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An Ethylene Over-Producing Mutant of Tomato (*Solanum lycopersicum*), Epinastic, Exhibits Tolerance to High Temperature Conditions

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Abstract

Above-optimal temperatures reduce yield in many crops, including tomato, largely because of the heat-sensitivity of their reproduction process. A full understanding of heat-stress (HS) response and thermotolerance of tomato reproduction is still lacking. Recently, using external application of the plant hormone ethylene, it was demonstrated that ethylene plays a role in heat-tolerance of tomato pollen (the male reproductive cells). In order to expand our understanding on involvement of ethylene in tomato pollen thermotolerance, we analyzed the response of wild type and ethylene-related tomato mutant plants to HS, at physiological and molecular levels. We report that mild chronic HS conditions highly reduce the number of viable and germinating pollen grains as well as the production of seeded fruits in wild type tomato plants, while no significant reduction was detected/observed in pollen quality, number of seeded fruits and seeds per fruit in plants of the ethylene over-producer mutant epinastic. Our findings suggest that ethylene is involved in thermotolerance of tomato reproduction, pointing to an effect on pollen viability and germination potential, highlighting candidate genes involved in pollen response to HS (like *SlHSP17*, *SlHSP101*, *SlMBF1*) and suggesting directions for further studies.

Keywords

Ethylene, Mild Chronic Heat Stress, Pollen Grains, Reproduction, *Solanum lycopersicum*, Thermotolerance

1. Introduction

High temperature conditions constitute a major environmental stress, affecting yield and quality of many crops including vegetable crops. Heat stress (HS), defined as temperatures above the normal optimum needed for growth, is expected to increase in the coming years [1] [2], thus being an important factor to consider for maintaining food security. This problem is crucial in tomato because a significant proportion of production is achieved in greenhouses (to avoid insect-transmitted virus infections) where the daily mean temperatures are high, especially during the warmer seasons. We showed previously that developing pollen grains, the male reproductive cells, are most sensitive to both mild chronic and short-term acute HS conditions [3] [4] [5]. We found that heat-tolerant tomato genotypes, that exhibited higher yield under HS, produced also a larger number of high quality pollen grains under HS compared with the tested heat-sensitive genotypes [3]. The physiological and molecular bases for pollen grains' HS response and heat tolerance (thermotolerance) are, however, still not fully understood [6] [7] [8].

Looking into pollen HS response, previous results from our laboratory revealed high HS regulation of heat-shock protein genes (*HSPs*; including small *HSPs* and *HSP101*), HS transcription factors (*HSFs*, including *HSFA2*) and additional factors that do not belong to the classical HS-responsive genes [9]. Involvement of ethylene in pollen HS response was suggested due to the high HS-induced expression, observed in developing tomato pollen grains, of several ethylene-responsive genes, including *SlER24*/*SlMBF1* (transcriptional coactivator multi-protein bridging factor; [9]). There is limited understanding, however, about ethylene involvement in pollen development and in pollen HS response [4] [5] [10] [11]. Available data from our laboratory indicate that external application of an ethylene releaser (ethephon) to tomato plants, prior to their exposure to HS conditions, increased significantly the number of germinating pollen grains per flower [11]. Involvement of ethylene in plant thermotolerance was previously reported in studies that dealt with vegetative tissues [12] [13] [14].

In the present study, in order to look into and further our understanding on the involvement of ethylene in pollen thermotolerance and in the production of seeded fruits under HS conditions, the response of tomato plants to HS conditions was investigated, in wild type (WT) and in two ethylene-related mutants, using physiological and molecular approaches.

2. Materials and Methods

The following mutants were used in the background of the tomato cultivar Micro-Tom [15] [16]: 1) *Never ripe* (*Nr*), a tomato mutant defective in the ethylene receptor (ETR3), a semi dominant ethylene receptor mutant [17]. 2) Epinastic (*epi*), being an ethylene over-producer [18]. The mutants were a gift from Dr. Peres (University of Sao Paulo, Brazil). The effect of mild chronic HS (MCHS) conditions was tested on flower ethylene production, pollen quality, production of seeded fruits and on expression levels of genes (known to be involved in ethy-

lene production and signaling, as well as genes previously shown to be involved in plant thermotolerance) in isolated pollen grains.

