Mol Breeding (2013) 32:687–697
DOI 10.1007/s11032-013-9901-5

Constitutive overexpression of small *HSP24.4* gene in transgenic tomato conferring tolerance to high-temperature stress

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Received: 4 December 2012 / Accepted: 3 June 2013 / Published online: 12 July 2013

Abstract Acquired thermotolerance in plants refers to the ability to cope with lethal high temperatures and it reflects an actual tolerance mechanism that occurs naturally in plants. Tomato (*Solanum lycopersicum* syn. *Lycopersicon esculentum* L.) is sensitive to high temperature at all stages of its growth and development. Considering the important role of the heat shock protein gene (s*HSP24.4* gene) in imparting tolerance to high temperature stress in the cells and tissues, we isolated small *HSP24.4* (*MasHSP24.4*) cDNA from wild banana (*Musa accuminata*) and introduced it into the cultivated tomato cv. PKM1 by using *Agrobacterium tumefaciens*-mediated genetic transformation. Stable integration and expression of the transgene in the tomato genome was demonstrated by Southern, Northern and Western blot analyses. There was no adverse effect of transgene expression on overall growth and development of the transgenic plants. The genetic analysis of the transgenic T2 lines showed that the transgene segregated in a Mendelian ratio. We compared the survival of T2 transgenic lines compared to the control plants after exposure to different levels of high temperature. The gene *MasHSP24.4* was expressed in root, shoot and stem tissues under 45 °C treatment and conferred tolerance to high-temperature stress as shown by increased seed germination, healthy vegetative growth and normal fruit and seed setting. The transgenic tomato plants showed significantly better growth performance in the recovery phase following the stress. This thermotolerance appeared to be solely due to overexpression of the s*HSP24.4* gene. Thus, the transgenic tomato plants developed during the present investigations can be grown at high temperatures.

Keywords *Solanum lycopersicum* · Small heat shock proteins (sHSPs) · Heat shock response (HS) · *Agrobacterium*-mediated transformation · Thermotolerance

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Introduction

The small heat shock proteins (sHSPs), ubiquitous stress proteins proposed to act as chaperones, are

encoded by an unusually complex gene family in plants. Plants synthesize multiple sHSPs encoded by six nuclear multigene families; each gene family encodes proteins found in a distinct cellular compartment, e.g. the cytosol (Siddique et al. 2008, Jiang et al. 2009; Basha et al. 2010; Zhou et al. 2012), chloroplast (Wang and Luthe 2003; Shakeel et al. 2011), mitochondria (Liu and Shono 1999; Waters et al. 2008) and endoplasmic reticulum (Zhao et al. 2007; Mamedov and Shono 2008). The high diversification of plant sHSPs probably reflects a molecular adaptation to stress conditions that is unique to plants. Plant sHSPs respond to a wide range of environmental stresses including heat, drought, salinity, oxidative and cold stress. The abundance of sHSPs in plants and their functional characteristics of binding and stabilizing denatured proteins suggest that sHSPs play an important role in plants' acquired stress tolerance (Wang et al. 2003; Valcu et al. 2008; Shakeel et al. 2011, 2012).

Recently, several reports have appeared in which it has been possible to address the issue of developing plants tolerant to high temperature stress by manipulating the heat shock response components. Xue et al. (2010) have observed that the overexpression of the *HSP26* gene in transgenic *Arabidopsis* produced increased amounts of proline during enhanced thermotolerance. Similarly, Murakami et al. (2004) found that increased levels of sHSP17.7 protein were found in transgenic rice plants under high temperature stress and UV-B tolerance.

Tomato (*Solanum lycopersicum* L.) is the most important major vegetable crop in the developed and developing countries. Tomato is rich in precursors of vitamin A, vitamin B6, vitamin C, potassium, niacin and folate. It is a very good source of lycopene which can act strongly against cancer, especially colon and prostate cancer (Rao and Agarwal 2000). Several abiotic stress factors such as high temperature, drought, salinity and heavy metal stress that could affect the cultivation of tomato are on the increase (Boyer 1982; Grover and Minhas 2000; Foolad 2007). These factors are detrimental to both the vegetative and reproductive stages of tomato (Satake and Yoshida 1978; Pareek et al. 1998); thus the development of improved tomato cultivars with enhanced tolerance to high temperature stress could help in solving the problem.

