



Characterization of high-temperature stress-tolerant tomato (*Solanum lycopersicum* L.) genotypes by biochemical analysis and expression profiling of heat-responsive genes

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Abstract

High-temperature stress severely impacts both yield and quality of tomato fruits, and therefore, it is required to develop stress-tolerant cultivars. In the present study, two tomato genotypes, H88-78-1 and CLN-1621, identified through preliminary phenotypic screening were characterized by analysis of molecular, physiological, and biochemical traits in comparison with a susceptible genotype Punjab Chhuhara. Phenotypic stress tolerance of both the genotypes was validated at biochemical level as they showed higher amount of relative water content, photosynthetic pigments, free cellular proline, and antioxidant molecules while less amount of H₂O₂ and electrolyte leakage. Expression analysis of 67 genes including heat shock factors, heat shock proteins, and other stress-responsive genes showed significant up-regulation of many of the genes such as 17.4 kDa class III heat shock protein, HSF A-4a, HSF30, HSF B-2a, HSF24, HSF B-3 like, 18.1 kDa class I HSP like, and HSP17.4 in H88-78-1 and CLN-1621 after exposure to high-temperature stress. These candidate genes can be transferred to cultivated varieties by developing gene-based markers and marker-assisted breeding. This confirms the rapid response of these genotypes to high-temperature stress. All these traits are characteristics of a stress-tolerance and establish them as candidate high-temperature stress-tolerant genotypes that can be effectively utilized in stress tolerance improvement programs.

Keywords High-temperature stress · Tolerance · Tomato · Gene expression · Heat shock proteins

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Introduction

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops cultivated all over the world in different climatic conditions. However, the growth and development of tomato being severely hampered when it experiences high-temperature stress which ultimately affects the quality and yield of fruits (Pressman et al. 2002). The scenario of global environmental changes suggests a future increase in aridity and in the frequency of extreme temperatures in many areas of the world. In arid and semiarid regions of the world, high temperature or heat stress is an important constrain in the productivity of agricultural crops, more particularly vegetables. Therefore, it is extremely important to improve the high-temperature stress tolerance of crops through molecular manipulations. The preliminary requirement for the development of high-temperatures tress-tolerant crops is to identify the genotypes having such desired traits and understand the stress tolerance mechanism of such genotypes. The stress tolerance in a genotype is characterized by

its quick and optimal response to a stress condition in the form of molecular, biochemical, and physiological response that ultimately leads to whole-plant response.

The primary effects of high-temperature stress are denaturation of heat-labile proteins and accumulation of harmful reactive oxygen species in plant cells (Grover et al. 2013; Mittler et al. 2012). To protect from such heat-induced damages, plants have a repertoire of heat shock proteins (HSP). Heat shock transcription factors (HSFs) play a critical role in high-temperature stress response by regulating the expression of HSPs and other temperature stress-related proteins (Lin et al. 2011). Several high-temperature stress-inducible proteins have been identified in tomato species (Ashraf and Harris 2004). The plant's adaptive response to high-temperature stress is mainly through the expression of these HSPs (Feder and Hoffman 1999). Plants have enormous diversity in HSPs in terms of molecular weight which ranges from 10 to 200 kDa. These HSPs function as chaperone proteins and are also involved in various signaling pathways during stress (Schoffl et al. 1999). Expression of HSPs during stress confers tolerance to plants by modulating numerous processes in plants such as water and nutrient use efficiency, membrane stability, photosynthesis, and assimilate partitioning (Camejo et al. 2005; Ahn and Zimmerman 2006; Momcilovic and Ristic 2007). Such mechanisms facilitate the growth and development of plants possible under high-temperature stress. Analysis of these different mechanisms in stress-tolerant genotypes is an important aspect for establishing any genotype a true high-temperature stress-tolerant one to utilize it in breeding programs.

The present study was done with the objective of identifying and characterizing a high-temperature stress-tolerant tomato genotype that can be effectively utilized in future breeding programs. Preliminary screening for high-temperature stress tolerance by phenotyping identified two genotypes H88-78-1 and CLN-1621. The screening for high-temperature tolerance was carried out during 2013–2016 at field conditions as well as temperature gradient tunnel at ICAR—Indian Institute of Vegetable Research, Varanasi. The stress tolerance was assessed based on physiological and biochemical attributes along with pollen viability and percentage of fruit setting (Singh et al. 2017). H88-78-1 was identified as moderately tolerant with 30% fruit setting and CLN1621 was identified as tolerant with 60–70% fruit setting, whereas there was less than 10% fruit setting on a susceptible genotype Punjab Chuhara. CLN1621 is also a notified genotype by world vegetable center, Taiwan for high-temperature stress tolerance. These genotypes were characterized by analysis of molecular, biochemical, and physiological parameters to establish them as a source of high-temperature stress tolerance traits for breeding purposes. For expression analysis, a total of 67 genes have been selected which included heat shock factors, heat shock proteins, and other important heat-responsive proteins based

on previous reports (Yang et al. 2016), sol genomics network (<https://solgenomics.net/search/locus>), and the other stress-related genes that have been earlier used in our laboratory. Furthermore, we have localized the important genes identified here on the physical map of 12 tomato chromosomes to show their close proximity to chromosomes according to functions. This information will be helpful in marker-assisted breeding programs.

