Transgenic Res (2015) 24:859–873
DOI 10.1007/s11248-015-9881-9

ORIGINAL PAPER

Ectopic expression of GroEL from *Xenorhabdus nematophila* in tomato enhances resistance against *Helicoverpa armigera* and salt and thermal stress

Punam Kumari · Gagan Kumar Mahapatro · Nirupama Banerjee · Neera Bhalla Sarin

Received: 7 January 2015 / Accepted: 29 April 2015 / Published online: 10 May 2015

Abstract The GroEL homolog XnGroEL protein of *Xenorhabdus nematophila* belongs to a highly conserved family of molecular chaperones/heat shock proteins (Hsps). XnGroEL was shown to possess oral insecticidal activity against a major crop pest *Helicoverpa armigera*. Under normal conditions, the Hsps/chaperones facilitate folding, assembly, and translocation of cellular proteins, while in stress conditions they protect proteins from denaturation. In this study, we describe generation of transgenic tomato plants overexpressing insecticidal XnGroEL protein and their tolerance to biotic and abiotic stresses. Presence of XnGroEL in the transgenic tomato lines conferred resistance against *H. armigera* showing 100 % ($p \leq 0.001$) mortality of neonates. In addition, XnGroEL provided thermotolerance and protection against high salt concentration to the tomato plants. Expression of XnGroEL minimized photo-oxidation of chlorophyll and reduced oxidative damage of cell membrane system of the plants under heat and salt stress. The enhanced tolerance to abiotic stresses correlated with increase in the anti-oxidative enzyme activity and reduced H_2O_2 accumulation in transgenic tomato plants. The variety of beneficial properties displayed by XnGroEL protein provides an opportunity for value addition and improvement of crop productivity.

Keywords *Helicoverpa armigera* · Heat stress · Insect resistance · Insecticidal XnGroEL · Salt stress · *Xenorhabdus nematophila*

Introduction

Plants grow and survive in diverse climatic habitats and are often exposed to stressful conditions of both biotic and abiotic nature. Environmental stresses such as salinity, extreme temperatures, drought, chemical toxicity, oxidative stress, and insect infestation are some of the serious threats to agriculture and result in crop losses worldwide, reducing average yields of most crop plants by more than 50 % (Wang et al. 2003; Vinocur and Altman 2005). To overcome these limitations and to improve production, it is necessary to develop crop cultivars that are capable of alleviating stress.

Electronic supplementary material The online version of this article (doi:10.1007/s11248-015-9881-9) contains supplementary material, which is available to authorized users.

P. Kumari · N. Banerjee (✉) · N. B. Sarin (✉)
School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India
e-mail: nirupamaban@yahoo.com

N. B. Sarin
e-mail: neerasarin@rediffmail.com

G. K. Mahapatro
Division of Entomology, Indian Agricultural Research Institute, New Delhi 110012, India

In nature, animals, pests, and pathogens thrive on plants as a source of subsistence. Agricultural productivity losses caused by herbivorous insects have been estimated to be 10–20 % for major crops grown worldwide (Ferry et al. 2004; Kumari et al. 2014). Among these herbivorous insect, *H. armigera* is the most destructive species. *H. armigera* is a serious polyphagous pest and has developed resistance to a number of chemical pesticides (Reed and Pawar 1982; Whalon et al. 2004). In India, losses in the yield of tomato crop due to *H. armigera* alone amount to 22–38 % (Dhandapani et al. 2003) or one thousand crore rupees per annum (Padmanaban and Arora 2002; Arora et al. 2012, 2014).

Temperature and salinity are the other major environmental factors that adversely affect crop productivity around the world. The exposure of plants to multiple stress factors results primarily in cell damage, leading to secondary effects, such as, osmotic or oxidative stress (Wang et al. 2003; Mittler 2006; Li et al. 2014). Disruption of cellular homeostasis induced by exposure to these stresses often causes protein dysfunction. Therefore, cells must employ efficient mechanisms to preserve and maintain proteins in their native conformation and prevent aggregation of denatured proteins. One of the universal cellular responses to abiotic stress is induction of chaperone protein (GroEL) and other heat shock proteins (Hsps). These accumulate in both, prokaryotic and eukaryotic cells in response to exposure to various stress conditions (Forreiter et al. 1997; Lee et al. 1997; Heckathorn et al. 1998; Low et al. 2000; Scharf et al. 2001; Kotak et al. 2007) and help the system to endure the environmental distress. As molecular chaperone, GroEL is a key component contributing to cellular homeostasis under both optimal and adverse growth conditions. It is responsible for protein folding, assembly, translocation, and degradation in a broad array of normal cellular processes; it also functions in the stabilization of proteins and membranes, and can assist in protein refolding under stress conditions (Lindquist 1986; Lindquist and Craig 1988). Studies demonstrating overexpression of Hsps in plants conferring tolerance to various stressful conditions have been reported (Song and Ahn 2011; Mu et al. 2013; Ruibal et al. 2013; Personat et al. 2014).

A GroEL homolog XnGroEL is secreted by *X. nematophila*, a Gram-negative bacterium residing in the gut of a soil nematode of the genus *Steinernema* as a symbiont (Akhurst et al. 1993; Forst et al. 1997; Herbert and Goodrich-Blair 2007). The bacteria-nematode association is highly toxic to many insect species and causes rapid larval death. The protein was orally insecticidal to the major crop pest, *H. armigera*. The XnGroEL protein was found to bind to chitin in the peritrophic membrane and cause cessation of growth and development of the larvae (Joshi et al. 2008). In our earlier report, XnGroEL was shown to confer resistance against *H. armigera* in transgenic tobacco (Kumari et al. 2014).

In this investigation, we report the development of transgenic tomato plants by *Agrobacterium* mediated transformation with DNA sequence encoding XnGroEL protein and examine the response of transgenic plants constitutively expressing XnGroEL to various abiotic stresses and the crop pest *H. armigera*. Our results demonstrate that this technology could be expanded to many other crop species to improve their tolerance against environmental stresses. To the best of our knowledge, this is the first report showing that the overexpression of a GroEL homolog from *X. nematophila* confers tolerance to salt and heat stress as well as to insect attack in transgenic tomato plants.

Materials and methods

Construction of expression vector and transformation of tomato

The 1.7 kb gene encoding the 58 kDa XnGroEL protein was isolated from *X. nematophila* genome (Accession no. AY184491) as described in our previous report (Joshi et al. 2008). Our previous study described the cloning of *XnGroEL* gene into a plant expression vector pBI121 (Kumari et al. 2014). The construct was called pBI121-*XnGroEL*. The gene construct was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Hofgen and Willmitzer 1988). The tomato seedlings were raised by germinating seeds of tomato (*c.v*. Pusa Early Dwarf) soaked for 1–2 h in tap water in egg trays containing soil: vermiculite (1:1) under controlled growth conditions. Tomato transformation was performed via *Agrobacterium*-mediated transformation method using cotyledon explants as described by

Madhulatha et al. (2007). After inoculating with *A. tumefaciens*, the plant cultures were maintained at 25 °C under a 16 h photoperiod. After 6 weeks, regenerated shoots were transferred to rooting medium. The plantlets were watered with Hoagland solution (Hoagland and Arnon 1950) and hardened for 2 weeks before transfer to soil in pots. After hardening, plants were maintained in the glasshouse for flowering and seed set. The temperature of the greenhouse was maintained within a range of 25–30 °C.

DNA isolation, PCR and Southern blot analysis

Tomato genomic DNA was isolated from fresh leaf tissue of transgenic, untransformed control (UC), and vector control (VC) plants using the CTAB method (Rogers and Bendich 1994) and quantitated spectrophotometrically. The presence of the transgene *XnGroEL* in the T_1 transgenic lines was confirmed by PCR analysis using primers: FP 5′-GGATCCATGG-CAGCTAAAGA-3′ and RP 5′-GAGCTCTCACAT-CATGCCG-3′. The amplified products were separated by electrophoresis on a 0.8 % agarose gel.