High temperature is a major factor limiting productivity and adversely affects the vegetative and reproductive stages of tomato and ultimately reduces the yield and fruit quality (Dinar and Rudich 1985; Sato et al. 2001). During the reproductive period of the plants, fruit set and fruit yield was significantly decreased by extreme temperatures (Hall 1992; Singh et al. 2007). Under the stress, plants are induced to express sHSP genes for the production of sHSPs.

As there is no report on transgenic tomato plants cultivated for thermotolerance, the present investigations report on the development of transgenic cultivated tomato cv. PKM1 plants by using the *sHSP24.4* gene to confer thermotolerance.

Materials and methods

Plant material

The seeds of tomato (*S. lycopersicum* cv. PKM1) were collected from Tamilnadu Agricultural University (TNAU), Chennai, India. For in vitro seed germination, the seeds were washed under running tap water for 10 min and surface sterilized with 0.02 % (w/v) aqueous $HgCl_2$ for 1–2 min and then washed for 5–10 rinses in sterile distilled water. The sterilized seeds were dried on sterile tissue paper and cultured on modified Murashige and Skoog (1962) medium (1/2 strength vitamins; 1/2 strength micronutrients; 1/4 strength macronutrients; without $CaCl_2$; 1 % sucrose; 0.75 % agar–agar), ~50 mL medium per six seeds. All the cultures were kept in the dark for 2 days andtransferred to light after germination. The pH was adjusted to 5.8 with either 0.1 N HCl or 0.1 N NaOH before autoclaving. The cultures were maintained at 25 ± 1 °C under a 16/8-h light/dark photoperiod provided with fluorescence light intensity of 60 μmol m^{-2} s^{-2}.

Construction of plant expression vector

To overexpress the *MasHSP24.4* (*Musa acuminata* small heat shock protein) gene in cultivated tomato cv. PKM1, the plasmid pTOPO-*MasHSP24.4* was digested with *Xba*I and *Kpn*I restriction enzymes and cloned in the corresponding sites of cloning vector pUC19/35 AMV Nos that was flanked by CaMV 35S promoter, *uid*A-gus cassette and the poly-adenylation signal to create pUC19/35 AMV Nos-*MasHSP24.4*. This expression cassette was excised with *Xba*I and *Kpn*I digestion and sub-cloned subsequently at *Xba*I

and *Kpn*I sites in the binary vector pCAMBIA 2301 (Fig. 1), and was confirmed by restriction analysis and DNA sequencing. The resultant recombinant vector pCAMBIA2301-*MasHSP24.4* carrying the small *HSP24.4* gene (GenBank accession number: JQ867401; Protein ID: AFK24464S), *uid*A-gus gene and the neomycin phosphotransferase gene (*npt*II) for kanamycin (Kan) resistance was transformed into *Agrobacterium tumefaciens* LBA4404 strain.

In-vitro regeneration

Fully expanded cotyledons grown in vitro from 8-day-old axenic seedlings were used for the *A. tumefaciens* LBA4404-mediated genetic transformation experiments. Before infection of cotyledons, the decapitated cotyledons (1 cm^2 in area) were pre-cultured on shoot induction medium (SIM) containing 2 mg/L BAP for 5 days. These pre-cultured cotyledon explants were used for *Agrobacterium*-mediated genetic transformation.

Agrobacterium tumefaciens-mediated genetic transformation of tomato plants

The *A. tumefaciens* LBA4404 strain carrying the desired gene (*pCMasHSP24.4*) was grown at 28 °C in 50 mL LB medium containing 50 mg/L Kan and 25 mg/L rifampicin to post log phase. The bacterial suspension was then pelleted at 5,000 rpm for 10 min. To this, half-strength liquid MS medium (1:2, v/v) was added followed by 200 mM of acetosyringone.

