Containment evaluation, cold tolerance and toxicity analysis in *Osmotin* transgenic tomato (*Solanum lycopersicum* L. cv. Pusa Ruby)

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Abstract

The present study reports engineered cold tolerance and toxicity analysis in genetically modified tomato (*Solanum lycopersicum* L. cv. Pusa Ruby) developed through constitutive over expression of *Nicotiana tabacum Osmotin* gene. Rate of seed germination, seedling establishment and growth remained unaffected in the transgenic tomato in response to a low temperature (15 °C) treatment, but were significantly ($P \leq 0.05$) reduced in the wild type. At reproductive stage, the wild type plants failed to recover at the low temperature (4.0 °C) treatment for 10 days but the transgenic plants survived successfully without any leaf senescence or other visible chilling injury symptoms. The quantitative transcript expression analysis confirmed up regulation of the transgene by 55% in the transgenic plants on cold treatment for 2 h whereas, the transcripts were not detected in the wild type. Containment evaluation under normal environmental conditions revealed similar morphology in both the transgenic and wild type tomato plants however an average fruit yield was higher in the transgenic plants (725.91 ± 39.27 g) than the wild type (679.84 ± 28.80 g). The composition of mature fruits in terms of element content was at par in both the transgenic and wild type except significantly higher Ca and Mg contents in the transgenic fruits than that of the wild type. Further, acute and sub-acute toxicity tests conducted in the adult female Wister rats revealed no mortality or significant changes in general and psychological behaviour, at par food intake and body weight and, normal biochemical, and hematological parameters for animals fed with the wild type or transgenic tomato fruits as compared to the control group, confirming its safety for animal consumption.

Keywords Genetic engineering · Cold stress · Germination · TaqMan[®] probe · Toxicity

Introduction

Environmental stresses such as extreme temperature, salinity and drought severely affect agriculture thereby posing a great challenge for ensuring food security globally. An exposure to low temperature stress is associated with a series of morphological, physiological, biochemical and molecular changes that negatively influence survival, growth and productivity in sensitive plants (Goyary 2009; Patade et al. 2013). Tomato (*Solanum lycopersicum* L.) being of subtropical origin, its

optimal temperature for greenhouse cultivation and fruit set is 20 °C (Van der Ploeg and Heuvelink 2005). Temperature below the optimal range severely affects the plant growth and fruit yield in tomato (Dominguez et al. 2005; Ntatsi and Savvas 2014). Research efforts to breed low temperature tolerance through conventional crop improvement approaches are limited by the complex polygenic nature of the trait and availability little genetic diversity for the trait. Therefore, genetic engineering techniques, developed to overcome the limitations of the conventional approaches, are being used for improvement in low temperature tolerance in sensitive crop plants. Earlier research involving over expression of *Osmotin* gene in transgenic plants have reported improvement in tolerance against various biotic and abiotic stresses including cold, salt and drought (Goyary 2009; Parkhi et al. 2009; Goel et al. 2010; Das et al. 2011; Subramanyam et al. 2011).

At Defence Institute of Bio-Energy Research (DIBER), *Osmotin* transgenic tomato (*Solanum lycopersicum* cv. Pusa

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Ruby) has been developed with an objective to enhance the cold tolerance in the important vegetable crop and thereby enabling its cultivation in high altitude areas under low temperature conditions (Goyary 2009; Patade et al. 2013). Screening of the independent transgenic lines was carried out in the earlier generations (T2-T8) at various locations including Defence Institute of High Altitude Research (DIHAR), Leh (11,500' asl), India, DIBER Field Station, Auli (9000' asl), India and DIBER Haldwani (1400' asl), India under containment conditions to identify and advance a desired line. The high altitude containment evaluation trials have established better performance of the transgenic line in terms of survival over the wild type. However, the systematic study on cold tolerance at germination and maturity stage, and yield attributes under contained conditions is not reported yet in the *Osmotin* transgenic tomato. Further, safety assessment of food and feed derived from genetically modified crops is of paramount importance for seeking approval for the environmental release in India and other countries. Therefore, the present study was designed to evaluate cold tolerance, yield attributes and bio-safety of the *Osmotin* transgenic tomato.