Southern blot analysis was performed according to the standard procedures (Sambrook et al. 1989). Genomic DNA (20 µg/lane) from UC, VC, and PCR positive plants was digested with *Eco*RI overnight at 37 °C and fractionated on 0.8 % agarose gel, denatured, neutralized, and blotted onto a nylon membrane (Amersham Pharmacia, USA). The probe for the *XnGroEL* gene was amplified from a pBI121 vector containing *XnGroEL* gene by PCR and was labeled with α-[^{32}P] dCTP using a random primer labeling kit (Amersham Pharmacia Biotech, UK) and used for hybridization of the membrane at 54 °C using standard procedures (Sambrook et al. 1989).

RNA extraction and RT-PCR analysis

Total RNA was extracted from the mature leaf tissue of transgenic, UC, and VC tomato plants using the Plant RNeasy extraction kit (Qiagen, Valencia, USA) and RT-PCR amplification was done using an RT-PCR kit (AccuScript, Stratagene, USA) in accordance with the manufacturer's instructions. Total RNA (1 µg) was used for generation of first-strand cDNA using Moloney Murine Leukaemia Virus-Reverse Transciptase (MMLV reverse transcriptase). The gene-specific primers used for amplification of *XnGroEL* cDNA were as described above and primers for tomato actin gene (FP 5′-TGGACTCTGGT-GATGGTGTC-3′ and RP 5′-CCTCCAATCCAAA-CACTGTA-3′) were used as internal control. The RT-PCR products were visualized after electrophoresis on 1.2 % agarose gel.

Western blot analysis

Mature leaves (100 mg fresh weight) from transgenic, UC, and VC tomato plants were ground to a fine powder in liquid nitrogen, homogenized in 200 µl protein extraction buffer (20 % v/v glycerol, 62.5 mM Tris-HCl, pH 6.8, 5 % v/v 2-mercaptoethanol, 0.1 % w/v SDS, plant protease inhibitor cocktail; G Biosciences, St Louis, MO, USA) and centrifuged at 13,000 rpm for 15 min at 4 °C. Protein concentration was estimated in the supernatant by the method of Bradford (1976). The samples were fractionated on a 12 % SDS-PAGE (Sambrook et al. 1989) and transferred to nitrocellulose membrane (Towbin et al. 1979) using a semi-dry transfer system (Owl Separation Systems, UK) according to the manufacturer's instructions. After blocking, the membrane was immunoblotted with rabbit antisera against XnGroEL (1:10,000 dilution) for 3 h (Joshi et al. 2008; Kumari et al. 2014) and secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (1:10,000 dilution; Sigma-Aldrich) for 1 h. The membrane was developed with BCIP/NBT (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine and nitro-blue tetrazolium chloride). All the dilutions of primary and secondary antibodies were made in TBST containing 1 % BSA.

Insect bioassay of transgenic plants against *H. armigera*

H. armigera cultures were reared on a semi-synthetic diet (Gupta et al. 2010). Young leaves of three transgenic (Xn2, Xn3, and Xn4), UC and VC plants were selected for the insect bioassays. The detached leaves were briefly washed with sterile distilled water, dried and placed on moist Whatman no.1 filter paper discs in petri dishes. Ten newly hatched larvae (neonates) of *H. armigera* were released on a leaf of transgenic, UC, and VC plants, respectively. Three replicates for each transgenic line, UC, and VC plants were set up. The plates were sealed with parafilm and

maintained for 5 days in a controlled environment growth chamber at 27 ± 2 °C (16 h light) with 75 % relative humidity. Larval mortality, leaf damage, weight scores, and pupation rate were recorded. For leaf damage, every leaf was assessed for percent damage using a scale 1–9 (where 1 equals to 10 % damage, 2 equals 11–20 % damage and so forth). The experiment was carried out three times under identical conditions.

Leaf disc senescence assay and measurement of chlorophyll content

Leaf discs of 1 cm diameter were excised from healthy and fully expanded tomato leaves of similar age from transgenic, UC, and VC plants and kept in half strength Hoagland solution containing NaCl (200, 400, and 600 mM), mannitol (200, 400, and 600 mM), and $CdCl_2$ (10, 20, and 40 mM), respectively, for 3–4 days. For cold stress, leaf discs were exposed to 4 °C for 5 days while heat stress was given for 10 h at 42 °C. Leaf discs kept in half strength Hoagland solution were taken as control. The chlorophyll content was measured spectrophotometrically after extraction in 80 % acetone (Arnon 1949). The experiment was repeated thrice with three different transgenic lines.

Testing transgenic tomato plants under salinity and heat stress

The 2-weeks-old seedlings were transferred to plastic pots and maintained under 16 h light/8 h dark and 25 °C, either in absence or presence of 200 mM NaCl for 2 weeks. The transgenic plants were also exposed to a high temperature of 42 °C for 16 h. Three plants from each transgenic, UC, and VC lines were taken for this analysis.

Biochemical analysis of XnGroEL transgenic lines

Estimation of relative water content (RWC), lipid peroxidation, electrolytic leakage, Fv/Fm ratio, and hydrogen peroxide (H_2O_2) was carried out as described below. Estimation of various antioxidant enzymes, including superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) was performed following the well-established standard protocols.

Measurement of relative water content (RWC)

The procedure of Barr and Weatherley (1962) was followed for determination of RWC, fresh leaves were detached and weighed immediately to record fresh weight (FW), followed by dipping them in distilled water for 12 h. The leaves were then blotted to wipe off excess water, weighed to record turgid weight (TW) and subjected to oven drying at 70 °C for 24 h to record dry weight (DW). The RWC was determined by the equation:

$$\mathrm{RWC} = (\mathrm{FW} - \mathrm{DW}) \times 100/(\mathrm{TW} - \mathrm{DW})$$

Measurement of MDA content

The malondialdehyde (MDA) content in leaves was measured according to the protocol described by Heath and Packer (1968). The tomato leaves were ground to a fine powder in liquid nitrogen. 3 ml of 10 % trichloroacetic acid was added to 0.2 g of the powder and left at 4 °C overnight. After centrifugation at $1000 \times g$ for 20 min, the supernatant was transferred to a new tube for measurements. 2 ml of 0.6 % thiobarbituric acid (TBA) was added to 2 ml of the supernatant. The mixture was vortexed thoroughly, heated in boiling water for 15 min, cooled immediately and centrifuged. Absorbance values of the supernatant were detected at wavelengths of 532 and 450 nm using water as blank. The MDA content was calculated using the following formula:

$$\text{MDA content } (\mu\text{mol/g fresh weight}) = 6.45 \times \mathrm{OD}_{532} - 0.56 \times \mathrm{OD}_{450}$$

Measurement of electrolyte leakage

Electrolyte leakage was determined according to the method described by Sairam and Srivastava (2002). Leaves were collected and washed with deionized water thrice in order to remove electrolytes adhering to the surface. They were then placed in test tubes and immersed in 10 ml of deionized water and the electrical conductivity (EC1) was measured. After incubation at 55 °C for 30 min, the EC2 was determined again. The samples were heated in boiling water for 1 h before the total conductivity was measured in the solution (EC3). Relative ion leakage

was expressed as a percentage of the total conductivity:

$$\text{Relative Electrolyte Leakage} = [(EC2 - EC1)/EC3] \times 100$$

Fv/Fm ratio

Photosynthetic efficiency of tomato plants was determined by measuring the chlorophyll fluorescence. Chlorophyll fluorescence from the adaxial side of the leaf was monitored using a portable chlorophyll fluorometer (Handy-PEA, Hansatech Instruments, Ltd., UK). Photochemical efficiency of leaves, as determined by chlorophyll fluorescence ratio (Fv/Fm), was monitored before and after salt and heat treatments. Measurements were taken during the light cycle on the leaves using the saturation pulse method after 30 min of dark adaptation (white light 8000 μmol/m^2 s^{-1} for 0.8 s).

In planta histochemical detection of O_2^- and H_2O_2

Accumulation of O_2^- and H_2O_2 was examined based on histochemical staining by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively, as described earlier (Kumar et al. 2013). For detection of O_2^-, the leaves from UC, VC, and XnGroEL-expressing plants grown under normal and stress conditions, respectively were picked and floated in 50 mM sodium phosphate (pH 7.5) containing 0.2 % NBT. The dark blue insoluble formazan compound formed by reaction of NBT with O_2^- was visualized and photographed. For detecting H_2O_2, the leaves were immersed overnight in a solution of DAB (1 mg ml^{-1}, pH 3.8). Thereafter, chlorophyll was removed by boiling in ethanol for 10 min and the reddish-brown coloration denoting the H_2O_2 content was photographed.

