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Spermidine affects the transcriptome responses to high temperature stress in ripening tomato fruit*#

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Abstract: Objective: High temperature adversely affects quality and yield of tomato fruit. Polyamine can alleviate heat injury in plants. This study is aimed to investigate the effects of polyamine and high temperature on transcriptional profiles in ripening tomato fruit. Methods: An Affymetrix tomato microarray was used to evaluate changes in gene expression in response to exogenous spermidine (Spd, 1 mmol/L) and high temperature (33/27 °C) treatments in tomato fruits at mature green stage. Results: Of the 10 101 tomato probe sets represented on the array, 127 loci were differentially expressed in high temperature-treated fruits, compared with those under normal conditions, functionally characterized by their involvement in signal transduction, defense responses, oxidation reduction, and hormone responses. However, only 34 genes were up-regulated in Spd-treated fruits as compared with non-treated fruits, which were involved in primary metabolism, signal transduction, hormone responses, transcription factors, and stress responses. Meanwhile, 55 genes involved in energy metabolism, cell wall metabolism, and photosynthesis were down-regulated in Spd-treated fruits. Conclusions: Our results demonstrated that Spd might play an important role in regulation of tomato fruit response to high temperature during ripening stage.

Key words: *Solanum lycopersicum* L., Spermidine, High temperature, Microarray, Gene expression
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1 Introduction

High temperature (HT) is one of the main environmental constraints to agricultural productivity worldwide. Many efforts have been made to explain the mechanisms of HT tolerance in plants through molecular and genomic approaches, and a number of genes involved in HT stress response at the transcriptional level have been reported (Wahid *et al.*, 2007). Immediately after exposure to HT, changes at the molecular level alter the expression of many genes involved in various pathways, thereby leading to the synthesis of stress-related proteins as a stress-tolerance strategy (Iba, 2002). Some of these genes might play important roles in protecting plants from heat stress through stress perception, signal transduction, and transcriptional regulatory networks in cellular responses.

Tomato (*Solanum lycopersicum* L.) is an important commercial crop and has been proved to be a highly useful model system for fruit development and ripening. HT is a major factor limiting the productivity in warm seasons and adversely influences the vegetative and reproductive phases of tomato, which ultimately reduces in yield and quality (Sato *et al.*, 2001; Pressman *et al.*, 2002). Exposure to HT

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adversely affects tomato ripening and nutrient and flavor quality, inducing poor fruit coloring, vacuous fruit, low sugar/acid ratio, and decreasing lycopene content (Yakir *et al.*, 1984; Paull and Chen, 2000), and finally causing tissue damage and physiological disorder (Inaba and Crandall, 1988). Furthermore, heat treatment leads to an inhibition of expression of tomato-ripening genes and thus has a negative impact on fruit quality (Picton and Grierson, 1988). The mRNA levels of 1-aminocyclopropane-1-carboxylic acid oxidase, phytoene synthase, and polygalacturonase decreased dramatically during the heat treatment but recovered afterwards, whereas the mRNA of heat shock protein (HSP) 17 increased during the HT treatment and then decreased when fruits were removed from heat stress (Lurie *et al.*, 1996). So the inhibition of fruit ripening by HT might occur at the level of gene expression. However, the entire molecular events occurring in the fruit under HT stress were not investigated until now.

Aliphatic polyamines [putrescine (Put), spermidine (Spd), and spermine (Spm)] are ubiquitous compounds involved in various physiological processes, including plant growth and development, flowering, fruit growth and development, and stress response, senescence, and fruit ripening (Mariani *et al.*, 1989). In recent years, a protective role against stress has been attributed to polyamines, both in the free and soluble conjugated forms, during mineral nutrient deficiency, and osmotic, salt, drought, heat, chilling, and oxidation stresses (Liu *et al.*, 2000; Perez-Amador *et al.*, 2002; Nayyar and Chander, 2004; Renaut *et al*., 2005; Todorova *et al.*, 2007). Manipulation of polyamine biosynthesis may lead to improvement of plant tolerance against multiple environmental stresses. Transgenic rice plants expressing an oat ADC cDNA exhibited an increased polyamine accumulation, which enhanced plant biomass under salinity (Roy and Wu, 2001). Transgenic tomato seedlings overexpressing yeast *S*-adenosyl-l-methionine decarboxylase gene would improve the tolerance to HT stress as compared to wild plants (Cheng *et al.*, 2009). Furthermore, Mattoo *et al.* (2002) proved that higher polyamines enhanced the accumulation of lycopene in the tomato. Since polyamines have been described as anti-senescence agents and a higher endogenous level of polyamines is associated with delayed fruit ripening (Valero *et al.*, 2002), many attempts have recently been made to explain the role of exogenous polyamines on fruit ripening. Pre-harvest and post-harvest applications of polyamines have been demonstrated to delay the fruit ripening and extend shelf life in mango, peach, plum, apple, and tomato (Law *et al.*, 1991; Pérez-Vicente *et al.*, 2002; Torrigiani *et al.*, 2004). However, controversial results on the effect of exogenous polyamines on fruit ripening have also been obtained (Wang C.Y. *et al.*, 1993; Escribano and Merodio, 1994). Therefore, much deeper insight is still required to understand the role of polyamines in fruit development and ripening.

In recent decades, there is increasing evidence demonstrating that polyamines act as antioxidants under environmental adverse conditions (Groppa *et al.*, 2001; Kakkar and Sawhney, 2002). Exogenous application of polyamines reduced the H_2O_2 level and malondialdehyde content and increased the level of antioxidants in chickpea plants subjected to water deficiency and cold stress (Nayyar and Chander, 2004). Exogenous Put improved Indian mustard seedling growth by preventing lipid peroxidation and denaturation of macromolecules through the induction of antioxidative enzymes and the increase of glutathione and carotenoid under NaCl stress (Verma and Mishra, 2005). Conversely, polyamines may act as a mediator or secondary messenger to activate a vast genetic network with a potential to provide defense against biotic and abiotic stresses (Paschalidis and Roubelakis-Angelakis, 2005; Cona *et al.*, 2006). It has been proved that a wide array of genes regulating transcription, translation, signal transduction, stress protein biosynthesis, ethylene biosynthesis and action, isoprenoid and flavonoid biosyntheses was activated by polyamines. However, there are still many doubts concerning the role of polyamines in stress tolerance (Groppa and Benavides, 2008).

More recently, high-throughput screening techniques such as microarray analysis have been used to monitor the expression of genes that respond to biotic and abiotic stresses (Zhang H. *et al.*, 2010; Lang *et al.*, 2011). Investigating how polyamines alleviate the HT injury could facilitate a better understanding of the genetic bases of heat tolerance. Thus, in the present study, to investigate the effects of HT stress and Spd application on the gene expression in tomato fruit during ripening, mature green tomatoes were harvested and treated with Spd under different temperature

regimes. The transcription profiles were compared using Affymetrix microarray analysis, and the differentially expressed genes involved in primary metabolism, stress response, and hormone biosynthesis were identified after HT and Spd treatments.

