002; Baldwin, 2001; Neuhaus and Emes, 2000). Much work remains to understand how starch biosynthesis and granule formation, size, and number are regulated in the wheat endosperm.

5. Effects of environment on starch biosynthesis

Reductions in starch accumulation at high temperatures account for significant losses in grain yield (Tashiro and Wardlaw, 1989; Bhullar and Jenner, 1985). The decline in starch content for Australian varieties exposed to temperatures greater than 35 °C was associated with a decrease in rate of conversion of sucrose to starch (Bhullar and Jenner, 1986a). Limitations in supply of sucrose to the head and the endosperm were not observed (Sofield et al., 1977b; Nicolas et al., 1984; Bhullar and Jenner, 1986b). High temperatures decreased levels of fructose, hexose phosphate, and sugar nucleotides (Jenner, 1991) and reduced the activity of some enzymes in the starch biosynthetic pathway, especially soluble starch synthase (Keeling et al., 1993; Rijven, 1986). Diminished rates of starch production in wheat endosperm at high temperatures were hypothesized to be due mainly to heat inactivation of starch synthase, a key enzyme in the starch biosynthetic pathway.

The effects of temperature on starch accumulation are probably more complex than can be explained by just heat inactivation of starch synthase. In a number of studies, the rate of starch accumulation was not affected by very high temperature regimens. Instead, high temperatures shortened the duration of starch accumulation. For example, the rate of starch accumulation was essentially the same in grain from plants grown under 24/17 °C, 37/17 °C, and 37/28 °C day/night temperature regimens (Altenbach et al., 2003; Hurkman et al., 2003). The observed decrease in amount of starch per kernel resulted from a decrease in duration of starch accumulation, which was 6 days less under the 37/17 °C regimen and 13 days less under the 37/28 °C regimen. Guedira and Paulsen (2002) observed that either high shoot or high root temperature reduced starch accumulation in the grain, mainly through an effect on duration of starch accumulation, suggesting that high temperature influenced factors that regulate the duration of grain fill, even when the grain itself was not exposed to high temperature. Although temperatures above 30 °C had negative effects on in vitro rates of starch synthase, the actual rate of accumulation in intact grains was stimulated for several days by exposing shoots or roots to 30 °C. Both Altenbach et al. (2003) and Guedira and Paulsen (2002) observed that accumulation of starch began earlier when plants were exposed to higher temperatures during grain fill.

Environment also influences expression of genes for the enzymes involved in starch biosynthesis. Temperature affected the temporal pattern of ADP-glucose pyrophosphorylase, granule bound and soluble starch synthases and starch branching enzymes, and high temperature reduced transcript levels for all of the enzymes, particularly the soluble starch synthases (Hurkman et al., 2003). Environment also may affect formation of starch granules and amylose/amylopectin ratios. Exposure to high temperatures was reported to increase the proportions of A granules and decrease the proportions of B granules in wheat and barley (Bhullar and Jenner, 1985; Hurkman et al., 2003; Blumenthal et al., 1995; MacLeod and Duffus, 1988; Tester and Karkalas, 2001), an effect consistent with a shorter duration of starch accumulation.

6. Storage proteins

Mature wheat grains contain 8–20% protein, including the gluten storage proteins that are enriched in proline and glutamine. The abundant gluten proteins constitute up to 80% of total flour protein, and confer properties of elasticity and extensibility that are essential for functionality of wheat flours (Shewry et al., 1994, 1995a,b,; Shewry, 1995). The gluten proteins consist of monomeric gliadins and polymeric glutenins. The gliadins constitute from 30 to 40% of total flour protein and are a polymorphic mixture of proteins soluble in 70% alcohol. They range in size from about 30–60 kDa and can be separated into α, γ and ω subgroups, each containing many closely related proteins (Anderson and Greene, 1997). In addition, coding regions for several low molecular weight proteins related to gliadins have been identified in genome sequencing projects, although only one of the proteins has been characterized (Clarke et al., 2000; Anderson et al., 2002a). The glutenin polymers consist of low molecular weight glutenin subunits (LMW-GS) of about 40 kDa linked by interchain disulphide bonds to high molecular weight glutenin subunits (HMW-GS) of about 90 kDa. The LMW-GS most closely resemble γ-gliadins in sequence (Müller et al., 1998) and comprise about 20–30% of the total protein (Gupta et al., 1992) while the HMW-GS account for about 5–10% of the total protein (Payne, 1986). Three to five HMW-GS and 15–20 different LMW-GS proteins are recognized in 2D gels of hexaploid wheat (Lew et al., 1992). The roles of the individual gluten components in dough functionality are complex (Gupta et al., 1992; MacRitchie et al., 1991; Khatkar et al., 2002). Although

