



Heterologous Expression of *Heliotropium thermophilum* XTH Genes Improves Tomato Tolerance to Combined Heat and Drought Stress

Asim Kadioğlu¹ · Karam Mostafa^{2,3} · Dilek Ünlüer Birinci^{1,5} · Mohamed Farah Abdulla⁴ · Bayram Ali Yerlikaya² · Seher Yerlikaya² · Aykut Sağlam⁶ · Utku Avcı⁷ · Musa Kavas²

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Abstract

High temperature and drought are among the primary abiotic stresses restricting crop growth. Under the progression of global climate change, the incidence of their combined occurrence is anticipated to intensify. Elucidating the mechanisms underlying thermotolerance is critical for breeding climate-resilient crops. *Heliotropium thermophilum*, native to geothermal regions characterized by extreme soil temperatures, provides an ideal model for investigating plant responses to high-temperature stress. Xyloglucan endotransglucosylase/hydrolases (XTHs) are key enzymes involved in cell wall remodeling, and their critical functions in cell expansion and morphogenesis indicate a pivotal role in mediating plant stress responses. In this study, we identified and overexpressed *HtXTH-7* and *HtXTH-15* in tomato to examine their roles in tolerance to heat, drought, and combined heat-drought stress. Analysis of the XTH protein domains showed that all sequences possessed both GH16_XET and BglS domains, except for *HtXTH15*, which contained only the GH16_XET domain. Under all stress conditions, HtXTH7.4 (OE-1) and HtXTH15.7 (OE-2) lines showed significantly elevated levels of chlorophyll a, total chlorophyll, and total carotenoids compared to wild-type plants. Chlorophyll b, however, increased significantly only in OE-1 under combined stress and OE-2 under heat and combined stress. Moreover, both transgenic lines showed the most pronounced reduction in TBARS under heat stress relative to other treatments, while OE-2 consistently exhibited the highest proline accumulation across all stress conditions. Regarding antioxidant enzyme activity, both SOD and APX were elevated in response to stress, exhibiting an inverse correlation with H₂O₂ levels. RT-PCR confirmed *HtXTH* expression in all transgenic lines, with HtXTH7 showing 5–50 fold and HtXTH15 8–62 fold under control condition. The expression profiles of HtXTH7 and HtXTH15 were consistent with those of established abiotic stress marker genes, including RD22 (Responsive to Dehydration 22), HSFA (Heat Shock Transcription Factor A), HSP90, HSP70, and NCED (9-cis-epoxycarotenoid dioxygenase). Our findings highlight the roles of *HtXTH7* and *HtXTH15* in tomato responses to heat and drought stress, providing a basis for future functional and mechanistic investigations of *HtXTHs*.

Keywords *Heliotropium thermophilum* · Xyloglucan endotransglucosylase/hydrolases (XTHs) · Heat stress · Drought stress · Tomato

Asim Kadioğlu, Karam Mostafa and Mohamed Farah Abdulla contributed equally to this work and shared first authorship.

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✉ Musa Kavas
musa.kavas@omu.edu.tr

¹ Department of Biology, Faculty of Science, Karadeniz Technical University, Trabzon, Turkey

² Department of Agricultural Biotechnology, Faculty of Agriculture, Ondokuz Mayıs University, Samsun, Turkey

³ The Central Laboratory for Date Palm Research and Development, Agricultural Research Center (ARC), Giza 12619, Egypt

⁴ Protium Technologies, Sharjah, United Arab Emirates

⁵ Graduate School of Natural and Applied Sciences, Karadeniz Technical University, Trabzon, Turkey

⁶ Department of Molecular Biology and Genetics, Faculty of Science, Karadeniz Technical University, Trabzon, Turkey

⁷ Department of Agricultural Biotechnology, Faculty of Agriculture, Eskisehir Osmangazi University, Eskisehir, Turkey

