

Tomato (*Lycopersicon esculentum*) Response to Heat Stress: Focus on Pollen Grains

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ABSTRACT

Exposure to higher than optimal temperatures, heat-stress (HS), reduces yield and decreases the quality of many crops, including tomato. Pollen development is one of the most heat-sensitive developmental stages in a large variety of plant species. Impaired fruit set in response to HS conditions may thus be largely due to HS-induced changes in pollen characteristics. This review emphasizes the effect of HS on the developing pollen grains, though the available data are still very limited. Recently accumulated data on the molecular control of pollen development are included, which indicate the processes important for the production of functional pollen grains. These recent studies are put in the context of data on HS effects on the other parts of the tomato plant (whole plant, leaves, anther cells) likely to affect the development and functioning of the gametes. Among other effects, HS was found to affect membrane integrity, Ca^{2+} flux, protein conformation and production of reactive oxygen species (ROS) which, in turn, elicit defense mechanisms that include the production of heat shock factors, heat shock proteins and ROS-scavenging enzymes. In the developing pollen grain HS directly affects sugar and starch metabolism – effects that are associated with reduced pollen quality. In parallel, defense mechanisms are elicited, including the induction of heat shock proteins. The full understanding of the heat sensitivity of pollen, on the one hand, and of the mechanisms that may enable the pollen to cope with HS, on the other hand, requires additional data, including data on other components of the chaperone network, as well as data on other protective mechanisms (such as ROS scavengers and compatible solute accumulation).

Keywords: carbohydrate metabolism, heat shock proteins, microgametogenesis, microsporogenesis, molecular and physiological aspects, pollen quality, reactive oxygen species scavenging, yield

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INTRODUCTION

Most of the world's crops are exposed to heat stress (HS) during some stage of their life cycle, and HS is expected to become a more frequent problem; each year of the current decade has been one of the 15 warmest of the last 100 years (Sato *et al.* 2000). Exposure to higher than optimal temperatures reduces yield and decreases the quality of many crops, including cereals (Maestri *et al.* 2002; Boote *et al.* 2005; Prasad *et al.* 2006), grain legumes (Prasad *et al.* 2003) and vegetable crops (Wien 1997; Kinet and Peet 1997; Sato *et al.* 2006). Furthermore, as the world population grows exponentially there is a need both to increase agricultural productivity and to extend the area under agriculture into warmer climates. The prevalence of high ambient temperatures [generally over 35°C] in a significant proportion of the tomato-growing areas of the world is one of the most crucial problems in tomato production. Foolad (2005) pointed out that, although tomato plants can grow in a wide range of climatic conditions, their vegetative and reproductive growths are severely impeded at high temperatures, resulting in reduced yields and impaired fruit quality. Chronic HS even of a mild degree (day/night temperatures of 32/26°C) and/or short waves of acute high temperatures (40°C), have been shown to disrupt the normal development of the gametes, especially the microgametes, and thereby to impair fruit set (Peet *et al.* 1998). In Israel, summer production of tomatoes is adversely affected by high temperatures (day/night temperatures of 35/25°C), sometimes to the stage of a total lack of fruits in the market. This problem becomes crucial in greenhouses, where the daily mean temperatures may rise high above the optimum (40-45°C), especially during the warmer seasons. Such HS conditions are common in tomato-growing regions such as southern European countries and the southern USA.

Pollen development is one of the most heat-sensitive developmental stages in a large variety of plant species, including vegetable crops (Peet *et al.* 1998; Aloni *et al.* 2001; Sato *et al.* 2002). Impaired fruit set in response to HS conditions may thus be largely due to HS-induced changes in pollen characteristics. However, the physiological and molecular basis for the high sensitivity of developing pollen grains to HS, on one hand, and pollen heat-tolerance, on the other, is poorly understood.

Recently it has been demonstrated that high temperature sensing and response may involve a wide range of molecu-

lar mechanisms (Kotak *et al.* 2007). The temperature levels that cause stress, and what forms and magnitude of acquired tolerance or resistance a given plant possesses depend on the plant, tissue, or cell type in question (Sung *et al.* 2003). Even among *Lycopersicon* species it is possible to find variations in high-temperature sensitivity. For example, Fender and O'Connell (1990) have demonstrated that the cultivated tomato, *Lycopersicon esculentum* (*Solanum lycopersicon*, <http://sgn.cornell.edu>) differed from the species *Lycopersicon pennellii* in its HS response.

In order to develop strategies for coping with the damaging effects of HS on crop yield, it is essential to know what stages of development and what parts of the plant are most sensitive, and what are the most sensitive biochemical and molecular processes and, at the same time, to look for heat-tolerance-inducing mechanisms and heat-tolerant genetic material. Such data will enable the development of appropriate breeding programs as well as the creation of heat-tolerant plants by genetic engineering techniques.

This review will emphasize the effect of HS on the most heat-sensitive part of the tomato plant – the developing pollen grains – though the available data are still very limited. Recently accumulated data on the molecular control of pollen development are included, which indicate the processes important for the production of functional pollen grains. These recent studies are put in the context of data on HS effects on the other parts of the tomato plant (whole plant, leaves, anther cells) likely to affect the development and functioning of the gametes.

PRACTICAL ASPECTS OF TOMATO CULTIVATION UNDER HIGH TEMPERATURE CONDITIONS

Two, currently grown, main tomato types were described by Kinet and Peet (1997) experiencing differences in exposure to high temperature conditions, caused by direct or indirect sun irradiation or cultivation practices. The first is the 'determinate' or 'bushy' tomato which is grown outdoors and used mainly for processing. The 'determinate' tomato type has a short flowering period that is followed by a period of fruit development. The second type is the 'indeterminate' or 'vine' tomato, which is largely used for production of fresh fruits in greenhouses and home gardens. The 'indeterminate' type produces inflorescences, flowers and fruits continuously throughout the plant life. The indeterminate glasshouse plants (*SP_*, *pw9-2-5/pw9-2-5* or *SP_*, *PW9-2-5*)