For applying HS conditions, WT and mutant plants were grown in two temperature-controlled greenhouses at the Volcani Center in Rishon LeZion, Israel, under natural light conditions (day length of 13.5 - 14 h) and day/night temperatures of 26/22˚C ± 2˚C for a month. Afterwards, one of the greenhouses was set to day/night temperatures of 32/26˚C ± 2˚C (designated MCHS) while the other greenhouse was maintained at 26/22˚C ± 2˚C (control conditions) and the plants were kept, in both greenhouses, for three additional months, with continuous production of flowers and fruits. These MCHS conditions were used in order to mimic the effect of summer conditions. Sampling of flowers and pollen grains was done from plants exposed to either "control" or "MCHS" conditions after at least 14 days from the time of applying the heat conditions.

Flower ethylene production was determined as described by Jegadeesan *et al.* [4]. Pollen quality was evaluated as described [4], by sampling mature pollen grains, to determine the number of viable and non-viable pollen grains. The population of viable pollen grains constituted of germinating and non-germinating pollen. All ethylene production and pollen quality results were the mean of at least three biological replicates. For ethylene measurements, flowers, 0.5 g per sample/replicate were collected/used. For pollen quality evaluation, each replicate consisted of pollen derived from at least eight flowers collected from four plants. For looking into the effect of the applied MCHS conditions on fruit and seed production, tomato fruits were harvested, from both greenhouses, at 110 days after transplanting and evaluated for fruit weight, percent of seeded fruits and number of seeds per fruit. Average number of red (ripe) fruits was 6 per plant in WT and ethylene mutants. Tomato fruits were collected from 16 plants, exposed to either "control" or "MCHS", and number of seeded fruits and average fruit weight were calculated. For each tomato genotype (WT, *Nr*, *epi*) and treatment, seeds were collected from 40 fruits and average seed number per fruit was calculated.

For gene expression analyses, pollen grains were isolated from plants grown at "control" and "MCHS" conditions and maintained at −80˚C until use. Gene expression was evaluated using the Biomark HD system (Fluidigm, USA). Fluidigm 48.48 dynamic array chip was used following the manufacturer's ADP37 Fast GE (http://www.fluidigm.com/user-documents) protocol. Gene expression was calculated using the $2^{-\Delta\Delta ct}$ method, following normalization with the 18S gene. Primer specificity and reference genes were validated prior to analysis. Heat map was generated using MultiExperiment Viewer, MeV v4.9 software, and expression profiles were obtained from $2^{-\Delta\Delta ct}$ data, where Hierarchical clustering of genes was based on Spearman correlation, allowing gene clustering according to their expression patterns.

3. Results and Discussion

Ethylene production results indicate that the amount of ethylene produced by

flowers of the ethylene over-producer mutant, *epi*, was 2.9- and 1.5-fold higher than that produced by WT and *Nr* flowers, respectively (Figure 1(A)). The applied MCHS conditions caused a further increase in ethylene levels produced by *epi* flowers (Figure 1(A)). Mild chronic HS conditions caused a significant decrease in pollen quality of WT and *Nr* mutant plants as evidenced by reduction in the number of viable as well as germinating pollen grains, while *epi* plants showed no significant reduction in either the number of viable or germinating pollen grains, exhibiting thermotolerance at the level of pollen quality (Figure 1(B) and Figure 1(C), respectively). These results are in line and substantiate previous results from our laboratory, showing that external application of an ethylene-releasing substance, ethephon, prior to HS exposure, increased tomato pollen quality, pointing to involvement of ethylene in pollen thermotolerance [5] [11]. It should be noted that, under optimal conditions, total number of viable and germinating pollen grains per plant was lower in *epi* than in WT plants (Figure 1(B), Figure 1(C)).