Materials and methods

Seeds of high-temperature stress-tolerant tomato genotypes H88-78-1 and CLN-1621 and a high-temperature stress susceptible variety Punjab Chuhara were germinated and grown in a greenhouse at 25 ± 2 °C with a photoperiod of 14 h light and 10 h dark. Plants were exposed to high-temperature stress at 42 °C by transferring them to a growth chamber after 25 days of transplanting. The leaf samples were collected from the plants after 0, 16, 32, and 48 h of stress treatment, frozen immediately in liquid nitrogen, and stored at -80 °C until RNA isolation and other biochemical analysis; however, relative water content and electrolyte leakage were measured immediately.

Measurement of relative water content (RWC) and electrolyte leakage (EL)

The method described by Khare et al. (2010) was used to measure RWC and EL of leaf tissues. In brief, 12 leaf discs were weighed (FM) and rehydrated in water for 6 h. The fully turgid leaf discs were then surface dried and reweighed (TM). The dry mass (DM) was weighed by the oven drying of turgid discs at 80 °C for 24 h. The following equation was used to calculate RWC:

$$\text{RWC} [\%] = \frac{(\text{FM} - \text{DM})}{(\text{TM} - \text{DM})} \times 100.$$

To measure EL, 10 leaf discs were kept in 25 ml of Milli Q water and conductivity was measured after 4 h at room temperature (*a*) by conductivity meter (CM-180, Elico, India). Furthermore, the leaf discs were autoclaved at 121 °C for 30 min, and again, conductivity was measured (*b*). The EL was calculated as: $\text{EL} [\%] = a/b \times (100)$.

Estimation of photosynthetic pigments

Chlorophyll (Chl) and carotenoid (Car) content was estimated according to the method given by Lichtenthaler and Buschmann (2001). For this, leaf samples (300 g) were crushed in chilled acetone and the supernatant was used for measurement of absorbance at 663, 645, and 470 nm (UV-Vis 1601 Shimadzu, Japan). Chl and Car contents

[mg g^{-1} (DM)] were calculated using the formula: $\text{Chl a} = [(12.7 \times A663) - (2.69 \times A645)]$; $\text{Chl b} = [(22.9 \times A645) - (4.68 \times A663)]$; $\text{Car} = \{[(1000 \times A470) - (3.27 \times \text{Chl a} + \text{Chl b})] / 227\}$.

Estimation of H_2O_2 , lipid peroxidation, and proline

The process described by Jana and Choudhuri (1981) was used to determine the H_2O_2 content in leaf tissues. In brief, approximately 200 mg leaf sample was crushed in 5 ml of 50 mM sodium phosphate buffer (pH 6.5). The supernatant was collected and mixed in 1 ml of 0.1% (w/v) titanium sulfate in 20% (v/v) H_2SO_4 .

The mixture was then centrifuged at $6000 \times g$ for 15 min and absorbance was measured at 410 nm (UV–Vis 1601 Shimadzu, Japan). To measure lipid peroxidation, the method described by Heath and Packer (1968) inferred by malondialdehyde content (MDA) by the thiobarbituric acid reaction was used. The process includes crushing of leaf sample in 4 ml of 0.1% (v/v) trichloroacetic acid mixed with 0.5% (v/v) butylated hydroxytoluene and 1% (w/v) polyvinyl pyrrolidone (PVP). The supernatant (2.5 ml) from the crushed sample was then mixed with 0.5% (v/v) and 20% thiobarbituric acid and trichloroacetic acid, respectively. The mixture was boiled for 30 min before recording the absorbance at 532 nm and 600 nm (UV–Vis 1601 Shimadzu, Japan). The later absorbance was used for correcting the non-specific turbidity by subtracting it. Proline estimation was done by homogenizing the leaf samples (200 mg) in 3% (v/v) sulfosalicylic acid. The supernatant was collected by centrifuging ($13,000 \times g$ for 10 min) it and 0.5 ml of the supernatant was incubated at 100°C for 60 min with 0.5 ml glacial acetic acid and 0.5 ml freshly prepared ninhydrin reagent. Absorbance was measured at 520 nm after adding 1 ml toluene (UV–Vis 1601 Shimadzu, Japan).

Determination of antioxidant enzyme activity

To measure catalase activity, the method described by Rai et al. (2012) was used. For this, 200 mg leaf sample was crushed in 5 ml of 50 mM Tris-NaOH buffer (pH 8.0) containing 2% PVP, 0.5 mM EDTA, and 0.5% (v/v) Triton X-100. The assay mixture consisted of 1 ml of 100 mM K_2HPO_4 buffer (pH 7.0), enzyme extract (100 μl), and 200 mM H_2O_2 (400 μl). To assess CAT activity, H_2O_2 degradation was recorded at 240 nm and was expressed as μmol (H_2O_2 oxidized) $\text{min}^{-1} \text{mg}^{-1}$ (protein). To measure superoxide dismutase activity, the method described by Shah et al. (2001) was used. The process includes crushing of leaf sample (200 mg) in 5 ml of 100 mM potassium phosphate buffer (pH 7.5) and centrifuging it at $22,000 \times g$ for 10 min at 4°C .