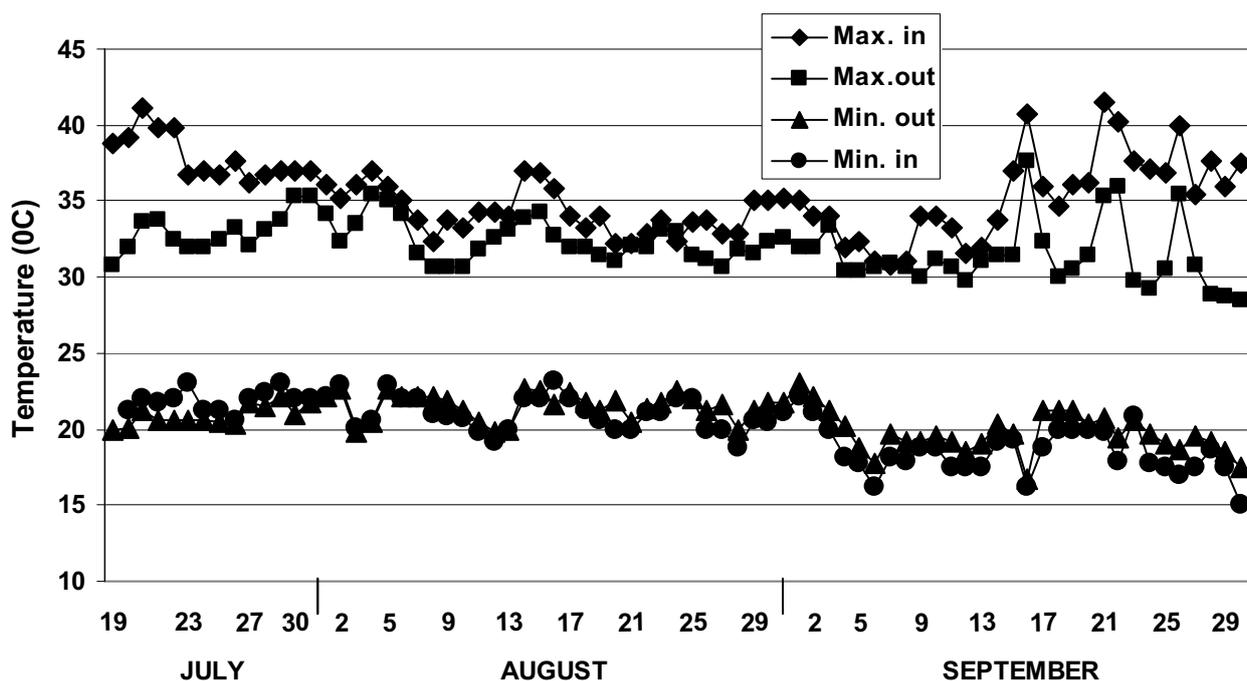


Fig. 1 Mean indoor (in) and outdoor (out) maximum and minimum temperatures in a tomato greenhouse at the Besor region, Israel, during the summer of 2005.

show an unrestricted number of sympodial units, each consisting of three leaves and an inflorescence, whereas the determinate (*sp/sp*, *pw9-2-5/pw9-2-5*) open-field plants terminate with the fifth inflorescence, after showing a progressively reduced number of leaves per sympodial unit (Fridman *et al.* 2002). A third type – ‘semi-determinate’ growth habit (*sp/sp*, *PW9-2-5/*) is also used. It terminates after eight inflorescences with two leaves per unit (Fridman *et al.* 2002) and was found to be suitable mainly for small greenhouses or high tunnels.

The need to protect greenhouse tomatoes from virus infestation during the summer forces the farmers to grow the plants in constructions covered with 50-mesh nets to prevent the white fly (the major vector of the *Tomato yellow curl leaf virus* (TYCLV)) from reaching the plants. The use of this net causes a marked increase in temperatures which are already high in the summer. Measurements conducted during the summer of 2006 in the Besor region of Israel (H. Weksler, KANAT, Israel, pers. comm.), revealed differences of 5 to 10°C in day temperatures between inside and outside the greenhouse, respectively. Night minimum temperatures inside the greenhouse were the same as those outside (Fig. 1). These high temperature values are not unique to Israel, during the summer, in a large number of countries, including Mediterranean countries, southern European countries and the US, maximum temperatures inside the greenhouse could reach over 40°C at midday. Growing tomatoes under such conditions was found to affect their inflorescences and flowers: numbers decreased and flowers and fruits in the cluster were irregular (Fig. 2); and intrusion of the style and stigma through the anther cone (Fig. 3) were observed, which prevent or hinder pollination.

The practical approach to reduce the temperature is to reduce solar irradiation penetrating the greenhouse by means of shading nets (in addition to the 50-mesh nets), which may cause significant reductions in light intensity, to below the recommended values. Farmers also use more expensive means of cooling, such as desert coolers. In such cases one should be aware of a temperature gradient across



Fig. 2 A normal tomato truss (left) and a HS-affected truss (right).



Fig. 3 A normal tomato flower (left) and a flower with HS-induced stigma protrusion (right).

the greenhouse, which depends on the construction, size and type of the greenhouse, and on the efficiency of the cooling system. Since the major effect of HS is significant reduction in fruit set Abdul-Baki and Stommel (1995), farmers use pollinators, for which bumble bees are very efficient and commonly used for tomatoes, since they perform buzz pollination (Velthuis and Doorn 2006), or they apply hormones, mainly auxins (Ramin 2003) to induce fruit set. However, to the best of our knowledge, in contrast to winter-grown tomatoes, the response of summer-grown tomatoes to auxins is only partial, and the quality of the resulting fruits is relatively low [Pressman, personal data].

PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ASPECTS OF THE TOMATO PLANT RESPONSE TO HEAT STRESS

Heat stress affects several different parts of the plant, including roots, stem, leaves, flowers and the reproductive parts. Tomato flowers were found to respond to HS by induced flower bud drop, splitting of the anther cone, style exertion and reduction in the quantity and functioning of the gametes, especially of the male gametes, all of which contributed to low fruit set (Levy *et al.* 1978; Abdul-Baki 1991).

At the cellular level, the HS response of plants was found to involve multiple cellular components, including membranes (cellular and organellar), specialized components such as the photosynthesis apparatus, and organelles (mitochondria, chloroplasts, peroxisomes), and it may also affect the cytoskeleton components. The HS response also involves biochemical and molecular responses, which include enzyme pathways, transcription and translation of proteins, as well as protein conformation (including that of regulatory and structural proteins). The available data are, however, still very limited and somewhat sporadic. In a recent review, Sung *et al.* (2003) presented a model that summarized the various components thought to be involved in HS tolerance, as well as molecular events that may underlie high temperature perception, signal transduction and transcriptional activation. Kotak *et al.* (2007) stressed the complexity of the HS response in plants, indicating the involvement of factors other than classical HS-responsive genes in thermotolerance. They summarized data that indicate that plant hormones and reactive oxygen species also contribute to HS signaling. Most of the accumulated data, however, come from plant species other than *Lycopersicon*, and relate to the vegetative parts of the plant. Very few data are available concerning the effect of HS and heat-tolerance mechanisms in the most heat-sensitive part of the plant: the developing pollen grains. The effect of high temperatures on the vegetative parts of the plant may, nonetheless, contribute to the observed reduction in pollen quality. Pollen develops inside the anther, surrounded by the mother tissues, and is nourished by assimilates that are transported to the anther from source leaves. Indeed, most male-sterile mutants of tomato are thought to be defective in genes that function in the sporophyte (Gorman and McCormick 1997).