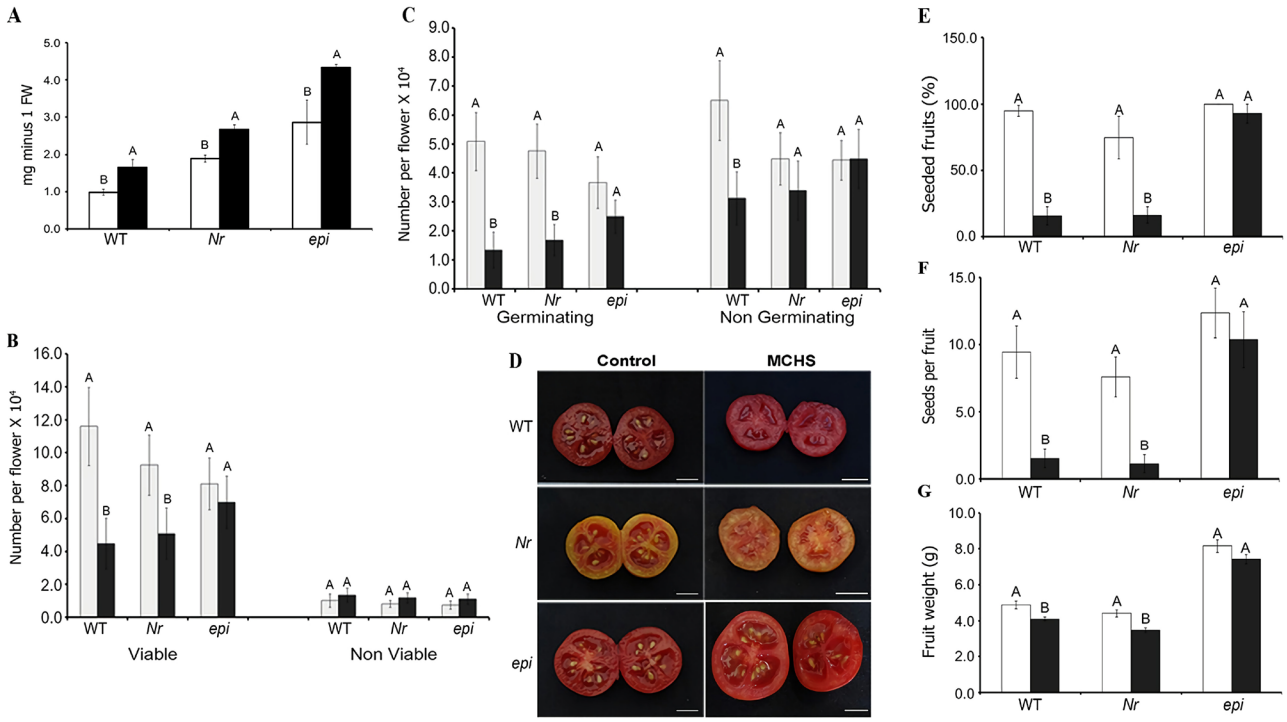

Figure 1. Effect of mild chronic HS on ethylene production, pollen quality and seeded-fruits production in wild type and ethylene-related mutant tomato plants. Ethylene production data (A) represent the amount of ethylene produced by flowers at anthesis, derived from tomato (*cv.* Micro-Tom) plants exposed to either control (C, white bars, 26/22˚C ± 2˚C day/night temperatures) or mild chronic HS (MCHS; black bars, 32/26˚C ± 2˚C day/night temperatures) conditions. Ethylene production was evaluated as described by Jegadeesan *et al.* [4]. Results are presented as mean values ± SE of three biological replicates. Pollen quality was evaluated by collecting mature pollen grains from wild type (WT) and ethylene-related mutant plants (*Nr* and *epi*), exposed to either control or MCHS conditions. Pollen quality data are presented as mean values ± SE (using three biological replicates) of number of viable, non viable (B), germinating and non germinating (C) pollen grains per flower. Representative fruits of WT, *Nr* and *epi* exposed to either control or MCHS are presented in (D). Percent of seeded fruits (E), number of seeds per fruit (F) and fruit weight (G) were evaluated and data are presented as mean values ± SE. Different letters indicate statistical significance by Tukey HSD test (p-value ≤ 0.05) between treatments (C and MCHS). Scale bars = 1 cm.

The outcome of successful fertilization is the production of seeded fruits. Representative pictures of fruits produced under optimal and MCHS conditions in WT, *Nr* and *epi* plants are presented in **Figure 1(D)**. Under optimal growth conditions ("control") percent seeded fruits and number of seeds per fruit in *epi* plants were comparable to those in WT plants, indicating that the number of pollen grains produced by *epi* was sufficient for fulfilling the plant's reproductive potential (**Figure 1(E)**, **Figure 1(F)**). Mild chronic HS conditions highly reduced the production of seeded fruits in both WT and *Nr* plants, while in *epi* plants there was no reduction in either the percentage of seeded fruits or the average number of seeds per fruit (**Figure 1(E)** and **Figure 1(F)**, respectively). Fruit weight was also maintained in *epi* plants under HS (**Figure 1(G)**).

