



Molecular genetics of heat tolerance and heat shock proteins in cereals

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Abstract

Heat stress is common in most cereal-growing areas of the world. In this paper, we summarize the current knowledge on the molecular and genetic basis of thermotolerance in vegetative and reproductive tissues of cereals. Significance of heat stress response and expression of heat shock proteins (HSPs) in thermotolerance of cereal yield and quality is discussed. Major avenues for increasing thermotolerance in cereals via conventional breeding or genetic modification are outlined.

Introduction

Most of the world crops are exposed to heat stress during some stages of their life cycle (Stone, 2001). Exposure to higher than optimal temperatures, or heat stress, reduces yield and decreases quality of cereals. Furthermore, as the world population grows exponentially, there is a need to both increase agricultural productivity and to expand productive areas of the world into warmer climates. Both of these goals require significant breeding efforts to improve high-temperature tolerance of cereal yield and quality. Meanwhile, cereal breeding to date had utilized a limited number of progenitor germplasms and emphasized a high yield potential under favourable environmental conditions narrowing down genetic diversity of stress resistance traits, including heat stress tolerance (Holden *et al.*, 1993). Hence, there is a strong need to elucidate molecular and genetic basis of heat tolerance in cereals, to identify beneficial genes and alleles, and to utilize them in the molecular breeding programmes targeted to produce superior cereal cultivars in the future.

Heat stress-induced decrease of the duration of developmental phases leading to fewer organs, smaller organs, reduced light perception over the shortened

life cycle, and perturbation of the processes related to carbon assimilation (transpiration, photosynthesis and respiration) are most significant for losses in cereal yields (Stone, 2001). In this review, we summarize the current knowledge of genetic basis and molecular mechanisms of heat tolerance in cereals. Cereals hereby include cultivated species belonging to the genera oats (*Avena*), barley (*Hordeum*), millet (*Pennisetum*), rice (*Oryza*), rye (*Secale*), sorghum (*Sorghum*), wheat (*Triticum*) and maize (*Zea*). Where relevant, data obtained on other species are discussed. Furthermore, we outline major avenues for improvement of high-temperature tolerance in cereals, in terms of both yield and quality, by conventional breeding or molecular genetic modification.

Genetics of thermotolerance

Heat tolerance is not controlled by a single 'thermotolerant' gene in cereals. Different components of tolerance determined by different sets of genes are critical for heat tolerance at different stages of the life cycle and in various tissues. Quantitative trait loci analysis, correlation and co-segregation approaches,

and the use of genetic stocks are most applicable to the dissection of the genetic basis of thermotolerance in cereals.

Genetic components of heat tolerance

Quantitative trait loci (QTL)

QTL analysis to dissect high-temperature tolerance in cereals was used by Ottaviano *et al.* (1991) who detected six QTLs accounting for 53% of the genetic variability for inherent cellular membrane thermostability (CMS) in a maize recombinant inbred line (RIL) population. The capacity to acquire thermotolerance in maize seedlings in this experiment was not analysed and the population size was limited to just 44 RILs. Later, in the same RIL population, five QTLs for pollen ability to germinate at high temperature and six QTLs for pollen tube growth at high temperature were identified (Frova and Sari-Gorla, 1993; Frova, 1996).

Co-segregation analysis

In a RIL population of spring wheat, acquired thermotolerance of wheat seedlings in terms of CMS was correlated with yield of RILs grown under hot summer conditions in Israel (Blum *et al.*, 2001). CMS correlated well with seedling thermotolerance in four hot environments in Mexico, Sudan, India and Brazil in wheat (Fokar *et al.*, 1998) and in pearl millet (Howarth *et al.*, 1997). Mitochondrial respiration activity under heat stress as measured by the triphenyl-tetrazolium chloride (TTC) reduction assay and wheat seedling thermotolerance did not correlate significantly (Fokar *et al.*, 1998).

Causal genetic involvement of heat shock proteins (HSPs) in thermotolerance was suggested in a co-segregation analysis of a small subset of RILs of winter wheat (Joshi *et al.*, 1997). Similar evidence of significance of chloroplast-localized HSPs for high-temperature tolerance was obtained in nine genotypes of tomato (Preczewski *et al.*, 2000) and accessions of creeping bentgrass (Park *et al.*, 1996). In maize, expression of a 45 kDa HSP correlated with thermotolerance (Ristic *et al.*, 1998).

Genetic stock analysis

There is accumulating evidence that HSPs play an important role in thermotolerance. A wheat ditelosomic line lacking the long arm of chromosome 1B, DT1BS, was able to acquire thermotolerance at lower induction temperatures and was therefore better protected from heat stress damage (O'Mahony and Burke,

2000). Coincidentally, expression of HSPs in this line was induced at temperatures 4 °C lower than those required for HSP induction in the original wheat cultivar Chinese Spring. Hence, the long arm of chromosome 1B is likely to carry genes repressing both the heat shock response at lower temperature and thermotolerance. In contrast, a ditelosomic line missing the long arm of chromosome 7D was heat-sensitive and exhibited reduced expression of some HSPs; it was concluded that it carries genes necessary for induction of several HSPs and the capacity to acquire thermotolerance (O'Mahony *et al.*, 2000). Analysis of available genetic stocks in wheat, rye, barley and other cereals, including nullisomic and ditelosomic lines, addition, recombination, translocation lines and hybrids can be further employed for dissection of the genetic basis of thermotolerance in cereals.