Estimation of H_2O_2 content

The accumulation of H_2O_2 in leaves was determined using the method of Velikova et al. (2004). Frozen leaf tissue (0.5 g) was ground to a fine powder in the presence of liquid nitrogen and 5 ml of 0.1 % (w/v) TCA was added to it. The homogenate was centrifuged at 12,000×*g* for 15 min at room temperature. To 5 ml of the supernatant obtained, 0.5 ml of 1 M potassium phosphate buffer (pH 7.0) and 1 ml of potassium iodide (1 M) were added. The absorbance of the mixture was taken at 390 nm after vortexing. The amount of H_2O_2 was calculated using the equation:

$$H_2O_2(\mu mol/g \text{ fresh weight}) = 1 + 227.8 \times OD_{390}$$

Measurement of the activities of antioxidant enzymes

Two-weeks-old plants were treated with 200 mM NaCl for 2 weeks. For heat stress, 2-weeks-old plants were kept at 42 °C for 16 h after which the leaves were harvested. The specific activities of various antioxidant enzymes in the leaves of UC, VC, and transgenic plants were determined following the well established standard protocols.

Superoxide dismutase (SOD) activity

SOD activity was assayed by measuring the inhibition of photochemical reduction of NBT spectrophotometrically at 560 nm using the procedure described by Gupta et al. (1993). A 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 μM methionine, 63 μM NBT, 1.3 μM riboflavin, and 100 μl of the enzyme extract. Riboflavin was added last and the tubes were shaken and placed 30 cm below two 15-W fluorescent tubes for 10 min. One unit of SOD activity is defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT.

Ascorbate peroxidase (APX) activity

APX activity was determined using the method of Chen and Asada (1989). Leaves were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate, 10 % glycerol, and 1 mM EDTA. The activity was determined in 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM ascorbate, 0.3 mM H_2O_2, and 50 μl of the enzyme extract. The decrease in absorbance of the reaction mixture was recorded at 290 nm. Enzyme activity was expressed as units per milligram of protein. One unit of APX activity is defined as the amount of enzyme required to reduce 1 μmol of H_2O_2 min^{-1} under the assay conditions.

Catalase (CAT) activity

The activity of CAT was measured following the procedure of Aebi (1984). The assay mixture contained 3.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 100 µl of enzyme extract in a total volume of 3 ml. The CAT activity was estimated by a decrease in absorbance of H_2O_2 at 240 nm. One unit of CAT is defined as the amount of enzyme dismuting 1 nmol of H_2O_2 per minute.

Statistical analysis

All the experiments were performed at least thrice independently. For bioassay and various growth parameters of the UC, VC, and XnGroEL T_1 transgenic plants, values are presented as means of three replicates. Here, the 'mean of three replicates' represents the 'mean of three independent plants'. The results were assessed by Student's t test. Significance was defined as $p \leq 0.05$ (*) and $p \leq 0.001$ (**).

Results

Development and characterization of tomato plants transformed with *XnGroEL*

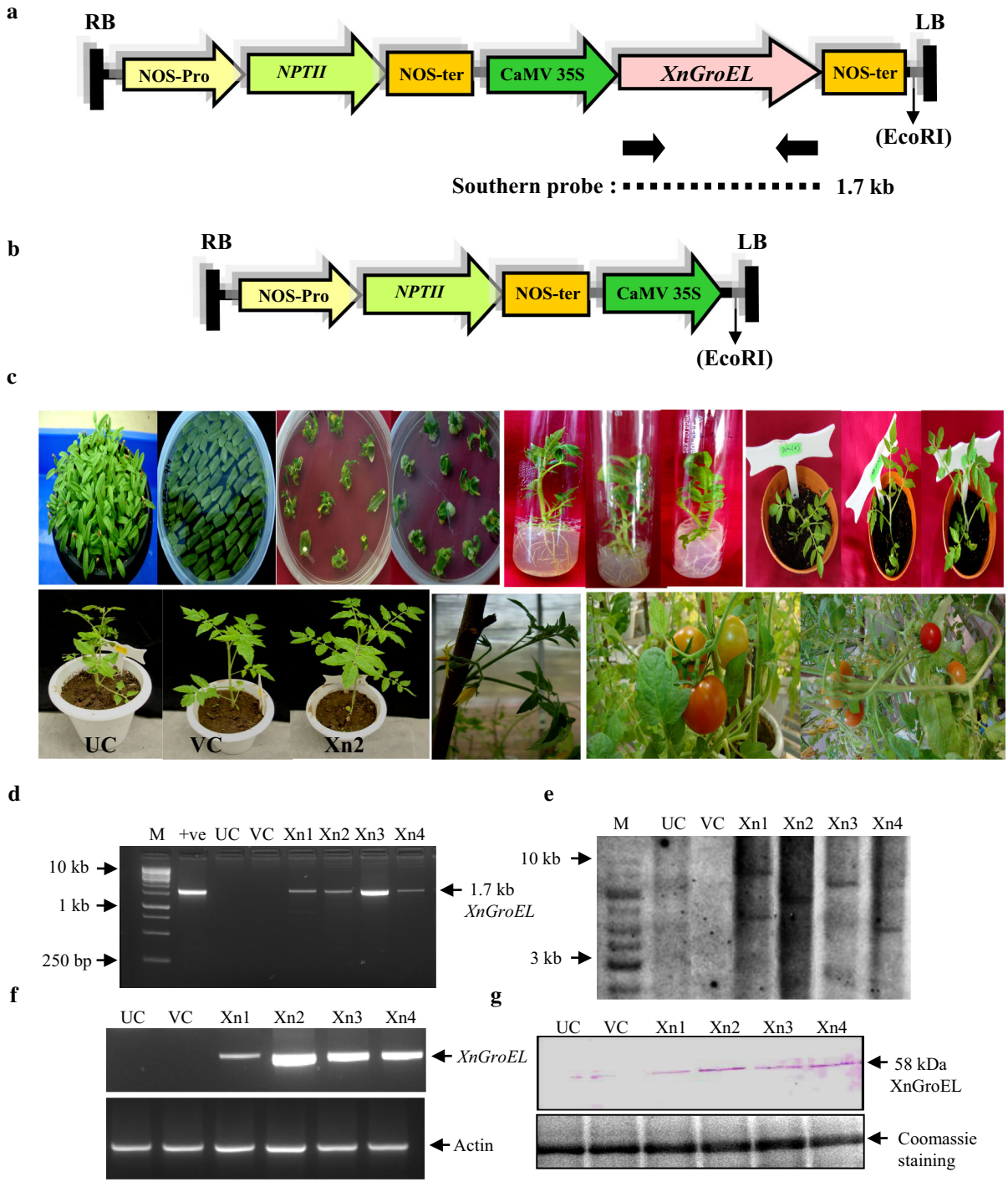
The tomato lines (Xn1, Xn2, Xn3, and Xn4) were produced by incorporating the DNA sequence encoding XnGroEL (Fig. 1a) in the genome. The vector control plants were also regenerated with a vector control construct (Fig. 1b). Phenotypically there were no significant differences amongst the vector control (VC) and the transformed lines (Xn1–Xn4), as compared with the untransformed control (UC) plants. The transformed plants grew normally in the glasshouse where they flowered and produced seeds. Seeds from T_0 generation of transformed plants were harvested (Fig. 1c) and presence of the *XnGroEL* in the T_1 transgenic tomato plants was confirmed by PCR using gene-specific primers (Fig. 1d).

Southern blot analysis of genomic DNA from T_1 transformed plants confirmed integration of the transgene in the tomato plants (Fig. 1e). In the lines Xn2, Xn3, and Xn4, a single-copy transgene insertion was observed, while in Xn1 line two copies were found to be integrated in the genome. The RT-PCR analysis showed that mRNA of *XnGroEL* was expressed in the transgenic lines, but not in the UC and VC plants (Fig. 1f). Expression of XnGroEL protein in transgenic tomato plants was demonstrated by western blot analysis using anti-XnGroEL antibody (Fig. 1g). Western blot analysis confirmed the presence of a single polypeptide of predicted molecular weight of 58-kDa protein reacting positively with the antibody. A very faint band corresponding to the XnGroEL protein was detected in the UC and VC plants. We assume that the polyclonal antibodies might have cross-reacted with the endogenous Hsps as they are conserved in nature.