PHYSIOLOGICAL AND BIOCHEMICAL ASPECTS

Photosynthesis

The optimal temperature for higher plant photosynthesis generally matches the average daytime temperature encountered in the natural environment. Significant inhibition of photosynthesis occurs at temperatures above the optimum, resulting in considerable loss of potential productivity (Salvucci and Crafts-Brandner 2004). Heat treatment (42°C in the dark) caused a complete and irreversible inhibition of photosystem II in well-watered tomato leaves, but a small and fully reversible reduction of the photochemical efficiency of photosystem II in drought-stressed leaves (Havaux 1992). Inhibition of photosynthesis by HS has been attributed to an impairment of electron transport activity, caused

in part by changes in membrane fluidity (Raison *et al.* 1982; Havaux 1993; Murakami *et al.* 2000 – cited by Salvucci and Crafts-Brandner 2004). Thermostability of the oxygen-evolving complex and the reaction center of PSII have been recently suggested to be involved in thermotolerance (Yang *et al.* 2006). Yang *et al.* (2006) further suggested that increased thermotolerance, induced by accumulation of glycinebetaine *in vivo* was associated with the enhancement of the repair of PSII from heat-enhanced photo-inhibition. Others support the idea that the initial site of inhibition is associated with a Calvin cycle reaction, specifically the inactivation of Rubisco (Weis 1981a, 1981b; Kobza and Edwards 1987 – cited by Salvucci and Crafts-Brandner 2004). Salvucci and Crafts-Brandner (2004) recently considered species from contrasting environments (Antarctic hairgrass, *Lysipomia pumila* and spinach, as well as jojoba, tobacco and cotton), and suggested that deactivation of Rubisco is the primary cause of inhibition of photosynthesis under moderate HS and that the upper temperature limit of photosynthesis is determined by the thermal properties of activase.

In tomato also, Nkansah and Ito (1994) indicated that yield significantly and positively correlated with photosynthesis, transpiration and stomatal conductance. HS has been found to decrease the activity of Rubisco in the sensitive cultivar Roma, causing a reduction in photosynthetic activity, whereas the heat-tolerant cultivar Saladette was not affected (Markus *et al.* 1981). Bar-Tsur *et al.* (1985a, 1985b) reported on a decrease in apparent photosynthesis in both heat-sensitive and heat-tolerant cultivars after exposure of plants to temperatures of 35 to 40°C for various lengths of time, but the heat-tolerant cultivar Saladette was less affected by the HS. More recently it has been demonstrated that HS (45°C for 2 h) caused a reduction in the net photosynthesis rate of young plants of the heat-sensitive genotype Campbell-28, because of effects on the Calvin cycle and also in the PSII functioning, but not in Nagcarlang, a wild thermotolerant genotype (Camejo *et al.* 2005). Gent (1986), however, found that total nonstructural carbohydrates and soluble sugar concentration were not affected by high temperatures. Similarly, Sato *et al.* (2000) reported that photosynthesis and night respiration did not limit tomato fruit set under chronic, mild HS. In a more recent study, Camejo *et al.* (2006) exposed another cultivated tomato var. Amalia to 45°C for 3 h. They suggested that heat injury observed in var. Amalia may involve chlorophyll photo-oxidation mediated by reactive oxygen species (ROS) and more severe alterations in the photosynthetic apparatus. These effects were not evident when the wild genotype Nagcarlang was exposed to the same HS conditions. A correlation between photosynthetic thermotolerance and production of specific HS (or heat shock) proteins (Hsps) was demonstrated in tomatoes (Preczewski *et al.* 2000). Positive relationships were observed between production of chloroplast Hsp24 and Hsp60 and P_N (net photosynthesis rate) thermotolerance. In addition, Neta-Sharir *et al.* (2005) reported that chloroplast sHsp, Hsp21, protected PSII from temperature-dependent oxidative stress.

Assimilate transport, partitioning and metabolism

Dinar *et al.* (1983) found that increasing the day temperature from 25 to 36°C resulted in a marked decrease in starch levels in the leaves, and inhibition of the export of radioactive carbon from the leaves of two tomato cultivars; they also found callose formation on phloem sieve tube plates of leaf petioles exposed to high temperatures for 72 h. This callose may have been the reason for reduced carbon transport. More sieve tube plates were covered with a thicker callose layer in a heat-sensitive cultivar than in a heat-tolerant one. In an additional study, Dinar and Rudich (1985a) showed that transport of carbon to the trusses and apex was inhibited by HS (exposure to day/night temperatures of 36/25°C for 24 h) in the heat-tolerant cv. 'Saladette', but es-

pecially so in a heat-sensitive cultivar, 'Roma VF'. Assimilate partitioning within the young truss was also affected by HS in both cultivars, but the effect was more pronounced in cv. 'Roma VF'. Heat stress (38/25°C) has been found to inhibit import of assimilates by tomato flower buds (Dinar and Rudich 1985b), and the authors concluded that sucrose accumulation and decrease in starch levels in the source leaves when temperatures increased were due to the reduction in assimilate transport. However, inhibition of sucrose hydrolysis in the flower buds could not be ruled out.

In contrast to the conclusions of Dinar and Rudich (1985a, 1985b) we (Pressman *et al.* 2002, and Pressman, unpublished data) have recently demonstrated that high temperatures, which dramatically reduce starch and sugar levels in the developing and mature pollen grains, respectively, do not affect the sugar levels in the locular fluid that surrounds and feeds the developing pollen. These data indicate that sugars do arrive in the flower buds throughout development and that, even though photosynthesis and sugar translocation are affected by HS, enough sugar arrives in the locular fluid.

Metabolites/osmolytes

Recently, data have accumulated with respect to the temperature stress metabolome in plants that indicate the involvement of a large number of metabolites in the temperature stress response (Kaplan *et al.* 2004). In tomato, Rivero *et al.* (2004) have suggested that the use of NH_4^+ as an N source increases heat tolerance in tomato plants, because of increased accumulation of proline and choline, which can act as osmoprotective solutes. Thus, plant nutrition could play an important role in HS tolerance in plants. Comprehensive data on the temperature stress metabolome of tomato are, however, still lacking but are expected to become available during the next few years.

Brassinosteroids (BRs)

Brassinosteroids are a group of naturally occurring plant steroidal compounds with wide-ranging biological activity that offer the unique possibility of increasing crop yields both by changing plant metabolism and by protecting plants from environmental stresses (Krishna 2003). Dhaubhadel *et al.* (2002) showed that *Brassica napus* and tomato seedlings grown in the presence of 24-epibrassinolide (EBR) were significantly more tolerant to a lethal heat treatment than control seedlings grown in the absence of the compound. In addition, they demonstrated that after the applied HS, heat shock protein (Hsp) accumulation was higher in EBR-treated than in untreated seedlings. The possibility that Hsps contributes, at least in part, to thermotolerance in EBR-treated seedlings has been suggested (Dhaubhadel *et al.* 2002).

Oxidative damage and reactive oxygen species scavenging

Rainwater *et al.* (1996) have demonstrated that, following exposure to HS conditions (daily temperature of 34°C compared with average daily temperature of 26°C) five tested heat-tolerant cultivars exhibited greater antioxidant activity than the tested heat sensitive cultivar: superoxide dismutase activity, for example, increased up to nine-fold in the heat-tolerant cultivars but decreased in the heat-sensitive one. In addition, all tested heat-tolerant cultivars exhibited significantly lower oxidized ascorbate/reduced ascorbate ratios, greater reduced glutathione/oxidized glutathione ratios, and greater alpha-tocopherol concentrations than the heat-sensitive cv 'Floridade'. The authors concluded that the more heat-tolerant cultivars had an enhanced capacity for scavenging active oxygen species and a more active ascorbate-glutathione cycle, and suggested that there was a strong correlation between the ability to up-regulate the antioxidant

defense system and the ability of tomatoes to produce greater yields when grown under HS (Rainwater *et al.* 1996).