HMW-GS constitute no more than 10% of total flour protein, they may be the most important determinants of breadmaking quality because of their importance in forming the glutenin polymer. Because it is nearly impossible to extract the intact glutenin polymer from flour, it is difficult to obtain a true estimate of the amount and size of the glutenin polymer. Nonetheless, the amount of unextractable protein, mainly glutenin polymer, the amount of polymer extracted by sonication, and the amount of HMW-GS contained within the glutenin polymers have all been reported to correlate with breadmaking quality (Gupta et al., 1992; Bean et al., 1998; Field et al., 1983). Breadmaking quality also 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). Recent studies using transgenic plants demonstrated that alteration of HMW-GS composition impacts breadmaking quality (Barro et al., 1997). LMW-GS contributed greatly to pasta-making quality of tetraploid durum wheats (Masci et al., 2000).

Water-soluble albumins and salt-soluble globulins constitute from 10 to 22% of total flour protein (Pence et al., 1954; Singh and Skerritt, 2001). Predominant albumins and globulins such as alpha-amylase/trypsin inhibitors (Shewry et al., 1984; Buonocore et al., 1985; Buonocore et al., 1977; Garcia-Maroto et al., 1990), serpins (Østergaard et al., 2000) and purothionins (Garcia-Olmedo et al., 2002) may have dual roles as nutrient reserves for the germinating embryo and as inhibitors of insects and fungal pathogens prior to germination. Triticin is related to storage globulins in oats, rice and legumes (Singh et al., 1991, 1993), and puroindolines influence grain hardness (Morris, 2002). Generally, albumins and globulins are not thought to play a critical role in flour quality, although the ratio of albumin to globulin was reported to correlate with breadmaking quality in one of the few studies to make such a determination (Pence et al., 1954). Also, one study suggests a relationship between alpha-amylase/trypsin inhibitors, misidentified as LMW-GS, and pasta quality for flour from durum wheat (Kobrehel and Alary, 1989a,b).

All seed storage proteins are secreted into the endoplasmic reticulum, where the transit peptide is removed and other post-translational processing may take place. Some wheat storage proteins then appear to follow the secretory pathway from ER to Golgi to protein bodies. However, other proteins accumulate in the ER and then are incorporated into vacuole-like compartments that also engulf the protein bodies (Levanony et al., 1992). Unlike rice or maize, there are no recognizable protein bodies in the endosperm of the mature wheat grain. Instead, protein in the vacuole-like compartments is compressed between the starch granules, with the loss of recognizable compartmentation (Levanony et al., 1992; Jiang et al., 2001; Shy et al., 2001; Chrispeels and Herman, 2000).

7. Effect of environment on synthesis and accumulation of storage proteins

Field studies indicate that environmental conditions, particularly fertilizer and temperature, affect the amount, composition and/or polymerization of the gluten proteins (Wrigley et al., 1984; Ciaffi et al., 1996; Graybosch et al., 1995; Borghi et al., 1995; Wieser and Seilmeier, 1998; Panozzo and Eagles, 2000; Selles et al., 1998; Luo et al., 2000; Johansson et al., 2001; Vaughan et al., 1990). Increases in grain protein content and in gliadin to glutenin and HMW-GS to LMW-GS ratios were observed with increased nitrogen fertilizer (Gupta et al., 1992; Wieser and Seilmeier, 1998; Zhu et al., 1999; Jia et al., 1996). Relative amounts of albumin and globulin were reported to decrease (Pence et al., 1954; Wieser and Seilmeier, 1998; Doekes and Wennekes, 1982). Large increases in ω-gliadins together with lesser increases in HMW-GS were also reported under conditions of sulphur deficiency (Wrigley et al., 1984). The polymeric nature of gluten makes it difficult to separate and quantify the components, however, and it is difficult to compare results from studies using different methods of protein fractionation and analysis. Wieser and Seilmeier (1998) used RP-HPLC to conduct a detailed quantitative study of the effects of nitrogen fertilizer on individual gliadin and glutenin components in 13 varieties of hexaploid wheat. With increased fertilizer, amount of protein per mg of flour increased 44–68%. There were 2- to 3-fold increases in amount of ω-gliadins per mg flour and increases of 56–101% in HMW-GS, whereas little change was observed in α and γ-gliadins and LMW-GS as a proportion of total flour protein. In the same study, amount of albumin plus globulin per mg of flour was unaffected by fertilizer and the proportion of albumins and globulins decreased relative to total flour protein. Although total albumin and globulin tend to be insensitive to applied fertilizer, no studies have determined whether this is true for individual albumin and globulin components.