2 Materials and methods

2.1 Plant materials and growth conditions

Tomatoes [*Solanum lycopersicum* L. (formerly *Lycopersicon esculentum* Miller)] cv. Zhongshu No. 6 obtained from the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China were grown in a commercial greenhouse (21–29 °C day, >12 °C night) at the experimental farm in Zhejiang University, China. Seeds were sown in pots (30 cm×20 cm) containing potting media (mixture of peat and vermiculite) at the end of Jan. 2008, and seedlings with five growing leaves were transplanted to a field in a Venlo-type greenhouse. The plants were arranged at a density of four plants per m^2. Plant nutrition, pest and disease controls were in accordance with commercial practices.

2.2 High temperature and exogenous spermidine treatments

Tomato fruits were harvested at the mature green stage approximately four months after sowing in June 2008. Only fruits at a firm, mature green color classification with uniform shape and size and free from fungal infection were selected. After harvest, fruits were washed in tap water, air-dried at 25 °C, and individually labeled.

Mature green fruits were randomly divided into two batches. One batch was immersed in 1.0 mmol/L Spd solutions with 0.1% Tween-20 surfactant (5 L, 20 °C) for 30 min, and the other in distilled water containing 0.1% Tween-20 surfactant for 30 min. After air-drying for 10 min, the fruits of Spd-treatment and control were respectively subdivided into two sub-batches with 60 fruits each, and furthermore, each sub-batch was kept under normal (26/20 °C) or heat temperature (33/27 °C) condition. Finally, there were four different treatments, including normal temperature (C26) and HT (C33) without Spd treatment, Spd pre-treatment under normal (Spd26) and HT (Spd33) conditions. Detached mature green fruits were incubated in controlled environment chambers with 16/8 h (light/dark) period and 85% relative humidity (RH). The fruits were sampled at 1, 6, and 12 h, respectively. One gram of fresh peel and flesh were taken at the fruit shoulder position, immediately frozen in liquid nitrogen, and stored at −80 °C for further use.

2.3 Microarray analysis

Total RNA was extracted from the fruits of C26, C33, Spd26, and Spd33 at 1, 6, and 12 h, respectively, using TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Each pooled RNA sample was generated by mixing equal amounts of RNA from three intervals and then the mRNA was purified from 80–90 μg mixed total RNA using the RNeasy Plant mini kit (QIAGEN, Germany) according to the instructions. Then the double stranded cDNA was synthesized using the GeneChip® two-cycle cDNA synthesis kit. Tomato genome array (Affymetrix, CA, USA), designed specifically to monitor gene expression in tomato, was used. All procedures for probe preparation, hybridization, washing, staining, and scanning of the GeneChip® tomato arrays, as well as data collection, were performed at Affymetrix custom service (CapitalBio, Beijing, China) by following the standard protocol (http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Normalization was performed according to the standard Affymetrix protocol to allow the comparison of the samples from each set of experiments. Fold changes and *P*-values of probe sets were calculated using limma nested *F*-test, and the *P*-values for multiple testing were corrected using the false discovery rate (Benjamini and Hochberg, 1995). The Excel add-in for significance analysis of microarrays was used to identify differentially expressed genes between different treatments.

2.4 Databases for tomato functional genomics analysis

The data described here have been deposited in the Tomato Expression Database (http://ted.bti.cornell.edu/) and are available for public access (Fei *et al.*, 2006). Nucleic acid sequence and annotation data pertaining to the TOM1 microarray are available via the sol genomics network (SGN) database (Mueller *et al.*, 2005) (http://sgn.cornell.edu/).

2.5 Quantitative real-time polymerase chain reaction (PCR)

To validate the expression patterns revealed by microarray results, 15 genes identified through microarray analysis, which represent up-regulated, down-regulated, and unchanged genes, respectively, were analyzed using quantitative real-time PCR. The RNA samples for microarray analysis were also used for quantitative real-time PCR. Equal amounts of total RNA for each sample from three sampling time points (1, 6, and 12 h) were mixed and then the first-strand cDNA was synthesized from the mixed total RNA using ImProm-II™ reverse transcription system (Promega, Madison, USA) according to the manufacture's instructions. Specific primers (Table 1) for selected genes were designed for 80–120 bp amplicon with melting temperature at 50–52 °C by DNAMAN software. The cDNA obtained (1 μg) was subjected to real-time PCR in a final volume of 20 μl containing 12.5 μl SYBR Green Master Mix Reagent (TaKaRa, Japan) and specific primers (3 pmol). Two biological and three technical replicates for each sample were performed in an iCycler iQ™ real-time PCR system (Bio-Rad, USA) programmed to heat for 4 min at 95 °C, followed by cycling conditions (melting step for 30 s at 95 °C, annealing for 30 s at 54 °C, and extension for 30 s at 72 °C) repeated for 40 cycles. To normalize the total amount of cDNA present in each reaction, *Ubi3* gene was co-amplified as an endogenous control for calibration of relative expression. Melting curves were performed using Dissociation Curves software (Applied Biosystems, Foster City, CA) to ensure only a single product was amplified. The PCR efficiency was estimated by the data obtained from the exponential phase of each individual amplification plot and the equation $(1+Eff)=10^{slope}$ (Ramakers *et al.*, 2003). The comparative cycle threshold ($\Delta\Delta C_t$) method of relative gene quantification recommended by Applied Biosystems was used to calculate the expression level of different treatments.

Table 1 Primers for real-time PCR analysis

Accession No.		Primer sequence (5′→3′)
BG629220	F	CTTCAAACCTTGGCTCTT
	R	TCATCTATCCAGCACCAG
BM535342	F	CTCACCTCACTTCGCTTC
	R	GAGATGGTCAGGTGAGAGAG
U66300	F	AAGCGGAACTGAAGAATG
	R	GGACATCAATCACCTTTTTCTC
BF112635	F	GGGGATTCTTTGAGGTGA
	R	GCCTTTACTTGCCACCAT
BE459581	F	ACAGGAGTTGCTGGTTCATTT
	R	TTGAGAAAGCCAATGAAGAC
BG628248	F	GTTTATCCTTACGGTTTCTCC
	R	GCGGCTGATAAAGGTGAG
AY128100	F	CAAGAATTGCTTTGCCTG
	R	AACACTTTGGGATTGCTACT
AI781668	F	TGCTGTATCAATGCCTGTG
	R	TGAACAGACATGAACCCT
BE432137	F	CAGAGGATGTTGGAGGCA
	R	GACACGCAACATTTACCC
BG129203	F	GGCAAAGTTCACTGGGATA
	R	TTTCCCGTGGTTGGTT
X95296	F	GGCACATACAAACAAAGGAG
	R	CCATCGGAGACGACAAC
BT014421	F	CATTCGTGAAGGGTGATC
	R	TGGATAAAGTTCCGAGGC