MOLECULAR ASPECTS

Kinases

Little is known about the initial events that follow HS. Link *et al.* (2002) have demonstrated that the application of HS in a naturally occurring temperature range resulted in a fast and transient activation of a 50-kDa mitogen-activated protein (MAP) kinase both in a tomato photo-autotrophic cell suspension culture and in leaves of mature plants. The heat activation of the MAP kinase was shown to be calcium dependent. The specific phosphorylation of tomato HS transcription factor HsfA3 by a partially purified preparation of the heat-activated MAP kinase suggests a physiological role of the identified kinase activity in transducing the HS signal. These data fit into a more general scheme whereby calcium-mediated signal transduction of temperature stresses involves protein phosphorylation and activation of various transcription factors (Sung *et al.* 2003).

Heat shock factors (hsfs)

The key regulators of the HS response are the heat shock transcription factors encoded in plants by a large gene family (Nover *et al.* 2001; Port *et al.* 2004). Stress-induced gene expression leads to the rapid accumulation of heat shock proteins (Hsps) that share conserved promoter elements (Pelham 1982; Pelham and Bienz 1982; Nover 1987 – cited by Port *et al.* 2004). Many of them act as molecular chaperones, with important functions not only in the protection of proteins against stress damage but also in their folding, intracellular distribution and degradation (Dougan *et al.* 2002; Young *et al.* 2003). Expression of Hsps is mediated by heat shock factors (Hsfs). Out of the overall total of more than 20 plant Hsfs, detailed analyses have mainly addressed only tomato and Arabidopsis, and three important representatives of the family (Hsfs A1, A2 and B1). These three Hsfs represent examples of functional diversification specialized for the three phases of the HS response (triggering, maintenance and recovery). This is best exemplified in the tomato Hsf system. (i) HsfA1a is considered the master regulator responsible for HS-induced gene expression that includes synthesis of HsfA2 and HsfB1; it is indispensable for the development of thermotolerance. (ii) Although functionally equivalent to HsfA1a, HsfA2 is found exclusively after HS induction, and it represents the dominant Hsf of the HS response in plants subjected to repeated cycles of HS and recovery in a hot summer period. Tomato HsfA2 is tightly integrated into a network of interacting proteins (HsfA1a, Hsp17-CII, Hsp17-CI) that influence its activity and intracellular distribution. (iii) HsfB1 acts as a coregulator that enhances the activity of HsfA1a and/or HsfA2, but in addition, it cooperates with other, yet to be identified, transcription factors in maintaining and/or restoring house-keeping gene expression (Mishra *et al.* 2002; Kotak *et al.* 2007).

Heat shock proteins (hsps)

One of the main aspects of the HS response is the synthesis of Hsps. Hsps can be classified into five major groups on the basis of their molecular masses: Hsp100, 90, 70, 60, and small molecular weight Hsps (sHsps) (Vierling 1991; Guy and Li 1998; Wang *et al.* 2004) and are found in both the cytoplasm and the different subcellular compartments (organelles), such as the nucleus, mitochondria, chloroplasts and ER. Most of the Hsps act as molecular chaperones that help other proteins to maintain or regain their native conformation by stabilizing partially unfolded states (Bessinger and Buchner 1998; Low *et al.* 2000). They do not contain specific information for correct folding but rather prevent unproductive interactions (aggregation) between non-native

proteins. Experimental data indicate that members of different Hsp families act together in multi-subunit complexes to generate a network for protein maturation, assembly and targeting. The significance of Hsps in thermotolerance was first hypothesized on the basis of correlative evidence (for reviews, see Vierling 1991; Maestri *et al.* 2002). Recently, causal involvement of several Hsps in acquired thermotolerance of plants has been demonstrated (Burke *et al.* 2000; Hong and Vierling 2000; Queitsch *et al.* 2000).

Small Hsps family

Small Hsps (sHsps) are the most abundant stress-induced proteins with up to 20 different members in higher plants.

Chloroplast sHsp: There is evidence that the small chloroplast Hsp is involved in plant thermotolerance, but its site of action is unknown. Functional disruption of this Hsp by means of anti-Hsp antibodies or addition of purified Hsp to chloroplasts indicated that (a) this Hsp protects the thermolabile photosystem II and, consequently, whole-chain electron transport during HS; and (b) this Hsp completely accounted for heat acclimation of electron transport in pre-heat-stressed plants. Therefore, this Hsp was hypothesized to be a major contributor to adaptation to acute HS in plants (Heckathorn *et al.* 1998).

Cytosolic sHsps: In the cytoplasm, two different classes could be distinguished (Low *et al.* 2000). Two cDNA clones from tomato *Lycopersicon peruvianum* (L.) Mill., each coding for one of the cytoplasmic sHsp subfamilies, were analyzed with respect to their transcript and protein expression, genome organization and chaperone activity. Neither type was present under control conditions but both appeared in fruits under HS. Protein analysis using class-specific antibodies revealed identical expression patterns of both corresponding proteins. Transient expression in an *Arabidopsis thaliana* (L.) Heynh. cell culture showed that, despite the difference in their amino acid sequences, both classes are functionally active *in vivo* as chaperones, as shown by their ability to prevent thermal inactivation of firefly luciferase in a cellular environment (Low *et al.* 2000).

Nucleoplasmic sHsp: Siddique *et al.* (2003) have recently described a new class of plant sHsps with dominant nuclear localization (Hsp17-CIII), and they demonstrated that proteins of the sHsp classes CI, CII, and CIII interact with each other, thereby influencing the oligomerization state and intracellular localization.

Mitochondrial sHsp (mt-sHsp): The role of mt-sHsp in the HS response has been recently demonstrated by introducing the tomato (*L. esculentum*) mt-sHsp gene into tobacco (*Nicotiana tabacum*), under the control of the 35S promoter, and examining the thermotolerance of the transformed plants. Irrespective of the orientation – sense or antisense – of the gene, the transgenic plants exhibited normal morphology and growth rate in the vegetative growth stage. When 4-week-old seedlings were exposed to sudden HS, the sense plants, which overexpress the mt-sHsp gene, exhibited thermotolerance, whereas the antisense plants, in which the expression of the gene is suppressed, exhibited susceptibility (Sanmiya *et al.* 2004). Involvement of Hsp22 in adaptive mechanisms that lead to mitochondrial protection from stress has been previously suggested by Banzet *et al.* (1998).