Introduction

The plant cell wall plays a central role in determining cell morphology, enabling the formation of diverse cell types such as trichomes, root hairs, and pavement cells. (Ivakov and Persson 2013). There has been growing interest in studies that associate the roles of key enzymes with the architecture and dynamics of the plant cell wall. Members of the xyloglucan endotransglucosylase/hydrolase (XTH) family, belonging to glycoside hydrolase family 16 (GH16), are hypothesized to regulate cell wall extensibility and remodeling by mediating the cleavage and rejoining of xyloglucan chains, as suggested by indirect experimental evidence (Eklöf and Brumer 2010; Han et al. 2016; Miedes et al. 2013). XTH proteins display two established enzymatic functions: redefined xyloglucan endotransglucosylase (XET) activity and xyloglucan endohydrolase (XEH) activity (Ishida and Yokoyama 2022; Rose et al. 2002). These genes encode enzymes that modify xyloglucan, a major component of the plant cell wall, facilitating cell wall extensibility. In the conventional model, XTHs exhibit transglycosylase activity that modifies xyloglucan linkages, thereby altering the spacing between cellulose microfibrils by adjusting the length of the tethering xyloglucan chains. This process is crucial for cell elongation, organ growth, and overall plant morphology (Hayashi and Kaida 2011). Functional studies have demonstrated that overexpression or disruption of specific *XTH* genes results in significant alterations in cell wall composition and mechanical properties (Genovesi et al. 2008; Takahashi et al. 2021). Ectopic expression of the *BcXTH1* gene from *Brassica campestris* in *Arabidopsis* resulted in enhanced stem elongation through the stimulation of cell expansion and elongation (Shin et al. 2006). Additionally, elevated activity of *SIXET1* modifies xyloglucan structure during fruit ripening and softening in transgenic tomato (Miedes et al. 2010). In addition, overexpression of *GhXTH1* in cotton facilitates cell wall loosening and fiber elongation by cleaving xyloglucan-cellulose linkages (Lee et al. 2010; Shao et al. 2011). However, several recent studies have presented conflicting findings that challenge the traditionally proposed roles of xyloglucans and XTHs in cell wall remodeling and plant cell expansion. For example, Cavalier et al. (2008) illustrated that the *xxt1xxt2* double mutant, which lacks functional xyloglucan xylosyltransferases and is consequently deficient in xyloglucan, displayed overall normal growth and development, with only minor phenotypic alterations such as slightly shortened root hairs (Cavalier et al. 2008). Additionally, enzymatic degradation of xyloglucan in planta did not result in detectable cell wall loosening, suggesting that xyloglucan modification alone may not be sufficient to drive wall extensibility (Park and Casgrove 2012). Furthermore, the absence of xyloglucan

in the *xxt1xxt2* mutant did not impair the assembly of the cellulose network during protoplast cell wall regeneration (Kuki et al. 2020).

In light of these findings, we conclude that XTHs play diverse roles in plant development and immune responses to both biotic and abiotic stresses, extending beyond their established function in remodeling the plant cell wall. In the maize primary root elongation zone, XET activity facilitates cell wall loosening under low water potential conditions, a process that is partially regulated by abscisic acid (ABA) (Moore et al. 2008; Wu et al. 1994). Low water potential reduces XET activity in the hypocotyl elongation zone of etiolated soybean seedlings (Wu et al. 2005). Treatment of maize primary roots with polyethylene glycol solution led to a reduction in XET activity within the basal 5–10 mm region, which corresponded with decreased cell wall extensibility and inhibited cell elongation in that zone (Pritchard et al. 1993). Overexpression of the *GmXTH1* gene in soybean (*Glycine max*) increases salt stress resistance. The OEAs demonstrated enhanced antioxidant enzyme activities and reduced malondialdehyde (MDA) levels, indicating mitigated oxidative damage, while maintaining elevated chlorophyll content, reflecting improved photosynthetic efficiency (Song et al. 2024). Moreover, an exhaustive study of oilseed rape (*Brassica napus*) uncovered 80 *BnXTH* gene family members. Gene expression studies showed these genes respond differently to several abiotic stresses, including aluminum (Al), alkaline environments, salinity, and drought (Chen et al. 2024a, b). Three XTH-like genes (*CaXTH1*, *CaXTH2*, and *CaXTH3*) were identified from drought-stressed hot pepper plants. Multiple abiotic stresses, including drought, salinity, cold, and ethylene, simultaneously induced their expression, suggesting a role in the early stress response mechanisms. (Cho et al. 2006). Regarding plant hormone signaling, *OsXTH8* expression in rice is upregulated by gibberellic acid, particularly in the vascular bundles of the leaf sheath and young nodal roots; conversely, knockdown of *OsXTH8* leads to suppressed growth in transgenic plants (Jan et al. 2004). Loss-of-function mutations in *AtXTH31* decrease ABA sensitivity and promote accelerated germination in *Arabidopsis* (Endo et al. 2011). The downregulation of *XTH8* and *XTH31* contributes to reduced leaf cell expansion in the *Arabidopsis* *siz1* mutant, acting through a salicylic acid (SA)-dependent pathway (Miura et al. 2009).