Recent experimental data obtained by means of *transgenic, reverse-genetics and mutation* approaches in non-cereal species confirm causal involvement of HSPs in thermotolerance in plants. Four genomic loci determining the capacity of *Arabidopsis* to acquire thermotolerance were recently identified using a panel of heat-sensitive mutants (Hong and Vierling, 2000). A gene encoding HSP101 was assigned to one of these loci, the study of others is in progress. Expression of HSP70 was shown to be causally involved in the capacity to acquire thermotolerance in *Arabidopsis* by a reverse-genetics approach (Lee and Schöffl, 1996). Transgenic derepression of the activity of a heat shock factor, the transcription factor mediating transcription of mRNAs encoding HSPs, led to an increase of thermotolerance of *Arabidopsis* plants (Lee *et al.*, 1995; Prandl *et al.*, 1998). Burke *et al.* (2000) showed the absence of a HSP of 27 kDa in another thermosensitive *Arabidopsis* mutant. In carrot suspension cultures, a cytosolic low-molecular-weight HSP, HSP17, was identified as a factor of acquired thermotolerance in the study of transgenic cells with HSP17 in sense and antisense orientation (Malik *et al.*, 1999).

In cereals, transgenic and reverse-genetics approaches are rarely feasible because of genome size, polyploidy, long generation time and difficulties in transformation technology. Mutants of barley and wheat can be exploited (for example, Mullarkey and Jones, 2000).

Genetic diversity of heat tolerance

Genetic variability for thermotolerance and expression of individual HSPs was noted in numerous studies

in cultivated cereals (e.g. Howarth, 1989; Jorgenson and Nguyen, 1995; Joshi *et al.*, 1997; Fokar *et al.*, 1998). Recently, these investigations were taken to a new level taking advantage of larger collections of cultivars, landraces and wild germplasm.

Studies of genetic diversity among cultivars, wild accessions and ecotypes of cereals are useful for discovery of novel QTLs and alleles which can be further exploited in the programmes of thermotolerance improvement in cereals. Populations of wild species frequently harbour high intra-species variation for tolerance traits which is extinct in the modern cultivars (Nevo *et al.*, 1997). This may be particularly true for self-pollinated crops such as wheat in which the level of genetic variation constitutes only a small fraction of that available in nature (Pingali and Heisey, 1996).

Moreover, genetic make-up of wild populations is overall highly variable, including other tolerance and productivity traits such as disease resistance and seed protein composition (Johnson and Waines, 1997), making investigations into wild germplasm even more appealing. Recently, in a study of more than 1200 Mexican wheat landraces collected from areas with diverse thermal regimes, a highly significant correlation between leaf chlorophyll content and 1000-seed weight was observed and a group of superior accessions were identified (Hede *et al.*, 1999). A search of beneficial 'wild' alleles for important agronomic traits is especially urgent because of the fast erosion of the gene pool of cultivated cereals and rapid extinction of many natural habitats due to soil erosion and cultivation.

Polymorphism of HSP gene sequences and concomitant variability of thermotolerance traits were detected in a set of barley cultivars (Marmioli *et al.*, 1989a, b, c; 1994). We have observed a high degree of restriction fragment length polymorphisms at the *Hvhspl7* gene locus (Marmioli *et al.*, 1993), encoding a cytoplasmic class I low-molecular-weight HSP, within 26 European cultivars of *Hordeum vulgare* with diverse genetic background (Marmioli *et al.*, 1998). Spring and winter cultivars could be effectively separated in the dendrogram depicting genetic similarities. There are two possible explanations for the observed relationship between HSP gene polymorphisms and growth habit in barley. First, these HSP genes could be involved in the genetic determination of growth habit. Second, they could be in linkage disequilibrium with the genes that determine growth habit. Two polymorphic *Sgh* loci differentiate winter and spring barleys of European origin. From several syntenic

maps, it is known that the genomic region surrounding *Sgh2* on chromosome 7(5H) also contains other stress-related genes, including *ABA2* (Quarrie *et al.*, 1994), *Vrn1* (Laurie *et al.*, 1995), QTLs for winter hardiness (van Zee *et al.*, 1995) and for drought response (Quarrie *et al.*, 1994), and a gene encoding low-molecular-weight HSP (Dubcovsky *et al.*, 1995). This genetic configuration represents a cluster of stress response-related genes.

Molecular basis of thermotolerance in vegetative tissues

Application of exogenous compounds or transgenic manipulations has been widely used to determine molecular factors critical for thermotolerance. Here, we will discuss several major classes of molecules thought to be involved in determination of thermotolerance at the vegetative stage of development in cereals. These include phytohormones, antioxidants, membrane lipids and HSPs. In addition, thermotolerance of translation and thermostability of key enzymes are important.