F: forward; R: reverse

3 Results

3.1 Differentially expressed genes in response to high temperature stress

Using a microarray platform consisting of approximately 10101 tomato probe sets, we found that a total of 249 genes exhibited significant up-regulation (>2.0-fold), while 112 genes exhibited down-regulated expression in HT-treated fruits (C33) compared with those under normal condition (C26). The Affymetrix identification (ID) number, National Center for Biotechnology Information (NCBI) accession number, GenBank homologue, and *Arabidopsis* genome initiative number of the best hit from translated BLAST (TBLASTX) search are listed in Tables 2–5 and Tables S1–S2 using a significance threshold of 10^{-4}.

The differentially expressed genes identified were functionally annotated and subsequently classified into ten functional categories according to their putative functions (Fig. 1). Many loci encoding translational machinery, transcription factors, signal transduction components, in addition to genes associated with primary metabolism, photosynthesis, cell wall metabolism, and hormone responses were involved in tomato fruit responses to HT stress. The genes in the categories such as "defense responses", "signal transduction" and "metabolism" were mostly up-regulated.

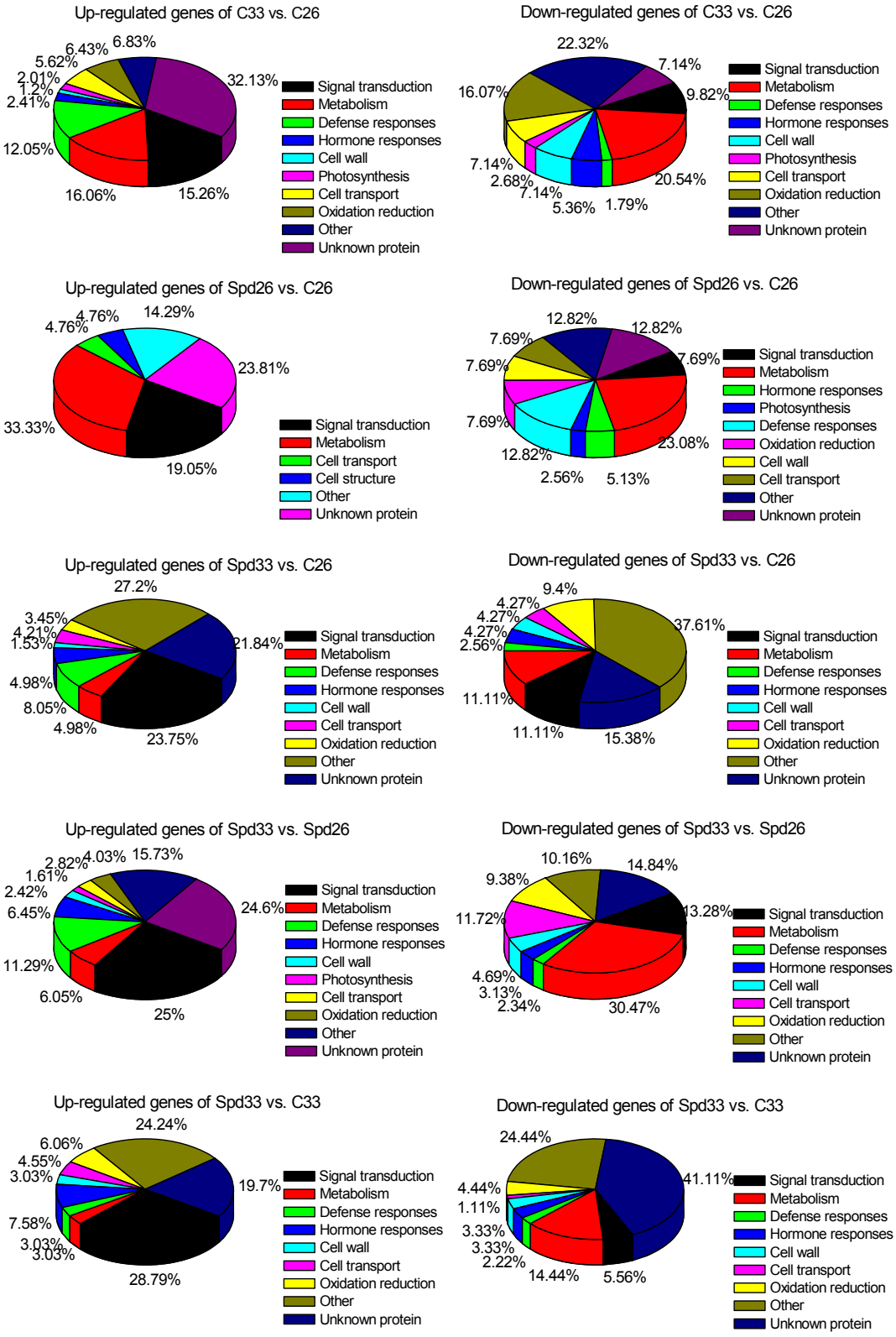

Fig. 1 Functional categorization of differentially expressed genes in response to different treatments

Among 361 HT-regulated genes, 32 genes were previously shown to be involved in stress response genes in other plant systems (Sun and Callis, 1997; Bohnert *et al.*, 2006) (Table 2). Different kinds of HSP genes were accumulated, while thaumatin-like protein (PR-5x) gene, cold shock domain protein (CSDP) transcript, was down-regulated. Previous reports indicated that the tolerance conferred by HSPs resulted in improved physiological phenomena such as photosynthesis, assimilate partitioning, and membrane stability (Momcilovic and Ristic, 2007). In addition to specific members of the classical HT-responsive genes, a major proportion of the genes presented in Table 2 are those that were only recently implicated in heat shock response (HSR) and/or HT stress signaling in other plant systems (Kotak *et al.*, 2007). Functional classification of the 361 heat stress-regulated genes indicates that 49 loci (13.57%) share homology with signal transduction including the genes of calcium binding protein, PP2C, protein kinases or transcription factors including C2H2 zinc finger, MYB, heat stress transcription factor (HSF), NAC, AP2, bHLH, and WRKY (Table 3). The majority of those genes involved in transcription regulation and carbohydrate metabolism were also stress- or defense-related genes. Another group of HT-regulated genes seems to be involved in hormone metabolism or hormonal response (Table 4). Microarray data indicate that the genes of AUX/IAA and jasmonate-zim-domain protein (JAZ) were up-regulated, while the genes of salicylic acid methyltransferase, ACC oxidase (ACO), auxin-responsive family protein, ornithine decarboxylase (ODC), and β-carotene hydroxylase were down-regulated.