Heliotropium thermophilum is a plant that exhibits remarkable resistance to elevated temperatures. *H. thermophilum* is a notable thermophilic flowering plant species that thrives at soil temperatures ranging from 55 °C to 65 °C. This plant has evolved remarkable adaptations to survive in harsh settings. The studies on *H. thermophilum* provides a comprehensive examination of temperature

tolerance mechanisms in plants (Bülbül et al. 2024; Ozturk et al. 2020; Sezgin Muslu and Kadioğlu 2021). Understanding these mechanisms can facilitate the development of heat-resistant crops, which is more vital due to global warming and elevated soil temperatures. This may also lead to enhanced agricultural productivity in arid and semi-arid regions. In our prior work on the plant *H. thermophilum*, RNA-seq analysis revealed that two transcripts encoding XTH proteins (*HtXTH7* and *HtXTH15*) were synthesized in significantly elevated quantities under high-temperature circumstances (Sezgin Muslu et al. 2025). Consequently, we hypothesized that these two XTH proteins may be integral to the high-temperature tolerance of *H. thermophilum*. *HtXTH7* and *HtXTH15* genes were introduced and over-expressed in the tomato plant to validate our theory. Subsequently, these produced lines were subjected to elevated temperatures, drought conditions, and combinations of both abiotic stressors. Consequently, the functions of these two genes in stress tolerance were examined in the current study. It is shown that transgenic tomato plants overexpressing the two *H. thermophilum* transcripts encoding *HtXTH7* and *HtXTH15* are much more resistant to high temperature and to drought than the wild-type lines.

Materials and Methods

Identification and Comparative Analysis of XTH Genes Across Diverse Organisms

Protein sequences of xyloglucan endotransglucosylase/hydrolase (XTH) genes from *Heliotropium thermophilum*, *Solanum lycopersicum*, *Oryza sativa*, and *Arabidopsis thaliana* were retrieved from the Phytozome v13 database (<https://phytozome-next.jgi.doe.gov/>) (Table S1) (Goodstein et al. 2011). To construct the phylogenetic tree, all protein sequences were aligned using ClustalW with default parameters through the MEGA 12 alignment tool (Kumar et al. 2024). The resulting alignment file in FASTA format was used to construct a maximum likelihood phylogenetic tree via the IQ-TREE web server (<http://iqtree.cibiv.univie.ac.at>) (Trifinopoulos et al. 2016). The best-fit model was automatically selected, and the tree was inferred with 1,000 bootstrap replicates. The resulting Newick tree file was extracted for further visualization.

Conserved domain analysis was performed using the NCBI Conserved Domain Database (CDD) tool (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>), which predicted functional domains across the protein sequences. Conserved motifs were identified using the MEME Suite web tool (<https://meme-suite.org/>) (Bailey et al. 2015), with the maximum number of motifs set to 10 and other parameters

kept at default. The final figure, including the phylogenetic tree, conserved domain architecture, and motif distribution, was generated using the TBtools II software (Chen et al. 2023).

Plant Material and Growing Conditions

Leaf samples were collected from fully expanded, mature leaves of *Heliotropium thermophilum* plants grown for a period of 60 days under controlled greenhouse conditions at Karadeniz Technical University (KTU), located in the Trabzon district of Turkey. The growth conditions and the heat stress of these plants were described in detail in a previous study (Sezgin Muslu Kadioğlu 2021). Leaf material was subsequently transported to Ondokuz Mayıs University (OMU) in an ice box to preserve RNA integrity and was stored at -80°C until RNA isolation. Total RNA was extracted from leaf tissues of *Heliotropium thermophilum* using the FavorPrep™ Total RNA Isolation Kit (Favorgen, Taiwan), following the manufacturer's instructions. The samples were first ground in liquid nitrogen to ensure complete homogenization and preserve RNA integrity from phenolic compounds. RNA purity and concentration were assessed using a NanoDrop™ 2000/2000c spectrophotometer and verified by electrophoresis on a 1.5% (w/v) agarose gel. First-strand cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instructions. The resulting cDNA was used for further genetic transformation.

