

RESEARCH PAPER

# Developmental and heat stress-regulated expression of HsfA2 and small heat shock proteins in tomato anthers

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## Abstract

The high sensitivity of male reproductive cells to high temperatures may be due to an inadequate heat stress response. The results of a comprehensive expression analysis of HsfA2 and Hsp17-CII, two important members of the heat stress system, in the developing anthers of a heat-tolerant tomato genotype are reported here. A transcriptional analysis at different developmental anther/pollen stages was performed using semi-quantitative and real-time PCR. The messengers were localized using *in situ* RNA hybridization, and protein accumulation was monitored using immunoblot analysis. Based on the analysis of the gene and protein expression profiles, HsfA2 and Hsp17-CII are finely regulated during anther development and are further induced under both short and prolonged heat stress conditions. These data suggest that HsfA2 may be directly involved in the activation of protection mechanisms in the tomato anther during heat stress and, thereby, may contribute to tomato fruit set under adverse temperatures.

**Key words:** Anther development, heat stress, HsfA2, Hsp17-CII, pollen, tomato.

## Introduction

Exposure to environmental stresses, such as high temperature, can severely reduce the productivity/yield of tomato plants grown under field conditions (Bar-Tzur *et al.*, 1985). Decreased fruit set due to heat stress (hs) has been associated with several alterations in the morphology of tomato flower structures and with physiological imbalances in stress-protective metabolites, such as carbohydrates, polyamines, and proline (Pressman *et al.*, 2002; Song *et al.*, 2002; Sato *et al.*, 2006). The major factor responsible for the failure of tomato fruit to set under suboptimal temperature conditions is considered to be the higher sensitivity of flower developmental processes to temperature changes, with the anthers being reported to be more vulnerable than the female organs (Peet *et al.*, 1998; Sato *et al.*, 2000). Alterations in tomato anthers, such as a failure of adequate

dehiscence and of tapetum development, have been found when hs occurs during the early phases of pollen development, i.e. 7–15 d before anthesis (Sato *et al.*, 2000, 2002). However, hs also affects late pollen development. Yang *et al.* (2009) recently demonstrated that *TMS1*, encoding a Hsp40 heat shock protein with DnaJ and PDI domains, is required for thermotolerance in growing pollen tubes in *Arabidopsis*. A mutation in this gene led to the reduction of seed production when plants were exposed to high temperatures.

The sensitivity of pollen development to hs has also been attributed to reduced thermotolerance due to the inability of the pollen to provide a strong hs response and, consequently, to produce large quantities of heat shock proteins (Hsps) as compared with vegetative tissues

(Mascarenhas and Crone, 1996). However, both low and high molecular weight Hsps have been found to be expressed in the early and late stages of pollen development in various plant species (Becker *et al.*, 2003; Honys and Twell, 2003; Volkov *et al.*, 2005; Sheoran *et al.*, 2007; Frank *et al.*, 2009). One hypothesis is that the activation of hs gene expression during plant development is correlated to developmental programmes rather than to the response of the plant under stressful environmental conditions (Waters *et al.*, 1996; Sun *et al.*, 2002). According to this proposal, during pollen formation, Hsps may function as molecular chaperones for the folding/refolding of proteins involved in meiosis and tetrad formation (Bouchard, 1990; Atkinson *et al.*, 1993; Reynolds, 1997). The developmental regulation of Hsp expression has been well characterized in animals, where they are induced by distinct heat stress transcription factors (Hsfs) activated by endogenous stimuli (Pirkkala *et al.*, 2001). In contrast, few members of the large Hsf family in plants have been shown to be developmentally regulated (Gagliardi *et al.*, 1995; Honys and Twell, 2004; Frank *et al.*, 2009). Kotak *et al.* (2007) observed an elevated expression of HsfA9 during embryogenesis and seed maturation in *Arabidopsis thaliana*, and showed that its transcriptional control was strictly mediated by the ABI3 signal cascade. The function of other Hsf genes as potential transcriptional activators of Hsp expression in plant developmental programmes, however, remains poorly understood.

Researchers have expended the most effort on analysing the network of Hsfs activated in plant tissues, such as leaves, under hs conditions (von Koskull-Döring *et al.*, 2007). In particular, one of the components of the tomato Hsf family, HsfA2, shows a number of striking characteristics that suggest it would be an ideal potential transcriptional activator during the hs response (Baniwal *et al.*, 2004). Mishra *et al.* (2002) reported that HsfA2 was strongly accumulated in tomato cells under conditions of high temperature, following its induction by the master regulator, HsfA1, and that it became the dominant Hsf component in thermotolerant cells. Three major functional states of HsfA2 are distinguished: a soluble nuclear form, a soluble cytoplasmic form, and a stored form (Nover *et al.*, 1989; Scharf *et al.*, 1998; Heerklotz *et al.*, 2001; Port *et al.*, 2004). After the plant has sensed hs, HsfA2 is accumulated in the nucleus of tomato cells in the presence of HsfA1, and both Hsfs then form hetero-oligomeric complexes that markedly enhance the expression of hs genes (Chang-Schammet *et al.*, 2009). In contrast, during ongoing hs conditions, a considerable quantity of HsfA2 is present in large cytoplasmic aggregates (the hs granules). Ultimately, following the hs and during the recovery period, most of the HsfA2 is found in a soluble form in the cytoplasm. Port *et al.* (2004) demonstrated that Hsp17.4-CII acts as a specific repressor of HsfA2 activity, suggesting that this complex is responsible for the recruitment of HsfA2 into hs granules.