Materials and methods

Osmotin transgenic tomato

Osmotin transgenic tomato (*Solanum lycopersicum* cv. Pusa Ruby) was developed using *Agrobacterium* mediated genetic transformation method (Sarad et al. 2004). Transgenic nature of the tomato plants was confirmed with polymerase chain reaction (PCR), dot blot, Southern hybridization, real time PCR with hybridization probes and, Kanamycin spray (Sarad et al. 2004; Patade 2012; Patade et al. 2013, 2014). The seven independent transgenic lines were evaluated for morphological and yield attributing characters at different locations (1400–11,500' asl) under containment conditions (Sarad et al. 2004; Goyary 2009). The best performing *Osmotin* transgenic tomato line in T9 generation was used in the present investigation.

Cold tolerance analysis

For cold tolerance analysis at seeds germination stage, transgenic and wild type tomato seeds (20 per replicate) were sown on water saturated filter paper placed in glass Petri dishes. One set containing both the transgenic and wild type was incubated in a cooling incubator with a set temperature of 15 °C for 12 days. Cold treatment at 15 °C for germination was based on results of laboratory experiment with the tomato cultivar Pusa Ruby. Germination process in the tomato cultivar was inhibited in response to cold treatment

at temperatures less than 15 °C. At the end of cold treatment, the Petri dishes were incubated at room temperature to allow the recovery. The control set of the Petri dishes was incubated at a room temperature (25.0 ± 2.0 °C). Data on radical protrusion and opening of cotyledonary leaves was recorded daily for calculating the percentage germination and opening of cotyledonary leaves. Rate of opening of cotyledonary leaves was calculated based on modified Timson's Index (Patade et al. 2012). The observations were recorded up to 20 days after sowing. Fresh weight of transgenic and wild type seedlings (five per Petri dish) exposed to cold treatment was recorded at 15 days after sowing.

For cold tolerance analysis at flowering stage, the transgenic and wild type tomato plants (120 days after sowing) were treated at low temperature (4.0 °C) in a cooling incubator equipped with cool fluorescent light with a photoperiod of 16 h. The treatment was given for 10 days, after which, the plants were incubated in containment and allowed to recover. Survival of the plants was recorded.

Transcript expression analysis of *Osmotin* gene on cold exposure

Leaf samples were harvested from the low temperature (4.0 °C) treated and control transgenic as well as wild type plants at 2 and 24 h of exposure. The samples were immediately frozen in liquid nitrogen and stored at – 80 °C until RNA isolation.

All the glassware and plastic-ware were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma chemicals, USA) for 24 h followed by autoclaving for 1 h and oven drying at 80 °C for 48 h to make RNase free. Total RNA was isolated using TRIZOL reagent (Sigma chemicals, USA) following an earlier optimized protocol (Patade 2012). The quality and quantity of the isolated total RNA were checked with Agilent Bioanalyzer 2100. First-strand cDNA was synthesized from the isolated RNA (1.0 µg) using QuantiTect Reverse Transcription Kit (Qiagen) following the instructions in a user manual. The cDNA was tested for amplification using *18S rRNA* specific primers for 35 cycles.

Osmotin and *18S rRNA* gene specific primers and probes (Table 1) were designed using Primer3 software (Rozen and Skaletsky 2000) based on the available sequences at NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>). The reaction mixture was prepared using 10× PCR buffer, dNTP (10 mM) gene specific primers (10 picomol), Probe (10 picomol), *Taq* DNA polymerase (1.0 U), cDNA as a template and nuclease free molecular biology grade water to make the reaction volume of 50 µl. The thermal cycling program consisted: initial denaturation (95 °C, 5 min), followed by 30 cycles of denaturation (95 °C, 30 s) and primer annealing/extension (60 °C, 30 s). The qRT PCR reactions were carried out in a real time thermal cycler-Max3005P

Table 1 Design of gene specific primers and probes for transcript expression analysis

Primer/probe	Sequence (5'–3')	Length (bp)	T _m (°C)	Amplicon (bp)
<i>Osmotin</i> F	AATGCGCCACGAGGTACTAA	20	60.0	107
<i>Osmotin</i> R	AGGACTCCACCACAGTCACC	20	60.0	
<i>Osmotin</i> Probe (5'-FAM; 3'-TAMRA)	ATGGCACGTGTATGGGGCCG	20	70.0	
<i>18S rRNA</i> F	AGGGACTACGGCCTTTTAGG	20	60.0	157
<i>18S rRNA</i> R	CAGAAGGGGACAATTTCAAAGA	22	60.0	
<i>18S rRNA</i> Probe (5' HEX; 3'TAMRA)	CGCACGCGCGCTACACTGAT	20	70.0	

(Stratagene, Germany). The normalized transcript expression of the *Osmotin* gene was quantified based on Ct values using the $\Delta\Delta$ CT method (Livak and Schmittgen 2001). All experiments were carried out with no template control and negative (-RT) control and repeated thrice.