Fully expanded cotyledons grown in vitro from 8-day-old axenic seedlings were used for genetic transformation experiments. *A. tumefaciens* strain LBA4404 harbouring the binary vector *pCMasHSP24.4* gene was grown to 0.6–0.8 at OD_{600} (Horsch et al. 1985). The pre-cultured explants were subsequently submerged in *A. tumefaciens* suspension in half-strength MS liquid medium containing 200 mM acetosyringone for 10 min, dried on sterile tissue paper and co-cultured in the dark at 28 °C for 2 days on SIM consisting of 200 mM acetosyringone. Subsequently, the cotyledon explants were washed in half-strength MS liquid medium and dried on sterile tissue paper and co cultivated with *A. tumefaciens* for 2 days. Later these were transferred onto selection medium containing SIM with 200 mg/L cephotaxime and 50 mg/L Kan. After 3 weeks of incubation, these explants were shifted onto fresh SIM supplemented with 50 mg/L Kan.

In-vitro rooting

Kan-resistant healthy elongated shoots 4–5 cm long were cultured on rooting medium containing 0.5 mg/L indole-3-acetic acid (IAA) and 50 mg/L Kan. All the cultures were maintained at 24 ± 1 °C under a 16/8-h light/dark photoperiod with fluorescent light intensity of 60 μmol $m^{-2}s^{-1}$.

Plantlet establishment

The in vitro regenerated Kan-resistant plantlets were taken from the culture vessels and washed with sterile distilled water to remove the remains of agar, then covered with polythene bags to maintain relative humidity (RH; 80–90 %). All these were transferred to plastic pots containing soil and vermiculite (1:1) and kept in a culture room for acclimatization. After 4 weeks, all the plants were shifted to earthenware pots containing garden soil and maintained in the greenhouse.

Screening of Kan resistance

The seeds from T0 plants were collected through self-pollination and were germinated on MS medium containing Kan (50 mg/L) to select T1 seedlings; the same procedure was used to select T2 seedlings.

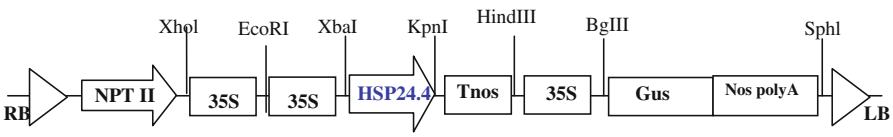

Fig. 1 Construct showing *MasHSP24.4* gene driven by CaMV35S promoter, *uid*A gene (GUS) and *nptII*-a gene for neomycin phosphotransferase

Molecular characterization of transgenic tomato plants

For the molecular characterization of putative T0 transformants, DNA samples of the transgenic and WT plants were obtained using the CTAB procedure (Doyle and Doyle 1990). PCR amplification and Southern hybridization were carried out using standard molecular protocols and Southern blots were hybridized with a radiolabeled α-^{32}P probe (Sambrook et al. 1989).

Northern blot analysis

For Northern blot analysis, the total RNA was extracted from the frozen samples prepared from heat stress treated and unstressed samples as well as from the tissues such as cotyledons, stems, leaves, roots and flowers using TRI reagent (Sigma, USA) according to the manufacturer's instructions. Total RNA (20 μg) was separated by electrophoresis at 90 V for 2–3 h through 1.2 % (w/v) formaldehyde agarose gel in formaldehyde gel buffer. The gel was washed in diethylpyrocarbonate (DEPC) water for 30 min. Transfer of RNA onto Hybond-N^+ nylon membrane, hybridization and washings were carried out at 42 or 65 °C as described in the protocol of Southern blot analysis.

All glassware, plasticware and buffer solutions used for RNA work was treated with 0.1 % (v/v) DEPC.

Segregation analysis of progeny

The seeds from T0 tomato plants (transformants) were germinated in vitro on modified MS medium and each plant was transferred to an earthenware pot. Genomic DNA was isolated from the leaf tissue of transgenic and control tomato plants using the CTAB procedure (Doyle and Doyle 1990). PCR screening of these plants was carried out using primers specific for the *MasHSP24.4* gene (forward primer: 5′-CTCCTAGAATGTCACTGGCTC-3′ and reverse primer: 5′-CGGGTACCTTACCGCACGTCA-3′) were designed according to the known Musa-*MasHSP24.4* sequence (GenBank accession number: JQ867401).