To characterize the effect of *epi* on tomato pollen HS response at the molecular level, we used a high throughput gene expression analysis (Biomark HD system, Fluidigm 48.48 dynamic array chip, Fluidigm, USA) as indicated in "Materials and Methods". Details on the Fluidigm array gene expression analyses and list of primers are given in **Supplementary Table S1**. Gene expression was calculated using the $2^{-\Delta\Delta ct}$ method, following normalization with the 18S gene (a reference gene; [4]). Heat map was generated using MultiExperiment Viewer, MeV v4.9 software, and expression profiles were obtained from $2^{-\Delta\Delta ct}$ data, where Hierarchical clustering of genes was based on Spearman correlation, allowing gene clustering according to their expression patterns. The list of genes used included: 1) Ethylene-biosynthesis and ethylene-signaling genes found by us recently to express in tomato pollen grains [4]. 2) Classical heat-stress genes (like *HSPs* and *HSFs*) known to play an important role in plant thermotolerance [19] [20] [21]. 3) Genes coding for reactive-oxygen species scavengers (like superoxide dismutase, ascorbate peroxidase) and 4) Translation initiation factors. The list of all gene primers is presented in **Supplementary Table S1**.

Gene expression results are presented as a heat-map (**Figure 2(A)**) highlighting a small group of genes exhibiting high and significant HS-upregulation in pollen of the ethylene overproducer mutant *epi* (**Figure 2(B)**). These genes include *SlER21* (an ethylene-responsive member of the *HPS70* family; [22]), *SlHSP101* and *SlHSP17* (suggested previously in numerous studies to play a role in plant thermotolerance; [8] [23]) as well as *SlMBF1* (shown in *Arabidopsis* to function upstream of salicylic acid and ethylene; [24] [25]) (**Figure 2(B)**).

Taken together, the data presented in this work, and schematically summarized in **Figure 3**, support the hypothesis that ethylene contributes to thermotolerance of tomato reproduction, as evidenced by the relative high number of germinating pollen grains, seeded fruits and seeds per fruit, detected in plants/flowers of *epi*, that were exposed to MCHS conditions. Furthermore, the relative high expression levels of HSPs (*SlER21*/*SlHSP70*, *SlHSP101, SlHSP17*) as well as *SlMBF1*, detected in pollen of *epi* plants exposed to MCHS, may protect pollen proteins from HS-induced damages and contribute to *epi* pollen thermotolerance (during pollen development and germination). Further studies are needed in order to better characterize the role of *SlMBF1* in pollen HS response and