Analyses of *HsfA2* and *Hsp17.4-CII* transcripts revealed a differential regulation during flower development under

normal and hs conditions (F Giorno and S Grillo, unpublished data). Frank *et al.* (2009) subsequently demonstrated the expression of these hs genes by transcriptional profiling of tomato microspores.

The results reported here were obtained from an in-depth investigation on the expression of these genes and the accumulation of their corresponding proteins in the tomato anther during its development under experimental hs conditions. Unravelling the transcriptional mechanisms that regulate Hsf and, thereby, Hsp expression during tomato flower development will undoubtedly be a major step towards improving genetic traits in tomato plants, such as pollen thermotolerance and fruit set.

## Materials and methods

### *Plant material, growth, and HS treatments*

Tomato seeds from *Solanum lycopersicum* cv Saladette, a heat-tolerant line that has normal fruit set under high temperature conditions (Rudich *et al.*, 1977), were germinated and grown at control temperatures (CT: 26 °C / 19 °C day/night) under a long-day photoperiod (16/8 h, day/night), with low-intensity light supplied by high-pressure sodium lamps (600W; Philips, Eindhoven, The Netherlands). Following initiation of the flowering period, tomato buds containing anthers 2, 4, 6, and 8 mm long, respectively, corresponding to the flower morphological stages described by Brukhin *et al.* (2003), were harvested from the plants. Mature microspores and dry pollen were obtained from the 8 mm long anthers (200–300) and from flowers at anthesis, respectively.

Heat stress (HS) was applied to plants in the form of high temperature (36 °C). Anthers at the 2 mm stage were collected after being subjected to short HS treatments at 36 °C for 1, 2, and 6 h, respectively (Fig. 4A, samples A, B, and C), after recovery at 26 °C and on the second day of the HS treatments (Fig. 4A, samples D, E, and F). Anthers at the 2 mm stage were harvested from the same treated plants (Fig. 4A, samples G, H and I) at day 7 after daily repeated cycles of mild HS and recovery (36 °C / 26 °C day/night). Recovery samples D and G (Fig. 4) were also harvested at 26 °C after 30 min of light acclimatization to avoid interference from the circadian rhythm.

From the same plants, flower buds containing anthers 4, 6, and 8 mm in length, mature microspores from 8 mm anthers, and dry pollen from the anthesis stage were collected after 2 h at 36 °C.

In this manuscript, the abbreviation HS refers to the heat stress treatment, while hs refers to heat stress as a stress condition.

### *RNA isolation and real-time PCR*

Total RNA was isolated from anther cone tissues ( $n > 6$ ) using a one-step RNA isolation method (TRIzol<sup>®</sup> Reagent; Invitrogen, Carlsbad, CA, USA). RNA quantity was measured spectrophotometrically, and its quality was checked by agarose gel electrophoresis. For reverse transcription, 1 µg of total RNA for each sample was incubated with RNase-free DNase (RQ1; Promega, Madison, WI, USA), and 400 ng of total RNA was used for reverse transcription according to the manufacturer's instructions (iScript<sup>™</sup> cDNA Synthesis Kit; Bio-Rad Laboratories, Hercules, CA, USA). PCR analyses were carried out in 25 µl volumes containing 0.125 µl of cDNA synthesis reaction mixture, 400 nM of each primer, and 12.5 µl of iQ SYBR Green Supermix (Bio-Rad Laboratories). The PCRs were performed in a 96-well thermocycler (Bio-Rad iCycler; Bio-Rad Laboratories) using a controlled temperature program starting with 3 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 45 s at 60 °C. To verify

the presence of a specific product, the melting temperature of the amplified products was determined. In addition, a fraction of each PCR mixture was analysed on a 2% agarose–ethidium bromide gel to verify the size of the amplified DNA fragment. The primers used for the real-time quantitative PCRs were designed using a computer program (BEACON DESIGNER 5.01; Premier Biosoft International, Palo Alto, CA, USA) to obtain primers that have close to identical melting temperatures and do not form secondary structures with each other under the given PCR conditions (Supplementary Table S1 available at *JXB* online).

The primers used to quantify *Hsp17.4-CII* and *Hsp17.6-CII* mRNAs were gene specific. Relative mRNA levels of the target genes were calculated following the Bio-Rad outlined methodology based on Vandesompele *et al.* (2002). The *LeEF1* and *18S rRNA* genes were used as references. For each sample, the mRNA quantity was calculated relative to the calibrator sample for the same gene. All reactions were performed on two independently collected series of RNA samples. The gene expression patterns were comparable in the two biological replicates. All figures show one series, with the error bars based on one technical repeat.