Hsp70 family

Family members are found to serve a variety of functions and include proteins that are induced by HS as well as constitutively expressed cognates (Hspcs). Proteins belonging to this family were shown to be involved in acquired thermotolerance (Lee and Schoffl 1996; Guy and Li 1998). Hsp70 proteins are found in all the major subcellular compartments of plant cells (Li *et al.* 1999), and induction of nine members of the Hsp70 family was recorded following a 1-h exposure of tomato seedlings to heat stress, and it was followed by down-regulation during extended high temperature exposure.

Hsp100 family

HSP100 proteins, also known as caseinolytic protease (Clp) proteins, are a class of diverse molecular chaperones, involved in a wide variety of essential metabolic processes throughout prokaryotes and eukaryotes (Schirmer *et al.* 1996; Yang *et al.* 2006).

HSP101 was recently shown to be a major factor in thermotolerance in *Arabidopsis* (Hong and Vierling 2000; Queitsch *et al.* 2000). Recently, the involvement of chloroplast Hsp100/clpB in acquired thermotolerance in tomato has been demonstrated (Yang *et al.* 2006). Chlorophyll fluorescence measurements showed that PS II in tomato Hsp100/clpB antisense lines were more susceptible to irreversible thermal inactivation than the untransformed and vector-transformed control plants. This study by Yang *et al.* (2006) provides an example of the contribution of the induction of chloroplast LeHSP100/ClpB to the acquisition of thermotolerance in higher plants.

THE RESPONSE OF DEVELOPING POLLEN GRAINS TO HEAT STRESS

Pollen grain development

Structural and biochemical aspects

Pollen development starts inside the anther 9-10 days before anthesis, when the sporogenous, pollen mother cells, undergo meiosis to form tetrads of cells. Each tetrad is enclosed in a thick callose wall. The microspores are freed by the action of callase, an enzyme produced by the tapetum, the innermost layer of the anther, that feeds the developing microspores. This phase is termed microsporogenesis. In the later stages of pollen development the tapetum disintegrates into the locular fluids that bathe the developing microspores. The microspores enlarge and undergo an asymmetric mitosis to form a bicellular pollen grain whose two cells have strikingly different fates: the larger cell is the vegetative cell, and the smaller is the generative cell. The vegetative cell does not divide again but eventually will form the pollen tube; the generative cell is engulfed in the cytoplasm of the vegetative cell. The generative cell undergoes a second mitosis to form two sperm cells. The pollen development phase of microspore formation is termed microsporogenesis, and the whole development, from microspores to mature pollen is termed microgametogenesis. In the late stages of development symplastic discontinuity requires that the individual microspores be programmed with appropriate signals, or at least be activated for major functions, including the two mitotic divisions, intracellular vacuolar biogenesis and several metabolic changes such as starch biosynthesis (Gorman and McCormick 1997 and references therein).

Molecular aspects

During the last few years novel data have accumulated, and significant advances have been made with respect to the molecular control of pollen development (Caryl *et al.* 2003; McCormick 2004; Scott *et al.* 2004; Rotman *et al.* 2005; Sheoran *et al.* 2007). These advances have resulted from gene expression studies, including high-throughput microarray analysis, and from analyses of male gametophytic mutants and of mutants in sporophytic genes required for male fertility. The novel tools that became available recently include: the genome sequences of *Arabidopsis* and rice; extensive EST data bases for many plants, including tomato; microarray hybridization procedures; and quantitative RT-PCR techniques that can produce reliable results with small amounts of RNA; and a large collection of mutants, mainly in *Arabidopsis* and rice but also in other plants, including tomato.

At least two major sets of genes were hypothesized to be involved in pollen development (Mascarenhas 1990):

'early' genes that are activated in unicellular pollen shortly after meiosis, with mRNA reaching a maximum of expression around the first pollen mitosis; and 'late' genes that are activated at the time of the first pollen mitosis and whose transcripts accumulate during pollen maturation. Many of the recent data on mRNAs and proteins present in pollen, especially during the post-meiosis stages of development, are summarized in a review by McCormick (2004) and in a paper by Volkov *et al.* (2005). Results of microarray experiments (Becker *et al.* 2003; Honys and Twell 2003) indicate that mRNAs that encode signal transduction and cell-wall biosynthesis proteins were highly represented, whereas transcription and translation proteins were underrepresented. Proteome analysis of mature pollen of *Arabidopsis* indicates that about half of the identified proteins are involved in metabolism (20%), energy generation (17%), or cell structure (12%); these percentages are similar to those determined for the pollen transcriptome and this similarity is consistent with the idea that, in addition to the mRNAs, the mature pollen grain contains proteins necessary for germination and rapid pollen tube growth (Holmes-Davis *et al.* 2005); similar data were obtained by Noir *et al.* (2005). In rice, Kerim *et al.* (2003), using proteomic analysis, identified proteins that are associated with sugar metabolism, cell elongation and cell expansion – cell activities that are essential to pollen germination. Dai *et al.* (2006) have recently used a proteomic approach to identify 322 unique proteins in mature pollen of *Oryza sativa* L. ssp. *japonica*. Overrepresentation of the proteins was related to signal transduction (10%), wall remodeling and metabolism (11%), protein synthesis, assembly and degradation (14%), and carbohydrate and energy metabolism (25%). Sheoran *et al.* (2007) have recently identified 133 proteins of mature tomato pollen grains. A number of proteins in tomato pollen were similar to those reported in the pollen of other species; however, several additional proteins with roles in defence mechanisms, metabolic processes and hormone signaling were identified. The defence-related proteins include proteins associated with both biotic and abiotic stresses, such as Pto-disease resistance protein, Pto-like serine/threonine kinase, reactive oxygen species scavengers such as ascorbate peroxidase and superoxide dismutase and several Hsps. Data on genes involved in the earlier pollen developmental stages are limited. Caryl *et al.* (2003) reviewed the recent progress in the isolation of meiotic mutants in *Arabidopsis*, and the contribution this is making to the study of meiosis. The genes that have been identified are involved in a wide range of meiotic processes: chromosome cohesion, recombination, synapsis, chromosome segregation, and cell-cycle regulation.

With regard to tomato, to the best of our knowledge, there are no transcriptomic data yet available on microspore and pollen development, and for genes that are expressed during tomato pollen development, Proels *et al.* (2003, 2006) have recently demonstrated that, among the three invertase isoenzymes expressed in floral tissues, *Lin5*, *Lin6* and *Lin7*, the expression of *Lin7* was restricted to the tapetum and pollen; histochemical analysis of β -glucuronidase (GUS) reporter activity showed *Lin7* expression in pollen and pollen tubes of corresponding transgenic plants. In addition, Proels *et al.* (2003, 2006) have demonstrated a link between gibberellin (GA) action and invertase function in the tapetum for pollen development, and they suggested that the GA requirements for pollen development, pollen germination and pollen tube growth are linked to energy metabolism via the regulation of an extracellular invertase that is a key enzyme for carbohydrate supply via an apoplasmic pathway. We have recently demonstrated (Pressman *et al.* 2006) temporal changes in the expression and activity of invertases and sucrose synthase during tomato anther development, which suggests a specific role for neutral invertase in anther cell growth, elongation and differentiation, and in support of microspore development towards the vacuolated-microspore stage. In addition, we have shown that the temporal pattern of *Lin7* expression, encoding the flower-speci-

fic apoplastic invertase (Godt and Roitsch 1997) in developing tomato anthers, resembles that of cell-wall-bound acid invertase activity. This suggests that apoplastic invertase activity in tomato anthers is controlled at the transcription level, which would be consistent with the results of Goetz *et al.* (2001). We have found that the temporal expression and activity patterns of soluble acid invertase during tomato anther development, like those of cell-wall-bound acid invertase, increased in parallel with the maturation of the anther and pollen grains (Pressman *et al.* 2006).