Fig. 1 Tomato transformation and molecular analyses of T_1 ▶ transgenic plants. **a** Schematic representation of the pBI121-*XnGroEL* construct used for tomato transformation. The location of primers used for PCR and RT-PCR is indicated by *arrows*. The region used as probe for Southern blot analysis is also indicated. **b** Schematic representation of the vector control (VC) construct used for tomato transformation. **c** Different stages of regeneration of transformed tomato explants. **d** PCR amplification of a 1.7 kb fragment using *XnGroEL* sequence specific primers, pBI121-*XnGroEL* construct was used as positive control (+ve). **e** Southern blot of genomic DNA from independent tomato transformants (Xn1, Xn2, Xn3, and Xn4). **f** RT-PCR analysis confirming expression of *XnGroEL* gene in young fully expanded leaves of transgenic tomato plants. Actin was used as an internal control (*lower panel*). **g** Western blot of transgenic plants Xn1, Xn2, Xn3, and Xn4 expressing XnGroEL protein. UC and VC plants were used as negative controls. *Lower panel* shows the coomassie stained SDS-PAGE gel for checking the equal loading of proteins

Resistance of transformed tomato plants to *H. armigera*

Biological activity of the XnGroEL protein was tested in detached leaves of transgenic plants. The effect on survival and development of the larvae on transgenic, UC, and VC plants was monitored throughout the course of the assay (Fig. 2). Mortality of *H. armigera* neonatal larvae was 96.7, 67, and 80 % on the leaves of transgenic lines Xn2, Xn3, and Xn4, respectively, compared to 6.7 % in UC and VC plants on day 2. The mortality increased to 100, 90, and 100 %, respectively, in the lines Xn2, Xn3, and Xn4 compared to 17.8 % in UC and VC plants on day 4 (Fig. 2a). The increased expression of XnGroEL protein was correlated with reduced insect growth and corresponding reduction in larval induced tissue damage by 90, 70, and 80 %, respectively (Fig. 2b). After 4 days, the



surviving larvae present on the transgenic plants (line Xn3) had remained stunted in growth, whereas those fed on the control plants (UC and VC) had developed to the 2nd instar stage, gained more body mass and resulted in healthy pupae. A reduction in larval weight by ~86 % with corresponding reductions in the rate

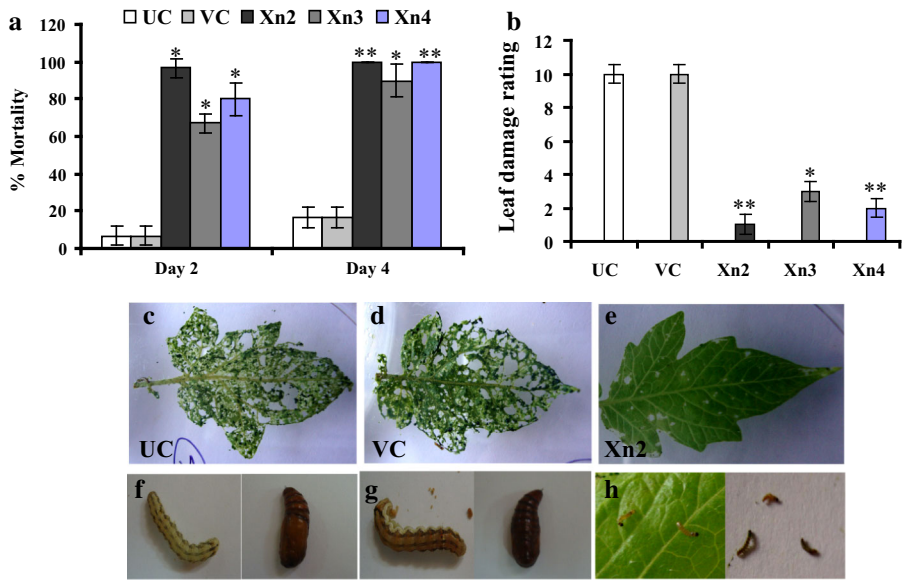


Fig. 2 Insect bioassay: evaluation of toxic effect of XnGroEL protein expressed in transgenic tomato plants on *H. armigera*. **a** Percent mortality on day 2 and day 4. **b** Leaf damage rating. **c** UC and **d** VC leaves showing damage by *H. armigera*. **e** Intact transgenic tomato leaf (Xn2) showing resistance to *H. armigera* attack. **f**, **g** Larvae on leaves of UC and VC plants resulting in development of healthy pupae, respectively. **h** Poor growth of larvae fed on transgenic leaf (Xn2) and photographed on day 4 after larval infestation. *, ** denote significance at $p \leq 0.05$ and $p \leq 0.001$, respectively

of pupation by ~90 % (Table S1) was observed in larvae feeding on the transgenic plants (line Xn3).

Stress tolerance of transgenic tomato plants expressing XnGroEL

Leaf disc senescence assay (see "Materials and methods" section) was used for assessing the tolerance of plants towards various abiotic stresses. This was performed with T_1 plants under salinity (200, 400, and 600 mM NaCl), dehydration (200, 400, and 600 mM mannitol), heavy metals (10, 20, and 40 mM $CdCl_2$), cold (4 °C) and high temperature (42 °C) stress. Leaf disc senescence assays showed a clear advantage of transgenic lines over UC and VC plants in overcoming the deleterious effects of NaCl. The control plants showed rapid senescence and extensive bleaching reflecting symptoms of injury due to salinity stress, while the transgenic lines performed better under similar conditions. Chlorosis was also less in transgenic lines under mannitol stress (simulating dehydration stress), $CdCl_2$ stress, and cold stress while the controls were bleached under similar conditions. At high temperature, the transgenic lines showed chlorophyll depletion, but in other respects they were better than the control plants (Fig. 3a).

The results of leaf disc senescence assays were confirmed biochemically by estimating the chlorophyll content, which in the leaf discs from all the transgenic plants was 1.2- to 1.7-fold more as compared to those from UC and VC plants at elevated NaCl concentrations. There was nearly complete loss of chlorophyll in the control plants, whereas the *XnGroEL* transformants showed relatively less reduction in total chlorophyll content at the highest NaCl concentration (600 mM) used (Fig. 3b). Similar, observations were recorded in response to other stresses. Figure 3c, shows 1.3- to 1.6-fold higher chlorophyll content in the lines Xn2, Xn3, and Xn4 when compared with UC and VC lines after exposure to mannitol. Chlorophyll content after exposure to heavy metals ($CdCl_2$ stress) was more than two fold higher in the transformed leaves as compared to the leaves from UC and VC plants (Fig. 3d). Furthermore, tolerance to both cold (4 °C) and heat (42 °C), was higher in the leaves from transformed plants as indicated by 1.4- and 1.6-fold higher chlorophyll contents on comparison with leaf dics from UC and VC plants, respectively (Fig. 3e, f). These results were found to be statistically significant. These observations established a positive relationship between the expression of XnGroEL and hardiness to the general stress conditions encountered by plants.