Semi-quantitative PCRs were performed using 5 µl of 25-fold diluted cDNA, buffer IV, 2.5 mM MgCl<sub>2</sub>, 0.5 U of Red Hot Taq DNA polymerase (all from ABgene Limited, Epson, Surrey, UK), 0.4 mM dNTPs (Fermentas, St. Leon-Rot, Germany), and 0.1 µM of primers. Amplification consisted of 30 cycles of 30 s at 95 °C (denaturation), 30 s at 58 °C (primer annealing), and 30 s at 72 °C (extension time).

#### *In situ* RNA hybridization

For *in situ* experiments, a 250 bp fragment was amplified from the pGEM5zf clone containing the total coding sequence of *Hsp17.4-CII*, previously amplified from tomato leaf cDNA. Primers 5'-TTTCGTGATGCTAAGGCAATG-3' (forward) and 5'-GCATTCTCCGGCAGACTAAA-3' (reverse) were used for the amplification. The 250 bp fragment was then inserted into pGEM-T easy. The sense and antisense digoxigenin (DIG)-labelled ribo-probes were generated by *in vitro* transcription using T7 and SP6 RNA polymerases according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN, USA). For the *in situ* experiments, 2 mm and 4 mm anthers were collected from tomato plants grown at CT and from those treated for 2 h at 36 °C. Anther fixation/paraplast embedding and *in situ* experiments were performed as described by Nitsch *et al.* (2009).

#### Protein isolation and immunoblotting

Proteins were extracted from anthers using TRIzol<sup>®</sup> Reagent (Invitrogen) according to the manufacturer's instructions. Following isolation, the protein concentration was measured using the bicinchoninic acid assay (BCA Assay Kit; Thermo Fisher Scientific, Waltham, MA, USA). One aliquot from each sample containing the same amount of total protein was precipitated with 4 volumes of acetone, and the proteins were dissolved in 1× SDS sample buffer. Aliquots of 25 µg of total proteins were separated on 14% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (pore size: 45 µm) (Protran; Schleicher and Schuell, Dassel, Germany). For immunodetection, the membranes were blocked with 5% (w/v) milk powder in phosphate-buffered saline (PBS) for 1 h and then incubated overnight at 4 °C with rabbit polyclonal antibodies against HsfA2 and Hsp17-CII, as described by Port *et al.* (2004). The blots were then rinsed and incubated with secondary antibody against rabbit IgG conjugated to horseradish peroxidase and were further processed for chemiluminescence detection using a kit as described by the manufacturer's protocol (Super Signal West Pico Chemiluminescent Substrate; Thermo Fisher Scientific).

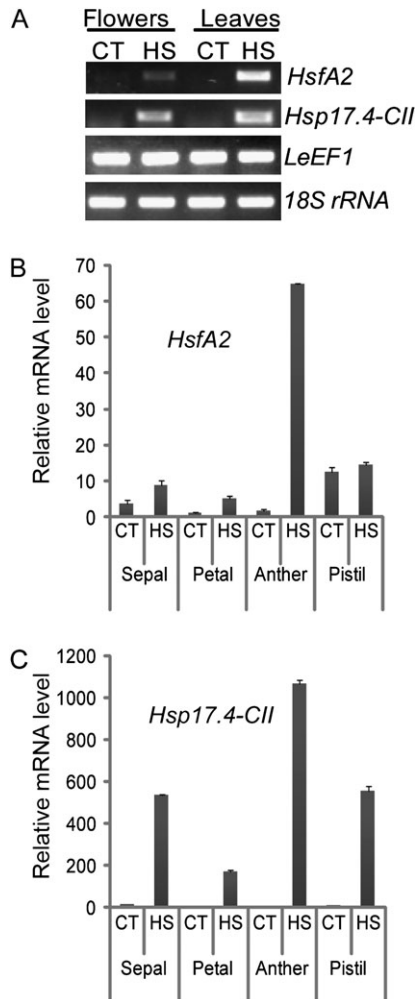
## Results

### Expression of *HsfA2* and *Hsp17.4-CII* in different tomato tissues and during anther development

Previous results (F Giorno and S Grillo, unpublished data) suggested that *HsfA2* and *Hsp17.4-CII* genes are induced in response to HS during tomato flower development, particularly at anthesis, when pollination occurs. The expression of these genes in tomato microspores has also been reported (Frank *et al.*, 2009). The influence of HS on the expression of these genes in the flower and during anther development was therefore studied here in more detail. As shown in Fig. 1A, *HsfA2* and *Hsp17.4-CII* were induced in the tissues of flowers at anthesis and in leaves harvested from cv Saladette treated with daily repeated cycles of mild HS and recovery for 3 weeks (HS: 36 °C / 26 °C, day/night). The real-time PCR (qRT-PCR) results showed that *HsfA2* and *Hsp17.4-CII* were more highly induced in the anther than in the other flower tissues (Fig. 1B, C). The observation that both *HsfA2* and *Hsp17.4-CII* were more highly expressed in the tomato male reproductive organs suggests that they may play a role in the protection system of sporophytic and/or sporogenic tissues in the anther. The timing of the activation of this protection system during anther development was investigated here by first determining the transcript levels of *HsfA2* and *Hsp17.4-CII* in 2, 4, 6, and 8 mm long anthers using semi-quantitative PCR (Fig. 2). As shown in Fig. 2B, the transcripts of *HsfA2* and *Hsp17.4-CII* were more detectable during development at the 2 mm stage under CT conditions, while both mRNAs were present at all stages of development after HS for 2 h at 36 °C.