Thermotolerance and hormonal regulation

Hormones govern all aspects of plant metabolism (Salisbury and Ross, 1992). When plants are subjected to heat stress during vegetative growth stage, among other things, it alters hormone homeostasis, including hormone stability, content, biosynthesis and compartmentalization. Some of the heat-induced processes at cell, organ and whole-plant levels can be hormone-mediated; others can be the consequence of a new hormonal status altered by heat stress (Hoffman and Parsons, 1991). These complex interactions and cause effect relationships have been rarely addressed. Effects of high temperatures on plant hormone receptors at the gene expression and protein steady-state levels are mostly unknown. Meanwhile, better thermotolerance may be achieved if hormonal control of plant processes in the thermotolerant state is optimized. Abscisic acid (ABA) is implicated in plant osmotic stress responses and mediates one of the intracellular dehydration signalling pathways (Davies and Jones, 1991). In the field, where heat and drought stresses frequently occur simultaneously, ABA induction can be an important component of thermotolerance. Indeed, in maize (*Zea mays*) exogenously applied ABA could mimic water stress in increasing thermotolerance of

photosystem II (Gong *et al.*, 1998); additional application of calcium in maize acted synergistically with ABA (Gong *et al.*, 1998). In sorghum (*Sorghum bicolor*) leaves, thermotolerance of photosystem II was protected by salt stress (Lu and Zhang, 1998). Several HSPs are ABA-inducible (Pareek *et al.*, 1998).

The effect of gibberellins on high temperature tolerance is the reverse of that of ABA. An inherently heat-resistant dwarf mutant of barley (*Hordeum vulgare*) impaired in the synthesis of gibberellins was rendered heat-sensitive by application of gibberellic acid, whereas application of the triazole paclobutrazol, a gibberellin antagonist, conferred heat tolerance (Vettakkorumakankav *et al.*, 1999). Recently, brassinosteroids were shown to confer thermotolerance to tomato (*Lycopersicon esculentum*) and *Brassica napus* (Dhaubhadel *et al.*, 1999). This was not confirmed in cereals. Overall, our understanding of phytohormone involvement in thermotolerance in cereals is far from being complete.

Antioxidants

A well known consequence of elevated temperatures in plants is oxidative damage caused by a heat-induced imbalance of photosynthesis and respiration (Fitter and Hay, 1987). Initially, heat stress may reduce activities of antioxidant enzymes as observed in maize seedlings (Gong *et al.*, 1997). The antioxidant defence mechanism is part of heat stress adaptation, and its strength is correlated with acquisition of thermotolerance. Accordingly, in a set of wheat (*Triticum aestivum*) genotypes, the capacity to acquire thermotolerance correlated with activities of catalase and superoxide dismutase, a higher ascorbic acid content, and less oxidative damage (Sairam *et al.*, 2000). Exogenous application of the compounds signalling oxidative damage – salicylic acid, acetylsalicylic acid and hydrogen peroxide – to mustard seedlings and micropropagated potato plants increased their thermotolerance (Dat *et al.*, 1998; Lopez Delgado *et al.*, 1998). Salicylate and reactive oxygen species are known to induce transcription of HSPs, including mitochondria-localized HSP22 and HSP70 (Banzet *et al.*, 1998; Cronje and Bornman, 1999); hence, their effect on thermotolerance may be mediated by HSPs. Calcium is a probable common secondary messenger in the heat, dehydration and oxidative stress signal transduction chains as its exogenous application improved oxidative stress tolerance while application of calcium

chelators or membrane transport blockers reversed the effect (Gong *et al.*, 1997, 1998).

Cell membranes and membrane-based processes

High temperature causes modifications in membrane functions mainly due to alteration of membrane fluidity. In plant cells, membrane function is especially important for membrane-based processes such as photosynthesis and respiration. Three commonly used assays of heat tolerance in plants are related to membrane-based processes (Blum, 1988): plasmalemma (CMS assay), photosynthetic membranes (chlorophyll fluorescence assay), and mitochondrial membranes (cell viability assay based on TTC reduction).

Membrane lipid saturation is considered an important element in high-temperature tolerance. In a mutant wheat line with increased heat resistance, heat treatment increased relative quantities of linolenic acid among galactolipids and of *trans*- Δ -3 hexaldecenoic acid among phospholipids compared with the wild-type wheat (Behl *et al.*, 1996). Currently, it remains unclear whether a higher or lower degree of membrane lipid saturation is beneficial for high-temperature tolerance (see Klueva *et al.*, 2001 for discussion).

Organelle-localized low-molecular-weight HSPs have been identified and their association with membranes upon heat stress was reported (Adamska and Kloppstech, 1991; Restivo *et al.*, 1986), suggesting that HSPs play a role in protecting photosynthetic electron transport, also under high-light conditions (Debel *et al.*, 1995). Addition of purified chloroplast-localized HSP conferred heat tolerance to the photosynthetic electron transport chain in isolated chloroplasts (Heckathorn *et al.*, 1998). This result was not confirmed in another laboratory (Harndahl and Sundby, 2001). The mitochondrial electron transport chain may be protected from heat damage by an addition of low-molecular-weight HSP *in vitro* (Downs and Heckathorn, 1998). More evidence for a role of chloroplast-localized HSPs in high-temperature tolerance was obtained in two different species, tomato (Preczewski *et al.*, 2000) and creeping bentgrass (*Agrostis palustris*, Park *et al.*, 1996).

In chloroplasts, heat decreases the photochemical efficiency of photosystem II, the most heat-sensitive link in photosynthesis (Al-Khatib and Paulsen, 1999). In the mitochondrial electron transport chain, NADH:ubiquinone oxidoreductase has been identified as one of the thermolabile components

(Downs and Heckathorn, 1998). The contribution of lipid and protein components to membrane function under heat stress needs further study.