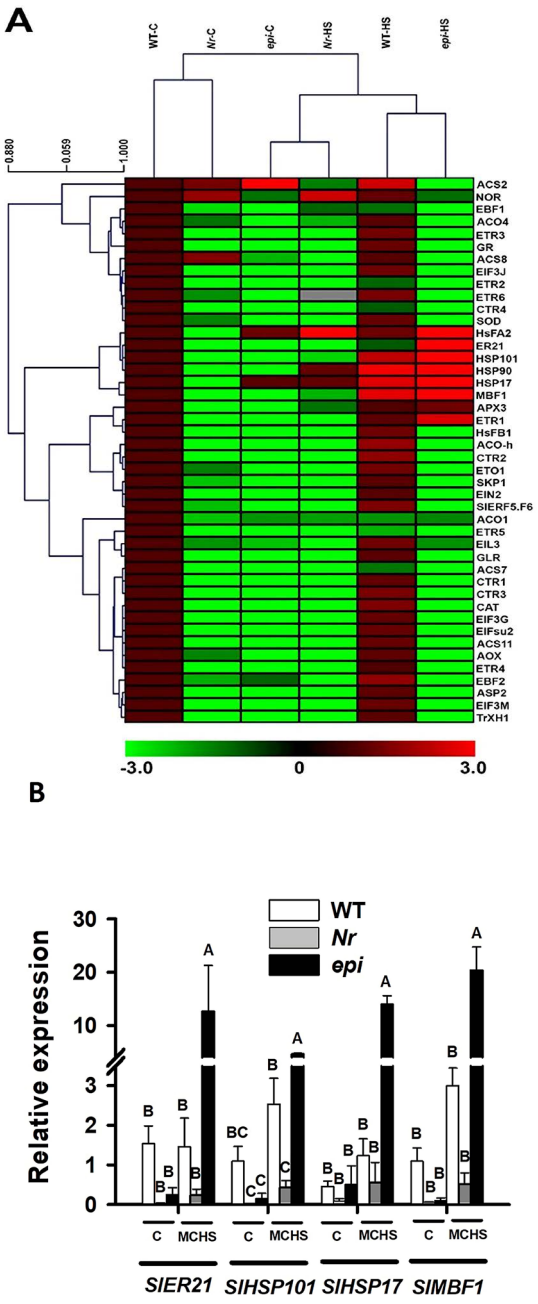

Figure 2. Effect of mild chronic HS on gene expression of pollen derived from WT and ethylene mutant plants. Gene expression was evaluated using the Fluidigm dynamic array chip. Pollen grains were isolated from flowers at anthesis, derived from plants that were exposed to either control (C, 26/22˚C ± 2˚C day/night temperatures) or mild chronic HS (MCHS, 32/26˚C ± 2˚C day/night temperatures) conditions as described in [4] [26]. Gene expression was calculated as detailed in "Materials and Methods" section. Heat map and hierarchical clustering of genes was done according to gene expression patterns as detailed in "Materials and Methods" (A). Fold change was calculated relative to expression values in pollen of WT plants at control conditions. Expression profiles of specific genes are given (B). Expression data are the means (±SE) of three biological replicates, each replicate containing pollen grains derived from 40 - 60 flowers/flower buds. For each gene, bars with different letters are significantly different by Tukey HSD test (p-value ≤ 0.05) across lines and treatments.

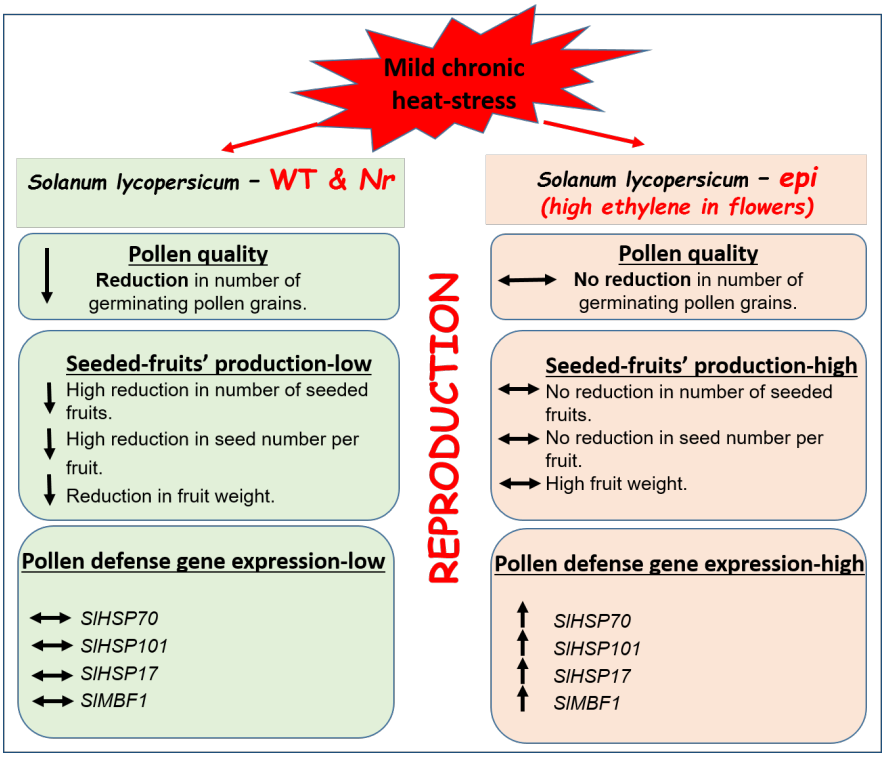

Figure 3. A scheme summarizing the effect of the *epi* mutation on tomato pollen quality, production of seeded fruits and expression of thermotolerance-associated genes under mild chronic HS conditions. Under mild chronic HS conditions, the ethylene-overproducer *epi* mutant plants of tomato (*cv.* Micro-Tom) exhibit relative high number of germinating pollen grains, seeded-fruits per plant and seeds per fruit, as compared to wild type plants. In addition, relative high expression levels of heat-tolerance-associated genes (*SlHSP70/SlER21*, *SlMBF1*, *SlHSP101* and *SlHSP17*) are detected in pollen grains of *epi* plants under mild chronic HS conditions, suggesting their involvement in pollen thermotolerance.

thermotolerance and identify additional regulators, downstream to *epi*, that may activate pollen thermotolerance mechanisms. It should be noted that that HS conditions applied in the current study were MCHS, lasting for at least 14 days prior to pollen collection and may have imposed heat acclimation and activation of acclimation mechanisms.