### *HsfA2* and *Hsp 17-CII* are differentially modulated in young anther stages

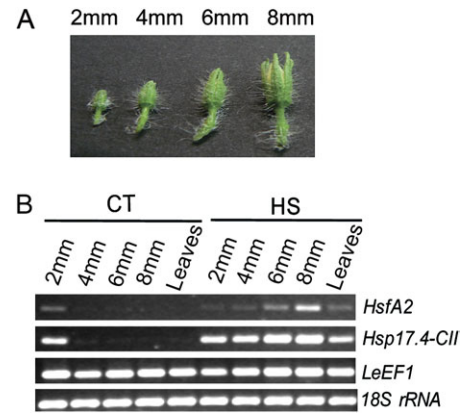
The observation that 2 mm anthers from CT plants had the highest expression of *HsfA2* and *Hsp17.4-CII* led to a more detailed investigation of transcript levels and protein accumulation under CT conditions. As shown in Fig. 3A, *HsfA2* expression in 2 mm anthers from CT plants was higher than that in older ones. Correspondingly, the amount of the protein was higher at the 2 mm and 4 mm anther stages, subsequently decreasing in 6 mm and 8 mm anthers (Fig. 3D). The *Hsp17.4-CII* gene was also expressed during anther development (Fig. 3B), particularly at the younger stages, when the accumulation of Hsp17-CII proteins was also observed (Fig. 3D). However, because tomato small Hsps, such as *Hsp17.4-CII* and *Hsp17.6-CII*, belong to the same subfamily and differ only by a few amino acid residues, it was impossible to discriminate them by the antibody used in this study (Port *et al.*, 2004). This led to an analysis of the *Hsp17.6-CII* transcript profiles in order to distinguish the *Hsp17.4-CII* and *Hsp17.6-CII* mRNAs that are translated into the respective proteins. As observed for *Hsp17.4-CII*, the *Hsp17.6-CII* gene was also expressed during anther development under CT conditions, particularly in 2 mm anthers (Fig. 3C), indicating that the



**Fig. 1.** Transcriptional changes of *HsfA2* and *Hsp 17.4-CII* in tomato tissues under HS. Total flower tissues at anthesis and leaf tissues were harvested from cv *Saladette* treated with daily repeated cycles of mild HS and recovery for 3 weeks (HS: 36 °C / 26 °C, day/night) or maintained under control conditions (CT: 26 °C / 19 °C day/night). From these same treated and untreated plants, flowers harvested at anthesis were dissected into sepal, petal, anther, and pistil. (A) Semi-quantitative PCR shows increased mRNA levels of *HsfA2* and *Hsp17.4-CII* in tomato flowers and leaves under prolonged HS conditions. (B) Relative transcript levels of *HsfA2* were measured by qRT-PCR using *LeEF1* and *18S rRNA* as housekeeping genes to normalize the data. The highest induction of *HsfA2* was identified in the anther tissues treated at high temperatures. (C) A similar transcript profile to that of (B) was also observed for *Hsp17.4-CII*, which was strongly induced in the male reproductive organs under HS.

*Hsp17.4-CII* and *Hsp17.6-CII* proteins may be detected together with the *Hsp17-CII* antibody.

An HS time course experiment was designed to analyse the influence of HS on *HsfA2* and *Hsp17-CII* expression modulations in the youngest anther under stressful conditions. The results are shown in Fig. 4. Plants were grown in the growth chamber at CT. At the beginning of flowering, temperatures were raised to 36 °C during the day and



**Fig. 2.** Expression analyses of *HsfA2* and *Hsp17.4-CII* during anther development under CT and HS conditions. (A) Tomato flower buds were harvested from cv *Saladette* and used to obtain different sized anthers (2, 4, 6, and 8 mm). (B) mRNAs of *HsfA2* and *Hsp17.4-CII*, from anthers at different stages and leaves treated with HS (2 h at 36 °C) or kept at CT conditions (26 °C / 19 °C, day/night) were analysed by semi-quantitative PCR. *LeEF1* and *18S rRNA* transcript levels were monitored as controls.