Heat shock proteins

Significance of HSPs in thermotolerance was first hypothesized on the basis of correlative evidence (for a review, see Vierling, 1991). Recently, causal involvement of several HSPs in acquired thermotolerance of plants has been demonstrated (Burke *et al.*, 2000; Hong and Vierling, 2000; Lee *et al.*, 1995; Malik *et al.*, 1999; Queitsch *et al.*, 2000, discussed above under Genetics of thermotolerance), but to date no causal involvement of HSP in thermotolerance has been demonstrated in cereals. For in-depth reviews of HSP and their relevance to high-temperature tolerance in plants, the reader is referred to Vierling (1991) and Klueva *et al.* (2001). Here, we would like to point out that in polyploid cereals molecular diversity of HSP species within each plant is increased due to the contribution of the subgenomes. Furthermore, HSP gene families have more members in even diploid cereals than in dicots contributing to the molecular diversity. Should causal involvement of an HSP in thermotolerance in cereals be confirmed, allelic composition and molecular diversity of HSP families could be significant for determination of cultivar thermotolerance.

High-molecular-weight HSPs (HSP70, HSP90, HSP101) are characterized by a high level of sequence similarity within the plant kingdom. However, molecular diversity within families of high-molecular-weight HSPs allows one to hypothesize that even closely related members of HSP gene families or alleles may vary in their specific functions. Furthermore, HSP homologues of the same families may function in different cell compartments (see discussion below and Boston *et al.*, 1996).

HSP101 was recently shown to be a major component of thermotolerance in *Arabidopsis* (Hong and Vierling, 2000; Queitsch *et al.*, 2000). This has not been confirmed in cereals to date. We and others have recently cloned three members of HSP101 family in hexaploid wheat (Campbell *et al.*, 2001; Wells *et al.*, 1998). Here, we will refer to the wheat HSP101 cDNA reported by Wells *et al.* (1998) as *Tahsp101a*. The other two wheat HSP101-encoding cDNAs (*Tahsp101b* and *Tahsp101c*) originate from cv. Mustang (Campbell *et al.*, 2001). Structural comparison of the predicted proteins indicates that while

there is significant degree of sequence similarity, particularly, in the ATP-binding domains of these proteins, *Tahsp101a* (Wells *et al.*, 1998) significantly diverged in the second ATP-binding domain from the Mustang-derived HSP101 alleles and from other HSP100 of monocots (Campbell *et al.*, 2001). More research needs to be done towards the analysis of genetic variability within HSP families and its functional implications (Burke, 2001).

Mitochondria-localized HSPs were described in Gymnosperms and in both di- and monocotyledonous angiosperms, including wheat and maize (Basha *et al.*, 1999; Lund *et al.*, 1998). In our laboratory (Marmioli), fingerprinting analysis of heat-acclimated *Arabidopsis* plants able to survive a normally lethal heat shock showed a number of cDNA fragments exclusively expressed in these conditions and not expressed during mild or abrupt heat shock. One of these cDNAs encoded mitochondrial low-molecular-weight HSP, which was subsequently isolated as genomic clone (Visioli *et al.*, 1997). A survey of dbEST (January 2001) for DNA sequences similar to the isolated cDNA produced 41 matches: *Triticum aestivum* (5), *Triticum turgidum* (1), *Oryza sativa* (28) and *Zea mays* (7). Thus, experimental data from model plants can be efficiently used for rapid 'in silico' cloning of HSP genes that could be significant for thermotolerance in cereals.

Thermotolerance of translation

An important component of thermotolerance is heat tolerance of gene expression. Heat stress is known to swiftly alter the pattern of gene expression, inducing the HSP complement and inhibiting expression of many genes expressed under normal temperature conditions (Yost and Lindquist, 1988). The mRNAs encoding non-heat-stress-induced proteins are destabilized during heat stress (Gallie *et al.*, 1995). Heat stress inhibits splicing and it was hypothesized that HSP-encoding mRNAs can be processed properly due to the absence of introns in the corresponding genes. However, subsequently it was shown that some HSP-encoding genes have introns and, under heat stress conditions, their mRNAs are correctly spliced nevertheless (Osteryoung *et al.*, 1993; Visioli *et al.*, 1997). Hence, the mechanism of preferential translation of HSP-encoding mRNA under heat stress remains unclear.

Our laboratory (Marmioli), in collaboration with Dr Ralph Sinibaldi (currently at Operon Technolo-

gies, Inc.), utilized maize protoplasts for transient gene expression assays of the promoters of heat shock genes (maize HSP18 and HSP82; Sinibaldi and Mettler, 1992). Reporter gene (GUS) expression driven by these promoters was optimal at 39 °C and was enhanced in the presence of an intron between the transcription start site and the coding sequence; at 41 °C the intron inhibited reporter gene expression. Heat acclimation of protoplasts conferred transcriptional thermotolerance as GUS expression at 45 °C from constructs with introns remained high. These experiments demonstrate that thermotolerance of a splicing mechanism can be a component of overall thermotolerance in cereals.

Recently, a maize HSP with a molecular mass of 45 kDa, which was earlier shown to be associated with thermotolerance in the field (Ristic *et al.*, 1998), was identified as a nuclear-encoded, chloroplast-localized elongation factor, EF-Tu (Bhadula *et al.*, 2001). In bacteria, EF-Tu functions as a molecular chaperone, and it may possess a similar function in maize chloroplasts, protecting newly synthesized proteins from thermal denaturation.