Cloning of *H. thermophilum* XTH Genes and Development of Expression Constructs

To develop tomato lines overexpressing *HtXTH7* (*c120134_g1_i*) and *HtXTH15* (*c133931_g3_i1*), the coding sequences of *HtXTH7* (882 bp) and *HtXTH15* (865 bp) were amplified by PCR using cDNA from *H. thermophilum* subjected to heat stress and gene-specific primers, as detailed in Table S2. Following purification, the amplified *HtXTH7* and *HtXTH15* PCR product was first inserted into the pENTR/D-TOPO entry vector using pENTR™/D-TOPO™ Cloning Kit according to the manufacturer's protocol. Primary confirmation of the insert and its orientation was performed by Sanger sequencing. Subsequently, the *HtXTH7* and *HtXTH15* coding sequences were individually transferred into the destination vector pIPKb004 (Accession No. EU161570) under the control of constitutive CaMV 35 S promoter using the Gateway® LR recombination reaction (Invitrogen, UK) (Figure S1B). Positive colonies were screened using gene-specific primers and M13 forward primer (Table S2). Final confirmation of the insert and its orientation was performed using Sanger sequencing. All

cloning steps were virtually planned to use SnapGene software (www.snapgene.com) to ensure efficient design and assembly. Next, the overexpression vectors were sent for Sanger sequencing. The recombinant plasmids were subsequently transferred into *Agrobacterium tumefaciens* strain GV3101 via electroporation using the MicroPulser™ system (Bio-Rad, USA).

Agrobacterium-Mediated Transformation of Tomato and Molecular Characterization of Transgenic Lines

Tomato cultivar ‘Crocker’ was selected as the plant material for the overexpression of the *HtXTH7* and *HtXTH15* genes. Tomato genetic transformation was performed according to the protocol previously established in our lab (Bahadır et al. 2024; Secgin et al. 2021). Briefly, tomato seeds were surface-sterilized in 70% (v/v) ethanol for 1 min, followed by treatment with 20% (v/v) commercial bleach supplemented with 0.02% Tween-20 for 20 min. Subsequently, the seeds were rinsed thoroughly five times with sterile distilled water to remove residual disinfectants. Sterilized seeds were then germinated on half-strength Murashige and Skoog (MS) medium. When the seedlings reached approximately 8–10 days of age, cotyledons were aseptically excised from sterile tomato seedlings. Both basal and apical portions were trimmed, and the resulting cotyledon segments were used as explants for transformation.

The *A. tumefaciens* strain GV3101 carrying the recombinant plant expression vector was first transferred by electroporation and subsequently cultured in liquid LB medium until the OD₆₀₀ reached approximately 0.6. The cultures were then centrifuged to pellet the cells, resuspended in liquid MS medium to adjust the final OD₆₀₀ to 1.0. Cotyledon explants were then immersed in the bacterial suspension and gently agitated for 20 min. After infection, excess bacterial solution was removed by blotting the explants onto sterile filter paper. The explants were then transferred to a co-cultivation medium of MS media with vitamins supplemented with 2 mg L⁻¹ 6-benzylaminopurine (BAP), 0.5 mg L⁻¹ indole-3-acetic acid (IAA), 100 mg L⁻¹ ascorbic acid, and 200 µM acetosyringone. Petri dishes containing the infected explants were incubated in the dark at room temperature for two days. Following co-cultivation, explants were washed sequentially in liquid MS medium containing 200 mg L⁻¹ timentin and then in 100 mg L⁻¹ cefotaxime to eliminate the *Agrobacterium*. The washed explants were blotted again on sterile filter paper and transferred to selection medium of MS medium with vitamins, supplemented with 2 mg L⁻¹ BAP, 0.5 mg L⁻¹ IAA, 100 mg L⁻¹ ascorbic acid, 100 mg L⁻¹ timentin, and 10 mg L⁻¹ hygromycin for

plant selection. Plants were cultivated in an environmentally controlled growth chamber maintained at 25 ± 2 °C under a 16-hour light/8-hour dark photoperiod, with a light intensity of 140 µmol m⁻² s⁻¹. Explants were subcultured every 10–12 days until shoot regeneration occurred. Then, the shoots were transferred to a rooting medium containing MS medium with 0.5 mg L⁻¹ IAA, 100 mg L⁻¹ timentin, and 10 mg L⁻¹ hygromycin for plant selection. Regenerated plantlets were acclimatized under a humid environment and then transferred to the greenhouse. To confirm successful transformation, genomic DNA was extracted from T0 seedlings and subjected to PCR using *HtXTH*-specific primers (Table S2). The resulting amplicons were subjected to agarose gel electrophoresis to verify the successful incorporation of transgenes.