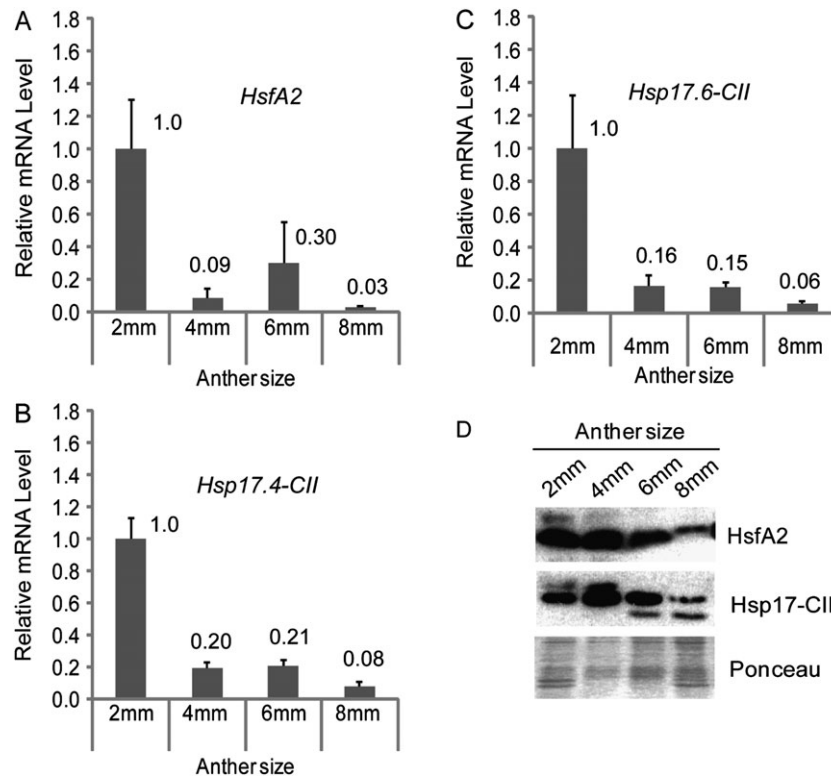
26 °C during the night. This treatment was repeated for 7 d to mimic the long-day hs condition that can occur in the field. Anthers at the 2 mm stage were collected at various intervals (samples CT-I, Fig. 4A). Controls (untreated samples) were harvested during the day at 26 °C, and samples D and G were harvested at the end of the recovery periods, after HS, at 26 °C and after 30 min of light. The results show that *HsfA2* was rapidly induced in cv *Saladette* after 1 h of HS but that the mRNA levels transiently declined quickly after 2 h HS (Fig. 4B, samples A and B). Interestingly, the *HsfA2* transcript was more strongly induced during the second HS treatments (sample E in Fig. 4B) and was still detectable in the recovery periods and under prolonged HS treatments (samples D, G, H, and I in Fig. 4B). However, its protein level remained comparable with that in the control sample (samples D, G, H, and I in Fig. 4E). The mRNA expression patterns of both *Hsp17-CII* were similar to that of *HsfA2* (Fig. 4C, D), but *Hsp17-CII* protein accumulation was particularly strong only after prolonged HS regimes (samples F, G, H, and I in Fig. 4E).

#### *HsfA2* and *Hsp17-CII* expressed in immature pollen

*In situ* RNA hybridization on anthers harvested at various developmental stages was performed to determine in which anther tissues the *HsfA2* and *Hsp17-CII* transcripts were present.

A riboprobe corresponding to a fragment of the coding sequence of *Hsp17.4-CII* was synthesized. However, some sequence domains of *Hsp17.4-CII* and *Hsp17.6-CII* are known to be highly conserved, implying that both messengers may hybridize to this probe.

As expected, *Hsp17-CII* mRNA was detected in anthers from plants subjected to HS for 2 h at 36 °C, but not in the



**Fig. 3.** Expression analyses of HsfA2 and Hsp17-CII during anther development. (A–C) Relative mRNA levels of *HsfA2* (A), *Hsp17.4-CII* (B), and *Hsp17.6-CII* (C) in different sized anthers under CT conditions (26 °C / 19 °C, day/night). Expression data were normalized using *LeEF1* and *18S rRNA* as housekeeping genes. The mRNA levels of the target genes in 4, 6, and 8 mm anthers are relative to that from 2 mm anthers (value 1). The experiment shown here is one of two biological replicates, which showed a comparable expression pattern. Each graph represents the data of two technical repeats. (D) Immunoblotting analyses of the proteins isolated from the same samples as in A, B, and C using specific antisera against HsfA2 and Hsp17-CII. Ponceau staining of total protein was used as control for equal loading.

controls (Fig. 5). In contrast, HsfA2 transcripts could not be detected in any of these stages, most probably due to low mRNA abundance.