RT-PCR for the expression analysis of *MaHSP24.4* gene

For RT-PCR analysis, 5 μg of total RNA prepared from heat stress treated and from unstressed samples as well as from the tissues such as cotyledon, stem, leaf, root and flowers were reverse transcribed using reverse transcriptase and Oligod$T_{(18)}$ following the manufacturer's instructions (Sigma, USA). RT-PCR analyses were performed by using gene-specific forward and reverse primers (forward primer: 5′-CTCCTAGAATGTCACTGGCTC-3′ and reverse primer: 5′-CGGGTACCTTACCGCACGTCA-3′) and *actin* was used as a loading control to check the expression levels of transgenes. The primer sequences of *actin* were forward: 5′-CCACCACAGCCGAACGGG-3′ and reverse: 5′-ACCCGGGAACATGGTGGAACC-3′. For PCR, 2 μL of the reverse transcription reaction was used as DNA template and the amplification was performed using cycling conditions of 94 °C for 1 min (denaturation), 58 °C for 30 s (annealing) and 72 °C for 45 s (elongation). The amplification reaction for *MaHSP24.4* was carried out for 35 cycles.

Functional analysis of tomato transgenic plants

Whole plant assay for dehydration tolerance

We measured the water-withholding capacity of the control plants to assess the plant growth levels during mid-summer in Indian Territory conditions (35–50 °C) in the research field by altering the water supply. In this experiment, we assayed three tomato lines (25 plants each) by keeping one line of plants as a positive control (with regular supply of water); the second line of plants was watered at 3-day intervals and the third line of plants was kept as a negative control (without water supply). In contrast to the above, another line of plants was grown at normal field-suitable conditions to compare the data on drought analysis during the crop season, i.e., November–January, and the experimental data was monitored regularly.

For the drought tolerance assay, wild-type (WT) and transgenic plants (4 and 16), grown in soil pots at 8 weeks old were treated by withholding water prior to growth and flowering for 4 weeks. Watering of plants

was resumed after 4 weeks and recovery was monitored.

Thermotolerance assay of transgenic plants

T2 seeds from 12, 15 and 45 transgenic lines and control (C_2) plants were germinated on MS modified medium for 8 days (24 ± 1 °C). The seedlings were transplanted into small earthenware pots containing soil and vermiculite (1:1) mixture and were kept in the greenhouse. The seedlings were supplied with nutrients regularly to ensure proper development. Potted plants of 45 days old were exposed to high temperature stress (45 and 50 °C) during mid-summer (according to Indian Territory conditions) and in addition pots containing plants were placed in trays filled with pre-heated water kept in an incubator maintained at different temperatures (45 and 50 °C). Stress treatments were continued for different intervals under continuous light. After exposure to 45 °C for 3 h, plants were returned to the growth chamber at 28 °C for recovery. In the case of the plants exposed to 50 °C stress for 40 min, leaves were excised and plants were kept in the growth chamber for re-growth at 28 °C. Recovery was monitored and plants were photographed after 5–10 days.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (12 %) electrophoresis was performed according to the protocol of Laemmli (1970). Protein concentrations were measured using Bradford's (1976) procedure.

Custom primer synthesis

All the primers used in this study were custom synthesized from Bioneer Corporation, Korea, MWG Biotech, Bangalore, Sigma, Bangalore, India and Bioserve, Hyderabad, India.

Custom sequencing

All the recombinant clones present in pCAMBIA2301 (Fig. 1) were sequenced from Macrogen, Korea and MWG Biotech, Bangalore, India.

Data analysis

All the cultures were observed on a visual basis and the shoot primordia were counted under the microscope.

Results

In-vitro regeneration of putative transgenic tomato plants

Shootbuds were induced after 2 weeks of incubation on selection medium. The Kan-resistant healthy elongated shoots 4–5 cm long were rooted on root induction medium (RIM) containing 0.5 mg/L IAA and 50 mg/L Kan. After rooting, the in vitro regenerated transgenic plantlets were taken from the culture vessels and shifted to plastic cups containing sterile soil and vermiculite (1:1) and covered with polythene bags to maintain RH (80–90 %). After 4 weeks of acclimatization, all the plants were shifted to earthenware pots containing garden soil and maintained in the greenhouse. The seeds from T0 tomato plants were collected after self-pollination and were germinated on MS medium containing Kan (50 mg/L) to select T1 seedlings; the same procedure was used to select T2 seedlings.