Application of Abiotic Stress Treatments in Putative Transgenic Tomato Plants

Seeds from wild-type and putative overexpressing tomato lines were sown in pots containing a 1:1 (v/v) mixture of peat and perlite. Plants were cultivated for four weeks under controlled environmental conditions at 21 ± 2 °C in a growth chamber (DigiTech PG42) with a 16-hour light/8-hour dark photoperiod, 60–70% relative humidity, and a light intensity of 150 µmol photons m⁻² s⁻¹.

Evaluation of Plant Responses to Heat, Drought, and Combined Heat–Drought Stress Conditions

Unstressed control seedlings were grown at room temperature at 21 ± 2 °C for six weeks under identical photoperiod and light intensity conditions to those used for stress treatments. Wild-type (WT) and putative overexpressed (OE) tomato plants were subjected to heat stress by exposing the 6-week-old seedlings to a 38 ± 2 °C temperature for five hours per day over a period of 14 days, as described by Mishra et al. (Mishra et al. 2018). Additionally, six-week-old WT and OE tomato plants were subjected to drought stress for 14 days by irrigating with 40% of the water volume used for full field capacity as described by Jiang et al. (Jiang et al. 2012). Control plants of both WT and OE lines were watered to 100% field capacity. WT and putative OE tomato plants were subjected to combined heat and drought stress by exposing them to 38 ± 2 °C for five hours per day and irrigating 40% of the water volume used for full field capacity. Both treatments were applied simultaneously for 14 consecutive days, as described by Zinta et al. (Zinta et al. 2018). Control plants were watered to 100% field capacity and maintained under normal growth conditions at 21 ± 2 °C.

Tissue Sampling and Biochemical Characterization of Plant Stress Responses

Plant Sampling for Analysis

Three days after the stress treatments, the fifth fully expanded leaf from the plant's apex was harvested from the WT and putative OE plants subjected to control and stress conditions. Sampling was carried out simultaneously to ensure experimental consistency across all treatment groups. Each group consisted of three biological replicates. Samples used for physiological assays and gene expression analyses were collected from the same plants at the same time point. The collected samples were labeled according to their treatment type and immediately stored at -80°C for the subsequent biochemical and molecular analyses.

Determination of Chlorophyll and Carotenoid Contents

Total chlorophyll and carotenoid contents were determined following the method of Arnon (1949) (Arnon 1949). Fresh leaf samples (0.25 g) were homogenized in 5 mL of 80% (v/v) acetone. The homogenate was then centrifuged at $5000 \times g$ for 5 min at room temperature, and the absorbance of the supernatant was measured at 663, 645, and 450 nm using a spectrophotometer. The concentrations of chlorophyll and carotenoids (mg pigment per gram fresh tissue) were calculated using the following equations:

$$\text{Chl a (g L}^{-1}\text{)} = 0.0127 \times A_{663} - 0.00269 \times A_{645},$$

$$\text{Chl b (g L}^{-1}\text{)} = 0.0129 \times A_{663} - 0.00468 \times A_{645},$$

$$\text{Total Chl (g L}^{-1}\text{)} = 0.0202 \times A_{663} - 0.000802 \times A_{645},$$

$$\begin{aligned} \text{Total Chl (g L}^{-1}\text{)} &= 4.07 \times A_{450} \\ &- (0.0435 \times \text{Chl a content} + 0.3367 \times \text{Chl b content}) \end{aligned}$$

Determination of Thiobarbituric Acid Reactive Substances Content

The level of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation, was determined according to the method described by Heath and Packer (1968). Fresh leaf tissue (0.1 g) was homogenized in 1.8 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at $15,000 \times g$ for 10 min. One milliliter of the resulting supernatant was mixed with 4 mL of 0.5% (w/v) thiobarbituric acid prepared in 20% (w/v) TCA. The absorbance of the mixture was recorded at 532 nm, and the non-specific

absorbance at 600 nm was subtracted. The TBARS concentration was calculated using the formula $A = E \times c \times l$.

Assay of Hydrogen Peroxide (H_2O_2) Content

Hydrogen peroxide (H_2O_2) content was determined following the method of Velikova et al. (Velikova et al. 2000). Fresh leaf tissue (0.1 g) was homogenized with activated charcoal in 1.8 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged. One milliliter of the supernatant was mixed with 1 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). The absorbance of the reaction mixture was measured at 390 nm using a spectrophotometer.