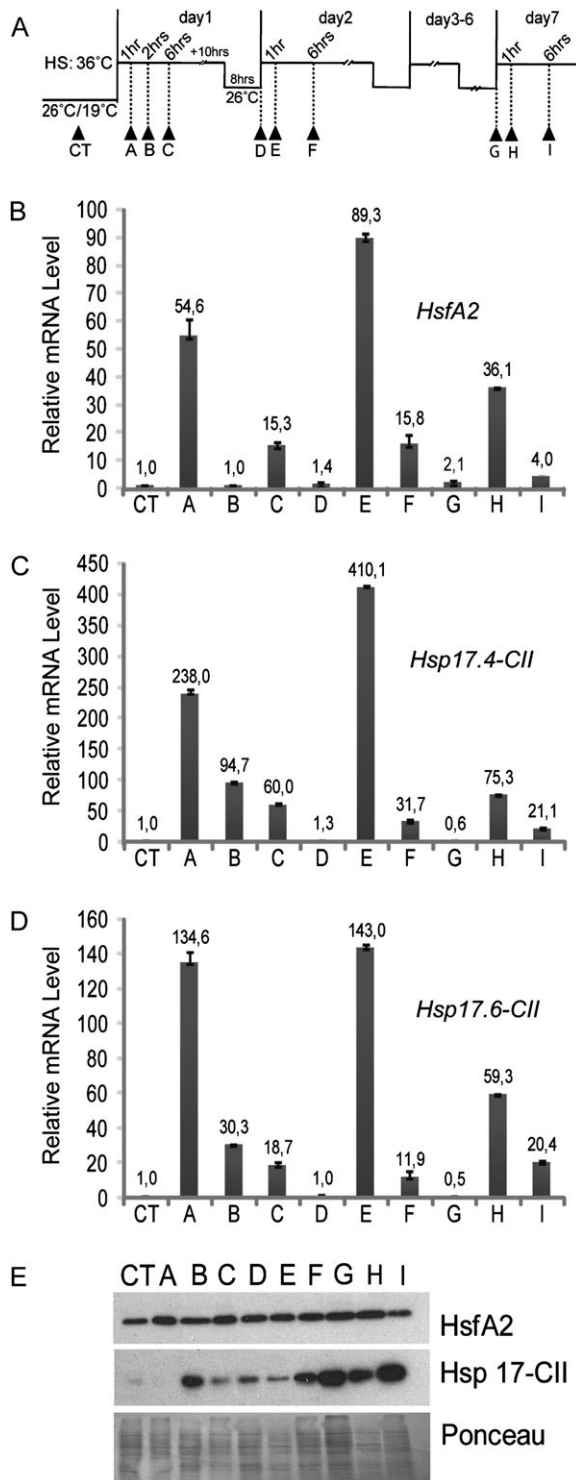
In agreement with the results of the qRT-PCR, *Hsp17-CII* transcripts were strongly accumulated in anthers at young stages, particularly in the pollen mother cells (Fig. 5B). A signal was detected in 4 mm anthers in the microspores, bound together as tetrads in the pollen sac (Fig. 5D, E). *Hsp17-CII* transcripts were not detected in 6 mm and 8 mm anthers (data not shown), which is in contrast to the results of the semi-quantitative PCR analysis reported in Fig. 2B. One possible explanation for this discrepancy is that mature microspores at these stages are surrounded by an exine wall that obstructs the penetration of the probe.

qRT-PCR and western blotting analyses were carried out on the developing pollen grains to confirm the presence of HsfA2 and Hsp17-CII. An increased level of *HsfA2* and *Hsp17.4 CII* transcripts was observed in mature microspores isolated from 8 mm anthers harvested from plants subjected to the HS treatment for 2 h at 36 °C (Fig. 6A). The expression of these genes in dry pollen was also tested, as dry pollen grains develop from the microspores and are released at anther dehiscence. However, mRNAs of *HsfA2* and *Hsp17.4-CII* were not detectable in the dry pollen from

flowers at anthesis (data not shown), whereas the mRNA of *Hsp17.6-CII* was expressed at this stage in the CT and HS samples (Fig. 6B). The accumulation of HsfA2 and the two Hsp17-CII proteins was observed in mature microspores in the CT and HS samples, as shown in Fig. 6C. In contrast, HsfA2 protein was not detectable in the dry pollen, whereas Hsp17-CII persisted, most probably as a product of *Hsp17.6-CII* mRNA (Fig. 6B, C).

## Discussion

High temperatures can potentially disrupt tomato fruit set by causing damage to developing pollen grains (Sato *et al.*, 2002). The high sensitivity of the developing pollen grain to hs is partly attributable to its incomplete hs response due to a defect in the accumulation of *Hsf* and *Hsp* mRNAs (Frova *et al.*, 1989; Gagliardi *et al.*, 1995; Mascarehans and Crone, 1996). *Hsp* genes are also induced in a number of developmental pathways, such as seed maturation, embryogenesis, and/or fruit maturation, in various other plant species (Waters *et al.*, 1996; Neta-Sharin *et al.*, 2005; Volkov *et al.*, 2005; Kotak *et al.*, 2007). However, the overproduction of Hsps can also interfere with normal developmental processes, as shown



**Fig. 4.** Expression analyses of *HsfA2* and *Hsp17-CII* in 2 mm anthers after HS treatments. (A) The pictogram shows the time course of HS treatments. Arrows indicate the time points when the 2 mm anthers were harvested (CT-I). (B-D) qRT-PCR of *HsfA2* (B), *Hsp17.4-CII* (C), and *Hsp17.6-CII* (D). Expression data were normalized using *LeEF1* and *18S rRNA* as housekeeping genes. The mRNA levels of the target genes are relative to that of the sample CT (value 1). (E) Immunoblotting analyses showing protein levels of *HsfA2* and *Hsp17-CII* in anthers at the same stages as in B, C, and D. Ponceau staining of total protein was used as control for equal loading.

by maize plants carrying a mutation in the *Empty Pericarp2* gene. This gene is normally a negative regulator of the heat shock response, but the presence of the mutation results in abortion or retarded embryogenesis (Fu *et al.*, 2002).