The use of tomato ethylene mutants is suggested as a valuable experimental tool for further research aimed at increasing our understanding on the involvement of ethylene in the heat-response and thermotolerance of tomato reproduction process (including pollen development and germination as well as the HS response of the female tissues) and fruit-setting.

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Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary

Supplementary Table S1. Details of the Fluidigm array gene expression analyses and list of primers.

Solyc ID	Gene name	Description	Forward primer	Reverse primer
Ethylene related genes				
Solyc01g095080	SlACS2	1 aminocyclopropane carboxylate synthase 2	CGATGCATTTTTAGTACCTTCACC	CGATGCATTTTTAGTACCTTCACC
Solyc02g063540	SlACS7	1 aminocyclopropane carboxylate synthase 7	CGTGTCTGAAAATCAAAAGAGG	TGTCTCATATCCACCCAACAAA
Solyc03g043890	SlACS8	1 aminocyclopropane carboxylate synthase 8	GCGCTACTTCCGCAAATGAA	TTGGAACTATTTCCGCCCCG
Solyc07g049530	SlACO1	1 aminocyclopropane carboxylate oxidase 1	ACAAACAGACGGGACACGAA	CCTCTGCCTCTTTTTCAACC
Solyc02g081190	SlACO4	1 aminocyclopropane carboxylate oxidase 4	TGATTGAGAAGACAGAAGAGGACA	GGAATTTGAGACCTGCATACAAC
Solyc12g011330	SlETR1	ethylene receptor1	TTCAAGGATTAAAGGTTTTGGTGAT	ATCACATCCAAGGTGTGTAAGCA
Solyc07g056580	SlETR2	ethylene receptor2	GCCGTCAGTGTACATGAGAAATTT	AGTTTTCTTTTGTCACTTGGTCAGTGT
Solyc09g075440	SlETR3	ethylene receptor/NR	AGGGAACCACTGTCACGTTTG	CTCTGGGAGGCATAGGTAGCA
Solyc06g053710	SlETR4	ethylene receptor4	GGTAATCCCAAATCCAGAAGGTTT	CAATTGATGGCCGCAGTTG
Solyc11g006180	SlETR5	ethylene receptor5	AGTCATCTTTTAGGAAACGCATGTT	AGGAGTACATGAAGGCCTCTGAA
Solyc09g089610	SlETR6	ethylene receptor6	ATTCCAAAGGCAGCCGTTAA	GGATGTGGATATGTGGGATTAGAAG
Solyc09g007870	SlEIN2	Ethylene-insensitive 2	AGA TGC GAG AGC TCA ATG GT	GAC CAC CTG CCC TTA GTG AA
Solyc01g096810	SlEIL3	EIN3-lke gene 3	GATGTCGCGTGCACAAGATG	GCACGAAGATTGTCCGAAGC
Solyc12g009560	SlEBF1		TCTCGCTTGCTGTCATTGGT	GACTGGAGGCCTTGAGCATT
Solyc08g060810	SlEBF2		GCAACAGATGTCCGACTTGC	TCAGTCACACCACGGATTGG
Solyc09g055310	SlETO1	Ethylene over producer 1	GCAAGGGCAGAGGTTTTTGG	GGGATCCAATCTGCTGGCAT
Solyc12g005960	SlERF5.F6	Ethylene response factor	TGCGCGTGCATTGTGTAAAA	CCACCACAGAAGACGACGAA
Solyc04g011440	SlER21	ethylene induced heat shock protein	AGGACCAGCGATCGGAATTG	CTCCAATCAACCGCTCCGTA
Solyc01g104740	SlMBF1	Multi protein bridging factor/ethylene response protein 21	CCA AGA CCT GAA GGA TCC AA	CCG CCT TCT TAT TCA AAC CA
Solyc10g006880	SlNOR	Non ripening	TCCATGGGAACTCCCTGCTA	GATGTTGCAGCCCGATTTGG
ROS scavangers				
Solyc09g007270	SlAPX3	Ascorbate peroxidase 3	CCGCCCTCTAGTCGAGAAAT	AGAACCAGACTGATCTCCAGAGA
Solyc04g082460	SlCAT	Calatase3	CTGATACCCAGAGACACCGC	TGACCTCTTCATCACGGTGC
Solyc06g067960	SlGR	Glutaredoxin family protein	CGTTTTGCAGCCTACCCTCT	GTGTCCGGATCACTAGGCAC
Solyc08g075540	SlAOX	Alternative oxidase	GTTGTCTTGGTTGTTCGGGC	ATACCCAAGTGGTGCTGGTG
Solyc02g084710	SlTRXH1	Thioredoxin H-type 1	GTGTGGTCCCTGCAAGTTCA	CTCCACGGCCCAATCAGTAG
Solyc09g091840	SlGR	Glutathione-disulfide reductase	ACCTGAAATCATGCAGGGCA	TGAAGAAATGCGCCTCGACT
HSFs and HSPs				