Protein thermostability

Protein thermostability, in part, is believed to be provided by chaperones, a specific class of proteins capable of assisting other proteins in proper post-translational folding and maintaining them in a functional state (Ellis, 1990). Chaperone function was identified as one of the major functions of HSPs. Moreover, specific structural features could be characteristic of thermostable proteins allowing them to maintain function at high temperatures. Correlation of key thermostable proteins with overall plant thermotolerance has been noted: tolerant sorghum cultivar E 36-1 possessed the highly thermostable photosynthetic enzymes rubisco (ribulose-bisphosphate carboxylase), phosphoenolpyruvate carboxylase, components of photosystem II, and higher levels of the chloroplast chaperonin 60 compared to thermosensitive cultivars (Jagtap *et al.*, 1998). A reduced thermal degradation of rubisco at 50 °C was evident in a thermotolerant cultivar of rice (*Oryza sativa*) compared with a thermosensitive cultivar (Bose *et al.*, 1999). Considerable differences in thermostability of rubisco activase from wheat and cotton (*Gossypium hirsutum*) were reported (Feller *et al.*, 1998). Rubisco activase is now considered a most thermosensitive photosynthetic enzyme. Higher sensitivity of wheat enzyme was at-

tributed either to genetic differences in the primary structure or to the expression of different isoforms (Feller *et al.*, 1998). Earlier, several enzymes from species with higher optimum growth temperatures were found to possess higher optimum temperatures *in vitro*. Transformation with a gene encoding a more thermotolerant enzyme led to an increase of the thermal kinetic window of that enzyme activity in the thermosensitive host plant (Burke *et al.*, 1988).

Reproductive tissues and specific developmental stages

Here we will discuss effects of high temperatures on reproductive organs, fertilization, zygotic embryogenesis, seed development, early germination, yield and yield quality in cereals. There are two major distinctions of reproductive tissues: (1) they are generally considered sensitive to the damaging effect of elevated temperature, and (2) expression of HSPs in many cases is developmentally regulated, and hence HSPs are present prior to heat stress. It is hypothesized that these developmentally regulated HSPs confer inherent heat tolerance to young seedlings.

Reproductive organs and embryogenesis

The temperature threshold for damage by high temperatures in reproductive organs is considerably lower than that in other crop organs. For successful seed establishment, pollen must remain viable and stigma receptive, pollen tubes must grow properly into the ovules, fertilization should succeed, and embryo and endosperm should develop properly. Some of these processes can be severely compromised by high temperatures frequently encountered by cereals in the field. Pollen viability and growth is one of the most heat-sensitive of these developmental stages in cereals (Saini and Aspinall, 1982; Stone, 2001). In maize, reduction in seed set occurs at temperatures higher than 38 °C mainly due to a reduction in pollen germination ability and tube elongation while silk emergence, receptivity and ovule are less affected (Dupuis and Dumas, 1990; Stone, 2001). Accordingly, wheat floret fertility could increase from 30% to 80% by pollinating heat-stressed pistils with unstressed pollen (Saini and Aspinall, 1982).

Pollen is unable to synthesize a full complement of HSPs (Cooper *et al.*, 1984; Mascarenhas and Crone, 1996; Xiao and Mascarenhas, 1985); this could explain pollen sensitivity to high temperatures. The heat

stress response of mature pollen is weak or absent compared with that of vegetative tissues (Mascarenhas and Crone, 1996). However, developmentally controlled HSPs were detected including low-molecular-weight HSPs (18 kDa), HSP90 (reviewed in Mascarenhas and Crone, 1996; Pechan and Smykal, 2001; Gallie, 2001; Stone, 2001), HSP70, and a heat shock factor (Gagliardi *et al.*, 1995). HSP70 accumulated in maize pollen to a significantly lower level than in vegetative tissues under heat stress. Recently, the classical heat stress response was detected in isolated developing maize microspores characterized by the repression of control protein synthesis and the expression of numerous HSPs belonging to each known major HSP class (Magnard *et al.*, 1996). The extent of this heat-shock response was limited in that some of the HSPs did not accumulate to high levels upon temperature elevation, while many of them were detected in microspores at control temperature. Qualitative differences in microspore HSP patterns among 5 maize genotypes were observed; allelic variation may have accounted for this variability.

The post-anthesis period in cereals is characterized by more frequent occurrence of higher ambient temperatures in the field; physiological and practical consequences of moderately and extremely high temperatures are different and have to be discussed separately (see below) as pointed out by Stone (2001). Embryo development can be halted if temperatures exceed the heat tolerance threshold. Because in cereals pollination is not synchronous even within a single ear, it is difficult to distinguish the effect of continuous high temperature upon fertilization from that on early embryo development.