Proline Content

Proline content was estimated according to the method of Bates et al. (Bates et al. 1973). Fresh leaf tissue (0.1 g) was homogenized in 3% (w/v) sulphosalicylic acid and centrifuged at $5,000 \times g$ for 5 min. Two milliliters of the supernatant were mixed with 1 mL of acetic acid and 1 mL of ninhydrin reagent. The mixture was incubated in a water bath at 100°C for one hour and then immediately cooled in an ice bath. The reaction mixture was extracted with 3 mL of toluene, and the absorbance of the upper phase was measured at 520 nm.

Assessment of Oxidative Stress Markers Through SOD and APX Enzyme Activity Measurements

Sample Preparation

For antioxidant enzyme analysis, fresh leaf samples (100 mg) were ground in liquid nitrogen and homogenized in 1.8 mL of extraction buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 1% PVPP). The homogenate was then centrifuged at $16,000 \times g$ for 20 min at 4°C , and the supernatant was used for subsequent enzyme assays. For ascorbate peroxidase (APX) activity, 5 mM ascorbic acid was added to the extraction buffer, as described by Demiralay et al. and Terzi et al. (Demiralay et al. 2013; Terzi et al. 2018).

Enzymatic Analysis of SOD and APX Under Stress

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined using the method of Beauchamp and Fridovich (1971) (Beauchamp and Fridovich 1971). The reaction was initiated by adding 2 μM riboflavin to 1 mL of reaction medium containing 50 mM potassium phosphate buffer (pH

7.8), 0.1 mM EDTA, 13 mM methionine, 75 μ M nitro blue tetrazolium, and 50 μ L of enzyme extract. A blank reaction mixture, which did not contain the enzyme, was also prepared. The mixtures were exposed to white light at 375 μ mol $\text{m}^{-2} \text{s}^{-1}$ for 10 min, and absorbance values were measured at 560 nm. SOD activity was expressed as units per milligram of protein ($\text{U mg}^{-1} \text{protein}$). Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was determined by measuring the decrease in absorbance at 290 nm, as described by Nakano and Asada (Nakano and Asada 1981). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 250 μ M ascorbate, 5 mM hydrogen peroxide (H_2O_2), and

100 μ L of enzyme extract. The activity was calculated using the extinction coefficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ for ascorbate.

Expression Profiling of Target Genes Using Quantitative Real-Time PCR

Total RNA was isolated from fully expanded leaves of wild-type and putative transgenic tomato plants using the GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, USA), following the manufacturer's protocol. The integrity and concentration of RNA were confirmed using a NanoDrop spectrophotometer and agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μ g of