Acetosyringone effect on transgenic plant recovery

The effect of acetosyringone on transformation has vital importance for transformed shootbud induction and shoot formation, since 200 mM acetosyringone was shown to be optimal for maximum nicking and transfer of the gene of interest from the nicked substrate (Culianez-Macia and Hepburn 1988). The plant transformation rate from this treatment was found to be enhanced. The number of confirmed transgenic plants per 100 resistant shoots was over 50 %, which was four times higher than that obtained without.

Molecular characterization of putative transgenic tomato plants

A total of 70 Kan-resistant plants were regenerated from co-cultivated cotyledon explants. Out of these, 32 transformants were analyzed at the molecular level.

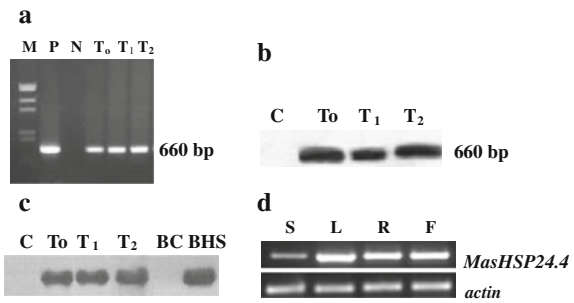

Fig. 2 **a** PCR gel picture showing the 660-bp amplified products of T0, T1 and T2 representing different transgenic lines of tomato cv. PKM1 for *MasHSP24.4* gene; *M* marker, *P* PCR positive control, *N* negative control. **b** Southern blot analysis of genomic DNA present in different transgenic lines of tomato cv. PKM1. Control plants (C) and T0, T1, T2 represent different transgenic lines. **c** Northern blot analysis of *MasHSP24.4* gene expression in T0, T1 and T2 transgenic lines. *C* control, *T0*, *T1*, *T2* transgenic tomato lines, *BC* banana control, *BHS* banana heat-stressed plants). **d** RT-PCR analysis of tissue-specific expression of sHSP transcript; *S* stem, *L* leaf, *R* root, *F* flower

The integration of *MasHSP24.4* (GenBank accession number: JQ867401; Protein ID: AFK24464S) cDNA was confirmed by PCR of genomic DNA of transformants. All of them were found to contain the transgene (Fig. 2a). All the transgenic plants were phenotypically normal and they grew well and were fertile. Southern blot hybridization was carried out to confirm the stable integration of the transgene and all the transgenic lines were found with single copy number (Fig. 2b).

T1 progeny of self-pollinated T0 plants were analyzed by PCR and Southern blot hybridization techniques for stability of the transgene. PCR screening of the T1 seedlings followed a Mendelian 3:1 segregation, suggesting that the transgene had integrated at a single locus. To confirm the copy number of the inherited transgene in T1 tomato plants, genomic DNA digested with *Xba*I and *Kpn*I was probed with the full-length *MasHSP24.4* cDNA. The tested T1 plants showed the presence of a single copy of the transgene (Fig. 2b) and an integration pattern similar to the respective parental T0 plants. Thus, the Southern analysis revealed the presence of a 660-bp band in all the transgenic lines, which was absent in the control plant (Fig. 2b). Total RNA (10 μg) was electrophoresed in a denaturing gel and processed further according to the protocol employed for Northern blot analysis. RNA from banana control (BC) and banana heat-stressed (BHS) plants were also loaded as positive control. Equal loading is shown by comparable intensities of methylene blue-stained RNA bands on the nylon membrane (Fig. 2c).

Soluble proteins were extracted from the leaves of control and T1 plants kept under control conditions at 28 °C as well as those subjected to high temperature stress (45–50 °C for 2 and 3 h). Western blot analysis using antibodies against MasHSP24.4 protein confirmed the presence of corresponding protein in all the transgenic T1 plants (unstressed and heat-stressed). In control plants, grown at 28 °C, no band was observed corresponding to MasHSP24.4. When control progeny were heat-stressed, a clear but faint band was seen. However, this band showed a lower molecular weight than the MasHSP24.4 band in transgenic plants. Possibly, this band corresponds to MasHSP24.4 of the host plant. In heat-stressed transgenic plants, there was no significant change in the intensity of the MasHSP24.4 band. On the other hand, endogenous tomato MasHSP24.4 showed distinct heat shock inducibility in control as well as transgenic plants when anti-wheat *sHSP24* antibodies were used for Western analysis (Fig. 3a, b). T2 plants along with the control plants were also tested for the level of transcript accumulation corresponding to *MasHSP24.4*. High levels of transcripts were found in all the transgenic lines, similar to the respective parental lines (Fig. 2d), showing stable inheritance of transgene expression.