Plants have complex mechanisms to regulate the balance of carbon, nitrogen and sulphur in their seeds (Galili et al., 2001; Tabe et al., 2002; Coruzzi and Zhou, 2001; Shewry et al., 2001). Responses of the various classes of storage proteins to nitrogen and sulphur fertilizer may be related to their amino acid compositions. Tatham and Shewry (1995) classified the glutamine- and proline-rich storage proteins into three groups based on sulphur content: the sulphur-poor ω-gliadins; the HMW-GS with intermediate amounts of sulphur; and the sulphur-rich α-gliadins, γ-gliadins and LMW-GS. Many albumins and globulins are rich in sulphur-containing amino acids. Because the ω-gliadins and HMW-GS are mainly composed of the metabolically inexpensive amino acids glutamine and proline they may be good storehouses for nitrogen when there is surplus nitrogen or insufficient sulphur.

Effects of temperature on storage protein composition are unclear, and may vary with genotype. High temperature was

reported to increase the proportion of gliadins to glutenins and decrease the proportion of large polymer in flour from several wheat varieties grown in controlled environment experiments (Blumenthal et al., 1995; Panozzo and Eagles, 2000; Corbellini et al., 1997). It was reported that genotypes having alleles for the HMW-GS pair 1Dx5, 1Dy10 generally showed less variability in storage protein composition in response to high temperature than genotypes with alleles for the HMW-GS pair 1Dx2, 1Dy12 (Blumenthal et al., 1995). However, the gliadin to glutenin ratio also increased, and flour breadmaking quality decreased, when plants of the US wheat 'Karl' were exposed to a 35/20 °C rather than a 20 °C/20 °C day/night temperature regimen from 10 DPA until maturity, even though 'Karl' has the 1Dx5, 1Dy10 HMW-GS pair (Gibson et al., 1998). Most studies of the effect of temperature on protein composition and flour quality have not taken the effects of temperature on flour protein percentage into account. When wheat plants do not receive high levels of fertilizer, protein content may be increased by heat because of differential effects of temperature on protein and starch deposition, and this may or may not lead to differences in flour protein composition and quality (Randall and Moss, 1990; Daniel and Triboi, 2000; Selles et al., 1998; Finney and Fryer, 1958). DuPont et al. (1998a) observed no significant differences in flour protein composition between plants grown under a 24/17 °C or a 37/17 °C day/night regimen when wheat plants received post-anthesis fertilizer, were well watered, and the flour had high protein levels. However, when plants were grown under the two temperature regimens without post-anthesis fertilizer, differences in protein amount and composition were observed. Protein content and levels of ω-gliadins were higher for flour from plants grown under the high fertilizer or high temperature regimens (DuPont et al., 2000).

8. Regulation of storage protein gene expression

The major storage protein genes are expressed specifically in endosperm tissue and are under strict temporal control. Coordinate accumulation of transcripts from the HMW-GS, LMW-GS, and α-, γ-, and ω-gliadin gene families occurs early in grain development (Lamacchia and Shewry, 2001; Altenbach et al., 2002; Thomas and Flavell, 1990; Reeves et al., 1986; Bartels and Thompson, 1986). Reverse-transcriptase polymerase chain reaction (RT-PCR) revealed that individual HMW-GS genes and at least seven LMW-GS genes exhibited identical patterns of temporal regulation (Altenbach et al., 2002). Initiation of transcription is regulated by transcription factors that bind to common elements found in promotor regions of prolamin genes. Promoter regions of genes for wheat LMW-GS and gliadins, barley hordeins, and rye secalins have a bipartite element, termed the endosperm box, consisting of two adjacent motifs, the GCN4-like motif (G-motif, GLM, or N-motif) and the prolamin or endosperm motif (E-motif)