Continued

Solyc08g062960	SlHsFA2	heat stress factor A2	TTCCACCACATTGTTGCCTA	GCAAGCACCAGATCCTTGTT
Solyc03g115230	SlHSP101	Heat shock protein 101	ACATAGATGCTGGACGTGGC	AGCATCATTCCTTGGCCCAT
Solyc03g007890	SlHSP17	Heat shock protein 17	AAGGTCCGGCACCAAAGAAT	CTCCGCTACGAGGTAAGCAG
Solyc06g036290	SlHSP90	Heat shock protein 90	GTTCGAGAGCTTGACGGACA	TCATGCCAATACCGCTGTCA
Solyc02g090820	SlHsFB1	heat stress factor B1	GTCCCAGTTCACGGACTTGT	TTGGCTCATGATACGGTTGA
Translation initiation factors				
Solyc02g089070	SlEIF3M	Eukaryotic translation initiation factor 3 subunit M	GCAAGATCTCCGCACGAAAC	TGCTAAACCTTGCACTGCCT
Solyc02g079060	SlEIF3J	Eukaryotic translation initiation factor 3 subunit J	CACGGCTGAATTGTTTGCCA	GCTTTTCTCAAACGGTCGCA
Solyc03g120010	SlEIF3su2	Eukaryotic translation initiation factor 3 subunit 2	TCCACAGCAAACGGGTCTAC	TTCTGCTCCACACCATCGAC
Solyc08g074790	SlEIF3G	Eukaryotic translation initiation factor 3 subunit 4	GCAGGGGCTGTTCTTATGGT	CCCTTGATGGTGGCCTATCC
X51576	*Sl18S-200bp*	18 S ribosomal gene	CTTCTGGAAGGGATGCATTT	CTCCGGAATCGAACCCTAAT

The Fluidigm 48.48 dynamic array chip was used following the manufacturer's ADP37 Fast GE (http://www.fluidigm.com/user-documents) protocol, which allows 2304 simultaneous real time PCR gene expression. Primer specificity and reference genes were validated prior to analysis. Pre-amplification of cDNA was performed on 1.25 µl of 50 ng·µl^{-1} samples using Fluidigm PreAmp Master Mix (Fluidigm, PN 1005581), and 2.7 µl of each pre-amplified cDNA was mixed with 3 µl of SsoFast EvaGReen Supermix with Low Rox (Bio-Rad, PN 1725211) and 0.3 µl of 20X Binding Dye Sample Loading Reagent (Fluidigm, PN 1001388). Individual primer pairs (50 µM) in a 1.08 µl volume mixed with 3 µl Assay Loading Reagent (Fluidigm, PN 85000736) and 1.92 µl of Low TE. Total 5 µl of each sample mix or each assay mix was then pipetted into individual sample inlet in the 48.48 Dynamic Array chip, and a (IFC) controller MX (Fluidigm) to prime the chip. The loaded chip was placed in the BioMark system for PCR at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Following each reaction in a specific inlet, the PCR amplification curve was generated and the chip was imaged. The dynamic array raw data was analyzed with the Fluidigm Real-Time PCR Analysis software. The gene expression was calculated using the $2^{-\Delta\Delta ct}$ method, following normalization with 18S gene.

Abbreviations

HS, Heat-stress;
HSF, Heat-stress factor;
HSP, Heat shock/stress protein;
MCHS, Mild chronic heat-stress;
MBF1, Multiprotein bridging factor1.