Containment evaluation and element content analysis

The transgenic as well as wild type tomato plants were evaluated for yield attributing traits under containment house conditions. All the standard bio-safety practices for growing transgenic plants were strictly followed. The dimensions of the containment facility are 55' × 25' × 15' (*l* × *b* × *h*). The frame structure of the containment facility is made of durable galvanized mild steel and is covered with FRP sheets. The facility is equipped with an evaporative cooling system to maintain set temperature. To provide proper physical containment for transgenic evaluation, the facility has double door system and all the outlets including cooling pads and exhaust fans are secured by fitting filter screens of 40 × 40 mesh made of durable plastic. The floor of the containment is tiled. The transgenic and wild type plants (50 each) were grown in earthen pots filled with potting mixture (sand: soil: FYM, 1:1:1). Transgenic nature of the plants was confirmed by an earlier standardized protocol in the laboratory (Patade et al. 2014). Fruits were harvested on maturity and data on the number of fruits, fruit yield and fruit weight per plant were recorded. The fruits were further processed for seeds and the rest of the plant biomass was disposed after autoclaving at the end of the trial. Element content (μg/g FW) in respect of Ca, Mg, Na, K, Mn, Zn, Pb, Cu, Al and Fe in the ripe transgenic and wild type tomato fruits were analyzed using Atomic Absorption Spectrophotometer at Indian Institute of Toxicological Research, Lucknow, India.

Acute toxicity study

Approval for conducting the toxicity study in female Wistar rats was obtained from Institutional Animal Ethics

Committee vide approval number 1306/C/09/CPCSEA/IAEC/08. The acute toxicity study of the transgenic and wild type tomato fruits was carried out as per Organisation of European Cooperation and Development-OECD Guidelines (Test Guideline 420 Acute Oral Toxicity—Fixed Dose procedure). The transgenic and wild type ripe tomato fruits (0.1, 0.5, 1.0, 2.0 g/kg body wt) were fed to adult female Wistar rats (240–260 g body wt, *n* = 5) by an oral route. Saline water (1 ml/rat) was orally administered to control group. All animals were observed for toxic effects and general behavioral changes at an interval of 1, 2.5 and 4 h after administration of transgenic and wild type fruits and then at least once in a day during the whole study period of 14 days. Food intake and body weight were also observed on the 1st day and the last 14th day in all treated animals. Mortality of animals was recorded during the entire study period.

Sub acute toxicity study

Transgenic and wild type ripe tomato fruits (2.0 g/kg body wt) were fed to adult female Wistar rats (220–260 g body wt, *n* = 5) by an oral route continuously for up to 28 days. Saline water (1.0 ml/rat) was administered orally to control group. All animals were observed for toxic effects and general behavioral changes at least once in a day during the whole study period of 28 days. Food intake and body weight of individual animal were recorded on 0, 14th and 28th day prior to administration of test substance. At the end of the experiment, blood samples were collected for hematological and serological examination. Animals were kept under overnight fasting before taking blood samples from the heart in sterilized disposable syringes (22 gauge needles) after anaesthetizing the rat with anesthetic ether. Blood samples were transferred to heparinized and non-heparinized tubes for hematological and serological examination, respectively. At the end of the study, the serum was stored at – 20 °C in a clean vial for further biochemical analysis using semi auto chemical analyzer (RA-50, BAYAR) and specific diagnostic kits from SIEMENS (AUTOPAK), India. The various parameters analyzed were total protein, albumin, cholesterol,

triglycerides, creatinine, alkaline phosphatase, total bilirubin, direct bilirubin, glucose, SGPT, SGOT, LDH, Uric acid, and BUN in all the groups of animals.

The hematological parameters like total count of RBC and WBC, hemoglobin-Hb (%) and Packed Cell volume-PCV (%) were recorded in all groups of animals. Total erythrocytic count and total leukocyte count were determined according to the method of Natt and Herrick (Natt and Herrick 1952) using Hayem blood diluting fluid and WBC diluting fluid, respectively. PCV (%) was analyzed following the earlier reported method (Jain 1986), whereas Hb content (%) was determined using Sahli's hemometer.