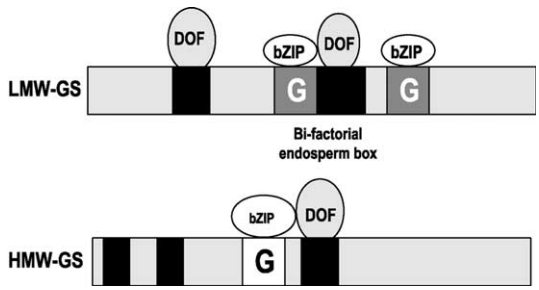

Fig. 3. Interaction of transcription factors and binding elements in promoter regions of gluten protein genes. G, GCN4 or G-box like motif; DOF, DNA binding with one finger; bZIP, basic leucine zipper binding factor. Black boxes indicate E-motifs and E-motif like elements. Diagram based on gene sequences for LMW-GS 1 (Conlan et al., 1999) and HMW-GS 1Dx5 (Norre et al., 2002).

(Fig. 3). The bipartite endosperm box is found approximately 300 bp upstream from the coding region, and additional copies of the motifs may be found upstream or downstream from the endosperm box (McKinnon and Henry, 1995). Similar, but not identical, motifs are found in genes for HMW-GS (Fig. 3) and some zeins. Prolamin promoters have been analyzed by transient analysis of promoter-GUS constructs in isolated endosperm, by expression of promoter-GUS constructs in transgenic wheat (Lamacchia and Shewry, 2001) and by expression of native and modified genes in tobacco plants, and functions of the regulatory elements have been identified by deletion analysis (Thomas and Flavell, 1990; Zheng et al., 1993). The E-motif was essential for endosperm-specific expression of prolamin genes in maize, barley and wheat, and in the cotyledon of tobacco seed. The GCN4 motif in yeast and other organisms is involved in the response to nutrients and environment, and the similar G motif in prolamin genes was suggested to be involved in the response to nitrogen by barley C-hordein and maize 22-kDa α-zein genes (Müller et al., 1997; Müller and Knudsen, 1993). Additional elements further from the coding region may contribute to stability of expression of genes for HMW-GS (Anderson et al., 2002b).

So far, two types of transcription factor have been identified that bind to the promoter region of prolamin genes (Vicente-Carbajosa et al., 1997; Marzábal et al., 1998; Hammond-Kosack et al., 1993) (Fig. 3). Proteins in the basic leucine zipper (bZIP) family bind to the G-motif. The opaque-2 or O2 protein from maize is the best characterized bZIP transcription factor associated with a prolamin gene. A similar protein in wheat is termed SPA (Albani et al., 1997; Conlan et al., 1999). Proteins in the DNA binding with one finger (DOF) family (Yanagisawa and Schmidt, 1999) bind to the E-box and were termed PBF (prolamin binding factor) in maize, BPBF in barley, and WPBF in wheat (Mena et al., 1998). DOF proteins are unique to plants and have been suggested to regulate genes involved in carbon metabolism (Yanagisawa, 2000). BPBF was reported to influence endosperm-specific expression of hordein genes during

grain development and to influence expression of aleurone-specific hydrolase genes during grain germination (Mena et al., 2002). There is evidence that bZIP and DOF transcription factors also interact with each other. Additional work is needed to understand developmental and environmental regulation of the transcription factors, how binding of these factors to the G- and E-boxes is mediated, to identify any other transcription factors that modulate gene expression, and investigate potential roles of phosphorylation (Ciceri et al., 1999).

Once transcribed, gluten protein mRNAs are present at high levels throughout the period of grain filling (Greene, 1983). At some point late in grain development, transcripts for all classes of storage protein genes disappear coordinately, suggesting that a common signal may trigger the decline of the gluten transcripts (Altenbach et al., 2002). This may be due to destabilizaton of the transcripts or changes in levels of transcript turnover relative to rate of transcription. Timing of the decline in mRNAs for the gluten proteins may have important consequences in the response of the developing kernel to the environment. However, this aspect has received little attention to date.

Genes for some of the albumin and globulin storage proteins also have been characterized (Dahl et al., 1996; Gautier et al., 1990, 1994; Sanchez de la Hoz et al., 1994). Temporal expression of a major group of 12-kDa albumins, the alpha-amylase/trypsin inhibitor family of CM proteins, was similar to that for the gliadins and glutenins (Gautier et al., 1990), whereas triticin transcripts peaked at 20 DPA in mid-grain fill (Singh et al., 1993).