The supernatant was used for assaying the SOD activity. In the supernatant, 50 mM sodium carbonate–bicarbonate buffer (pH 9.8), 0.6 mM epinephrine, and 0.1 M MEDTA were added to make the final volume of 3 ml. This assay mixture was used to record the adrenochrome formation at 470 nm (UV–Vis 1601 Shimadzu, Japan). One unit of SOD activity causes 50% inhibition of epinephrine oxidation and it is expressed as U mg^{-1} (protein). Similarly, the method of Nakano and Asada (1981) was used to measure the activity of ascorbate peroxidase. In 5 ml of 50 mM potassium phosphate buffer (pH 7.8), 200 mg leaf sample was homogenized. The buffer also consisted of 1.0 mM EDTA, 1% PVP, 1.0 mM ascorbic acid, and 1.0 mM phenylmethylsulfonyl fluoride. The final reaction mixture (3 ml) was composed of the extracted enzyme along with 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM EDTA, 0.5 mM ascorbic acid, and 0.2 mM H_2O_2 . The absorbance was measured at 290 nm (UV–Vis 1601 Shimadzu, Japan). The specific activity of APX was expressed as μmol (ascorbate oxidized) $\text{min}^{-1} \text{mg}^{-1}$ (protein). Protein in each enzyme preparation was measured by Lowry's method using bovine serum albumin (BSA) as the standard (Lowry et al. 1951).

RNA isolation and gene expression analysis

RNA isolation from all the leaf samples of control and high-temperature stress given plants was carried out using TRI reagent (Ambion, USA). DNA contamination was removed by RNase-free DNase treatment (Qiagen, Germany). cDNA was synthesized using a first-strand cDNA synthesis kit from 1 μg of total RNA in 20 μl reaction volume according to the manufacturer's instructions (Bio-Rad, Hercules, USA). Quantitative real-time PCR was performed in iQ5 thermal cycler (Bio-Rad) with iQ5 software using IQ SYBR Green Supermix (Bio-Rad) according to the manufacturer's protocol. Nucleotide sequence of all the heat shock factors and heat shock proteins of tomato available in different databases were retrieved and primers for quantitative polymerase chain reaction (qPCR) were designed using primerQuest tool (Integrated DNA Technologies, Inc.). The actin gene was used as an internal control for carrying out qPCR. Sequences of all the primers are listed in Supplementary Table 1. qPCR was performed with a reaction mixture containing 10 μl of SYBR Green qPCR Mix (Bio-Rad), 5 μl of cDNA, and 1 μl each of the specific forward/reverse primer (200 nM). The temperature cycles program used was: initial incubation at 95°C for 1 min followed by 35 cycles of 95°C for 30 s, at $55\text{--}60^\circ\text{C}$ (varied according to primer melting temperature) for 30 s, at 72°C for 40 s, and finally one cycle at 72°C for 5 min. Relative change in gene expression level was detected by the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001). $\Delta\Delta\text{CT}$ values reflect the relative fold change expression of the transcription of the target gene upon exposure to

high temperature. The qRT-PCR analysis was performed in triplicates for each cDNA sample.

In silico analysis of the candidate genes for sub-cellular localization, functional annotations, and physical map of chromosomes

Sub-cellular localization of all the candidate heat-responsive genes was predicted using the protein sub-cellular localization predicting SherLoc2 web server (<https://abi-services.informatik.uni-tuebingen.de/sherloc2/webloc.cgi>). Functional annotation information of all the genes was retrieved from Sol Genomics Network. Information of genes such as chromosome on which it is present and its position in base pairs, required to prepare the physical map was retrieved from the Sol Genomics Network resources and the National Center for Biotechnology Information (NCBI) Genome Data Viewer resources (<https://www.ncbi.nlm.nih.gov/genome/gdv/browser>). Using this locus information, a physical map of 12 chromosomes of tomato was developed with the help of MapChart tool (Voorrips 2002). Expression profiles of these candidate genes were also investigated by Geneinvestigator tool (Hruz et al. 2008).

Statistical analyses

The means and standard errors were calculated. The data were analyzed by a one-way analysis of variance (ANOVA) at ($p \leq 0.05$) using SPSS v. 16.0 (SPSS, Chicago, USA). Duncan's multiple range test was performed to compare the mean values when ANOVA results were significant.

Results

Relative water content and electrolyte leakage

The genotypes H88-78-1 and CLN-1621 exhibited the greater potential of maintaining tissue water than Punjab Chuhara (PBC) (Table 1). Under control condition, RWC in both genotypes was almost similar (85.9% and 85.6%), while in the case of PBC, it was 78.7%. As compared to control plants, RWC reduces with an increase in the duration of high-temperature exposure with maximum reduction in PBC under 48 h of high-temperature exposure (50.0%); however, under the similar condition, it was 63.6% and 59.5%, respectively, for the H88-78-1 and CLN-1621. Undamaged or unstressed plant cells maintain electrolytes within the cell membrane that are vital for proper cell functioning. In the present study, a significantly lesser extent of increase in EL was found in the H88-78-1 and CLN-1621 compared to PBC under 16 h, 32 h, and 48 h of high-temperature exposure.

Photosynthetic pigments

The concentration of photosynthetic pigments—total chlorophyll and carotenoid content—decreased with increase in duration of high-temperature exposure; however, total chlorophyll degradation was higher in PBC (reduced from 39.1 to 20.2) compared to H88-78-1 (reduced from 43.1 to 35.1) and CLN-1621 (reduced from 41.2 to 29.9) (Table 1). Similarly, carotenoid degradation was higher in PBC (reduced from 14.4 to 6.3) compared to H88-78-1 (reduced from 14.9 to 10.9) and CLN-1621 (reduced from 14.7 to 8.4).