Heat stress response

Pollen development is highly sensitive to heat-stress conditions: either chronic mild HS, or short periods of acute HS. Regarding chronic HS effects, studies used to relate to the effect of high night temperatures. Peet and Bartholemew (1996) for example, demonstrated that high night temperature in the range of 24-26 (when day temperature was 26°C) caused a reduced number of pollen as compared to night temperature of 18-22, although higher germination percentage was observed under the higher night temperatures. More recently, Peet *et al.* stressed the significance of mean daily temperature regarding HS effect on fruit-set (Peet *et al.* 1997) and pollen quality (Peet *et al.* 1998), where daily mean temperature of 29°C were shown to be detrimental compared to 25°C. The various stages of pollen development and functioning, including the release of the mature pollen grains from the anther were found to be HS sensitive to differing degrees as detailed below.

Pollen quality and germination

HS was found to affect pollen quality throughout anther development, but exhibited minimal effects on mature pollen grains. Flower buds at 9-5 days before anthesis were found to be highly susceptible to high temperature, when subjected to two daily exposures to 40°C for 3 h (Iwahori and Takahashi 1964). The greatest sensitivity to such exposures was found in the microspore mother cells in the meiosis stage, i.e., 9 to 8 days before anthesis, and sensitivity decreased as bud development advanced (Iwahori 1965). Similarly, Sato *et al.* (2002) demonstrated that flowers of a heat sensitive tomato cultivar were most sensitive to high temperatures 15-7 days before anthesis. Microscopic investigation of anthers in plants grown continuously under high temperatures revealed disruption of development in the pollen, endothecium, epidermis, and stomium. This disruption was reduced, but was still observable in plants relieved from high temperature for 10 days before anthesis. Pressman *et al.* (2002) and Firon *et al.* (2006) showed that continuous exposure of heat-sensitive and heat-tolerant tomato cultivars to mild high temperatures (daily mean of 29°C) led to a decreased number of pollen grains, and a significant reduction in pollen quality, as indicated by decreased germination capacity. However, under HS, heat-tolerant tomato cultivars, selected on the basis of higher yields under HS conditions, have been found to produce larger numbers of high-quality pollen grains than heat-sensitive cultivars. Both the microsporogenesis and the microgametogenesis phases were found to be HS-sensitive. Heat stress was also found to affect germination by arresting pollen tube elongation (Iwahori 1966).

Pollen release

In addition to the effect of HS on pollen production and functioning, Sato *et al.* (2000) found a significant relationship between the number of pollen grains released and the fruit-set percentage. In light of these studies they suggested that the pollen release mechanism and the quality of the pollen grain were closely related. Sato and Peet (2005) reported that high temperature stress reduced the number of pollen grains released, but not the timing of pollen release.

Other flower parts

Iwahori and Takahashi (1964) found that HS, in the form of two daily exposures to 40°C for 3 h, applied 9 d before anthesis, but not later, affected the pistils. Iwahori (1965) reported that macrospore mother cells degenerated under the same HS treatment. Ovules exposed to 40°C for 4 h, 18 h after pollination aborted, and in those exposed 24-96 h after pollination degeneration of the endosperm has been observed (Iwahori 1966). Peet *et al.* (1998) concluded that heat stress imposed during development of the female gametes and after pollination was deleterious, but less so than when it was imposed on the pollen grains.

Heat and humidity

The optimal relative humidity for tomato pollination is generally thought to be 70%. Peet *et al.* (2003) showed that fruit set, percentage of undeveloped flowers, total fruit weight and pollen germination percentage were most sensitive to high humidity (90%) at high temperature (31/25°C), and to high temperature under high humidity.

Fruit set

Weaver and Timm (1989) concluded that pollen germination and pollen tube length correlated positively with fruit set under high-temperature treatment, and that pollen viability had a major role in determining the fruit set in tomatoes at high temperatures. They suggested that pollen viability could serve as a basis for screening tomato plants for production of high yields in high-temperature environments. Fruit set varied between cultivars (Rudich *et al.* 1977; Levy *et al.* 1978; Abdul-Baki 1991; Fernandez-Munoz and Cuartero 1991). Abdul-Baki and Stommel (1995) found no fruit set in heat-sensitive genotypes and only 50% fruit set in heat-tolerant genotypes under day/night temperatures of 35/23°C. Nonetheless, they concluded that the response of pollen to heat treatment was genotype dependent and was not a general predictor of fruit set under high temperature stress. Peet *et al.* (1997) reported that reproductive characteristics, i.e., fruit set percentage, total number and weight of fruits per plant, and seediness index, deteriorated as the temperature rose. The primary parameter affecting these variables was mean daily temperature, with daily temperature having a secondary role. In a further study, Peet *et al.* (1998) demonstrated that no fruit at all developed on male-sterile plants that received pollen produced under a daily mean temperature of 29°C. In light of these findings, Sato *et al.* (2004) examined the effect of high temperature, i.e., mean daily temperature of 29°C, on nine cultivars, and found that one cultivar (FLA 7156) was the most tolerant, although under the high temperature conditions seeded fruit set in this cultivar was less than half that of the control.

Physiological and biochemical characteristics of HS effect on developing pollen

Abiotic stress conditions (including HS) have been shown, in diverse experimental systems, to affect sugar metabolism in the anther and in developing pollen. Normal starch accumulation during pollen development was inhibited in the anthers of water-deficient rice plants (Sheoran and Saini 1996; Saini 1997), in which it caused male sterility, and a similar effect was found in wheat (Dorion *et al.* 1996). Koonjul *et al.* (2005) have recently reported that water deficit during male meiosis in wheat (*Triticum aestivum* L.) caused pollen sterility; in seeking to identify the internal trigger for this failure, they found that water stress specifically impaired the activities of vacuolar and cell-wall invertases in anthers, prior to the arrest of pollen development. The enzymes were affected only when water deficit occurred around meiosis. Together their results show that the decline in invertase activity probably was regulated primarily at the transcriptional level in a gene-

and cell-specific manner. In sorghum, high temperatures, i.e., day/night temperatures of 36/26°C, caused reduced pollen numbers and reduced the percentage of starch-positive pollen by a factor of 8 (Jain *et al.* 2007). Aloni *et al.* (2001) have shown that high temperature conditions reduced the quality of pollen in pepper and affected acid invertase activity and pollen carbohydrate metabolism.