9. Effects of environment on expression of storage protein genes

Temporal expression of gluten protein genes was influenced by temperature during grain fill (Altenbach et al., 2002). Transcripts within all major gene families accumulated earlier and disappeared earlier in kernels developing under high temperature regimens or high temperatures combined with drought, when compared to kernels produced under a moderate temperature regimen. Similar changes in the timing of protein deposition during grain development were observed (Altenbach et al., 2003). Shifts in the temporal expression of the gluten protein genes may result from a developmental signal or directly from an environmental cue. Neither temperature nor drought uncoupled the coordinate expression of the gluten protein genes. In addition, there has been no convincing evidence thus far that temperature alters the levels of transcripts within any of the gluten protein gene families despite the presence of putative heat shock consensus elements in the upstream regions of several gliadin genes (Blumenthal et al., 1990). Little difference was found in steady-state levels of α-gliadin or HMW-GS transcripts when several Italian wheat varieties were subjected to high temperatures (Perrotta et al., 1998). In controlled growth experiments using a US wheat supplied with post-anthesis fertilizer, transcript levels for α-, γ- and ω-gliadins, LMW-GS and HMW-GS also showed little variation with temperature when assessed by hybridization analysis throughout grain development (Altenbach et al., 2002).

Accumulation of storage proteins in wheat and barley is affected by N (Shewry et al., 2001). The presence of the GCN4 motif in the promoter region of major wheat gluten genes suggests that availability of nitrogen may affect transcription of these genes. Effects of nitrogen on gene expression in isolated kernels or spikes was demonstrated for the C-hordein gene of barley (Müller and Knudsen, 1993) and the 22-kDa zein gene of maize (Müller et al., 1997). In intact wheat plants, transcript levels for ω-gliadin genes declined in the absence of post-anthesis fertilizer, but transcript levels for other classes of gliadin or glutenin genes were not affected (DuPont et al., 2000). This suggests that transcriptional regulation plays a role in the response of ω-gliadin genes to fertilizer. Post-anthesis fertilizer did not influence temporal expression of any of the gluten protein genes under moderate temperature regimens. In fact, in the absence of post-anthesis fertilizer, transcripts from all gluten protein classes were present for at least ten days beyond the point that protein accumulation ceased, suggesting that translational regulation plays an important role in cessation of protein accumulation under these conditions (Altenbach et al., 2002). Regulation of albumin and globulin genes in response to environment has received little attention. In barley, transcript levels for an albumin storage protein, β-amylase, did not increase in response to in vitro nitrogen, whereas transcript levels for a B-hordein did increase (Giese and Hopp, 1984).

Wheat endosperm genes encoding several proteins that may be involved in processing and post-translational modification of the storage proteins have been identified and characterized. Two of these proteins, binding protein (BiP) and protein disulphide isomerase (PDI), a foldase, reside in the endoplasmic reticulum and are required for synthesis, polymerization, and accumulation of storage proteins in many tissues (Bulleid and Freedman, 1988; Li et al., 1993), although their role in gluten deposition is not clear. Analysis of steady-state RNA levels in developing kernels demonstrated that BiP and PDI were developmentally regulated (Grimwade et al., 1996; DuPont et al., 1998b) and that mRNA levels were reduced in response to high temperature (Hurkman et al., 1998). Despite the decrease in transcripts, however, the amounts of corresponding protein in the developing kernels were not affected by the temperature treatment. Members of the HSP70 gene family are known to function as protein chaperones and are highly expressed in cereal endosperm and soybean seeds (Skylas et al., 2002; Cho et al., 2002). HSP70 transcript levels increased, and HSP70 protein accumulated in the endosperm in response to high temperatures during grain fill (Hurkman et al., 1996). Although it has been suggested that

excess HSP70 could alter folding and aggregation of gluten proteins during dough development, specific effects of HSP70 were not observed in dough-mixing experiments (Blumenthal et al., 1998). A number of small HSPs were shown to be upregulated in a heat-tolerant Australian cultivar in response to a three-day heat treatment at 40 °C during mid-grain development (Skylas et al., 2002).