Constitutive expression of several HSPs was prominent in developing seeds of dicot species in the absence of heat challenge (Almoguera and Jordano, 1992; Almoguera *et al.*, 1995; Coca *et al.*, 1994; Wehmeyer *et al.*, 1996). In rice, wheat and maize, significant levels of HSP101 were found in developing and mature grains (Singla *et al.*, 1998). We observed constitutive accumulation of HSP70, chloroplast-localized HSP26, and low-molecular-weight HSPs of both class I and class II in grains of 20 diverse wheat cultivars grown at 20/15 °C (day/night) under temperature-controlled conditions (Klueva, Blum and Nguyen, unpublished). Mature maize seeds differed from those of dicot species in that developmentally regulated HSP17 was localized strictly to nuclei while in dicots it was found both in nuclei and protein bodies (zur Nieden *et al.*, 1995). Notably, developmentally

regulated expression of the chloroplast-localized HSP was observed in developing grains of 20 diverse wheat cultivars (above), but not in pea (DeRocher and Vierling, 1994). Expression and functions of HSPs in developing seeds in monocot and dicot species may differ and need further study.

Germination and seedling growth: effects of heat and HSP expression

Mature seeds of all species studied to date contain significant quantities of HSP homologous proteins and corresponding mRNAs as a result of developmental and stress induction during seed development. Germinating cereal seeds can be exposed to super-optimal temperatures throughout the world's cereal-growing areas as bare soil surfaces at sowing can easily overheat in both temperate and subtropical areas, especially when dry (Howarth *et al.*, 1997; Stone, 2001). Soil temperatures of up to 45 °C at sowing for wheat and up to 55 °C for maize have been reported (Stone, 2001). Meanwhile, seedling growth and vigour are particularly temperature-sensitive. The final mass of wheat seedlings declines with temperatures above 20 °C while phasic development accelerates. This leads to reduced final yields even when the rest of the season's temperatures are at optimum. In maize, root and shoot mass decline by 10% per degree above 26 °C (after Stone, 2001). Hence, germination ability and early development of cereal seeds have to be tolerant to high temperatures from the onset of imbibition. Relevance of heat tolerance to the protein complement of the germinating seeds was studied in detail mainly in wheat and barley (Helm *et al.*, 1989; Helm and Abernethy, 1990; Kruse *et al.*, 1993; Lanciloti *et al.*, 1996). When imbibed, seeds initiate and maintain an expression of a specific set of proteins including 'constitutive' (or developmentally regulated) HSPs throughout the initial phase of germination. Accordingly, HSPs were detected in 2-day old seedlings (Kruse *et al.*, 1993). This initial period of seed imbibition of up to about 12 h was termed 'transition phase' (Abernethy *et al.*, 1989). During this phase, seeds possess high inherent thermotolerance. If subjected to heat stress at this stage, a typical heat stress response including HSP induction could be induced in both barley embryos and aleurone layers, but only the former were able to acquire thermotolerance. In the aleurone, both α -amylase expression level, its mRNA stability and overall intensity of protein synthesis were not protected by permissive heat treatment in spite

of the presence of HSPs (Lanciloti *et al.*, 1996). No direct comparison of HSP patterns or expression levels was made between aleurone layers and embryos; quantitative or qualitative differences between sets of HSPs in embryos and aleurones could explain the difference in ability of these tissues in acquired thermotolerance. In contrast to barley, imbibing wheat seeds were unable to acquire thermotolerance until 9 to 12 h after initiation of imbibition while still able to express a full complement of HSPs starting immediately upon imbibition (Abernethy *et al.*, 1989). We cannot directly compare these experimental results since there were significant differences in the timing of the experimental treatments.

The 'thermotolerance transition' period identified by Abernethy *et al.* (1989) may have evolved in cereals to allow efficient stress protection at the early critical stage of plant establishment. The first biochemical priority of imbibing seeds is DNA repair which is dependent on the *de novo* synthesis of DNA polymerase; the latter is known to be initiated ca. 6 h after imbibition (Osborne, 1983). Hence, transcription of DNA does not start *de novo* until at least 6 h after initiation of imbibition. Thus, early-imbibing seeds must rely on a transcription-independent pre-existing thermotolerance mechanism, should they encounter heat stress at this early stage.

Expression of early developmentally regulated HSPs gradually decline and disappear by about 12 h after imbibition. This second stage of seed germination is characterized by developmental induction of a new set of genes including several HSP homologues. Among these are HSP60 homologues, reported in maize (Prasad and Stewart, 1992), and homologues of low-molecular-weight cytoplasmic class I HSPs (in Douglas fir seedlings; Kaukinen *et al.*, 1996). Embryogenesis-specific developmentally induced HSP homologues could be induced by ABA application and signal was mediated by HSF in sunflower seeds (Almoguera *et al.*, 1998). Overall, expression of HSPs can be an important component of thermotolerance at the early stages of germination.

Yield, quality and HSPs

Heat stress on every stage of the plant life cycle can result in a loss of yield due to effects on plant metabolic processes. Due to space limitations, we will not discuss here multiple effects of heat stress during the vegetative phase of cereal development on the resulting yield. For a detailed recent review of these issues,

the reader is referred to Stone (2001). Here, we will address major aspects of high temperatures at the seed development stage as they relate to yield and quality and to HSP induction and accumulation in cereal grain.

General effects of high temperatures in developing cereal grains include a faster rate of grain development, a decrease of kernel weight, shrivelled seeds, reduced starch accumulation and alterations of polypeptide and lipid composition (see Stone, 2001). Long-term effects of heat stress in a developing seed are lower seed germination ability, delay of germination and loss of seed vigour (Grass and Burris, 1995). It is not known whether developmentally or heat-induced HSPs accumulating in developing seeds play a role in alleviation of such damage.