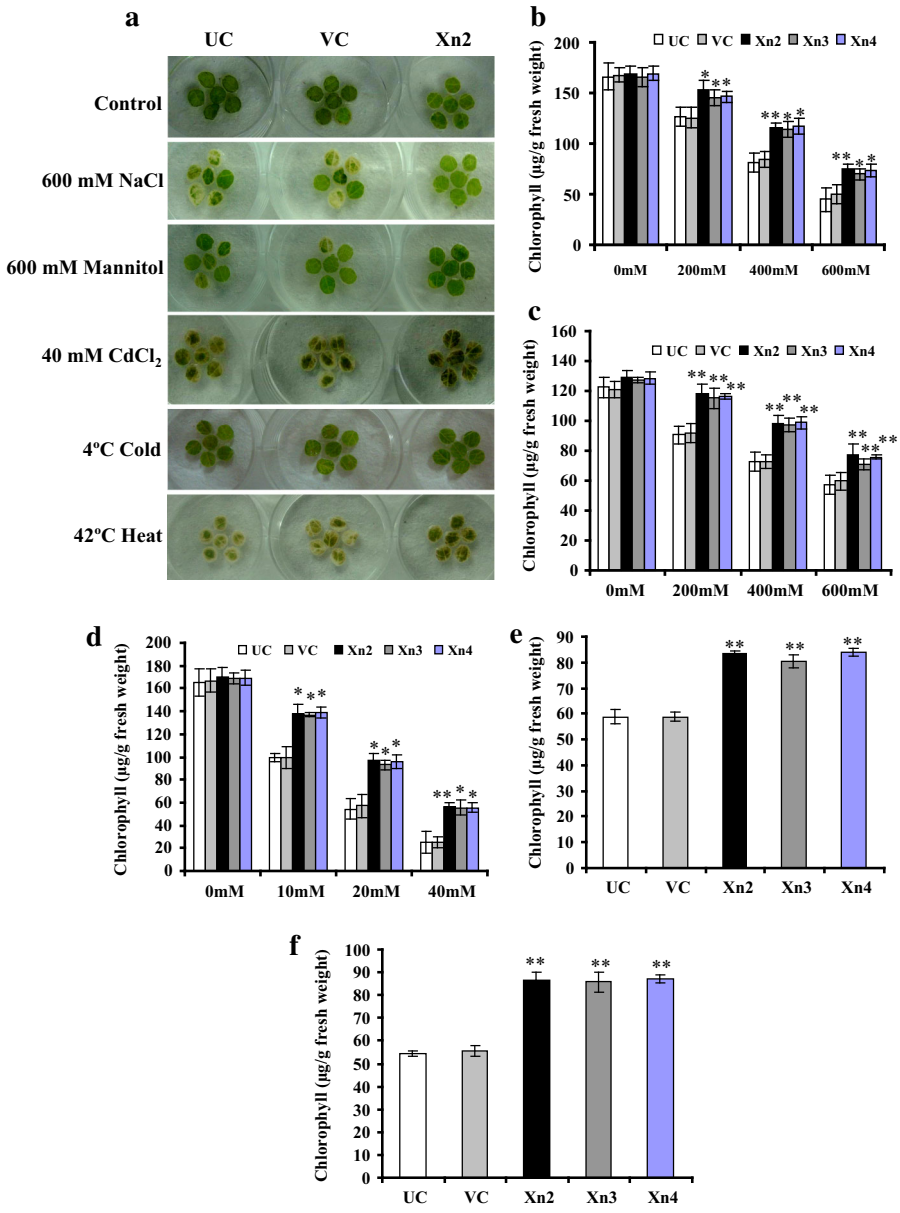


Fig. 3 Assessment of XnGroEL transgenic tomato plants for their tolerance towards various abiotic stresses. **a** Leaf disc senescence assays for abiotic stress tolerance in transgenic tomato plants. Experiments were performed on three transgenic lines (Xn2, Xn3, and Xn4) subjected to either NaCl (200, 400, and 600 mM) for salinity stress, mannitol (200, 400, and 600 mM) for dehydration stress, $CdCl_2$ (10, 20, and 40 mM) for heavy metal stress, 4 °C for cold stress or 42 °C for heat stress. Leaf discs floated in Hoagland solution served as the experimental control. The chlorophyll content (µg/g fresh weight) retained in corresponding leaf discs shown in **a** is depicted by histograms, **b** NaCl, **c** Mannitol, **d** $CdCl_2$, **e** 4 °C cold, and **f** 42 °C heat. The data represents mean ± SE of three biological replicates. *, ** denote significance at $p \leq 0.05$ and $p \leq 0.001$, respectively

Effect of XnGroEL expression on relative water content, membrane damage, and photosynthetic efficiency of transgenic plants

The performance of plants under extended exposures of salt and heat stress was also tested. One set of plants was irrigated with 200 mM NaCl solution for 2 weeks to induce salt stress. In another set, the plants were exposed to 42 °C for 16 h. In a control set, transgenic, UC, and VC plants were irrigated with water. Growth and development of the transgenic plants were indistinguishable from those of UC and VC plants. The transgenic plants (Xn2) showed better growth under salt and heat stress as compared to the UC and VC plants where the leaves showed curling, yellowing, and dropping characteristics (Fig. 4a). The other two transgenic lines (Xn3 and Xn4) showed similar performance as Xn2 under salt and heat stress (photographed not shown).

The salt and heat induced changes in relative water content, MDA (lipid peroxidation product), electrolyte leakage, and photosynthetic efficiency were also measured in the transgenic lines (Xn2, Xn3, and Xn4), UC, and VC plants. The water holding capacity of transgenic plants was determined by measuring the relative water content of leaves under salt and heat stress. The XnGroEL transgenic lines maintained the relative water content by ~1.7-fold higher when exposed to salt stress and ~2-fold higher on exposure to heat stress as compared to UC and VC plants (Fig. 4b).

The MDA content and electrolyte leakage were significantly reduced in XnGroEL transgenic lines by ~1.4- and ~2-fold, respectively, during these stress conditions, as compared with UC and VC plants (Fig. 4c, d).

The Fv/Fm ratio is a measure of the proportion of light absorbed by chlorophyll molecules associated with PSII in the photochemical reaction and thus, an indication of actual photosynthetic efficiency (Harbinson et al. 1989; Heckathorn et al. 1998). To examine salt and heat stress tolerance of the transgenic plants, changes in chlorophyll fluorescence was also measured. The initial chlorophyll fluorescence ratio (Fv/Fm), was similar in the transgenics and the control (UC and VC) plants. However, following stress treatment, the Fv/Fm ratio of XnGroEL expressing transgenic plants was higher by ~1.5-fold ($p \leq 0.05$) and ~1.7-fold ($p \leq 0.05$), compared to control plants

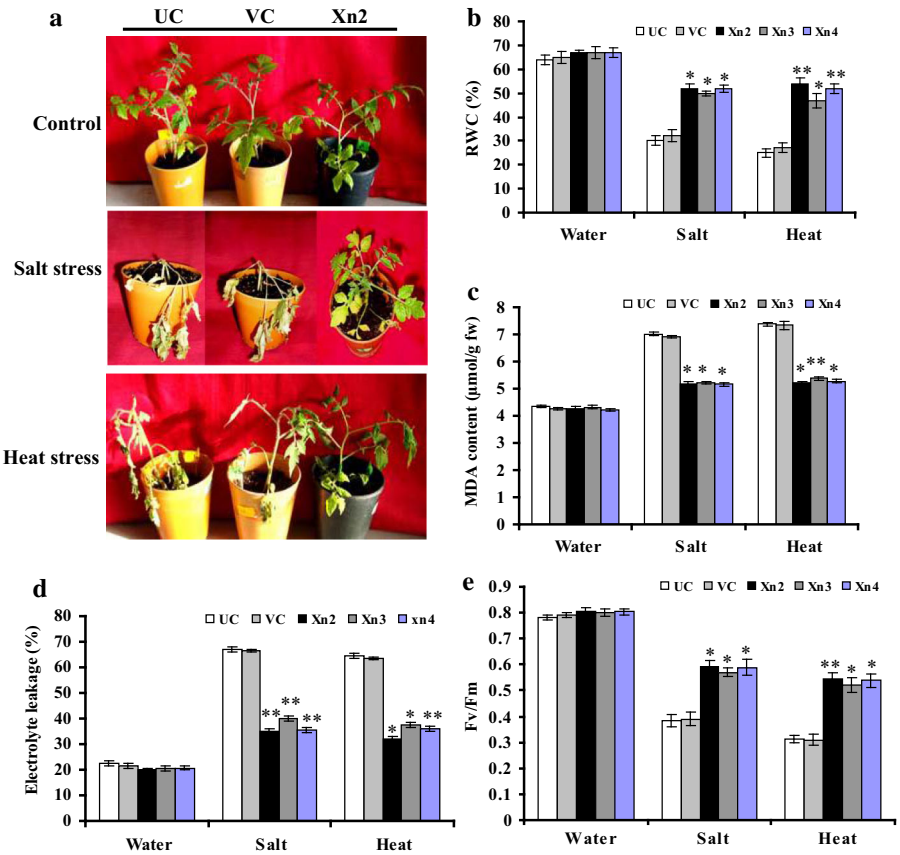


Fig. 4 Effect of exposure of transgenic and control plants to salinity and heat stress. **a** *Control panel* shows the phenotype of UC, VC, and transgenic tomato plants under non-stress condition. *Middle panel* shows response of transgenic, UC, and VC control plants after exposure to 200 mM NaCl for 2 weeks. *Bottom panel* shows the response of transgenic, UC, and VC control plants after 16 h of heat stress (42 °C). Biochemical analysis of transgenic and control plants when exposed to salt and heat stress. **b** Relative water content. **c** Lipid peroxidation expressed in terms of MDA content. **d** Percentage electrolytic leakage. **e** Chlorophyll fluorescence. *, ** denote significance at $p \leq 0.05$ and $p \leq 0.001$, respectively

when exposed to salt and heat stress, respectively (Fig. 4e).