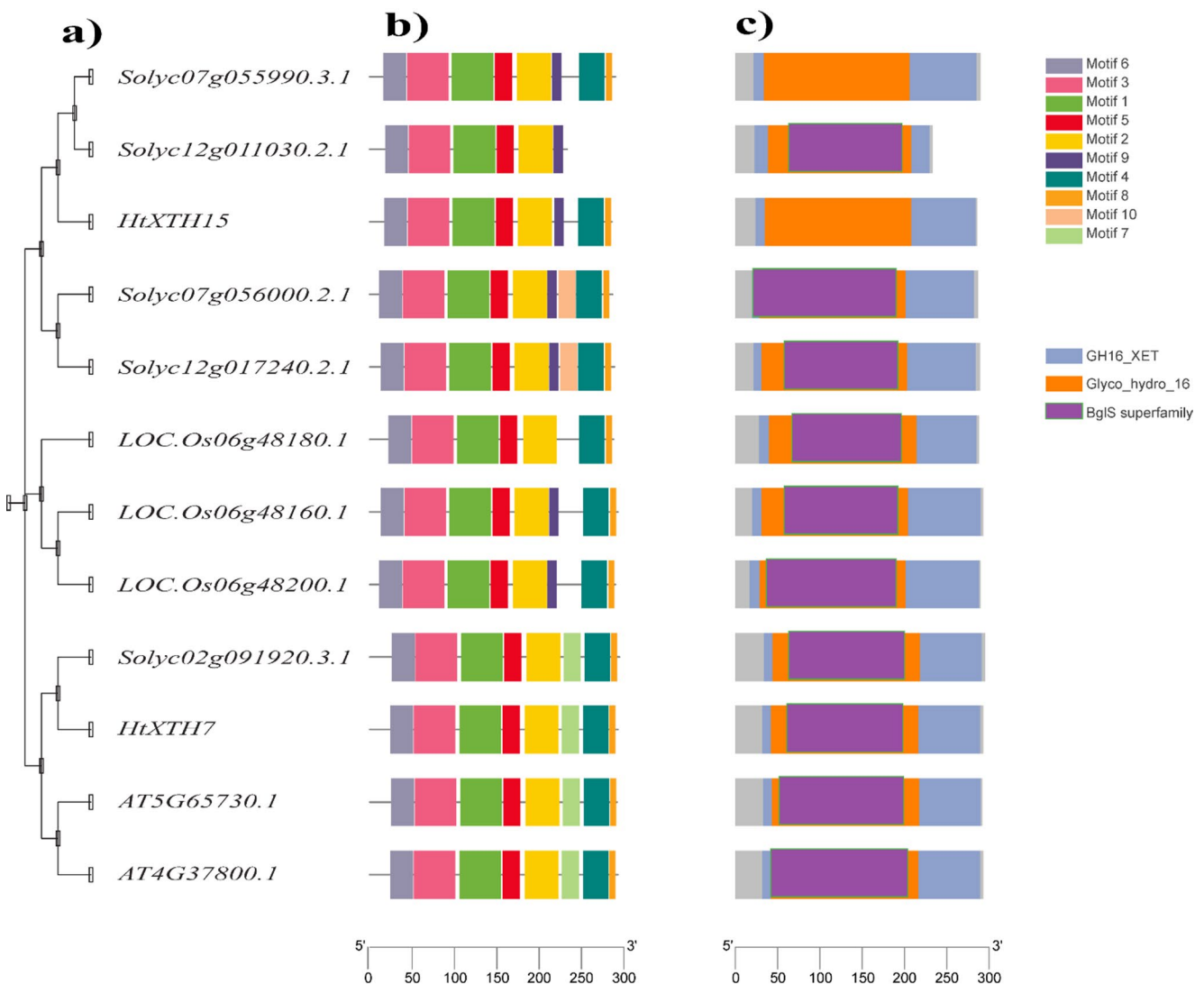


Fig. 1 Phylogenetic relationship, conserved motif organization, and domain composition of XTH proteins from *Heliotropium thermophilum*, *Solanum lycopersicum*, *Oryza sativa*, and *Arabidopsis thaliana*. **(a)** The unrooted phylogenetic tree (left) shows the evolutionary relationships among 12 XTH proteins, including *HtXTH7* and *HtXTH15* from *H. thermophilum*. **(b)** The middle panel depicts the conserved

architecture of the ten motifs. **(c)** The right panel illustrates protein domain composition, highlighting the GH16_XET domain (light-blue), Glyco_hydro_16 domain (orange) and BglS superfamily domain (purple). The conserved structure and clustering patterns suggest evolutionary and functional conservation among XTH family members

RNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, USA). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green Master Mix (Thermo Fisher Scientific, USA) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Tomato *SlActin* (*Solyc03g078400*) was used as the internal reference gene for normalization of expression data, it was previously validated and optimized in our earlier study as the most stable reference gene (Bahadır et al. 2024; Secgin et al. 2022). Gene-specific primers for *HtXTH* and four drought- and heat-responsive marker genes (*SINCE*, *SIRD22*, *SIHSP70*, *SIHSP90*, and *SIHSEA*) were designed using Primer3 software and are listed in the supplementary materials.

The qRT-PCR reactions were carried out in a total volume of 20 µL containing 10 µL of SYBR Green Master Mix, 0.5 µM of each primer, and 2 µL of diluted cDNA. The thermal cycling conditions included an initial denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. A melt curve analysis was performed at the end of each run to verify the specificity of the amplifications. Relative gene expression levels were calculated using the Log2FC method (Design 2009). Each sample was analyzed with three biological replicates and three technical replicates to ensure reproducibility. Results were expressed as fold changes relative to wild-type plants under control conditions.