10. Proteomic studies of grain development

Proteins involved in cellular metabolism and biosynthetic and regulatory processes during grain development are of low abundance compared to storage proteins. Many of these low abundance proteins, such as the enzymes needed for starch biosynthesis (Rahman et al., 1995), are essential for grain fill. New developments in proteomics make it possible to identify hundreds of endosperm proteins using mass spectrometry (Van Wijk, 2001). Identification of proteins is based on first separating proteins by 2D-PAGE, HPLC, or other methods, then subjecting them to protease digestion or fragmentation, measuring the masses of the resulting peptides and matching them to masses predicted from known gene and protein sequences. A complementary approach involves microsequence analysis of proteins or peptides. Both methods rely heavily on availability of extensive databases of gene and protein sequences from wheat, rice, maize, *Arabidopsis*, and other plants and organisms, and, just as importantly, on the quality of the sequences contained within those databases. Identification of rare protein species within complex mixtures may require enrichment by various fractionation methods prior to analysis.

A recent proteome analysis of rice grains identified 877 unique proteins (Koller et al., 2002). Approximately 1300 proteins extracted from wheat endosperm at mid-development were resolved by 2D-PAGE (Skylas et al., 2000). Of the 321 proteins analyzed by N-terminal microsequencing, 177 were identified, 55 had no match in sequence databases, and it was not possible to sequence the remaining 89. The proteins identified included many gliadins, HMW-GS, and alpha-amylase/trypsin inhibitors.

Quantitative differences in accumulation of proteins throughout grain development can be assessed using gel-analysis software to analyze 2D-PAGE images of proteins from different developmental stages. An initial study compared proteins from 17-DPA and 45-DPA wheat endosperm (Skylas et al., 2000). Proteome analysis of developing barley grains compared proteins at five developmental time points and yielded identification and expression profiles for 19 proteins. These included a number of alpha-amylase/trypsin inhibitors, several serpins, β-amylase, and PDI (Finnie et al., 2002).

To evaluate the effects of environment, protein profiles generated from kernels grown under different environmental conditions must be assembled and analyzed. Thus far, a comparison of proteins accumulated in 17-DPA and 45-DPA wheat grains subjected to a three-day heat treatment has been reported (Skylas et al., 2002). Of the 48 protein spots on 2D-PAGE that exhibited differential expression, 17 were identified, and all of these were members of families of small heat shock proteins.

Proteomics techniques promise to add greatly to information about patterns of protein accumulation and can provide information about pathways of signal transduction by identifying regulatory modifications of proteins, such as phosphorylation (Vener et al., 2001). Thus far, however, most of the proteins identified in wheat endosperm were abundant species. One of the challenges of proteomic studies will be to identify the less abundant proteins. A second challenge will be to determine the functions of newly described proteins, a formidable task given that about 40% of the predicted proteins in the *Arabidopsis* genome have no assigned function (Van Wijk, 2001). In any event, proteomic analyses will be most useful when combined with other functional genomics approaches such as microarray analysis.

11. Microarray analysis of grain development

Microarrays are an excellent tool for comparing global patterns of gene expression in two different samples (Cho et al., 2002). With the current technology, thousands of DNA fragments, each representing an expressed gene, are printed onto a microscope slide and hybridized in a competitive fashion to differentially labeled fluorescent probes that represent mRNAs from two tissue samples. The relative amounts of transcripts hybridising to each gene sequence are determined by scanning the slide with a laser detection system. Microarray analyses have been used to compare gene expression in different tissue types in maize (Cho et al., 2002; Fernandes et al., 2002) and *Arabidopsis* (Girke et al., 2002). In *Arabidopsis*, microarray analyses also have been used to compare genes expressed in whole plants in response to short periods of drought or cold stress (Seki et al., 2001). It is more complex to analyze developmental processes using microarray techniques, and there are few published studies thus far (Livesey, 2002). The complement of expressed genes must be surveyed at a number of time points between anthesis and harvest maturity in order to generate a profile of gene expression in the developing grain. Because microarrays measure relative proportions of a given transcript in two samples, comparisons must be made between each time point in a pair-wise fashion (Churchill, 2002). Alternately, mRNA from individual time points may be compared to a reference mRNA population. Under conditions where grain development occurs over a 45-day period, the analysis of six time points would generate a considerable amount of information, yet still leave large intervals between data points. More detailed analyses using a greater number of RNA

samples may be necessary to pinpoint those genes responsible for limiting kernel expansion, initiating and terminating starch and protein accumulation, and inducing apoptosis and desiccation. Such analyses can be done on selected genes using Northern blot analysis or quantitative RT-PCR.