Table 2 Defense responses and oxidation reduction genes with significant (P<0.01) differential expression (≥2.0-fold)

Contig ID	Accession No.	Annotation	Fold difference				
			C33 vs. C26	Spd26 vs. C26	Spd33 vs. C26	Spd33 vs. C33	Spd33 vs. Spd26
Affx.11839.1.S1_at	BI208864	NADH dehydrogenase subunit 3	2.08	1.25	−1.56	−3.24	−1.12
Affx.44224.1.A1_at	AI487590	NADH-ubiquinone oxidoreductase	4.29	1.44	1.68	−2.56	1.33
Affx.44474.1.S1_at	AJ831993	NADH dehydrogenase subunit 4	2.30	−1.09	1.30	−1.76	1.07
Affx.70450.1.S1_at	AW033442	NADH dehydrogenase subunit 6	3.85	−1.03	2.56	−1.50	1.23
Les.3311.3.S1_at	AI782246	NADP-isocitrate dehydrogenase	−2.52	−1.10	−1.56	1.61	−1.69
Les.3677.1.S1_at	AY128100.1	Small heat shock protein	3.50	−1.10	3.30	−1.06	5.24
Affx.10807.1.S1_at	BM408547	Heat shock protein 70	2.55	1.10	2.12	−1.20	1.82
Les.3739.1.S1_at	AB026983.1	Small heat shock protein	6.29	1.05	6.54	1.04	13.71
Affx.69957.1.S1_at	BM410870	26.5 kDa class I small heat shock protein-like	2.23	−1.23	2.66	1.20	3.82
Affx.63687.1.S1_at	CN384460	26.5 kDa class P-related heat shock protein	3.20	1.02	2.42	−1.32	2.82
Les.3550.1.A1_s_at	AF096251.1	Ethylene-responsive heat shock protein cognate	2.51	1.06	2.25	−1.12	1.49
Les.269.1.S1_at	U66300.1	Heat shock protein	2.94	1.35	3.29	1.12	5.48
Affx.9815.1.S1_at	CK715796	DNAJ-like protein	2.90	1.05	3.43	1.63	1.89
Affx.56637.1.S1_at	BI208173	DNAJ heat shock N-terminal domain-containing protein	3.40	1.12	2.31	−1.47	2.56
Affx.45577.1.A1_at	CN384902	DNAJ heat shock N-terminal domain-containing protein	−2.40	1.09	1.27	3.04	1.16
Affx.23109.1.S1_at	AI782520	Cold shock domain protein 1	−2.63	−1.03	1.01	2.66	−1.02
Les.124.1.S1_at	AY034473.1	CRT/DRE-binding protein 1	1.20	1.06	3.70	3.10	3.76
Les.4880.1.S1_at	BT012820.1	Cytochrome P450	2.83	1.01	2.47	−1.14	2.12
Les.3534.1.S1_at	AJ270961.1	Putative cytochrome P450	−1.04	−1.18	−5.29	−1.10	1.10
Affx.24042.1.S1_at	CN385197	Cytochrome P450 76A1	−2.28	1.02	1.61	3.67	1.61
Les.4528.1.A1_at	AJ635324.1	Polyphenol oxidase A	−6.22	1.06	−10.94	−1.76	−15.78

Table 3 Signal transduction genes with significant (P<0.01) differential expression (≥2.0-fold)

Contig ID	Accession No.	Annotation	Fold difference				
			C33 vs. C26	Spd26 vs. C26	Spd33 vs. C26	Spd33 vs. C33	Spd33 vs. Spd26
Affx.57054.2.A1_at	AI487567	Calcium-binding EF hand family protein	2.10	−1.05	3.10	1.48	2.37
Affx.16164.1.S1_at	CN385704	Calcium-binding EF hand family protein	2.73	−1.02	2.73	1.00	3.81
Affx.57454.1.S1_at	BI923132	Calmodulin	2.17	−1.29	2.94	1.35	2.57
Affx.70732.1.S1_at	AI897832	Calmodulin-related protein	11.77	−1.29	3.21	−3.67	1.18
Affx.9367.1.S1_at	CN385420	Ca^{2+}-binding protein 1	4.65	1.27	1.98	−2.35	2.16
Affx.66814.1.S1_at	AW621230	Calmodulin binding	2.02	−1.14	2.64	1.31	1.94
Affx.66436.1.S1_at	AW040280	Calcium binding protein	3.24	−1.11	3.30	1.02	4.47
Affx.50750.1.A1_at	AI771837	MYB1	2.25	1.16	3.49	1.55	2.35
Affx.53591.1.S1_at	AI899018	MYB domain protein 107	−4.78	−1.22	−3.01	1.59	−2.36
Les.5017.1.S1_at	BT013110.1	MYB domain protein 111	1.40	1.15	2.19	1.07	1.77
Les.2084.1.S1_at	BF097539	NAC domain protein	1.54	1.08	2.20	1.44	1.91
Les.4483.1.S1_at	AY498713.1	NAC domain protein	2.16	−1.04	1.63	−1.33	1.36
Affx.37343.1.A1 at	AI488497	NAC domain protein	2.51	1.06	3.02	1.20	1.83
Les.2667.3.S1_at	BE433811	WRKY-type transcription factor 2	2.62	−1.12	2.89	1.11	2.11
Les.3964.1.S1_at	AY157060.1	WRKY transcription factor IId-2	−3.73	1.10	−2.45	1.52	−1.78
Affx.64823.1.S1_at	AI778204	Zinc finger like protein	2.23	1.49	4.30	1.93	4.50
Affx.68645.1.S1_at	BM411685	Zinc finger protein	1.23	1.05	2.10	1.71	2.01
Affx.30683.1.S1_at	AI486655	Zinc finger protein	3.02	−1.16	3.25	1.08	2.82
Affx.70855.1.S1_at	AW220130	Zinc finger protein	1.01	−1.03	2.03	2.71	1.93
Affx.35255.1.S1_at	AW623792	Zinc finger protein 1	2.21	−1.29	2.14	−1.03	1.67
Les.5126.1.S1_at	BT013336.1	Zinc finger protein	−1.16	−1.07	2.17	2.52	2.30
Affx.71311.1.S1_at	CN384672	Zinc finger (C2H2 type) family protein	2.53	1.02	3.78	1.49	2.76
Les.5244.1.S1_at	BT013559.1	Zinc finger (DHHC type) family protein	2.74	−1.01	−1.05	−2.89	−1.28
Les.1287.2.S1_at	AW621773	Zinc finger (DHHC type) family protein	2.99	1.17	3.00	1.01	3.77
Les.1287.1.A1_at	BG630588	Zinc finger (DHHC type) family protein	2.64	1.05	2.37	−1.11	2.43
Affx.30683.2.S1_at	AI897122	Zinc finger (C3HC4 type) family protein	3.12	−1.12	3.13	1.00	2.62
Affx.53941.1.S1_at	BF176552	Zinc finger (C3HC4 type) family protein	4.10	−1.01	3.48	−1.18	2.12
Affx.3163.2.S1_at	BM412856	Zinc finger (AN1-like) family protein	1.68	−1.02	2.22	1.32	2.49
Les.3549.1.S1_at	AF096252.1	Ethylene-responsive catalase	1.71	−1.28	2.58	−1.22	1.29
Les.4102.1.S1_at	AY192368.1	Ethylene response factor 2	1.68	1.24	2.19	1.30	2.21
Les.4139.1.S1_at	AY192370.1	Ethylene response factor 4	1.57	1.13	2.05	1.31	1.00
Les.271.1.S1_at	CN384665	Ethylene-responsive methionine synthase	−1.76	1.23	−2.00	−1.13	−2.43
Les.3465.1.S1_at	AY079426.1	Ethylene receptor-like protein	−2.66	−1.37	−2.43	1.09	−2.20
Les.3766.1.S1_at	U77719.1	Ethylene-responsive late embryo-genesis-like protein	−7.48	−1.06	−4.21	1.78	−1.44
Affx.16424.1.A1_at	AI487449	Mitogen-activated protein kinase 3	−1.04	1.10	2.11	2.17	2.13
Affx.26003.1.S1_at	CK715553	Serine/threonine-protein kinase	2.22	1.09	−1.20	−1.13	−1.12