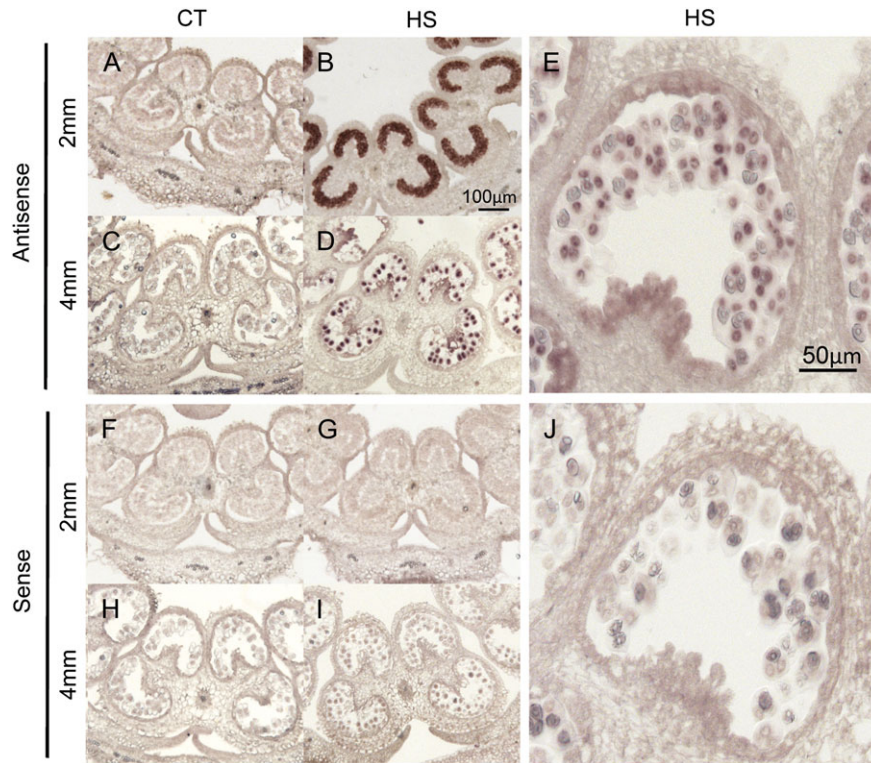
The results reported here on the temporal and spatial regulation of *HsfA2* and *Hsp17-CII* suggest possible roles for the *Hsf* and *Hsp* genes in developmental processes.

*HsfA2* was more highly expressed in anthers than in other organs and was strongly activated when exposed to hs; this is similar to its expression in leaves (Mishra *et al.*, 2002). *HsfA2* was expressed early in young anthers, at the stage when, according to Brukhin *et al.* (2003), pollen mother cells are developing. Frank *et al.* (2009) reported the expression of these genes at the post-meiotic stage, presumably in the tetrads, under non-hs conditions.

It has been suggested that during these developmental processes Hsps may function as molecular chaperones for the folding/refolding of proteins involved in meiosis and tetrad formation (Bouchard, 1990; Atkinson *et al.*, 1993; Reynolds, 1997). *HsfA2* is an activator of Hsp genes and is likely to be involved in the regulation of Hsp activation during tomato pollen/anther development (Nishizawa *et al.*, 2006; Schramm *et al.*, 2006; Oyawa *et al.*, 2007). A similar function of transcriptional activator is well documented for Hsf members in animals and also for *HsfA9* in *Arabidopsis*, where *HsfA9* induces Hsp expression during seed maturation coinciding with dormancy and desiccation tolerance (Kotak *et al.*, 2007). Honys and Twell (2004) meticulously analysed the transcriptome of the male gametophyte of *Arabidopsis* but were unable to identify transcripts of *HsfA2*. Given these data from earlier studies, the present results suggest that *HsfA2* expression is differentially modulated between tomato and *Arabidopsis*. Consequently, the species-specific functional diversification previously documented for *HsfA1* and *HsfB1* may also be extended to *HsfA2* in terms of its involvement in different developmental pathways (Bharti *et al.*, 2004; von Koskull-Döring *et al.*, 2007).

*HsfA2* is known to be markedly activated in young anthers in response to hs regimes. Frank *et al.* (2009) reported that *HsfA2* is strongly expressed in maturing tomato microspores experimentally treated with a short hs at 45 °C. However, in the present study, *HsfA2* was activated even with a mild hs (36 °C), and its expression was maintained for several days when plants were subjected to this type of stress. This result indicates that *HsfA2* may even be involved in the hs response to milder stresses in order to enhance the expression of Hsp genes and to protect the pollen grains during development. Scharf *et al.* (1998) and Schramm *et al.* (2006) examined the kinetic expression characteristics of *HsfA2* and demonstrated that this protein is also present at relatively high levels during the recovery period following the hs. As such, the behaviour of *HsfA2* resembles that of typical Hsp proteins in thermotolerant cells. In agreement with these data, in this study, the *HsfA2* protein also remained at high levels





**Fig. 5.** *In situ* RNA localization of *Hsp17-CII* at the 2 mm and 4 mm stages. Sections of 2 mm and 4 mm anthers from CT (26 °C / 19 °C, day/night) and HS samples (2 h at 36 °C) were hybridized with antisense (A–E) and sense (F–J) probes. Accumulation of *Hsp17-CII* transcripts was detected in HS samples, particularly in pollen mother cells at the 2 mm stage (B) and in tetrads at the anther stage of 4 mm (D, E).

in the anthers during the recovery periods. However, the decline in mRNA levels of *Hsp17-CII* observed during the recovery periods suggests that the HsfA2 protein was inactive. Port *et al.* (2004) observed that HsfA2 inactivation depends on its interaction with Hsp17.4-CII protein. The experimental data reported here suggest that a similar control mechanism may operate in anther/developing pollen grains.