Statistical analysis

The treatments and controls of the experiments were replicated five times. CropStat program developed at IRRI,

Table 2 Effect of cold exposure on seed germination and seedling establishment in the *Osmotin* transgenic tomato (T) and wild type (WT)

Treatment	Germination		Opening of cotyledonary leaves
	%	Timson's index	%
WT_Control	82.5b	76.8a	47.5b
WT_Cold	87.5b	55.8b	33.8c
T_Control	93.8a,b	84.3a	83.8a
T_Cold	100.0a	80.8a	88.8a
SE (M) ($n=5$)	3.7	4.3	3.7
5% LSD	11.8	13.7	11.8

The different letters in each column indicate significant differences at $P \leq 0.05$, according to Least Significant Difference (LSD) test. Standard error of the means, SE (M) and LSD values for each parameter are given in the table

Philippines was used for analysis of variance (ANOVA) for experiments laid out in Completely Randomized Design (CRD). Standard error of the means, SE (M) and least significant difference (LSD) values for each parameter were computed with the program. The treatment means were compared by LSD test at a significance level of $P \leq 0.05$.

Results

Cold tolerance analysis

On cold exposure, total seed germination as a radical protrusion was not affected in wild type. However, the rate of germination as a Timsons index was significantly ($P \leq 0.05$) reduced as compared to the control (Table 2). In case of transgenic, the total as well as rate of seed germination remained unaffected on cold exposure unlike in the control. Similarly, the opening of cotyledonary leaves was significantly reduced in wild type on cold exposure than the control whereas, the opening of cotyledonary leaves in transgenic remained unchanged on cold exposure than the control (Fig. 1). Growth on cold exposure, recorded at 15 days after sowing, of wild type seedlings as a fresh weight was significantly lower (0.1 g) than the transgenic (0.2 g).

Analysis of cold tolerance at maturity stage revealed differential post-recovery survival in the transgenic and wild type tomato plants after cold (4.0 °C) treatment for 10 days. All the wild type plants failed to recover the cold treatment and ultimately died (Fig. 2). No chilling injury symptoms were observed in transgenic plants and these plants successfully continued flower-fruit production.

Fig. 1 Effect of cold treatment on seedling development and survival in the transgenic (T9) and wild type tomato plants. Photographs were taken 8 days after sowing

Fig. 2 Effect of cold exposure (4.0 °C) on post-recovery survival in the *Osmotin* transgenic (T9) and wild type tomato plants at reproductive stage. The transgenic and wild type tomato plants were allowed to recover after cold treatment of 10 days

Fig. 3 Quantitative transcript expression analysis using the transgene specific TaqMan[®] probe in leaves of the transgenic (T9) tomato plants in response to cold treatment of 2 and 24 h. The transgene specific transcript expression was detected only in the transgenic plants, but not in wild type. The error bars indicate SE (M). Mean values marked with different letters are statistically significant ($P \leq 0.05$) according to LSD test

Transcript expression analysis of *Osmotin* gene on cold exposure

Quantitative transcript expression analysis with the transgene specific TaqMan[®] probe detected the gene transcripts only in leaves of transgenic but not in wild type plants. In response to cold exposure of 2 h, the transcript expression increased significantly ($P \leq 0.05$) by 60% than the untreated control whereas, on the cold treatment of 24 h, the transcript expression again reduced similar to that of untreated control in transgenic plants (Fig. 3).

Table 3 Evaluation of the *Osmotin* transgenic (T) tomato along with the wild type (WT) under physical containment condition

Average fruit yield per plant (g)	
T	725.91 ± 39.27
WT	679.84 ± 28.80
Average no. of fruits/plant	
T	57.37 ± 3.59
WT	51.87 ± 2.59
Average fruit wt (g)	
T	13.62 ± 0.53
WT	13.52 ± 0.65

Data on yield and the attributing traits were recorded. The values are mean ± standard error

Containment evaluation and element content analysis

The containment evaluation of the *Osmotin* transgenic tomato plants revealed similar morphology to that of the wild type plants. However, data on yield attributing traits suggested better performance of the transgenic plants than the wild type under containment conditions (Table 3). An average fruit yield per plant was higher (725.91 ± 39.27) in the transgenic plants than the wild type (679.84 ± 28.80). The higher fruit yield in the transgenic was associated with the higher number of fruits per plant (57.37 ± 3.59) than the wild type (51.87 ± 2.59). However, the average fruit weight was similar in the transgenic plants and the wild type. Analysis of element from the ripe tomato fruits from both the transgenic plants and the wild type plants revealed at par content except Ca and Mg, the content of which were higher in case of the transgenic tomato than the wild type (Table 4).