Table 4 Hormone-related genes with significant (P<0.01) differential expression (≥2.0-fold)

Contig ID	Accession No.	Annotation	Fold difference				
			C33 vs. C26	Spd26 vs. C26	Spd33 vs. C26	Spd33 vs. C33	Spd33 vs. Spd26
Affx.23969.1.S1_at	AA824862	Brassinosteroid-responsive ring-h2	2.00	1.07	1.16	−1.20	1.02
Affx.1251.1.S1_at	BG125851	Auxin-responsive family protein	2.17	−1.04	2.08	−1.04	1.43
Les.5138.1.S1_at	BT013365.1	Auxin-responsive family protein	−2.22	1.04	−2.00	1.11	−1.70
Affx.63209.1.S1_at	AW092854	Auxin-induced SAUR-like protein	1.55	1.01	2.87	1.85	2.54
Affx.71035.1.S1_at	BI207404	Auxin-induced SAUR-like protein	2.91	−1.08	1.81	−1.61	1.04
Les.3707.1.S1_at	AF022013.1	IAA2 protein	2.03	1.09	10.86	3.34	6.40
Les.3706.1.A1_at	AF022014.1	IAA3 protein	−1.20	1.18	2.73	3.28	2.97
Les.256.1.A1_at	AF022017.1	IAA6 protein	1.65	1.11	2.15	1.30	1.72
Les.4097.1.A1_at	AF022018.1	IAA7 protein	2.40	1.06	2.53	1.05	2.77
Les.5442.1.S1_at	BT013931.1	IAA19 protein	−3.35	−1.18	−2.51	1.56	−1.80
Les.5917.1.S1_at	AJ715790.1	1-Aminocyclopropane-1-carboxylate oxidase	1.83	1.19	2.36	1.29	2.99
Les.3225.3.S1_at	BF112635	1-Aminocyclopropane-1-carboxylate oxidase	−3.20	1.01	−2.05	1.56	−2.97
Les.3769.1.S1_at	AB013100.1	1-Aminocyclopropane-1-carboxylate synthase	2.48	−1.60	2.85	1.15	2.28
Les.3358.1.S1_at	AI771286	Arginine decarboxylase	1.64	−1.15	2.25	1.37	1.82
Les.3525.1.S1_at	AF029349.2	Ornithine decarboxylase (ODC)	−2.17	−6.06	−2.31	−1.06	−1.37
Les.3728.1.A1_at	AJ278743.1	Gibberellin 2 (GA2) protein	−1.82	−1.02	−2.19	−1.20	1.19
Affx.30832.1.S1_at	CN384615	Jasmonate-zim-domain protein 7	4.05	1.08	1.78	−2.28	2.04
Les.612.1.S1_at	BE459581	Salicylic acid methyltransferase	−3.95	1.21	−3.40	1.16	−2.37

3.2 Transcriptome profiling of ripening fruit in response to exogenous spermidine treatment

Under normal temperature, a total of 21 genes were up-regulated, whilst 40 genes were down-regulated in Spd-treated fruits (Spd26), compared with those in non-treated fruits (C26). The genes of ethylene-responsive catalase, ethylene response factor 4, calmodulin-related protein were up-regulated, whilst those of MADS-box protein, *S*-adenosyl-L-methionine, ethylene-responsive late embryogenesis-like protein, and gibberellin 2 (GA2) protein were down-regulated in Spd26 fruits.

Furthermore, the expression profiles in Spd-treated fruits under HT condition (Spd33) were compared with those in untreated fruits (C33). A total of 66 genes were up-regulated in Spd-treated fruits, whilst 90 genes were down-regulated. Among these Spd-regulated genes, 24 candidate regulatory factors were identified, including the genes of ethylene-responsive transcription factor, zinc finger, mitogen-activated protein kinase (MAPK), WRKY, auxin response factors (ARF), and calmodulin-like protein (Table 3). Twelve genes were involved in defense and oxidation reduction, including the genes of LeCBF1 protein, HSP, salt responsive protein, cytochrome P450, NADH dehydrogenase, and NAD(P)H-quinone oxidoreductase (Table 2). Eight genes were involved in hormone pathways, such as the genes of IAA, Spd synthase, and brassinosteroid sulfotransferase (Table 4).

3.3 Expression changes under both high temperature and spermidine treatment

A total of 261 genes were up-regulated in Spd-treated fruits under HT condition (Spd33), while 117 genes were down-regulated compared with those in control tomatoes (C26) (Fig. 2).

Functional classification indicates that the largest group consists of 75 candidate regulatory factors, many of which are likely to be regulators of specific genes or gene sets, including the genes of ethylene cascade protein, MAPK, NAC, MYB, WRKY, and auxin-responsive family protein (Tables 3 and 4).