The elucidated characteristics of HsfA2 make it an ideal and potent transcriptional activator in many environmental stress responses (Busch *et al.*, 2005; Nishizawa *et al.*, 2006; Zhang *et al.*, 2009). In tomato, Mishra *et al.* (2002) reported that the essential component of the tolerance to high temperature is HsfA1 but that HsfA2 is sufficient to restore the thermotolerance in HsfA1-silenced protoplasts.

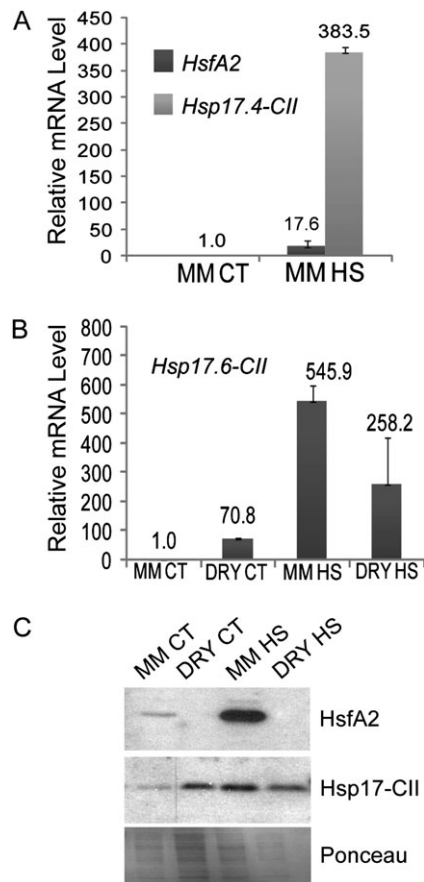
An *Arabidopsis* T-DNA insertion mutant lacking *HsfA2* expression is more sensitive to prolonged hs due to its decreased expression of Hsps and genes such as *ascorbate peroxidase 2* (Schramm *et al.*, 2006; Charng *et al.*, 2007). Meiri and Breiman (2009) recently demonstrated that HsfA2 contributes to the thermotolerance of *Arabidopsis* by mediating the translocation of the ROF1 and Hsp90.1 protein complex from the cytoplasm to the nucleus. Interference in this shuttle system severely affects the expression of Hsps during hs recovery periods in *Arabidopsis* mutant lines and therefore also hinders the acquisition

of thermotolerance following exposure to prolonged hs conditions. Recent evidence that maturing microspores of an hs-tolerant line have a higher HsfA2 basal level than those of an hs-sensitive line also suggests that this gene may contribute to pollen thermotolerance (Frank *et al.*, 2009).

However, it cannot be excluded that other Hsfs are active during microspore development in tomato and that HsfA2 is only one example of such transcription factors.

*HsfA2* expression in dry pollen was not observed here. However, this absence of expression may be due to the fact that dry pollen is metabolically inactive as the cytoplasm is greatly dehydrated; therefore, no further protection should be required at this stage. In contrast, the *Hsp17.6-CII* transcript was detected in the dry pollen. It is therefore possible that this latter gene is activated at this stage by other transcriptional factors that are, in turn, regulated by the desiccation processes, as has been observed for other Hsps in *Arabidopsis* during seed maturation (Kotak *et al.*, 2007).

In conclusion, the results from the study reported here show that HsfA2 and *Hsp17-CII* are activated early in the tomato diploid pollen mother cells before the microspores develop and that their expression is persistent under prolonged HS conditions until mature dry pollen is produced. The data suggest that HsfA2 may be directly involved in the activation of protection mechanisms in the



**Fig. 6.** HsfA2, Hsp17.4-CII, and Hsp17.6-CII expression analyses in mature microspores (MM) and dry pollen (DRY) under CT conditions (26 °C / 19 °C, day/night) and HS (2 h at 36 °C). (A and B) mRNA levels of *HsfA2* and *Hsp17.4-CII* (A) and *Hsp17.6-CII* mRNA (B) in MM and DRY pollen. The relative mRNA levels were measured using *LeEF1* and *18S rRNA* as housekeeping genes. (C) Protein levels of HsfA2 and Hsp17-CII in MM and DRY pollen. Ponceau staining of total proteins is given as the control for equal loading.

tomato anther during hs and, thereby, may contribute to tomato fruit set under adverse temperatures.

## Supplementary data

Supplementary data are available at *JXB* online.

**Table S1.** Primer sequences used for semi- and real-time quantitative PCR.

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