Lipid peroxidation, proline content, and hydrogen peroxide (H_2O_2)

The lipid peroxidation as indicated by MDA levels increased with increase in the duration of high-temperature exposure in all the studied genotypes (Table 1). PBC plants showed a higher increase in the MDA level under high-temperature exposure as expected. Lipid peroxidation increased from 20.1 to 31.2 and 23.7 to 30.4 in the case of H88-78-1 and CLN-1621, respectively; however, there was a large increase from 22.9 to 41.3 in PBC after 48 h of high-temperature exposure compared to control plants (Table 1). Proline which acts as an osmolyte got accumulated under high-temperature stress in all the genotypes and higher accumulation was observed in H88-78-1 and CLN-1621 compared to PBC plants (Table 1). A similar trend was observed when H_2O_2 content was estimated. It was found elevated under high-temperature stress conditions in both tolerant and susceptible lines compared to the control condition (Table 1). This increase was less in tolerant lines, while susceptible line PBC showed a greater increase in H_2O_2 content.

Catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) activity

The gradual and rapid increase in CAT activity was observed in tolerant genotypes H88-78-1 and CLN-1621 at 16 h, 32 h, and 48 h of high-temperature exposure, whereas PBC reported a relatively slow increase (Table 1). The maximum CAT activity was noticed after 48 h of heat stress treatment where it was ~3-times and 2.5 times higher in H88-78-1 and CLN-1621 genotypes, respectively, than in control, while it was only two times increased in PBC. SOD activity in all the three genotypes was comparable under control conditions. When plants were exposed to high-temperature stress, the activity of SOD was enhanced continuously up to 48 h; however, the increase was much more in H88-78-1 and CLN-1621 genotypes compared to PBC (Table 1). The peculiar results were obtained for APX activity where it was increased at 16 h and 32 h of treatment, but decreased onwards at 48 h of treatment in all the three genotypes

Table 1

Genotype	Duration of exposure	RWC (%)	EL (%)	Total Chl (mg g ⁻¹)	Carotenoid (mg g ⁻¹)	MDA (μmol g ⁻¹)	Proline (μg g ⁻¹)	H ₂ O ₂ (μmol g ⁻¹),	CAT (μmol of H ₂ O ₂ oxidized min ⁻¹ mg ⁻¹ protein)	SOD (units mg ⁻¹ protein)	APX (μmol of ascorbate oxidized min ⁻¹ mg ⁻¹ protein)
H88-78-1	0 h	85.9 ± 3.27 ^a	10.3 ± 0.65 ^b	43.1 ± 2.61 ^a	14.9 ± 1.47 ^a	20.1 ± 2.20 ^f	6.70 ± 0.98 ^g	15.9 ± 1.51 ^e	34.5 ± 2.61 ^d	5.50 ± 0.97 ^a	8.90 ± 1.06 ^g
	16 h	82.2 ± 2.25 ^a	14.5 ± 1.80 ^{gh}	41.3 ± 3.55 ^{ab}	12.7 ± 1.31 ^{ab}	24.8 ± 2.33 ^{def}	17.8 ± 1.60 ^{ef}	20.4 ± 2.65 ^d	62.4 ± 2.86 ^{bc}	12.8 ± 1.14 ^{de}	17.4 ± 1.71 ^{fg}
	32 h	73.8 ± 2.61 ^{bc}	18.1 ± 2.16 ^{efg}	38.1 ± 2.27 ^{abc}	10.9 ± 0.70 ^{bc}	33.4 ± 2.81 ^b	29.2 ± 2.94 ^b	25.7 ± 1.78 ^b	95.4 ± 4.12 ^a	21.8 ± 1.60 ^{bc}	29.8 ± 3.00 ^{ef}
	48 h	63.6 ± 1.94 ^{de}	25.5 ± 2.40 ^{cd}	35.1 ± 4.00 ^{bcd}	8.60 ± 0.82 ^{cd}	31.2 ± 3.47 ^{bc}	47.6 ± 5.80 ^a	48.4 ± 5.20 ^a	104.4 ± 5.42 ^a	30.5 ± 2.49 ^a	28.2 ± 2.10 ^c
CLN-1621	0 h	85.6 ± 3.20 ^a	12.5 ± 1.75 ^{gh}	41.2 ± 3.61 ^{ab}	14.7 ± 1.60 ^a	23.7 ± 2.49 ^{ef}	8.80 ± 0.90 ^g	16.8 ± 1.76 ^e	39.8 ± 2.80 ^d	6.20 ± 0.73 ^a	7.60 ± 0.41 ^g
	16 h	78.0 ± 2.17 ^{ab}	16.2 ± 2.65 ^{gh}	39.1 ± 4.30 ^{abc}	11.0 ± 1.51 ^{bc}	26.4 ± 2.70 ^{cde}	18.2 ± 2.53 ^{de}	26.2 ± 2.82 ^{cd}	65.3 ± 1.50 ^c	14.4 ± 2.10 ^{de}	15.9 ± 1.55 ^{ef}
	32 h	69.3 ± 3.33 ^{cd}	23.0 ± 2.98 ^{cde}	33.8 ± 2.96 ^{cd}	9.80 ± 1.34 ^{bc}	35.3 ± 2.38 ^b	27.5 ± 2.41 ^b	38.5 ± 3.96 ^b	94.2 ± 3.47 ^a	20.7 ± 2.57 ^{bc}	32.5 ± 4.40 ^d
	48 h	59.5 ± 4.76 ^e	28.6 ± 3.38 ^{bc}	29.9 ± 2.71 ^{de}	8.40 ± 1.71 ^{cd}	30.4 ± 2.08 ^{bcd}	46.8 ± 4.82 ^{ab}	64.2 ± 6.94 ^a	99.8 ± 4.77 ^a	28.6 ± 4.77 ^a	28.1 ± 4.00 ^b
Punjab Chhuhara	0 h	78.7 ± 4.20 ^{ab}	13.6 ± 1.59 ^{gh}	39.1 ± 4.24 ^{abc}	14.4 ± 1.92 ^a	22.9 ± 1.43 ^{ef}	8.20 ± 1.1 ^g	16.1 ± 1.88 ^e	35.7 ± 1.65 ^d	6.70 ± 0.90 ^a	7.60 ± 0.98 ^g
	16 h	69.5 ± 4.40 ^{cd}	21.8 ± 1.56 ^{def}	30.7 ± 2.53 ^{de}	10.0 ± 1.20 ^{bc}	30.7 ± 2.61 ^{bc}	15.3 ± 2.10 ^f	29.7 ± 3.20 ^d	55.4 ± 3.71 ^c	11.8 ± 1.71 ^e	14.2 ± 2.10 ^e
	32 h	60.7 ± 3.47 ^e	32.0 ± 2.99 ^b	24.4 ± 2.10 ^{ef}	7.90 ± 1.24 ^{cd}	44.6 ± 2.78 ^a	23.9 ± 2.20 ^c	55.3 ± 3.40 ^{bc}	77.4 ± 3.65 ^b	18.8 ± 2.57 ^{cd}	22.5 ± 3.60 ^c
	48 h	50.0 ± 5.47 ^f	47.9 ± 5.22 ^a	20.2 ± 1.47 ^f	6.30 ± 0.45 ^d	41.3 ± 3.63 ^a	31.4 ± 4.05 ^{cd}	94.6 ± 6.40 ^{cd}	71.6 ± 3.32 ^{bc}	15.2 ± 1.96 ^b	18.4 ± 2.41 ^a