Average wheat *yield loss* due to moderately high temperatures (Wardlaw and Wrigley, 1994) is estimated at 10–15% mainly due to the decrease of single-kernel weight. The latter is estimated at 4% for each °C above the optimum in wheat, at 3% per degree in maize, and at 1% per degree in rice. A heat wave of 3 or 4 days at 35–36 °C can modify grain morphology and reduce grain size in wheat (Wardlaw and Wrigley, 1994). There is significant genetic variability for high-temperature tolerance of yield (Hawker and Jenner, 1993; Stone and Nicolas, 1994). Heat-induced yield reductions were associated with shortening of grain growth period (Bagga and Rawson, 1977; Stone and Nicolas, 1995a) and impaired ability to synthesize starch. Soluble starch synthase has been reported as the most thermolabile component of the starch synthesis pathway in rice (Inaba and Sato, 1976; Sato and Inaba, 1976), maize (Cheikh and Jones, 1995; Singletary *et al.*, 1994), and wheat (Jenner, 1991a, b; Rijven, 1986).

Most significant heat-induced alterations of grain composition are quantitative and qualitative changes in the protein complement leading to the *deterioration of quality* (especially significant for wheat; see review by Stone, 2001). Protein complement of wheat grain include specific storage proteins, prolamins, which are classified into high-molecular-weight glutenins, low-molecular-weight glutenins, and gliadins (MacRitchie, 1984). Glutenins form high-molecular-weight polymers stabilized by intermolecular disulfide bonds, while gliadins are monomers. Bread-making quality (BMQ) is a quality of wheat flour to form good loaves of bread and it is mainly determined by high-molecular-weight glutenin components (MacRitchie, 1984). When heat stress is imposed on the developing wheat seeds

(after anthesis), reduced starch accumulation results in decreased kernel weight and increased relative protein content in the grain. Relative proportions of high-molecular-weight glutenins, low-molecular-weight glutenins, and gliadins change leading to an end product (flour) with changed consumer qualities (Stone, 2001).

While it is generally assumed that heat stress during the grain-filling period is detrimental to flour quality, it has been recently argued (Stone, 2001) that inconsistency of quality resulting from weather fluctuations during the grain filling period is more significant. Indeed, modestly high temperatures during the grain filling period led to an increased relative content of flour protein which may be beneficial, rather than detrimental, to the resulting bread-making quality (Stone, 2001). Wheat bread-making quality (BMQ) increased at temperatures up to 30 °C (Randall and Moss, 1990) and decreased at further temperature increases (above 35 °C) in a genotype-dependent manner (Blumenthal *et al.*, 1993a, Stone and Nicolas, 1995b).

Three factors are significant for flour quality in grain developing under heat stress: (1) allelic composition of high-molecular-weight (HMW) glutenin subunits; (2) polymerization state of HMW glutenin subunits; (3) changes of other flour constituents (starch and lipids). Heat-inducible accumulation of gliadins and HSPs was suggested to lead to deterioration of flour quality, but this still is to be proved.

Allelic composition of HMW glutenin subunit-encoding genes is widely accepted as the major factor of high BMQ. HMW glutenin subunits represent 5–10% of total seed protein. The importance of high-molecular-weight glutenin subunits 5+10 and, to a lesser extent, of subunits 1 or 2* for wheat BMQ has been demonstrated (Weegels *et al.*, 1996). Other alleles were far less beneficial; differential heat inhibition of various HMW glutenin alleles could be a factor in BMQ. Weegels *et al.* (1996) noted that beneficial glutenin alleles explained only a small portion of variation in flour quality insufficient to predict variations in flour quality for industrial processing. Nevertheless, these data could be used for plant breeding purposes. Recently, genetic engineering of wheat gluten became feasible and integration of some HMW glutenin subunit genes into wheat genomes were used to study wheat BMQ (see Vasil and Anderson, 1997, for a review).

A certain *proportion of high-molecular-weight glutenin polymer* in flour was closely associated with

quality traits and was the major determinant of dough strength, a major factor of BMQ. Several studies have associated the heat-induced decrease of glutenin polymerization state with decreased BMQ. In both durum and bread wheat grown either in the field or in the growth chamber, Ciaffi *et al.* (1995, 1996) did not detect any differences in amount of gliadin and total polymeric glutenin between control and stressed samples, while the formation of larger glutenin aggregates was lowered.

Heat-induced accumulation of gliadins (Blumenthal *et al.*, 1990, 1993b) was hypothesized to lead to a dough weakening effect; heat shock element-like sequences were discovered in the promoters of gliadin genes (Blumenthal *et al.*, 1990). We did not observe an increase of gliadin expression at the level of mRNA accumulation in the seeds developing under heat stress (Perrotta *et al.*, 1998; Treglia *et al.*, 1999). Expression of HMW glutenin subunits was reduced only at the late stage of grain filling. To our knowledge, there is no evidence to date of significant stress-induced gliadin accumulation in grains developing under heat stress and thus an increased proportion of gliadins is unlikely to account for decreased BMQ.