Thermotolerance assay of transgenic plants

T2 progeny of three T0 lines (12, 15 and 45) harbouring a single copy of the transgene as well as showing high levels of MasHSP24.4 protein were selected for the thermotolerance test. Control (C_2) and tomato transgenic plants 12, 15 and 45 at 45 days old were exposed to 45 °C for 3 h and subsequently placed at 28 °C for recovery (Fig. 3). Immediately after the heat stress, the extent of stress-induced damage in control appeared comparable to the transgenic plants. The leaves of these plants wilted and drooped. After 2 days of recovery, new leaf emerged in transgenic lines whereas control plants no signs of revival. After 5 days, C_2 plants wilted totally followed by death whereas transgenic plants 12, 15 and 45 were found to be green and healthy (Fig. 3).

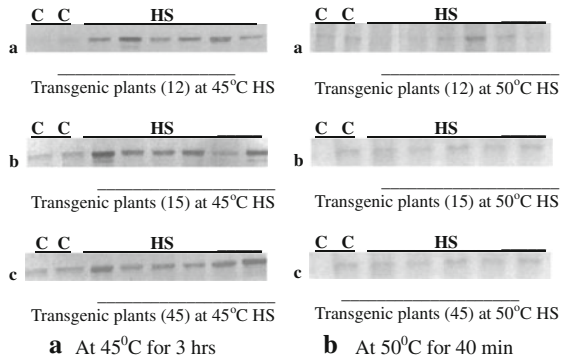

Fig. 3 **a** and **b** Western blot analysis of transgenic tomato cv. PKM1 plants assayed for thermotolerance. *Numbers below each panel* represent the plant line employed in this study. *C* Control plants, *HS* heat-stressed transgenic plants

In response to stress treatment at 45 °C for 3 h, leaves of control and transgenic lines were wilted. After this, the leaves were excised and at the same point of excision, the recovery was initiated in terms of re-growth of leaves immediately after stress (Fig. 3). However, after 8 days of recovery, lines 12, 45 (Fig. 4) and 15 of transgenic tomato plants exhibited considerable growth while the control (C_2) plants failed to recover. On day 10 of recovery, all the control plants died while the transgenic plants showed vigorous re-growth.

The performance of these transgenic plants was further tested under more severe stress conditions, i.e., at 50 °C for 40 min. We did not find any stress-induced damage in control or transgenic plants. After the stress, the leaves were excised and plants were placed at 28 °C to re-grow. All the plants showed slight initial elongation when kept for recovery. However, control (C_2) plants showed gradual drying and yellowing, culminating in their death on day 8 of recovery. In contrast, transgenic lines 12, 15 and 45 were found to have healthy growth showing emergence of new leaves in the post-stress recovery phase. The plants were photographed on day 12 after the stress (Fig. 4). All the transgenic plants kept for recovery after severe heat stress appeared noticeably green and healthy. These plants were subsequently monitored for growth and development. Although the development was slightly delayed compared to the unstressed transgenics at ambient temperature, transgenics that were stressed showed marked growth in time. Flowering and seed setting was also recorded in the recovered transgenic plants. The heat-stressed and unstressed transgenics were similar in seed setting.

Protein analysis of plants employed for the thermotolerance assay

To investigate thermotolerance in *MasHSP24.4* transgene-encoded Ma-sHSP24.4 protein levels in control and transgenic plants (under non-induced control conditions, after heat shock and in the post-stress recovery period), Western blot analysis was performed with antibodies against *MasHSP24.4* (Fig. 3). Control (C_2) plants showed no accumulation of MasHSP24.4 protein under both non-induced control conditions as well as upon subjecting seedlings to 45 and 50 °C heat stress. The transgenic plants 12, 15 and