Table 4 Data on element content ($\mu\text{g/g}$ FW) analyzed from the wild type (WT) and transgenic tomato (T) fruits

Fruits	Ca	Mg	Na	K	Mn	Zn	Pb	Cu	Al	Fe
WT	5.24	43.76	30.05	1055.95	0.81	3.96	0.44	0.28	0.59	6.12
T	10.42*	41.30*	30.26	1060.65	0.67	1.95	0.53	0.39	0.58	4.70
SE (M) ($n=5$)	0.81	0.44	3.69	14.39	0.08	1.13	0.10	0.02	0.05	2.23
5% LSD	3.64	1.99	16.52	64.46	0.37	5.07	0.44	0.10	0.23	10.00

Mean values marked with * indicate significant differences at $P \leq 0.05$, according to LSD test. Standard error of the means, SE (M) and LSD values for each parameter are given in the table

Table 5 Effects of the wild type (WT) and transgenic (T) tomato on food intake in the experimental animals

Group	Food intake (g)			
	Acute toxicity study		Sub-acute toxicity study	
	1st day	14th day	1st day	28th day
C	16.67a	17.33a	20.00a	21.75a
T	16.33a	18.33a	18.25a	18.50a
WT	17.00a	18.33a	17.75a	19.25a
SE (M) ($n=5$)	0.85	1.39	0.89	1.06
5% LSD	3.33	5.47	3.06	3.66

The food intake in the treatment groups (WT and T) was compared with the animals in the control (C) group. The mean values marked with the same letter in each column indicate non-significant differences at $P \leq 0.05$, according to LSD test. Standard error of the means, SE (M) and LSD values for each parameter are given in the table

Table 6 Effects of the wild type and transgenic tomato on body weight of the experimental animals

Group	Body weight (g)			
	Acute toxicity test		Sub-acute toxicity test	
	1st day	14th day	1st day	28th day
Control	254.67a	258.33a	240.50a	251.50a
Transgenic	256.33a	258.00a	247.50a	248.50a
Wild type	252.33a	248.33a	229.75a	250.25a
SE ($n=5$)	6.33	6.48	10.81	10.90
5% LSD	24.82	25.38	37.40	37.70

The mean values marked with the same letter in each column indicate significant differences at $P \leq 0.05$, according to LSD test. Standard error of the means, SE (M) and LSD values for each parameter are given in the table

Acute and sub acute toxicity test

In acute and sub acute studies, no mortality and significant changes in general and psychological behaviour of animals fed with the transgenic and the wild type fruits were observed during the entire study period. Further, no significant changes were observed in food intake (Table 5) and body weight (Table 6) of animals in the test groups as

compared to the control group. Moreover, an oral LD_{50} of the transgenic tomato and the wild type tomato was not found up to 2.0 g/kg body weight of the female rat.

In sub acute study, various biochemical parameters were analyzed in animals fed with the wild type and the transgenic tomato as compared to the control group. All the analyzed biochemical parameters (total protein, albumin, cholesterol, triglycerides, creatinine, alkaline phosphatase, total bilirubin, direct bilirubin, SGPT, SGOT, LDH, Uric acid and BUN) in the test as well as the control groups were within a normal range (Table 7). Total bilirubin and direct bilirubin contents were significantly higher in the animals fed with the transgenic tomato than the control and the wild type tomato. Albumin content was significantly low in the transgenic tomato fed animals than the control but higher than those animals fed with the wild type tomato. BUN content was significantly higher in the both test groups (transgenic tomato fed and wild type tomato fed animals) than the control. LDH was significantly higher in the animals fed with the transgenic fruits than those fed with the wild type and the control group. Despite the significant changes, all the biochemical parameters remained within a normal range in all the test as well as the control groups of animals (Table 7).

For all the tested hematological parameters (RBC, WBC count, hemoglobin and PCV), no significant changes were observed in the test group and the control group. These parameters were found within normal range in the blood samples of all the groups (Table 8).