O_2^- and H_2O_2 accumulation and ROS detoxification in transgenic and control plants

Salt and heat stresses are accompanied by the formation of reactive oxygen species (ROS) such as O_2^-, H_2O_2, and OH^- radicals that cause damage to biological membranes and macromolecules (Wang et al. 2003). The visual observation of accumulation of ROS (O_2^- and H_2O_2) was performed by histochemical staining of leaves with nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively. Formation of purple spots indicative of superoxide (O_2^-) accumulation increased in the leaves of control plants compared to leaves of transgenic plants (Fig. 5a).

Furthermore, the leaves of XnGroEL expressing plants displayed less brown colouration and diffused staining by DAB relative to the control (UC and VC) plants indicating less hydrogen peroxide (H_2O_2) accumulation in the transgenic lines (Fig. 5b). These results were corroborated by measuring the H_2O_2 content of the plants. The H_2O_2 level was reduced by $\sim$2-fold ($p \leq 0.001$) and $\sim$1.8-fold ($p \leq 0.001$) in the transgenic lines, as compared to UC and VC plants when exposed to salt and heat stress, respectively (Fig. 5c). These results indicate that overexpression of XnGroEL helps in reducing the accumulation of ROS in transgenic plants.

The effect of expression of XnGroEL on the activity of enzymes that help in quenching toxic radicals, e.g. superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) was also measured in plants. The activities of all these enzymes increased by approximately two to three fold in all the transgenic lines compared to controls on exposure to salt and heat stress (Fig. 5d–f). Together, these results suggest that XnGroEL increased tolerance of plants against salt and heat stress by activating antioxidant enzymes and as a consequence there was reduction in ROS accumulation in the transgenic tomato plants.

Discussion

Molecular chaperones are key components contributing to cellular homeostasis under both optimal and adverse conditions. Many molecular chaperones are stress proteins and are classified as Hsps (Lindquist 1986; Lindquist and Craig 1988; Fink 1999). Denaturation of enzymes and proteins is the primary signal of cellular distress. The chaperones/Hsps play a critical role in maintaining proteins in their functional conformation and preventing aggregation of non-native proteins during stress (Vierling 1991; Hendrick and Hartl 1993; Boston et al. 1996; Hartl 1996; Waters et al. 1996; Queitsch et al. 2000; Frydman 2001; Torok et al. 2001; Bosl et al. 2006; Goloubinoff and De Los Rios 2007). We have earlier reported insecticidal properties of XnGroEL, a molecular chaperone of bacterial origin, against *H. armigera* larvae (Joshi et al. 2008; Kumari et al. 2014). Here, we evaluate efficacy of XnGroEL in providing protection against biotic and abiotic stresses to transgenic tomato plants.

In *X. nematophila*, GroEL is expressed at a much higher level under normal conditions and transported outside the cell to perform its specific extracellular function. Insecticidal property of XnGroEL protein was shown earlier in in vitro bioassays (Joshi et al. 2008) and later in transgenic tobacco plants (Kumari et al. 2014). It was shown to bind chitin and brush border membrane of the larval gut (Joshi et al. 2008), however, the exact mechanism of action was not established. It was suspected that being a large heptameric molecule, it blocked access of chitinase to chitin in the larval gut peritrophic membrane, a critical step in remodeling of the gut membrane during development. As a consequence, metamorphosis and growth was adversely affected leading to death of the larvae. The detached leaf assay of XnGroEL transgenic tomato lines against *H. armigera* reaffirms its lethal action in a commercially important crop plant.

GroEL is a global stress protein and has orthologs across the spectrum of living organisms. In the backdrop of chaperoning activity of XnGroEL observed earlier (Joshi 2007; Joshi et al. 2008), we evaluated protective role of the protein in transgenic tomato plants against abiotic stress and ROS, a leading cause of cellular damage. Abiotic stresses like high salinity and high temperature cause oxidative stress due to disturbance in ROS production and scavenging (Zhu 2001; Mittler 2002; Xiong et al. 2002). The ROS are highly reactive and toxic to biological systems and cause membrane damage through lipid peroxidation (Gill and Tuteja 2010; Gill et al. 2012; Huang et al. 2010). They partially reduce or activate derivatives of oxygen and damage DNA, proteins, and other

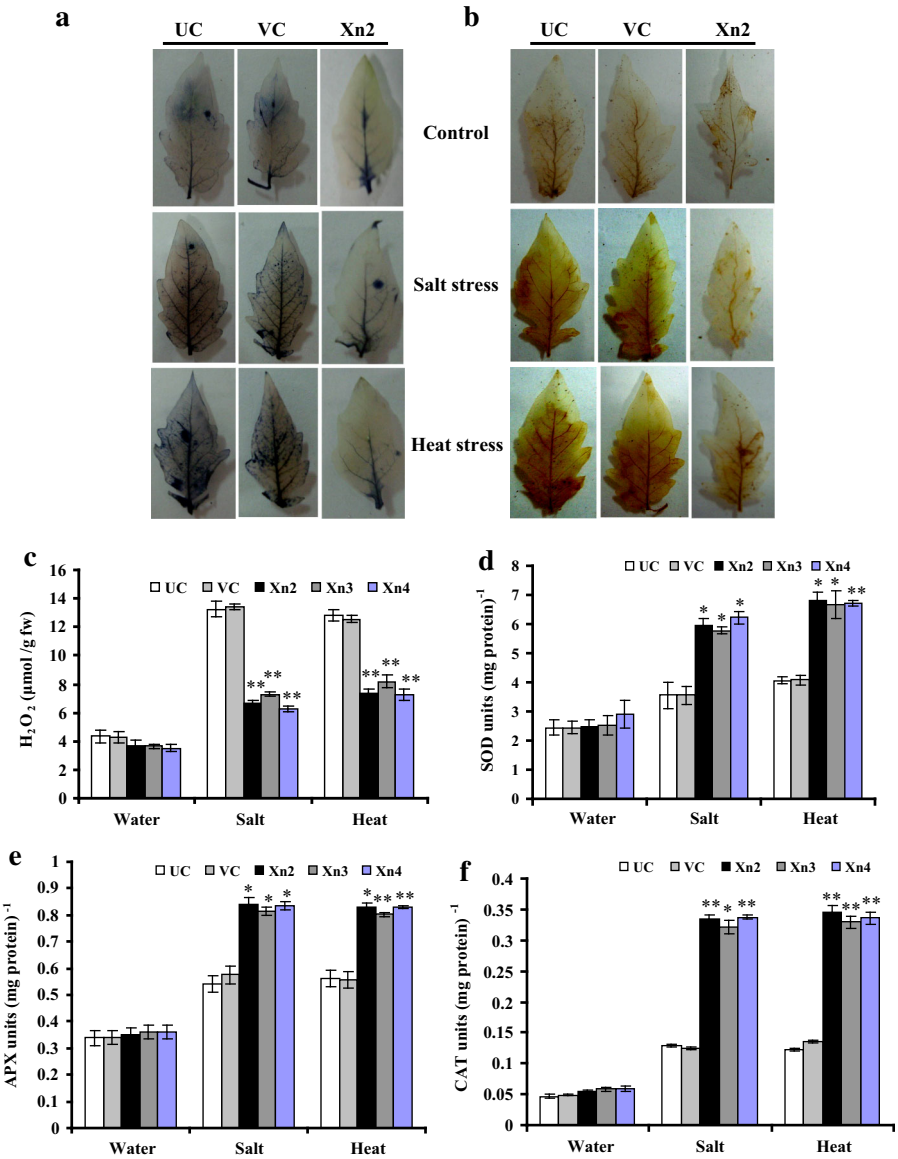


Fig. 5 ROS accumulation and antioxidant enzyme machinery in transgenic and control plants. Histochemical assessment of relative **a** superoxide (O_2^-) and **b** hydrogen peroxide (H_2O_2) accumulation in transgenic and control plants exposed to salt and heat stress. Plants irrigated with water served as the untreated control. **c** Changes in the level of H_2O_2 content in XnGroEL transgenic lines, UC, and VC were estimated. Effects of XnGroEL-expression on antioxidant enzyme activity under stress treatments. Treatment details are described in "Materials and methods" section. **d** Changes in the activity of superoxide dismutase (SOD) in transgenic, UC, and VC plants. **e** Changes in ascorbate peroxidase (APX) activity in transgenic, UC, and VC plants. **f** Catalase (CAT) activity in transgenic, UC, and VC plants. *, ** denote significance at $p \leq 0.05$ and $p \leq 0.001$, respectively

biomolecules, resulting in cell death (Mittler et al. 2004; Ouyang et al. 2010).