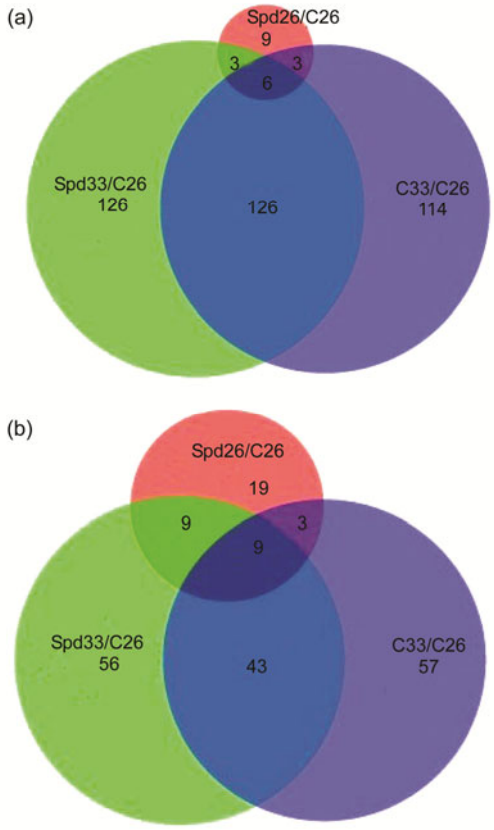

Fig. 2 Venn diagram showing the numbers of up- and down-regulated genes under different temperature conditions

The tomato Affymetrix GeneChip® contains 10101 probe sets. (a) Numbers of significantly up-regulated genes in Spd treatment under normal temperature (Spd26), high temperature (C33), and Spd treatment under high temperature (Spd33), compared with control treatment (C26), respectively. The number of up-regulated genes common between these treatments is shown within the overlapping circular. (b) Numbers of down-regulated genes in Spd26, C33, and Spd33, compared with C26, respectively. The number of down-regulated genes common between these treatments is shown within the overlapping circular

Members of the ethylene response element binding protein (EREBP) family may play important roles in the cross-talk of different kinds of abiotic stress and signaling pathways. The EREBP family genes are highly regulated by various kinds of abiotic factors and plant hormones, such as low temperature, drought, high salinity, ethylene, abscisic acid, and jasmonate (Zhang H.W. *et al.*, 2004).

Several HSPs were identified as the products of HT- and polyamine-regulated genes (Table 2). The HSPs, acting as molecular chaperons, play a crucial role in protecting plants against stress by re-establishing normal protein conformations and maintaining cellular homeostasis (Wang W.X. *et al.*, 2004; Ouyang *et al.*, 2007). In the suspension culture of tobacco and alfalfa, polyamines can affect the membrane system further to influence the HSP synthesis in response to HT stress (Königshofer and Lechner, 2002).

Many genes involved in hormone pathways changed in response to HT and Spd treatments (Table 4). The genes of IAA protein, early auxin-responsive (SAUR), ACC synthase (ACS), ACO, and ADC accumulated after treatment with Spd under HT condition. In particular IAA2 exhibited a 10.86-fold increase in expression. The ACO and ACS genes, which are crucial in ethylene biosynthesis, were also highly regulated by HT and Spd treatments, which indicated that Spd might play a role in ethylene signal pathway under HT stress.

3.4 Verification of the expression patterns with quantitative real-time PCR

To validate the expression differences detected by the microarray analysis, we analyzed expression patterns of 15 randomly selected genes using quantitative real-time reverse transcription (RT)-PCR (Fig. 3). These genes represent different groups with diverse change patterns, including up-regulation, down-regulation, and no significant changes. Real-time RT-PCR analyses confirmed the change of mRNA level. Out of 15 genes detected, the expressions of 12 (80%) genes agree with the microarray analyses, indicating that the expression patterns detected by microarray analyses are in good agreement with those detected by quantitative real-time PCR. HSP genes (AY128100.1, U66300.1 and BG129203) were significantly up-regulated by HT stress, but relieved by exogenous Spd treatment. The expression levels of 1-aminocyclopropane-1-carboxylate oxidases (BF112635 and BG629220) were significantly increased by Spd treatment. The mRNA levels of salicylic acid methyltransferase (BE459581), cytosolic ascorbate peroxidase (BE432137), and phenylcoumaran benzylic ether reductase (BT014421.1) were up-regulated by HT or Spd treatment. The mRNA level of the cell wall protein (BG628248) was also up-regulated in response to Spd and HT treatments.