Discussion

Being of the subtropical origin, temperature below the optimal range severely affects the plant growth and fruit yield in tomato (Domínguez et al. 2005; Ntatsi and Savvas 2014). In the present study, Timson's index, a measure of rate or velocity of germination, was reduced significantly in response to cold treatment in wild type tomato seeds indicating inhibition of the process. Further, the seedling establishment recorded in terms of opening of cotyledonary leaves and growth of seedlings was also significantly reduced in wild type tomato on cold exposure. As against, the rate of seed germination, seedling

Table 7 Effects of the wild type and transgenic tomato on biochemical parameters in the experimental animals

Parameter	Control	Transgenic	Wild type	SE ($n=5$)	Normal range
Total protein (g/dl)	8.08 ^a	9.20 ^a	7.78 ^a	0.54	5.0–8.5
Albumin (g/dl)	3.10 ^a	1.18 ^b	0.80 ^b	0.40	3.5–5.0
Alkaline phosphatase (U/L)	171.00 ^a	170.75 ^a	229.25 ^a	62.74	60–170
Creatinine (mg/dl)	0.65 ^a	0.68 ^a	0.58 ^a	0.08	0.5–1.1
Uric acid (mg/dl)	3.20 ^a	3.33 ^a	3.35 ^a	0.67	3.0–8.5
Cholesterol (mg/dl)	76.80 ^a	87.63 ^a	84.63 ^a	6.35	Below 200
Triglyceride (mg/dl)	21.45 ^a	41.07 ^a	31.70 ^a	9.63	Below 150
LDL (mg/dl)	22.21 ^a	25.30 ^a	13.52 ^a	7.21	Below 120
HDL (mg/dl)	27.95 ^a	32.70 ^a	28.20 ^a	6.04	20–70
Total bilirubin (mg/dl)	0.60 ^b	1.16 ^a	0.53 ^b	0.14	0.5–1.2
SGPT (U/L)	14.75 ^b	20.50 ^b	35.75 ^a	4.19	Below 40
SGOT (U/L)	102.25 ^a	98.75 ^a	106.50 ^a	14.67	Below 150
Direct bilirubin (mg/dl)	0.13 ^b	0.33 ^a	0.13 ^b	0.06	0.0–0.6
BUN (mg/dl)	20.33 ^b	26.80 ^a	27.35 ^a	1.55	–
Glucose (mg/dl)	92.68 ^a	87.48 ^a	80.65 ^a	11.75	70–110
LDH (U/L)	620.25 ^{a,b}	749.50 ^a	266.25 ^b	125.82	420–770

The values are mean of five replicates. The mean values marked with the different letters for a parameter indicates significant differences at $P \leq 0.05$, according to LSD test

Table 8 Effects of the wild type and transgenic tomato on hematological parameters in the experimental animals

Group	PCV %	Hb (gm %)	RBC (10^6 cells/mm ³)	WBC (10^3 cells/mm ³)
Control	44.25 ^a	14.63 ^a	8.56 ^a	11.10 ^a
Transgenic	44.00 ^a	14.25 ^a	8.60 ^a	12.28 ^a
Wild type	45.00 ^a	14.75 ^a	8.79 ^a	10.95 ^a
SE ($n=5$)	1.29	0.53	0.29	0.55
5% LSD	4.46	1.82	1.00	1.90

The values are mean of four replicates. The mean values marked with the same letter for a parameter indicate non-significant ($P \leq 0.05$) differences, according to LSD test. Standard error of the means, SE (M) and LSD values for each parameter are given in the table

establishment and growth remained unaffected in the transgenic tomato in response to the cold treatment. In response to the cold treatment at the reproductive stage, the wild type plants failed to recover and ultimately died whereas chilling injury symptoms were not seen in the transgenic tomato plants and these plants successfully produced fruits normally. Thus, the results confirmed the enhanced cold tolerance in the transgenic tomato plants at germination as well as reproductive stages. The cold tolerant response in the transgenic tomato was associated with significantly increased transcript expression of the transgene (*Osmotin*) than the untreated control on cold exposure of 2 h, whereas, on the cold treatment of 24 h, the transcript expression again reduced similarly to that of untreated control. The transgene specific transcripts were detected only in the leaves of the transgenic plants, but