In tomato, exposure to chronic, moderate HS caused a marked reduction in starch content in the maturing pollen grains (Fig. 4). Reduction in pollen germination ability, following the exposure of the plants to HS, has been associated with this reduction in starch content (Pressman *et al.* 2002; Firon *et al.* 2006; Pressman *et al.* 2006). Moreover, higher quality of pollen of heat-stressed, heat-tolerant cultivars was associated with higher levels of starch accumulation during pollen maturation, and higher levels of soluble sugars in the mature pollen grains (Firon *et al.* 2006). It should be emphasized that heat-tolerant and heat-sensitive cultivars, grown under chronic, moderate HS, i.e., day/night temperatures of 32/26°C, differed in starch and soluble sugars contents in the pollen grains only, and not in other parts of the anther (Pressman, unpublished data). These data indicate that the applied HS conditions did not impair the flow of sugars to the anther; rather, they affected the sugar metabolism itself, in the developing grains. In addition, it has been recently demonstrated that HS caused a reduction in cell-wall-bound acid invertase activity in the anthers of flower buds 5 days before anthesis (DBA), which correlated with reduced starch accumulation 2 days later, and an induction of sucrose synthase and soluble acid invertase activities in maturing anthers and pollen (Pressman *et al.* 2006). The HS-induced increases in soluble acid invertase and sucrose synthase activities close to anthesis were found to correlate with a threefold increase in the hexose:sucrose ratio in mature anthers, including pollen grains (Pressman *et al.* 2006). The HS conditions applied altered the gene expression profiles of the enzymes tested: steady-state level of the mRNA coding for cell-wall-bound invertase at 5 DBA decreased; expression of the soluble acid invertase gene at 5 DBA increased, and expression of the sucrose synthase gene at 5 and 3 DBA decreased. The data indicated that sucrose-cleaving enzymes in tomato anthers responded, at both the mRNA and enzyme activity levels, to high-temperature conditions, that the response was dependent upon the stage of flower development, and might have involved post-transcriptional control (Pressman *et al.* 2006). Song *et al.* (2002) have recently demonstrated that low activity of *S*-adenosylmethionine decarboxylase, probably because of impairment of protein synthesis or functional enzyme formation, could be a cause for poor performance of tomato pollen at high temperatures.

Recently, we (N. Firon and E. Pressman, unpublished data) have found that developing pollen grains of the *N. sylvestris* starchless mutant NS 458 (Hanson and McHale 1988) accumulated low levels of starch and were highly sensitive to HS: they exhibited a 50% reduction in germination capacity, compared with wild-type pollen. These findings further demonstrate the crucial role of starch metabolism in determining pollen quality.

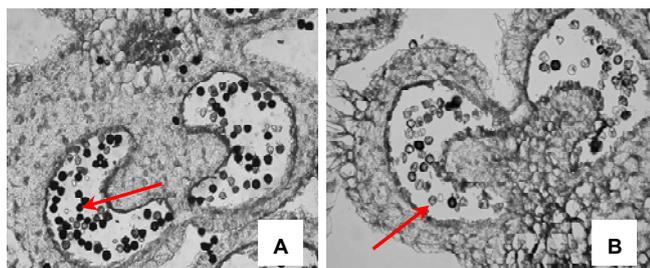


Fig. 4 Cross sections of the heat-sensitive tomato cv. 'NC 8288' flower buds at 3 days before anthesis, derived from plants grown at control (A) and mild-high temperature (B), stained with I/KI. Arrowheads point to developing pollen in the locular fluid.

However, the mechanism by which starch accumulation contributes to higher quality of mature pollen under HS is not well understood. Recently, Kaplan *et al.* (2004) suggested that metabolites (including sugars that may function as compatible solutes or in signaling) were involved in adaptation to thermal stress. Using the Arabidopsis starch-less mutant, Blasing *et al.* (2005) have demonstrated that sugars modify the expression of up to half of clock-regulated genes. Goetz *et al.* (2001) demonstrated in tobacco that targeted antisense repression of the cell-wall invertase gene in the anthers (the tapetum) and pollen caused significant reduction in pollen starch content, accompanied by male sterility, suggesting the involvement of carbohydrate metabolism and starch accumulation in determining pollen quality. Further, it is interesting to note that expression of the antisense SnRK1 sequence – a global regulator of carbon metabolism in yeast – in anthers and pollen, caused male sterility in transgenic barley (Zhang *et al.* 2001).

Heat stress effects on pollen gene expression

Data on the effect of HS on tomato pollen gene expression are very limited. It has been previously indicated, in various plant systems, that pollen is unable to synthesize a full complement of Hsps (Mascarenhas and Crone 1996; Maestri *et al.* 2002); this could explain pollen sensitivity to high temperatures. The HS response of mature pollen was shown to be 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) and Hsp90 (Mascarenhas and Crone 1996; Pechan and Smykal 2001; Gallie 2001; Stone 2001), Hsp70 and heat shock factors (Gagliardi *et al.* 1995; see also above in section *Molecular aspects of pollen development*).

Despite the amount of available information (Smykal *et al.* 2000; Volkov *et al.* 2005), data are still lacking with respect to the expression of the various *Hsp* families in developing pollen, and on their possible involvement in pollen thermotolerance, especially in the case of *Hsp101*, which recently has been shown to be a major factor in thermotolerance in *Arabidopsis* (Hong and Vierling 2000; Queitsch *et al.* 2000), though it has been shown not to be heat-stress induced in mature pollen of maize (Young *et al.* 2001). In tobacco, Volkov *et al.* (2005) have recently demonstrated that small HS proteins are differentially regulated during pollen development and following HS; they studied the pollen-specific expression of 11 cytosolic sHsp genes, and the results indicate that, for most sHsp genes, the overall induction level of mRNA was much lower in pollen than in leaves. Nevertheless, three genes of class CI (sHsp-6, -2B, -3C) were heat-induced with comparable efficiencies in leaves and mid-bicellular pollen, to an even higher level in mid-unicellular pollen, indicating tissue specificity of the transcriptional regulation of the HS response.

In tomato, we (N. Firon and E. Pressman) have recently obtained data demonstrating a high degree of induction of the steady-state mRNA levels, and also of the protein levels of a number of Hsps that belong to the small *Hsp* family (cytoplasmic class I and class II as well as mitochondrial, chloroplast and ER *sHsps*) in maturing tomato microspores derived from flower buds exposed to both a short period of acute HS (45°C for 90 min) and to chronic, mild high-temperature growing conditions, i.e., day/night temperatures of 32/26°C (Firon *et al.*, in preparation). In addition, we found that high steady-state mRNA levels of the *Hsp101* gene were induced in maturing tomato microspores upon exposure of flowers to acute HS (Fig. 5).

These data may indicate the involvement of these Hsps in protecting the developing pollen grains from HS conditions.