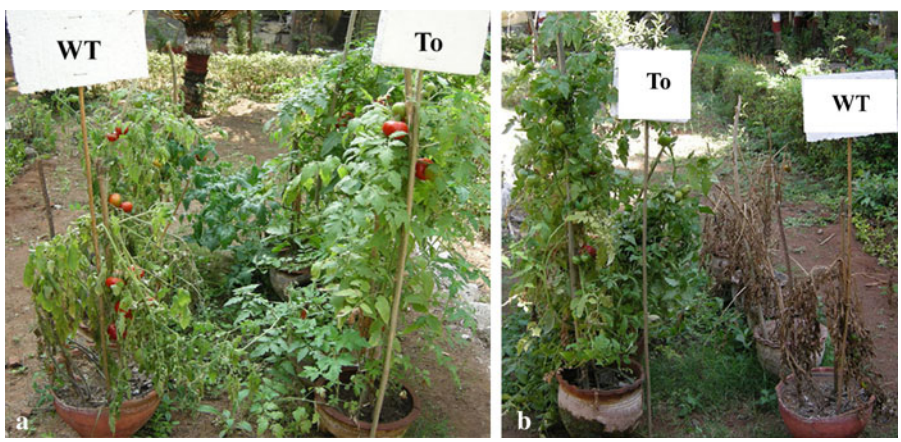

Fig. 4 Dehydration tolerance of 8-week-old WT and transgenic (T0) tomato lines. **a** Field trails of WT and T0 tomato plants after 8 days of assay. **b** Field trails of WT and T0 tomato plants after 15 days of assay

45 exhibited comparable high-level accumulation of MasHSP24.4 in non-induced and high-temperature-induced plants. Compared to 45 °C, induction of *MasHSP24.4* gene in control and transgenic plants at 50 °C showed much less recovery (Fig. 3). Further, transgenics subjected to 50 °C heat stress for 40 min were analyzed for the presence of MasHSP24.4 protein after 25 days of recovery. The *MasHSP24.4*-encoded MasHSP24.4 protein was observed as a strong band in all the plants that were examined (Fig. 3).

Abiotic stress tolerance of transgenic T1 tomato seedlings harboring *MasHSP24.4* gene

Dehydration stress tolerance

The dehydration stress response of the wild type and T1 transgenic plants was examined at seedling stage and also at mature stage using the leaf-disc senescence assay and by withholding water at the whole plant level among the greenhouse-grown plants. To assess the drought stress affecting plant growth and development, we conducted one set of pre-trial assays during the summer season (March–May). The plants grown during March–May were not healthy and vegetative growth and height was greatly affected and decreased by half (45–50 cm). A lower flowering percentage followed by decreased fruit yield (two fruits) was observed. Seed setting was also much less due to high temperature and dehydration stress. This is the critical observation during the mid-summer, whereas the plants showed healthy growth and development during January–February and all the plants showed better growth up to 110–112 cm height and had set stable flowering and fruiting with 4–6 fruits per branch and all were found to be set with fertile seeds.

Drought tolerance

For drought tolerance assay, we measured the water-withholding capacity of the control plants to assess the plant growth and development during mid-summer of Indian Territory conditions (35–50 °C) in the research field by altering the water supply. In this experiment, we assayed three tomato lines (25 plants each) by keeping one line as a positive control (with continuous water supply), the second line was watered at 3-day intervals and the third line was kept as a negative control (under non-water supply).

After 2 weeks of drought assay, the positive control plants (water-holding) were found to be healthy with normal flowering and seed set whereas the negative control plants (water-withholding) were completely wilted and died. However, in the positive control plants growth was affected (45–50 cm) because of temperature (stress) increase and fluctuations in comparison to the plants grown during winter (110–120 cm in height). In contrast to the above, all the plants (positive/negative controls) which were watered every 3 days withered in the gap days and maintained healthy re-growth after receiving water. A 1-week period without water was considered as the maximum for plants' non-growth if water can then be supplied, and a 3-day gap period was considered as the plants' recovery interval for further growth, though plants will grow if water can be supplied but will not re-grow if water can only be supplied after more than 1 week.

Based on the above observations, for the drought tolerance (dehydration stress tolerance) assay, WT and transgenic plants (lines 4 and 16) grown for 8 weeks in pots containing soil were treated by withholding water prior to growth and flowering for 4 weeks. Watering of plants was resumed after 4 weeks and recovery was monitored. The control plants (WT) did not grow healthy and withered after 3 days and subsequently wilted and died after 1 week of no water. In contrast, the tomato transgenic lines were not withered after 3 days and even after 1 week they began re-growth on being watered and showed flowering and fruit set with viable seeds (Fig. 4).