It was suggested that heat-inducible *accumulation of HSPs* in developing grain decreases BMQ (Blumenthal *et al.*, 1990). In Marmioli's laboratory, we observed a 3–5-fold increase in HSP17 and HSP70 at the level of mRNA expression in developing seeds of durum and hexaploid wheat under heat stress (Perrotta *et al.*, 1998; Treglia *et al.*, 1999). No protein levels were measured in this study. In Nguyen's laboratory, HSP accumulation in grain developed under heat stress was not consistent across several wheat genotypes (Klueva, Blum and Nguyen, unpublished). There is no clear evidence that HSP accumulation indeed occurs in developing seeds. It is not known whether heat-induced HSPs are functionally different from developmentally controlled HSPs that accumulate in seeds developing in the absence of heat stress. Addition of purified HSP70 to wheat flour did not result in alteration of dough quality, even though the quantity of additional HSP70 by far exceeded that theoretically present in naturally heated seeds (Blumenthal *et al.*, 1998). In conclusion, there is currently no clear evidence that HSP accumulation is related to wheat BMQ.

Content of starch and associated lipids in the grain affects the quality of bread, food additives and noodles (Shi *et al.*, 1994; Tester *et al.*, 1995). High temperatures increase starch and flour lipid contents

and moderately high temperatures decrease starch and non-starch lipid contents, which had a negative effect on loaf volume (Williams *et al.*, 1994).

In conclusion, recent research indicates that consistent grain quality and yield of wheat is determined by an optimal combination of alleles encoding beneficial high-molecular-weight glutenin subunits and heat tolerance of starch biosynthesis. Discovery and exploitation of new glutenin alleles contributing consistent quality to cereal grains independent of temperature fluctuations during grain development could lead to significant increases in grain quality. To date, accumulation of HSPs in wheat grain has not been associated with resulting bread-making quality.

Improvement of heat tolerance in cereals: future directions

According to a recent prognosis, global climate change will result in a reduction of productivity of the modern maize cultivars mainly due to increases of peak temperatures during growth (Southworth *et al.*, 2000). Several approaches should be actively exploited to improve heat tolerance in current cultivars including discovery and exploitation of new genes and alleles, improved breeding efficiency, marker-assisted selection and genetic modification. Further work is needed to characterize heat tolerance in wild germplasm adapted to hot, dry environments and to determine the genetic basis of this adaptation. QTL mapping and association genetic analysis (e.g. Cardon and Bell, 2001) should be useful in an effort to relate specific alleles to trait variation and to identify candidate genes.

Recently, a transgenic approach was successfully used for manipulating HSP expression and heat tolerance in model species (Lee *et al.*, 1995; Lee and Schöffl, 1996; Prandl *et al.*, 1998; Malik *et al.*, 1999). Novel genes and alleles from exotic germplasm and non-plant species can be exploited, as was recently demonstrated in *Arabidopsis* by transformation with a bacterial choline oxidase gene used to increase seed dehydration tolerance via accumulation of the osmoprotectant glycinebetaine (Alia *et al.*, 1998). Regulatory genes including transcription factors and signal transduction components can be used. The challenges of genetic transformation technology in cereals include (1) improving selection and quality of target genes, (2) precision targeting of recombination events to specific genomic locations, (3) better knowledge of

genome topography to identify suitable insertion loci, (4) more diverse and better quality of regulatory sequences, (5) improved delivery of larger fragments, and (6) genotype modelling for specific environments.

Further understanding and manipulation of heat tolerance mechanisms in cereals, either by transgenic approaches or by molecular breeding, will rely on achievements of genomics, proteomics and metabolic profiling (Stuber *et al.*, 1999; Thiellement *et al.*, 1999; Celis *et al.*, 2000).

A search for HSP genes from cereals currently available in the GenBank, EMBL and DDBJ databases (<http://www.ncbi.nlm.nih.gov>, January 2001) yielded a total of 83 full-length and 89 partial cDNA sequences encoding HSPs of wheat, barley, rice and maize. Simultaneously, more than 2150 expressed sequence tags (ESTs) homologous to HSPs of all classes were available in dbEST. The highest number of matches was to cytoplasmic HSP70 and HSP90. This example is representative of an overall progress in structural genomics to date.

Comprehensive EST sequencing projects in major cereal species (maize, wheat, rice and sorghum) have led to rapid accumulation of gene structure data, including the collections of ESTs from heat stress libraries. Data mining in the evolving EST databases is now warranted. Improved methods of identification of the rare and under-represented messages are needed. Full genome sequences of *Arabidopsis* and rice should assist in chromosomal assignments of ESTs and in pinpointing ORFs missing from data banks. Furthermore, development of unigene sets and functional genomics studies in cereals are urgently needed. In particular, seed development-related processes, including thermotolerance of seed development in cereals, could benefit from microarray-based genome-wide expression analysis.

Rice (*Oryza sativa*) is rapidly emerging as one of the key model organisms for the study of plant biology. Japanese researchers have developed a physical map and have initiated a large-scale genome sequencing effort and a cDNA sequencing effort. USDA/NSF/DOE have recently supported three groups in the USA to allow them to join the co-ordinated, international rice genome sequencing programme. Monsanto announced a rough draft of the rice genome sequence (Pennisi, 2000) and recently Syngenta announced the completion of the rice genome sequence (Davenport, 2001). The availability of high-density genetic and physical maps, ESTs, genomic sequences and mutant stocks (such as T-DNA insertional mutants by

Jeon *et al.*, 2000) have established rice as an excellent model for the study of heat tolerance in cereals. A high level of synteny and homology within the Poaceae family will facilitate transfer of identified QTLs and candidate genes from rice to other cereals.

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