RWC relative water content (%), EL electrolyte leakage (%), Chl chlorophyll, total chl (mg g⁻¹), carotenoid (mg g⁻¹), MDA malondialdehyde (μmol g⁻¹), proline (μg proline g⁻¹), H₂O₂ hydrogen peroxide (μmol g⁻¹), CAT catalase (μmol of H₂O₂ oxidized min⁻¹ mg⁻¹ protein), SOD superoxide dismutase (units mg⁻¹ protein), APX ascorbate peroxidase (μmol of ascorbate oxidized min⁻¹ mg⁻¹ protein)

(Table 1). There was a considerable difference in magnitude of change between tolerant and susceptible lines where increase was more in tolerant lines and less in susceptible line.

Gene expression analysis

Mining of different databases for retrieval of heat-responsive genes like heat shock factors and heat shock proteins resulted in a total of 67 different genes (Supp Table 1). All these heat-responsive genes were located on different chromosomes and their chromosomal locations on the corresponding chromosomes are shown in Fig. 1. To understand the differential response of these genes in high-temperature tolerant and susceptible genotypes, their expression was analyzed at different durations of high-temperature stress. Heat shock factors are transcription factors that control the expression of other heat-responsive genes. Expression

analysis of these genes in H88-78-1, CLN-1621, and PBC showed increased expression upon exposure to high-temperature stress (Fig. 2; Supp. Table 2). Some of the genes showed a gradual increase in expression relative to the duration of stress in heat-tolerant genotypes H88-78-1 and CLN-1621 compared to susceptible PBC genotype. Among the studied genes, 17.4 kDa class III heat shock protein-coding gene showed the highest expression in all three genotypes during heat stress conditions. The response to heat through the expression of this gene was better in the H-88-78-1 genotype (20.8-fold at 48 h of stress) compared to CLN-1621. The other important genes responsible for high-temperature stress tolerance in the tolerant lines were heat stress transcription factor A-4a, HSF30, HSF B-2a, HSF24, HSF A-4c, HSF19 (HSF B-3 like), 21.7 kDa class VI HSP, 18.1 kDa class I HSP like, and HSP17.4. Besides, the expression of several other genes coding for heat shock proteins, heat shock factors, and other stress-responsive genes under heat