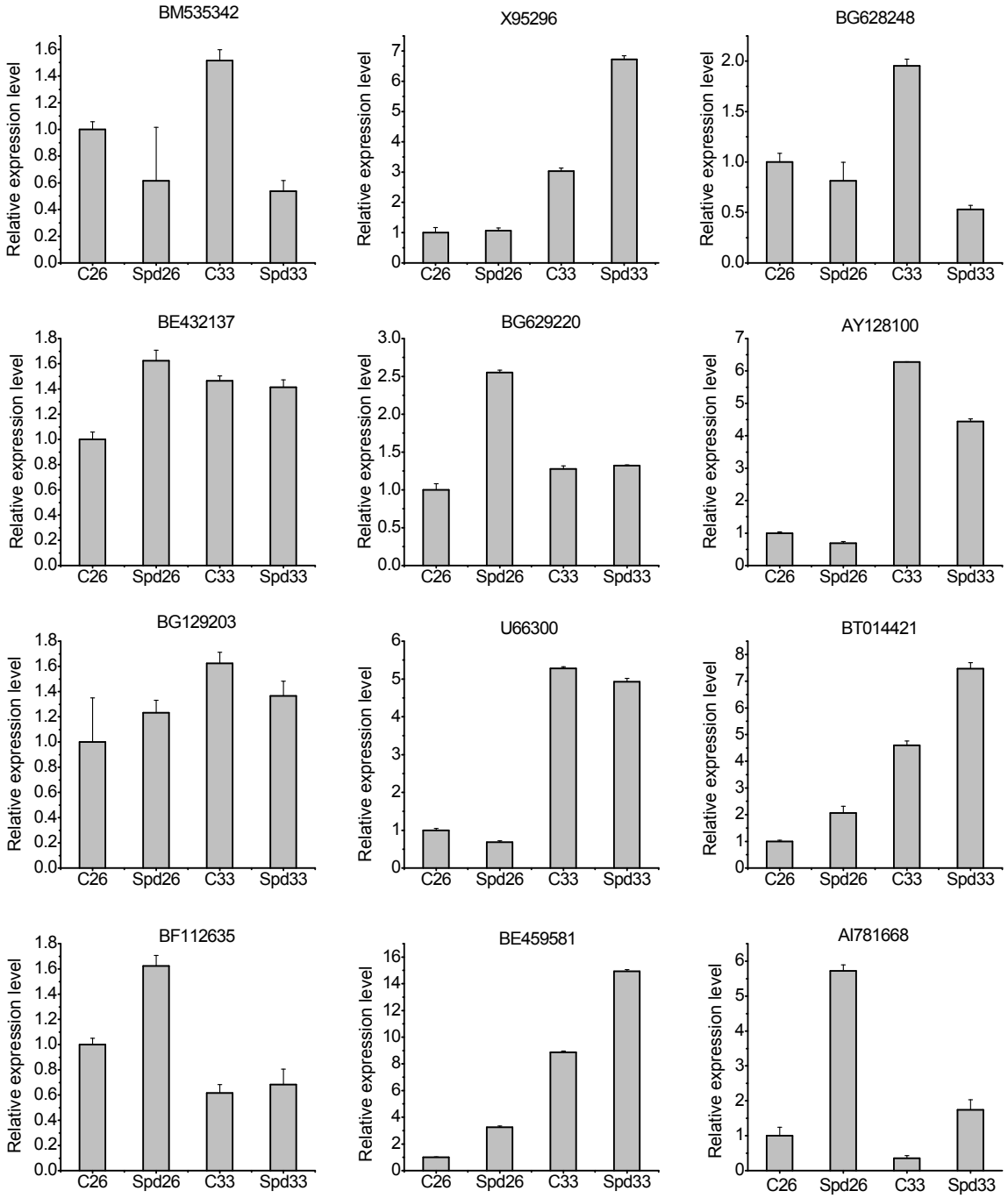

Fig. 3 Verification of microarray results by quantitative real-time PCR

Total RNA was isolated from tomato fruits of C26, Spd26, C33, and Spd33 for cDNA synthesis, respectively, then for quantitative real-time PCR. The presented data are the averages of two independent experiments [±standard error (SE)]. AY128100.1, U66300.1, and BG129203: heat shock protein genes; BF112635 and BG629220: ACO gene; BE459581: salicylic acid methyltransferase gene; BE432137: cytosolic ascorbate peroxidase gene; BT014421.1: phenylcoumaran benzylic ether reductase gene; AI781668: proteinase inhibitor 1 PPI3A4 gene; X95296: THM27 protein gene; BM535342: I2C protein gene; BG628248: calcium-binding EF-hand protein gene

4 Discussion

The ripening process of tomato fruit involves a complex and coordinated series of changes in pigmentation, flavor, texture, and aroma resulting from physiological and biochemical activities, which were caused by alterations in gene expression (Lurie *et al.*, 1996). HT has been found to inhibit ethylene synthesis and proteoglycan accumulation, to interfere with lycopene synthesis, and finally to inhibit fruit ripening. In this work, microarray approach was used to examine the gene expression profile of tomato mature green fruit in response to HT and exogenous Spd treatments. The transcript abundance changes were monitored and the HT- or/and Spd-regulated genes were identified in the early ripening stages. There were a total of 361 genes regulated in response to HT stress (33 °C), and only 61 genes exhibited regulation following the exogenous Spd-treatment. Meanwhile, 378 genes were simultaneously regulated by the HT and Spd treatments. A total of 185 genes changed together in both C33 and Spd33 (each normalized to C26). However, there were 114 and 126 specific genes up-regulated only in C33 and Spd33, respectively, while 57 and 56 genes down-regulated in C33 and Spd33, respectively (Fig. 2). All the differentially expressed genes were also functionally annotated and subsequently classified into ten functional categories according to their putative functions (Fig. 1). Most of these genes representing four groups of notably differentially regulated genes appear to be involved in the HSR of mature tomato fruits: genes involved in primary metabolism, defense responses, signal transduction, and hormone metabolism or hormonal response. The microarray analysis indicated that 49 differentially expressed genes were involved in signal transduction and reported as transcription factors in mature fruits in response to HT stress (C33) (Table 3), including the genes of EREBP family, MAPK, WRKY, MYB, NAC, LOB domain (LBD), zinc finger family protein, calmodulin-related protein, and serine/threonine-protein kinase. Some EREBP family genes also significantly accumulated in Spd-treated fruits under HT condition, including ethylene response factors, ethylene-responsive HSP cognate 70, ethylene-responsive late embryogenesis-like protein, ethylene receptor-like protein, and ethylene-responsive methionine synthase, which were not changed in response to HT. Interestingly, the expressions of MAPK family genes were up-regulated after the Spd treatment. MAPK cascades play an important role in signal transduction pathways in plants and function ubiquitously in many responses to external signals (Kaur and Gupta, 2005). In order to cope with heat stress, plants implement various mechanisms, such as MAPK cascades (Wahid *et al.*, 2007). Many genes of MAPK cascades changed in Spd33 other than in C33 indicated Spd might play important roles in alleviating HT injury by signal pathways. In a previous study, WRKY25 was involved in heat stress tolerance in *Arabidopsis thaliana* (Li *et al.*, 2009). MYB4 was found to be involved in drought, salt, UV, ozone, viruses, bacteria, and fungi stresses and represents a crucial knot in the cross-talk of stress signaling cascades through the activation of multiple components in rice (Vannini *et al.*, 2006). The NAC domain protein could interact with leaf curl virus in tomato (Selth *et al.*, 2005).

The microarray analysis indicated HT induction of many metabolism-related genes in mature fruits, including energy metabolism genes, amino acid and protein metabolism genes, nucleic acid metabolism genes, and secondary metabolism genes. Under HT treatment, the metabolism of development was deeply disordered, many genes involved were changed to adapt to HT stress. However, when the fruits were pretreated with exogenous Spd, the damage to metabolism was alleviated, and the number of those genes in response to stress was significantly decreased (Table 5).

Some genes involved in defense responses and oxidation reduction categories, were also decreased under HT and Spd treatments. There were 76 genes changed by HT stress, whilst 44 genes changed under HT and Spd treatments (Table 2). It indicated that Spd significantly promoted the plant response to HT at the molecular level to relieve the injury by heat stress.