The enhanced level of tolerance to multiple stresses is demonstrated through various markers like higher-chlorophyll, and relative water content, and reduction in lipid peroxidation, electrolyte leakage, and H_2O_2 production. A concomitant increase in the activity of antioxidant enzymes (SOD, APX, and CAT) in the transgenic plants further augments the cellular defence against unfavourable environmental conditions (Yeh

et al. 2002; Apel and Hirt 2004; Ogawa et al. 2007; Swindell et al. 2007; Jiang et al. 2009; Song and Ahn 2011; Li et al. 2014). Thus, increased antioxidant activity of the transgenic lines helps to suppress toxic ROS levels and their effect on membrane (lipid peroxidation). A direct consequence of the decreased MDA level (lipid peroxidation product) was a better photosynthetic performance (increased Fv/Fm ratio) of the transgenic tomato lines. Accumulation of MDA beyond a threshold level has been reported to cause irreparable damage to the photosynthetic machinery (Mishra and Singhal 1992). Overall, maintenance of better water balance, photosynthetic efficiency, and higher activities of antioxidant enzymes, the hallmarks of stress tolerance, most likely protect the XnGroEL transgenic lines from deleterious effects of oxidative damage caused by salt and heat stress. Moreover, analogous to GroEL, overexpression of XnGroEL is expected to safeguard general health of the transgenic plants by preventing protein/enzyme denaturation and activating the antioxidant machinery under biotic and abiotic stress conditions (Shi et al. 2001; Hall 2002; Murakami et al. 2004; Wang et al. 2004; Schramm et al. 2006; Jiang et al. 2009; Tripp et al. 2009, Mu et al. 2013).

In conclusion, this study demonstrates that the overexpression of the bacterial protein XnGroEL can lead to alleviation of biotic and abiotic stresses, thus, demonstrating its dual role. However, more comprehensive evaluation needs to be done before this technology could be applied more widely for protection of important agricultural crops against environmental stresses.

Acknowledgments The Grant from the Department of Biotechnology (No. BT/PR11260/PBD/16/819/2008) to Prof. Neera Bhalla Sarin and Dr. Nirupama Banerjee is gratefully acknowledged. P. K. acknowledges the financial support from the Council for Scientific and Industrial Research. Partial funds from Department of Science and Technology (D.S.T.-PURSE, D.S.T.-F.I.S.T.), U.G.C.-C.A.S., U.G.C.-R.N.W., and J.N.U are gratefully acknowledged.

References

Aebi H (1984) Catalase *in vitro*. Methods Enzymol 105:121–126

Akhurst RJ, Dunphy GB, Beckage N, Thompson S, Federici B (1993) Parasites and pathogens of insects, vol 2. Academic, New York, pp 1–23

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Arnon DI (1949) Copper enzymes in isolated chloroplasts: polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24:1–15

Arora S, Kanojia AK, Kumar A, Mogha N, Sahu V (2012) Biopesticide formulation to control tomato lepidopteran pest menace. Curr Sci 102:1051–1057

Arora S, Kanojia AK, Kumar A, Mogha N, Sahu V (2014) Biopesticide formulation to control tomato lepidopteran pest menace. Asian Agri-History 18:283–293

Barr HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficit in leaves. Aust J Biol Sci 15:413–428

Bosl B, Grimminger V, Walter S (2006) The molecular chaperone Hsp104—a molecular machine for protein disaggregation. J Struct Biol 156:139–148

Boston RS, Viitanen PV, Vierling E (1996) Molecular chaperones and protein folding in plants. Plant Mol Biol 32:191–222

Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248–254

Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 30:987–998

Dhandapani N, Umeshchandra SR, Murugan M (2003) Bio-intensive pest management (BIPM) in major vegetable crops: an Indian perspective. Food Agric Environ 1:333–339

Ferry N, Edwards MG, Gatehouse JA, Gatehouse MRA (2004) Plant-insect interactions: molecular approaches to insect resistance. Curr Opin Biotechnol 15:155–161

Fink AL (1999) Chaperone mediated protein folding. Physiol Rev 79:425–449

Forreiter C, Kirschner M, Nover L (1997) Stable transformation of an *Arabidopsis* cell suspension culture with firefly luciferase providing a cellular system for analysis of chaperone activity in vivo. Plant Cell 9:2171–2181

Forst S, Dowds B, Boemare N, Stackebrandt E (1997) *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. Annu Rev Microbiol 51:47–72

Frydman J (2001) Folding of newly translated proteins *in vivo*: the role of molecular chaperones. Annu Rev Biochem 70:603–647

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–939

Gill SS, Singh LP, Gill R, Tuteja N (2012) Generation and scavenging of reactive oxygen species in plants under stress. In: Tuteja N, Gill SS, Tiburcio AF, Tuteja R (eds) Improving crop resistance to abiotic stress. Wiley-VCH Verlag GmbH and Co. KGaA, Germany, pp 49–70

Goloubinoff P, De Los Rios P (2007) The mechanism of Hsp70 chaperones: (entropic) pulling the models together. Trends Biochem Sci 32:372–380

Gupta AS, Webb RP, Holaday AS, Allen RD (1993) Overexpression of superoxide dismutase protects plants from oxidative stress. Plant Physiol 103:1067–1073

Gupta GP, Birah A, Singh B, Mahapatro GK (2010) Methodology and composition of artificial diet for mass rearing of lepidopteran pests (in particular *Helicoverpa armigera*, *Spodoptera litura* and *Earias vittella*), Patent (IPA No. 1618/DEL/2008) The Patent Office Journal, Part I, 16th, pp 15

Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11

Harbinson J, Genty B, Baker NR (1989) Relationship between the quantum efficiencies of photosystems I and II in pea leaves. Plant Physiol 90:1029–1034

Hartl FU (1996) Molecular chaperones in cellular protein folding. Nature 381:571–580

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189–198

Heckathorn SA, Downs CA, Sharkey TD, Coleman JS (1998) The small, methionine rich chloroplast heat shock protein protects photosystem II electron transport during heat stress. Plant Physiol 116:439–444

Hendrick JP, Hartl FU (1993) Molecular chaperone functions of heat-shock proteins. Annu Rev Biochem 62:349–384

Herbert EE, Goodrich-Blair H (2007) Friend and foe: the two faces of *Xenorhabdus nematophila*. Nat Rev Microbiol 5:634–646

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Station Circ 347:1–32

Hofgen R, Willmitzer L (1988) Storage of competent cells for *Agrobacterium* transformation. Nucleic Acids Res 16:9877

Huang XS, Liu JH, Chen XJ (2010) Overexpression of *PtrABF* gene, a bZIP transcription factor isolated from *Poncirus trifoliata*, enhances dehydration and drought tolerance in tobacco via scavenging ROS and modulating expression of stress-responsive genes. BMC Plant Biol 10:230

Jiang C, Xu J, Zhang H, Zhang X, Shi J, Li M, Ming F (2009) A cytosolic class I small heat shock protein, RcHSP17.8, of *Rosa chinensis* confers resistance to a variety of stresses to *Escherichia coli*, yeast and *Arabidopsis thaliana*. Plant Cell Environ 32:1046–1059