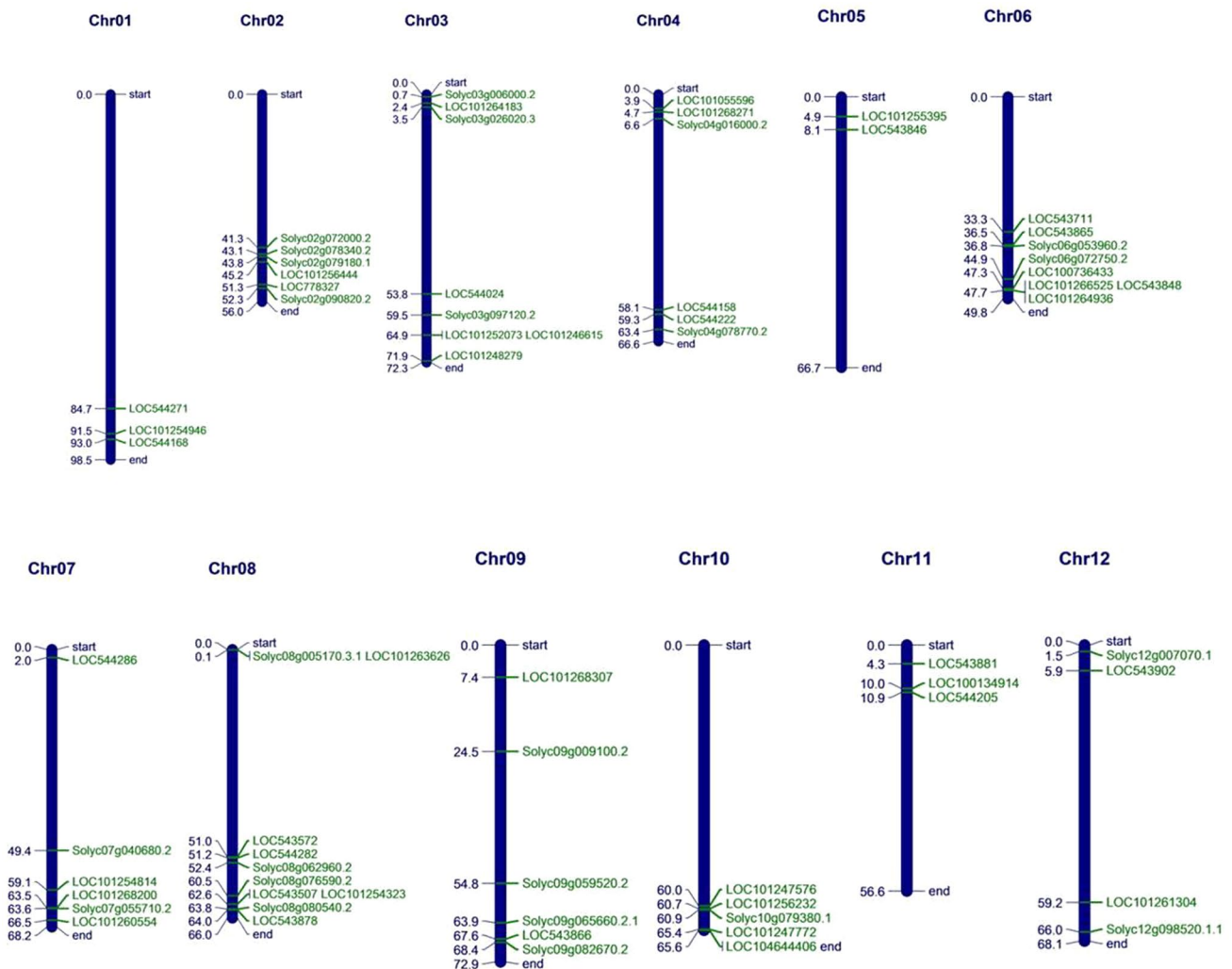


Fig. 1 Physical map of high-temperature stress-responsive genes on 12 chromosomes of tomato. Numerical values show size in mega base pairs

stress conditions was comparatively higher in H-88-78-1 and CLN-1621 genotypes than PBC confirming the tolerance phenotype of these genotypes.

In silico analysis of heat-responsive candidate genes

All the 67 genes studied here were localized on their respective chromosomes (Fig. 1). The genes were found to be distributed on all the 12 chromosomes with a peculiar distribution pattern. Interestingly, all these stress-responsive genes