The hormone metabolism was also involved in responses to HT stress (Table 4). Under HT and Spd treatments, ACS, ACC and IAA genes were up-regulated. Previous studies showed that polyamines were indispensable for IAA occurrence (Couée *et al.*, 2004). The action of auxin and polyamine may be closely related to each other (Rastogi and Davies, 1991; Nag *et al.*, 2001). Polyamines are able to replace auxin effects, suggesting that they could mimic

Table 5 Metabolism-related genes with significant (P<0.01) differential expression (≥2.0-fold)

Contig ID	Accession No.	Annotation	Fold difference				
			C33 vs. C26	Spd26 vs. C26	Spd33 vs. C26	Spd33 vs. C33	Spd33 vs. Spd26
Les.5934.1.S1_at	AI895164	Omega-6 fatty acid desaturase	−3.70	−1.05	−2.54	1.46	−3.32
Les.5240.1.S1_at	BT013554.1	Serine protease	−3.15	1.13	−2.94	1.07	−3.56
Les.5402.1.S1_at	BT013846.1	Serine carboxypeptidase	−2.53	1.04	−3.27	−1.29	−2.34
Les.3610.1.S1_at	BG629712	Glycine rich protein	−3.13	1.08	−3.06	1.02	−4.67
Les.3035.1.A1_at	BI423372	Cathepsin D inhibitor protein	−3.10	−1.22	−7.01	−2.26	−1.35
Les.2809.1.S1_at	BT014540.1	Putative glucosyltransferase	−2.66	−1.23	−3.35	−1.26	−2.77
Les.5832.1.S1_at	BT014414.1	UDP-glucoronosyl family protein	−2.31	−1.08	−1.08	2.13	2.60
Affx.15898.2.S1_at	BE434722	UDP-xylose phenolic glycosyltransferase	2.00	−1.01	2.39	1.20	2.03
Les.3696.1.S1_at	AF311943.1	UDP-galactose:myo-inositol galactosyltransferase	5.49	1.35	−1.48	−8.13	−3.55
Affx.51348.1.S1_at	AJ785026	Aspartyl protease family protein	−2.26	1.07	−2.19	−1.09	−1.78
Les.4317.1.S1_at	AW625684	Asparagine synthetase	−2.20	1.01	−2.83	−1.28	−1.21
Affx.58104.1.S1_at	AW036288	Globulin precursor	−2.16	1.14	−5.52	−4.62	−6.36
Affx.58041.1.A1_at	CN385714	Glycosyl hydrolase family 1 protein	−2.16	−1.01	−1.41	1.53	−1.32
Affx.19924.1.S1_at	BE459775	Glycosyl hydrolase family 17 protein	−2.10	−1.11	−1.94	1.08	−2.05
Les.2817.2.S1_at	BI933507	ATP-citrate lyase A-1	−2.05	1.34	−1.77	1.16	−1.72
Les.4868.1.S1_at	BT012795.1	ADP/ATP translocator	2.63	−1.22	2.44	−1.08	3.08
Affx.51975.2.S1_at	BI930488	Cytochrome c oxidase subunit	2.00	1.24	1.60	−1.25	1.21
Affx.51226.1.S1_at	AI779132	Cytochrome f	2.88	1.15	1.08	−3.11	−1.04
Affx.30946.1.A1_at	AJ785184	Cytochrome b6	3.02	−1.16	2.13	−1.42	1.73
Affx.9007.1.S1_at	CN385923	Lactoylglutathione lyase family protein	3.06	−1.01	3.53	1.15	2.68
Affx.51975.1.A1_at	AF362735.1	Succinate dehydrogenase subunit 4	3.35	−1.08	1.59	−2.11	1.16
Les.5956.1.S1_ at	CN385508	Proline dehydrogenase	3.74	−1.21	−1.07	−4.01	−1.05
Affx.71476.1.S1_at	AW036283	11S globulin seed storage protein 2 precursor	−1.41	1.04	−8.32	−5.90	−9.86
Les.3980.1.S1_at	U37839.1	Lipoxygenase	−1.82	1.04	−3.55	−1.95	−2.83
Les.3273.1.S1_at	BG627786	Proline rich protein	−1.44	1.66	−4.44	−3.08	−1.09
Affx.58104.1.A1_at	AJ785426	Globulin precursor	−1.20	1.14	−5.52	−4.62	−6.36

hormonal responses (Pal Bais and Ravishankar, 2002). Numerous reports have shown that different pathways are interconnected and together regulate the plant response to biotic and abiotic stresses (Ludwig *et al.*, 2005; Ma *et al.*, 2006). Abscisic acid and ethylene play an important role in the complicated story of abiotic stress and, consequently, cross-talk between these two kinds of plant hormone has been reported (Yamamoto *et al.*, 2005). The relationship between polyamine and ethylene was complex. Because of their common pre-requisite *S*-adenosylmethionine (SAM), there exists a competitive mechanism, which may be affected by species, environment, or limited SAM storage. Polyamines affect the levels of ACS and ACO gene transcriptions, thereby affecting the synthesis and conversion of ACC. Meanwhile, polyamines influence the nature of the ACO on the membrane system, so inhibit the conversion of ACC to ethylene. Polyamines, as an effective scavenger of free radicals, improve the protective enzyme activity and inhibit ethylene production (Apelbaum *et al.*, 1981; Walden *et al.*, 1997). Conversely, ethylene affected the activities of ADC, SAM decarboxylase (SAMDC) and other key enzymes in polyamine

biosynthesis (Thu-Hang *et al.*, 2002). Polyamines may have a dual function in plant stress tolerance, as a protectant in reactive oxygen species (ROS)-scavenging and a membrane-protecting compound and as a signaling regulator in stress signaling pathways that lead to the build-up of stress-tolerant mechanism (Kasukabe *et al.*, 2004). However, there are contradictory research data providing evidence for the lack of antioxidant activity and even prooxidant action of polyamines (Todorova *et al.*, 2007).

A large number of early response genes regulated by HT or/and Spd in this study encode unknown proteins, indicating that there is still a great deal of uncertainty with regard to the mechanism of the HT tolerance and in how polyamines affect the fruit ripening-related gene expression in tomato. This study has practical importance for subtropical and tropical tomatoes that are unable to ripen if grown under HT conditions.

5 Conclusions

The data presented here provide genome-wide expression profiles of mature green tomato fruit following their exposure to a short-term HT treatment and exogenous Spd application. An Affymetrix tomato genome array was successfully used to identify HT- or/and Spd-regulated genes representing the classical HSR and thermotolerance mechanisms. The results indicate HT regulates HSP and heat shock factor family members, carbohydrate metabolism genes, stress- or defense-related signal transduction genes, and hormone metabolism genes or hormonal response elements. Under normal temperature, the Spd-regulated genes were quite different with stress-related genes in response to HT. However, under HT conditions, when pre-treated with exogenous Spd, the number of genes involved in signal transduction was significantly increased. Many regulatory factors, ethylene-related genes, polyamine biosynthesis genes, hormone pathways genes, and oxidation reduction genes exhibited the regulation in response to Spd treatment. So our results indicated that Spd might alleviate the heat stress injury during tomato fruit ripening. However, more complete understanding of the molecular mechanisms that contribute to fruit thermotolerance requires additional data, including the functional analyses of a large part of the above differentially expressed genes, under both short-term HT and longer durations of moderate HT conditions.

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List of electronic supplementary materials

Table S1 Summary of other signal transduction genes with significant (P<0.01) differential expression (≥2.0-fold)

Table S2 Summary of other defense responses and oxidation reduction genes with significant (P<0.01) differential expression (≥2.0-fold)