It is not trivial to compare global patterns of gene expression in kernels from plants grown under different environmental regimens. Where environmental factors do not alter the timing of grain development, expression profiles of treated and untreated kernels of a similar age can be compared directly. Thus, effects of post-anthesis fertilizer on global patterns of gene expression can be evaluated in a relatively simple manner. Similarly, when the environmental stress is applied for a short period of time, for example, a three-day heat shock, there may be minimal changes in timing of grain development, and direct comparisons may yield valuable information. However, environmental factors such as temperature and drought tend to affect the timing of developmental processes, so that direct comparisons between kernels of a similar age grown under different conditions can be misleading. In these cases, developmental transcript profiles must be generated from kernels produced under each environmental regimen before comparisons can be made between treatments.

The availability of high quality databases of wheat gene sequences is essential for ensuring the success of functional genomic approaches. More than 400,000 wheat ESTs have been entered into public databases and can be accessed using the TIGR *Triticum aestivum* Gene Index at http://www.tigr.org/tbd/tgi/tagi/. Mathematical modelling estimates suggest that between 4500 and 8000 genes may be active in 8–12-DPA endosperm (Clarke et al., 2000). Undoubtedly, many of the genes that exhibit interesting expression profiles in developing endosperm tissue will not have been studied previously. Hence, a major challenge will be to assign functions to unknown gene sequences. Current approaches utilize reverse genetics, in which genes with unknown functions are targeted and disrupted and the resulting phenotype examined. It is easiest to carry out such experiments in a plant with a short life-span and a well-characterized genome, such as *Arabidopsis*. Such experiments also will be done with rice, barley and diploid wheat, especially for genes that do not have homologs in *Arabidopsis*. Gene silencing techniques may also work with tetraploid and hexaploid wheat, and chromosomal substitution lines will be useful in exploring the functions of wheat genes (Lafiandra et al., 1984).

12. Regulation of grain fill

It is likely that hormones mediate effects of environment on grain fill, and there are many studies of hormones and their function in signal transduction in dicot seeds and cereal caryopses. Most emphasize the role of hormones in the embryo or aleurone during seed development and germination (McCarty, 1995; Finkelstein et al., 2002). Cytokinin levels are maximal in grains immediately after anthesis, followed by peaks of gibberellin (GA) and auxin during the linear growth phase, and then of ethylene and abscisic acid (ABA) as the grains mature. Complex interactions between water stress, nitrogen, and levels of cytokininins, GA, auxin and ABA were observed in a detailed study of developing rice grains (Yang et al., 2001). There are few studies of the roles of hormones in the starchy endosperm. There are reports that cytokinins originating in the roots stimulate cell division in rice endosperm (Yang et al., 2002) and that auxins stimulate endoreduplication in maize endosperm (Lur and Setter, 1993). ABA and ethylene peak during grain fill and are reported to influence the timing of apoptosis in maize, rice and wheat endosperm (Herzog, 1986; Young and Gallie, 2000; Yang et al., 2001; White and Rivin, 2000; White et al., 2000).

Many other compounds produced in roots, leaves, and seeds are candidates for regulatory compounds that influence grain fill. The supply of photosynthate is a major factor that limits accumulation of dry weight. Sugars and amino acids from the leaves and stems not only serve as substrates for starch and protein synthesis, but also interact to regulate gene expression and metabolic pathways. Sugars act as growth regulators in seeds (Finkelstein and Gibson, 2002; Rolland et al., 2002), although little is known of their role in regulating endosperm development. The balance between nitrogen, sulphur, and carbon compounds may be involved in regulation of amino acid biosynthesis and catabolism and in protein accumulation (Herzog, 1986; Luo et al., 2000; Tabe et al., 2002; Coruzzi and Zhou, 2001). Plant and kernel water status also influence hormone status, as well as physical processes during grain fill (Egli, 1998; Westgate, 1994). Much work remains to be done before the precise roles of hormones and other growth regulating compounds in endosperm development are uncovered and the roles of signal transduction pathways that coordinate the response to the environment are understood.

13. Summary

Molecular processes involved in wheat grain development are as yet poorly understood despite the importance of cereals as a major source of nutrition for human kind. Genomics and proteomics techniques offer considerable promise for understanding these processes. Functional genomic and proteomic studies of developing wheat endosperm promise to reveal patterns of gene expression associated with key developmental events. By comparing profiles of gene expression and protein accumulation from endosperm developing under different environmental conditions, it should be possible to uncover basic molecular mechanisms that are influenced by environment and that affect productivity and quality. Global analyses of gene

expression and protein accumulation must rest on a solid foundation of grain developmental studies.

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