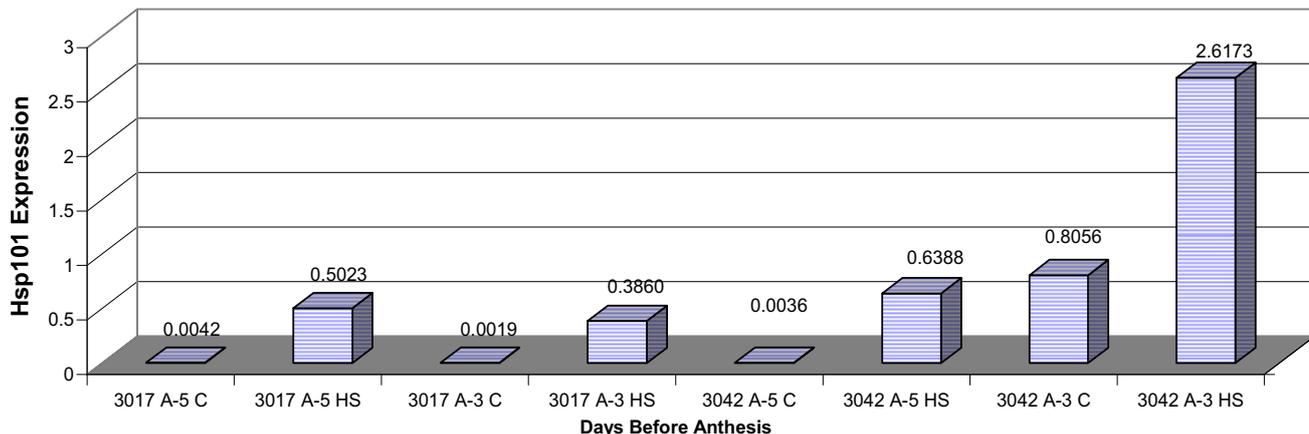


Fig. 5 Effect of HS (45°C for 90 min) on HSP101 mRNA abundance in developing tomato anthers at 5 (A-5) and 3 (A-3) days before anthesis of heat-sensitive cv. ‘3017’ and heat-tolerant cv. ‘3042’. Quantitative RT-PCR was performed and the values are normalized to expression of 18S rRNA. C – Control.

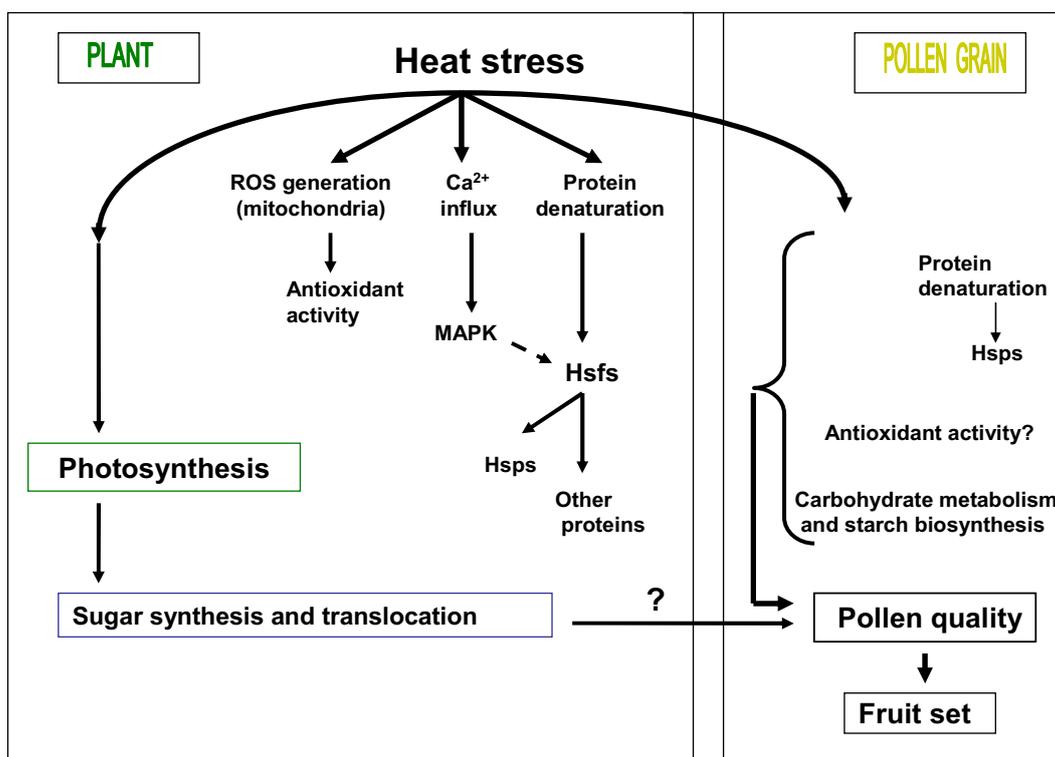


Fig. 6 Overview of the pathways and factors involved in the response of tomato to heat stress. HS simultaneously affects processes and components in the cytoplasm and the organelles in the various parts of the plant. HS affects membrane integrity, Ca²⁺ flux, protein conformation and production of reactive oxygen species (ROS) which, in turn, elicit defense mechanisms that include the production of heat shock factors, heat shock proteins and ROS-scavenging enzymes. Data suggesting that HS interferes with photosynthesis and sugar translocation and uptake, resulting in sugar limitation in the flowering buds are questionable (indicated by a question mark). In the developing pollen grain HS directly affects sugar and starch metabolism – effects that are associated with reduced pollen quality. In parallel, defense mechanisms are elicited in the developing pollen grains; these include the induction of Hsps and probably (?) antioxidant activity.

CONCLUSIONS

The response of the tomato plant, in general, and the most heat-sensitive part of the plant – the pollen grains, in particular, to HS is complex, involving a wide range of components and processes. **Fig. 6** summarizes the main components and processes that were shown to be affected by HS, and the defense system components elicited by it. The defense systems include the induction of Hsps and ROS scavengers.

The full understanding of the heat sensitivity of pollen, on the one hand, and of the molecular mechanisms that may enable the pollen to cope with HS, on the other hand, requires additional data, including data on other components of the chaperone network, including the involvement of heat-shock transcription factors, as well as data on other

protective mechanisms.

FUTURE PROSPECTS

Identification and characterization of pollen genes that are sensitive to HS, on the one hand, and of those involved in pollen defense mechanism(s) that enable microspores to cope with HS, on the other hand, will provide data that will enable enhancement of heat tolerance by improving breeding efficiency, and that will enable uses of marker-assisted selection and genetic modification, including the generation of the appropriate transgenic plants. There are still very limited data concerning pollen-specific gene expression in response to biotic or abiotic stresses in general, and to HS conditions in particular. This is true for Arabidopsis as well as for other plant species. Completion of the sequencing of the

tomato genome will enable the generation of a microarray chip that will include better representation of pollen-expressed as well as pollen-specific genes than possible with the currently available cDNA and Affymetrix microarray chips. The development of additional genomic tools in tomato, including tagged mutants, in parallel to additional experiments in tomato and in other plant systems, with efforts focused on the effects of HS on the microspore and pollen transcriptome, proteome and metabolome, will all contribute to the identification and characterization of genes that are involved in HS sensing and signal transduction, and of genes that can be used or manipulated for the production of more heat-tolerant and heat-resistant genotypes.

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