Discussion

Our results indicate that the developed transgenic cultivated tomato cv. PKM1 is tolerant to high-temperature stress through overexpression of the *MasHSP24.4* gene. Similarly, the positive role of sHSPs in enhanced tolerance against high-temperature stress has been reported in different plant species (Mahmood et al. 2010). More recently, Liping et al. (2012) reported that *ZmHSP16.9*, a cytosolic class I small heat shock protein in maize, confers heat tolerance in transgenic tobacco, as in the present investigation. We established that the transgenic

Fig. 5 T1 and T2 transgenic tomato cv. PKM1plants in the field. **a** and **c** T1 transgenic plants with subsequent fruit setting. **b** and **d** T2 transgenic plants with fruit setting and vertical section of a fruit, respectively

tomato plants ectopically expressing the *MasHSP24.4* gene conferring enhanced tolerance to high-temperature stress (HS) provides evidence that this banana sHSP functions during various abiotic stress responses (data not shown).

Interestingly, it was also observed that the constitutive expression of *MasHSP24.4* did not have any detrimental effect, since transgenic plants showed growth, development, flowering and seed setting comparable to control plants (WT) (Figs. 4, 5). Molecular analyses of transgenic plants carried out over the three consecutive generations (T0, T1 and T2) confirmed the transgene's stability and strong expression. Genetic analysis of the three lines (12, 15 and 45) tested in this study demonstrated Mendelian inheritance (3:1) of the transgene.

According to our present investigation, all transgenic tomato plants survived at high temperature (45–50 °C), exhibiting best growth during the subsequent recovery at 28 °C, whereas all WT plants died. These tests revealed that *MasHSP24.4* imparts basal high-temperature tolerance, possibly by acting in the post-stress recovery period. Hsp100 proteins have previously been described to exert their effect in a similar fashion (i.e., in the post-stress period) in yeast (Parsell et al. 1994; Agarwal et al. 2002) and *Arabidopsis* (Queitsch et al. 2000).

Based on protein expression studies, the constitutive overexpression of *MasHSP24.4* provided the improved thermotolerance capacity in the transgenic plants (Fig. 3), while similar sHSPs in rice such as *Oshsp17.3*and *Oshsp16.9* were constitutively overexpressed to enhance heat tolerance (Guan et al. 2003; Yeh et al. 1995; Malik et al. 1999; Murakami et al. 2004).

These studies indicate that sHsp24.4 provides protection against heat-induced damage at the cellular level, controlling protein metabolic machinery. Therefore, we expect that the overexpression of *MasHsp24.4* could protect the transgenic tomato plants regardless of their developmental stage.

We observed that under severe drought conditions, WT plants were severely affected in plant reproduction with no well-developed flowers, whereas transgenic plants had set fruits and viable seeds (Fig. 5) indicating the protective role of low-molecular weight sHSPs (sHSP24.4) at high temperatures and drought conditions. This might be due to the apparent antioxidant properties of the sHSP as suggested by our results from abiotic stress treatments. Based on these analyses, we conclude that *MasHSP24.4* as molecular chaperone may enhance plant stress tolerance through the protection of antioxidative enzymes and the reduction of reactive oxygen species.

For the first time, we have developed a high-temperature-tolerant transgenic tomato cv. PKM1 by overexpression of MasHSP24.4 protein. The increased high temperature tolerance may enable changing the seasons of tomato planting as desired to suit cultivation of preceding or succeeding crops, or extending the cultivation of tomato to areas that are currently not suitable due to constraints of high-temperature sensitivity. Considering the drastic increase in the global temperature due to increased concentration of greenhouse gases, these transgenic tomato plants may prove useful for the present need.

Acknowledgments Upender Mahesh is grateful to Andhra Pradesh Netherlands Biotechnology Research Programme sanctioned by Ministry of External Affairs, The Netherlands (BTU/PhD/Fellowship/2004-05/1372) for providing Junior and Senior Research Fellowships, and Department of Science and Technology, New Delhi, India for Young Scientist Fellowship and UGC for awarding Dr. S. Kothari Post-Doctoral Fellowship, India.

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