Joshi MC (2007) Characterization of GroEL homolog of *Xenorhabdus nematophila* and evaluation of its insecticidal potential in crop pests. Ph.D Thesis, School of Biotechnology, Jawaharlal Nehru University, India

Joshi MC, Sharma A, Kant S, Birah A, Gupta GP, Khan SR, Bhatnagar R, Banerjee N (2008) An insecticidal GroEL protein with chitin binding activity from *Xenorhabdus nematophila*. J Biol Chem 283:28287–28296

Kotak S, Vierling E, Baumlein H, Von Koskull-Doring P (2007) A novel transcriptional cascade regulating expression of heat stress proteins during seed development of *Arabidopsis*. Plant Cell 19:182–195

Kumar D, Yusuf MA, Singh P, Sardar M, Sarin NB (2013) Modulation of antioxidant machinery in α-tocopherol enriched transgenic *Brassica juncea* plants tolerant to abiotic stress conditions. Protoplasma 250:1079–1089

Kumari P, Kant S, Zaman S, Mahapatro GK, Banerjee N, Sarin NB (2014) A novel insecticidal GroEL protein from *Xenorhabdus nematophila* confers insect resistance in tobacco. Transgenic Res 23:99–107

Lee GJ, Roseman AM, Saibil HR, Vierling E (1997) A small heat shock protein stably binds heat denatured model substrates and can maintain a substrate in a folding competent state. EMBO J 16:659–671

Li S, Liu J, Liu Z, Li X, Wu F, He Y (2014) Heat induced TAS1 target1 mediates thermotolerance via heat stress transcription factor A1a-directed pathways in *Arabidopsis*. Plant Cell 26:1764–1780

Lindquist S (1986) The heat-shock response. Annu Rev Biochem 55:1151–1191

Lindquist S, Craig EA (1988) The heat-shock proteins. Annu Rev Genet 22:631–677

Low D, Brandle K, Nover L, Forreiter C (2000) Cytosolic heat stress proteins Hsp17.7 class I and Hsp17.3 class II of tomato act as molecular chaperones *in vivo*. Planta 211:575–582

Madhulatha P, Pandey R, Hazarika P, Rajam MV (2007) High transformation frequency in *Agrobacterium*-mediated genetic transformation of tomato by using polyamines and maltose in shoot regeneration medium. Physiol Mol Biol Plants 13:191–198

Mishra RK, Singhal GS (1992) Function of photosynthetic apparatus of intact wheat leaves under high light and heat stress and its relationship with peroxidation of thylakoid lipids. Plant Physiol 98:1–6

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405–410

Mittler R (2006) Abiotic stress, the field environment and stress combination. Trends Plant Sci 11:15–19

Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490–498

Mu C, Zhang S, Yu G, Chen N, Li X et al (2013) Overexpression of small heat shock protein LimHSP 16.45 in *Arabidopsis* enhances tolerance to abiotic stresses. PLoS ONE 8(12):e82264. doi:10.1371/journal.pone.0082264

Murakami T, Matsuba S, Funatsuki H, Kawaguchi K, Saruyama H, Tanida M, Sato Y (2004) Over-expression of a small heat shock protein, sHSP17.7, confers both heat tolerance and UV-B resistance to rice plants. Mol Breeding 13:165–175

Ogawa D, Yamaguchi K, Nishiuchi T (2007) High-level overexpression of the *Arabidopsis HsfA2* gene confers not only increased themotolerance but also salt/osmotic stress tolerance and enhanced callus growth. J Exp Bot 58:3373–3383

Ouyang SQ, Liu YF, Liu P, Lei G, He SJ, Ma B, Zhang WK, Zhang JS, Chen SY (2010) Receptor-like kinase OsSIK1 improves drought and salt stress tolerance in rice (*Oryza sativa*) plants. Plant J 62:316–329

Padmanaban N, Arora R (2002) Field evaluation of native NPV for the management of tomato fruit borer *Helicoverpa armigera*. Pestic Res J 14:113–119

Personat JM, Tejedor-Cano J, Prieto-Dapena P, Almoguera C, Jordano J (2014) Co-overexpression of two heat shock factors results in enhanced seed longevity and in synergistic effects on seedling tolerance to severe dehydration and oxidative stress. BMC Plant Biol 14:56

Queitsch C, Hong SW, Vierling E, Lindquist S (2000) Heat shock protein 101 plays a crucial role in thermotolerance in A*rabidopsis*. Plant Cell 12:479–492

Reed W, Pawar CS (1982) *Heliothis*: a global problem. In: Proceedings of the international workshop on *Heliothis* management, Nov 15–20 1981, ICRISAT, Patancheru, pp 9–14

Rogers SO, Bendich AJ (1994) Extraction of total cellular DNA from plants, algae and fungi. Plant Mol. Biol. Manual D1:1–8

Ruibal C, Castro A, Carballo V, Szabados L, Vidal S (2013) Recovery from heat, salt, and osmotic stress in *Physcomitrella patens* requires a functional small heat shock protein PpHsp16.4. BMC Plant Biol 13:174

Sairam RK, Srivastava GC (2002) Changes in antioxidant activity in subcellular fractions of tolerant and susceptible wheat genotypes in response to long term salt stress. Plant Sci 162:897–904

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor, New York

Scharf KD, Siddique M, Vierling E (2001) The expanding family of *Arabidopsis thaliana* small heat stress proteins and a new family of proteins containing alpha-crystallin domains (Acd proteins). Cell Stress Chaperones 6:225–237

Schramm F, Ganguli A, Kiehlmann E, Englich G, Walch D, Von Koskull-Doring P (2006) The heat stress transcription factor HsfA2 serves as a regulatory amplifier of a subset of genes in the heat stress response in *Arabidopsis*. Plant Mol Biol 60:759–772

Shi WM, Muramoto Y, Ueda A, Takabe T (2001) Cloning of peroxisomal *ascorbate peroxidase* gene from barley and enhanced thermotolerance by overexpressing in *Arabidopsis thaliana*. Gene 273:23–27

Song NH, Ahn YJ (2011) DcHsp17.7, a small heat shock protein in carrot, is tissue-specifically expressed under salt stress and confers tolerance to salinity. N Biotechnol 28:698–704

Swindell WR, Huebner M, Weber AP (2007) Transcriptional profiling of *Arabidopsis* heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. BMC Genom 8:125

Torok Z, Goloubinoff P, Horvath I, Tsvetkova NM, Glatz A, Balogh G, Varvasovszki V, Los DA, Vierling E, Crowe JH, Vigh L (2001) Synechocystis HSP17 is an amphitropic protein that stabilizes heat-stressed membranes and binds denatured proteins for subsequent chaperone-mediated refolding. Proc Natl Acad Sci USA 98:3098–3103

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354

Tripp J, Mishra SK, Scharf KD (2009) Functional dissection of the cytosolic chaperone network in tomato mesophyll protoplasts. Plant, Cell Environ 32:123–133

Velikova V, Edreva A, Loreto F (2004) Endogenous isoprene protects *Phragmites australis* leaves against singlet oxygen. Physiol Plant 122:219–225

Vierling E (1991) The roles of heat shock proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 42:579–620

Vinocur B, Altman A (2005) Recent advances in engineering plant tolerance toabiotic stress: achievements and limitations. Curr Opin Biotechnol 16:123–132

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity, and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218:1–14

Wang W, Vinocur B, Shoseyov O, Altman A (2004) Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 9:244–252

Waters ER, Lee GJ, Vierling E (1996) Evolution, structure and function of the small heat shock proteins in plants. J Exp Bot 47:325–338

Whalon M, Mota-sanchez D, Hollongworth L, Duynslager L (2004) Arthopod pesticide resistance database, (Michigan State University, East Lansing, MI). http://www.pesticideresistance.org/search/12/0/41/0/. Accessed 21 April 2009

Xiong L, Schumaker KS, Zhu JK (2002) Cell signaling during cold, drought, and salt stress. Plant Cell 14:165–183

Yeh CH, Chen YM, Lin CY (2002) Functional regions of rice heat shock protein, Oshsp16.9, required for conferring thermotolerance in *Escherichia coli*. Plant Physiol 128:661–668

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66–72