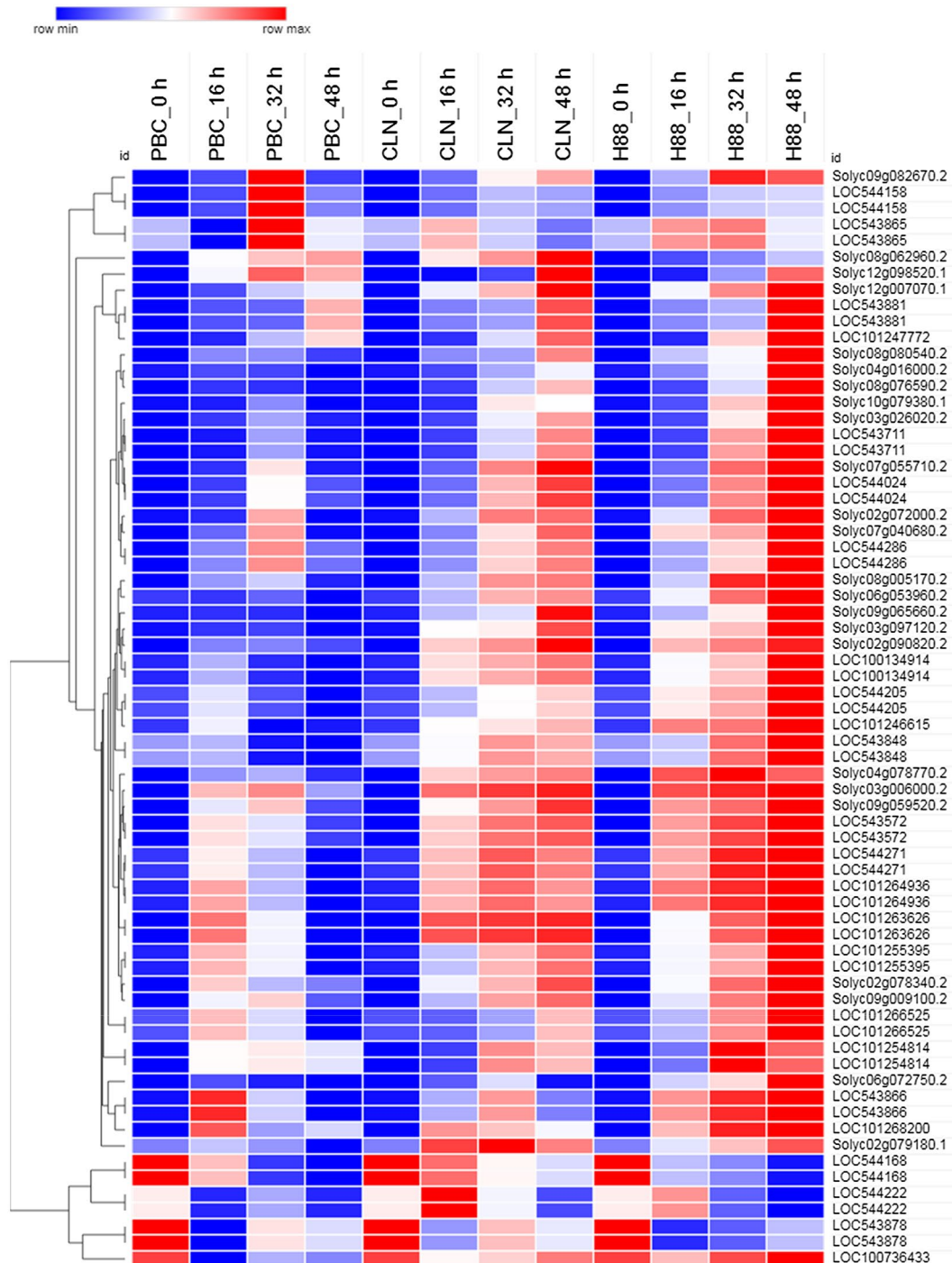


Fig. 2 Heatmap and hierarchical clustering showing expression pattern of 67 high-temperature stress-responsive genes in three tomato genotypes at different time intervals (0 h, 16 h, 32 h, and 48 h) of stress treatment. PBC: Punjab Chuhara, CLN: CLN1621, H88: H88-78-1

were present in clusters on terminal portions of the chromosomes. The number of genes on chromosomes varied from a minimum of 2 on chromosome 5 to a maximum of 10 on chromosome 8. In general, all the chromosomes were found to have a considerable number of high-temperature stress-responsive genes.

The information of sub-cellular localization and functional annotations has been provided in supplementary table 3. All the transcription factors were found to be localized in nucleus as expected. The heat shock proteins and other stress-responsive proteins were localized in cytoplasm and other cell organelles like mitochondria, chloroplast, endoplasmic reticulum, and peroxisomes based on their functions. Heat shock factors were annotated as DNA binding or protein binding with either transcription activation or repression activity. Many of the proteins are annotated as involved in protein folding, ATP binding, response to stresses like heat, drought, light intensity, hyperosmotic stress, etc. HSF30 and HSF24 which showed higher expression in the present study under heat stress and HSFA-2 like were found to be involved in response to water deprivation.

Expression analysis of the genes was conducted by Genevestigator tool where expression details of only seven genes were displayed (Supplementary Figs. 1 and 2). HSFA-1b was found to be expressed in only flowering stage. Two genes *er-sHSP* and *Le-HSP17.6* showed higher expression in fruit development and fruit ripening stages. DELLA protein GAI was highly induced in seedling and fruit development stages. *Er-sHSP*, *HSP20-1*, and *Le-HSP17.6* were highly expressed in heat-treated microspore and mature pollen samples. DELLA protein GAI, *Er-sHSP*, *HSP20-1*, *extension-3*, and *Le-HSP17.6* are involved in seed development, placenta development, and locular tissue development also (Supplementary Fig. 2).

Discussion

Rise in global temperature is one of the most serious concerns for agricultural production. High-temperature stress causes a reduction in the yield of several horticultural crops and the effect is more severe when stress is experienced at the reproductive stage of the plant. Tomato is particularly more sensitive to extreme temperatures, and therefore, identification of high-temperature stress-tolerant genotypes and their utilization in breeding programs is very essential for a sustainable yield of tomato. In the present study, two promising genotypes, H-88-78-1 and CLN-1621, which have been identified through phenotypic screening, were characterized through biochemical and molecular parameters. This will establish these genotypes as a source of genes for high-temperature stress tolerance. A plant genotype is said to be tolerant to a particular stress if the plant shows no/significantly

less signs of the effect of stress compared to the susceptible genotype. Effects of stress are generally assessed by studying different parameters like the content of osmolytes, antioxidants, membrane damage, chlorophyll content, and expression of stress-responsive genes. All these parameters have been analyzed in the two promising tolerant genotypes in comparison with susceptible genotype PBC. The foremost effect of high-temperature stress at the cellular level in plants is on the cell membrane and cellular macromolecules. Membrane damage results in electrolyte leakage leading to disturbance in the ion homeostasis. Stress tolerance is characterized by less electrolyte leakage which is the sign of less membrane damage due to high-temperature stress. Both H-88-78-1 and CLN-1621 genotypes were less affected by high-temperature stress even at 48 h of stress compared to PBC. Similar profiles were recorded for relative water content where H-88-78-1 and CLN-1621 retained more water during stress. High-temperature stress and moisture deficit stress are linked and the ultimate effect of high-temperature stress leads to water deficit. Thus, more retention of water by H-88-78-1 and CLN-1621 during stress correlates with their tolerant phenotype.