not in wild type using the specific TaqMan[®] probe. Thus, the results suggest that the cold tolerant response in transgenic tomato plants is the resultant of over expression of the *Osmotin* gene. Further, to assess the performance in terms of yield attributing traits, the transgenic tomato plants were evaluated under containment conditions. The transgenic tomato plants showed similar morphological features to that of wild type plants. However, data on yield attributing traits revealed higher fruit yield per plant in transgenic than the wild type. The higher fruit yield in transgenic plants was associated with the higher number of fruits per plant than the wild type as the average fruit weight was similar both in transgenic and the wild type. The seeds obtained from the ripe fruits were viable in both transgenic and wild types, as tested by the germination test. An earlier study on over-expression of tobacco *Osmotin* have shown enhanced salt and drought tolerance in transgenic tomato (Goel et al. 2010). Another study by Weber et al. (2014) reported enhanced drought tolerance through over expression of an osmotin-like protein from *Solanum nigrum* in transgenic soybean. Similarly, tolerance to salinity as well as fungal stress has been reported in *Osmotin* transgenic soybean (Subramanyam et al. 2012). In response to the biotic and abiotic factors, plants universally appear to suffer from osmotic and oxidative stresses (Mantri et al. 2010). The over expression of the *Osmotin* gene in plants is known to modulate transcript abundance and functional expression products of the other stress responsive genes involved in the stress alleviation functions such as osmotic adjustment and anti-oxidant defence (Patade et al. 2013). This could be the

reason for cross-stress protection against abiotic (salt, drought, cold) as well as biotic factors in the *Osmotin* transgenic plants. Thus, the *Osmotin* transgenic plants are likely to perform better under different environmental conditions than the wild types without compromising the growth and yield.

To confirm composition equivalence of the transgenic fruits, element contents from the ripe fruits were analyzed using Atomic Absorption Spectroscopy from both the transgenic and wild type. The results confirmed at par content of all the analyzed elements except Ca and Mg, the content of which was higher in case of transgenic tomato than the wild type. Thus, the element analysis revealed similar composition of the ripe fruits from the transgenic plants to that of the wild types. Safety assessment of food and feed derived from genetically modified crops is of paramount importance for seeking approval for the environmental release in India under “Rules for the Manufacture, Use, Import, Export and Storage of Hazardous Microorganisms/Genetically Engineered Organisms or Cells 1989” (Rules, 1989) notified under the Environment (Protection) Act, 1986. In the present study, acute and sub-acute toxicity tests were carried out in laboratory animals following the protocols prepared by Department of Biotechnology, Ministry of Science and Technology, Government of India based on OECD guidelines. No significant changes pertaining to the general and psychological behaviour, food intake and body weight were observed in animals fed with either transgenic or wild type tomato fruits as compared to that of the control. Triglyceride, total bilirubin, direct bilirubin, cholesterol, creatinine and LDH contents were slightly decreased in animal fed with tomato fruits either transgenic or wild types, as compared to the control group. Slight increase in uric acid, total protein, BUN and glucose levels was observed in treated animals than the animals in control group. These statistically significant differences among the test (animals fed with transgenic/wild type tomato) and the control groups may be due to mere chance and may not be biologically relevant as all the parameters remained within normal range in all groups of the animals. Further, hematological parameters viz. RBC, WBC, Hb, and PCV were found within a normal range in the blood of animals from all the groups. Thus, the acute and sub-acute toxicity tests confirmed the safety of the transgenic tomato fruits for an oral consumption in the experimental animals. As the toxic proteins generally exert their effect at low dosages and in a short time frame, the acute toxicity tests have been considered adequate for evaluating potential toxicity of the food/feed derived from genetically modified crops (EPA 2000). To the best of our knowledge, this is the first report of its kind revealing the safety of the *Osmotin* transgenic tomato fruits based on acute and sub-acute toxicity tests in the experimental animals.

Conclusions

The results of the study confirmed engineered cold tolerance at germination as well as reproductive stage in tomato through constitutive over-expression of the *Osmotin* gene, without any yield penalty. The cold tolerance may be attributed to the induced transcript expression of the *Osmotin* gene or changes thereof. Further, the toxicity analysis confirmed the safety of the transgenic fruits in laboratory animals as body weight, food intake, behaviour, serological and hematological parameters were within a normal range. Hence, the present study revealed engineered cold tolerance and food safety in the *Osmotin* transgenic tomato.

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Author contributions VYP conceived and designed the study. VYP, AG and SMG carried out cold tolerance analysis, containment evaluation, transcript expression and statistical analysis. HSM conducted toxicity tests. First draft of the manuscript was prepared by VYP, with inputs from HSM, AG and SMG. MN arranged funds and approved final version of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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