Photosynthesis is one of the primary processes affected by all kinds of stresses. High-temperature stress affected the photosynthesis process of all the tomato genotypes studied by causing a significant decrease in photosynthetic pigments chlorophyll and carotenoids. However, the tolerant genotypes H-88-78-1 and CLN-1621 were less affected in comparison with susceptible line PBC. Plants capable of carrying out photosynthesis in stress conditions without much effect are tolerant of such stress, and they reduce the ultimate effect of stress on growth and yield. Reduction in chlorophyll and carotenoid pigments under high-temperature stress is associated with the production of ROS, and thus, it is indirectly related with level of stress in the plant (Chalanika and Asaeda 2017). ROS are harmful to the cellular environment and they can damage important biomolecules such as proteins and nucleic acids. Hydrogen peroxide (H_2O_2) is a strong and reasonably stable ROS which regulates several biological processes during stressed conditions in plants (Bienert et al. 2007). To avoid the effect of stress on the cellular environment of the plant, it is essential to scavenge the excess H_2O_2 . Plants produce a number of antioxidant molecules to counteract or neutralize the ROS produced under stress (Wahid et al. 2007). The activity of different molecules like catalase (CAT), superoxide dismutase (SOD), and ascorbate peroxidase (APX) was gradually increased under the increased duration of high-temperature stress in both tolerant and susceptible genotypes. The higher increase in activity of these molecules in H-88-78-1 and CLN-1621 genotypes compared to PBC depicts their ability to scavenge the ROS during stress. This is also in correspondence with less accumulation of H_2O_2 in tolerant genotypes. Similar

results were also observed in the case of lipid peroxidase activity. More activity in H-88-78-1 and CLN-1621 genotypes confirms their tolerance to high-temperature stress. Another characteristic of stress-tolerant plants is an accumulation of free proline in the cytoplasm which functions as an osmolyte. The role of proline as an osmoprotectant has been established under exposure to different abiotic stresses in plants including tomato (Gujjar et al. 2018). More accumulation of free cellular proline in H-88-78-1 and CLN-1621 in comparison with susceptible PBC indicates their comparative tolerance against high-temperature stress.

High-temperature stress affects the large biomolecules of cells and the main targets are proteins that get denatured. Plants' defense response against high-temperature stress is mediated by the synthesis of heat shock proteins (Lindquist and Craig 1988). Stress tolerance is characterized by the quick response of plants by up-regulated expression of stress-responsive genes, heat shock proteins, or heat shock factors in case of high-temperature stress tolerance. 67 heat stress-responsive genes analyzed here showed better profiles in H-88-78-1 and CLN-1621 genotypes compared to PBC showing their tolerance at the molecular level, as well. Expression of heat shock factors increased immediately after exposure to high-temperature stress, while expression of heat shock proteins and other stress-responsive genes increased gradually with increased duration of stress. This is in accordance with the fact that heat shock factors are the primary transcription factors and induces the expression of other stress-responsive genes later. Besides we have identified the important genes such as HSP17.4, 17.4 kDa class III heat shock protein, transcription factor A-4a, HSF B-2a, HSF24, HSF30, and HSF19 (HSF B-3 like) which exhibited quick and higher expression pattern in tolerant lines. Functional annotation of HSF30 and HSF24 shows involvement in response to water deprivations. The heat stress ultimately causes water deficit stress, and thus, these two genes may function in providing tolerance to both heat and drought stress. HSF30 and HSF24 were also reported earlier to be induced by heat stress in *Solanum peruvianum* (Scharf et al. 1993). HSFA1 induced up to nine fold in H88-78-1 and up to eight fold in CLN1621 at 48 h of heat exposure is well known as a master regulator for thermotolerance in tomato and its over-expression in transgenic tomatoes resulted in significant tolerance against severe heat treatments (Mishra et al. 2002). HSFA2 transcription factor is slightly up-regulated under heat stress in the present study which further induces heat shock proteins and 17.4 kDa class III HSP is one of the main targets of HSFA2 in *Arabidopsis* (Nishizawa et al. 2006). 17.4 kDa class III HSP gene showed the highest expression under heat stress as almost 20-fold in H88-78-1 and 14-fold in CLN1621, suggesting its major role in high-temperature stress response in tomato. The genes such as GAI, er-sHSP, and Le-HSP17.6 that were found to

be induced in microspores and pollen tissues by Geneinvestigator tool were also slightly induced in leaf tissues in the present study. The DELLA protein GAI is involved in the number of plant processes like response to salinity stress, abscisic acid, and ethylene stimulus, etc. Up-regulation of GAI in high-temperature stress suggests its important role in environmental stress response in tomato. These genes can be utilized in developing gene-based markers for marker-assisted breeding programs to develop high-temperature stress-tolerant tomato cultivars.

Physical mapping of these high-temperature stress-responsive genes revealed an interesting fact that all these genes are present in small clusters preferably at the ends of the chromosomes. The presence of genes in clusters helps the regulatory machinery of the cell to regulate the expression of genes in response to environmental stimuli in an effective manner. Furthermore, the locations of these genes could help to identify different markers mapped in the same region by comparing the position of these genes and markers. It will help to narrow down the list of markers that can be used to validate their linkage with high-temperature stress response in stress-tolerant tomato genotypes CLN-1621 and H88-78-1. Markers identified in this way can be utilized for marker-assisted breeding for transferring the traits from these genotypes into superior tomato cultivars.

Conclusion

The global rise in temperature is a major threat to agriculture and food security. Tomato is particularly more sensitive to extreme temperatures, and therefore, continuous efforts are needed to develop the tomato cultivars tolerant to high-temperature stress. The present study has characterized two tomato genotypes, H-88-78-1 and CLN-1621, by molecular, physiological, and biochemical parameters for high-temperature stress tolerance. Both these genotypes showed better profiles for all the parameters including rapid and significantly higher expression of heat stress-responsive genes in comparison with a susceptible genotype PBC. All the studied genes are present in clusters on different chromosomes depicting their effective regulation during stress. Thus, the study establishes H-88-78-1 and CLN-1621 as high-temperature stress-tolerant genotypes that can be effectively used in breeding programs to transfer these traits to cultivated varieties.

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NR and PMS: funding acquisition, review, and editing; JS: project administration.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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