In Silico Protein-Protein Interaction (PPI) Network Construction and Functional Enrichment

To investigate the functional interaction networks and potential biological partners of *HtXTH7* and *HtXTH15*, a Protein-Protein Interaction (PPI) network was constructed using the STRING database (v12.0) (<https://string-db.org/>). The analysis was performed using *Solanum lycopersicum* (tomato) as the reference organism, with *HtXTH7* and *HtXTH15* (UniProt ID: A0A3Q7IA75) serving as the central nodes. A minimum required interaction score of 0.400 (medium confidence) was set to filter reliable interactions. The network was generated based on diverse evidence sources, where different edge colors represent the type of interaction: black lines for co-expression, magenta lines for experimentally determined interactions, light blue lines for curated databases, green lines for gene neighborhood, red lines for gene fusions, dark blue lines for gene co-occurrence, and yellow lines for automated text mining. To further refine and visualize the network, the data was exported to Cytoscape software (v3.10). Central hubs were identified based on their connectivity scores, and functional enrichment analysis, including Gene Ontology (GO) and KEGG pathways, was integrated to categorize the interacting proteins according to their biological processes and molecular functions.

Statistical Analysis

All experiments, including biochemical assays and gene expression analyses, were conducted with at least three biological replicates. Data are presented as mean ± standard error (SE). For physiological and biochemical parameters (chlorophyll content, carotenoids, H₂O₂, TBARS, and proline), statistical significance among genotypes (WT, OE-1, and OE-2) and treatments was determined using One-Way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test ($P < 0.05$). For gene expression analysis (qRT-PCR) and PPI-related expression profiles, statistical differences were evaluated using One-Way ANOVA followed by Tukey's post-hoc test ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$). In the graphical representations of expression data, asterisks directly above the bars indicate significance compared to the control, while brackets denote differences between specific stress groups. All statistical procedures were performed using GraphPad Prism (v9.0) and SPSS (v25.0).

Results

Phylogenetic, Domain, and Motif Relations between *Heliotropium*, *Arabidopsis*, *Tomato*, and *Rice*

To explore the structural conservation and evolutionary relationship of *HtXTH7* and *HtXTH15*, we performed a comparative analysis of the XTH gene family members across multiple plant species (Fig. 1). Homologous protein sequences from *H. thermophilum*, *S. lycopersicum*, *O. sativa*, and *A. thaliana* were analyzed to examine conserved motifs, domain composition, and phylogenetic grouping. The resulting unrooted phylogenetic tree grouped the 12 proteins into two major clades (Fig. 1a). *HtXTH7* clustered closely with *Solyc02g091920.3.1*, *AT5G65730.1*, and *AT4G37800.1*, indicating a close evolutionary relationship. *HtXTH15* was grouped with *Solyc07g056000.2.1*, *Solyc12g017240.2.1*, and the *O. sativa* proteins *LOC.Os06g48180.1* and *LOC.Os06g48160.1*, suggesting a distinct subclade within the XTH gene family. The conserved motif analysis revealed consistent motif architecture across all sequences (Table S3). Three motifs (namely, motifs 1, 2, 3, 5, and 6) were uniformly arranged in all proteins (Fig. 1b). Both *HtXTH7* and *HtXTH15* exhibited the same motif order and structure; however, motif 7 was uniquely present in *HtXTH7*, whereas motif 9 was specific to *HtXTH15*. The XTH protein domain analysis revealed that all sequences contained GH16_XET, Glyco_hydro_16 and BglS domains, except for *HtXTH15* and *Solyc07g055990*, which included only the Glyco_hydro_16 and BglS domains (Fig. 1c).

Assembly of Gene Constructs and Agrobacterium-Mediated Transformation in Tomato

To investigate the functional roles of *HtXTH* genes in tomato plants, we generated putative transgenic lines overexpressing two selected members of the XTH gene family, *HtXTH-7* and *HtXTH-15*. Two pENTR/D-TOPO vectors were constructed (Fig. S1). The resulting gene fragments were subsequently recombined into the plant expression vector pIPKb004 using the LR Clonase system, yielding the constructs pIPKb004_HtXTH-7 and pIPKb004_HtXTH-15. Sequence analysis using MEGA software for alignment confirmed the successful integration of the target genes into the intended vector regions (Fig. S2 and Fig. S3).

Agrobacterium-mediated transformation was employed to introduce the genetic constructs into young cotyledons of tomato plants. Approximately 83% of the precultured cotyledonary explants co-cultivated with *Agrobacterium* exhibited shoot bud induction on MS medium supplemented with 3 mg L⁻¹ BAP and 0.1 mg L⁻¹ IAA, in the presence of 15 mg L⁻¹ hygromycin for selection and 80 mg L⁻¹ Timentin for bacterial suppression. Shoots were induced from cotyledonary explants on the same medium after ten weeks of culture. The regenerated shoots were elongated and subsequently propagated as independent putative transgenic lines on MS medium supplemented with 2.0 mg L⁻¹ BAP.

In total, five putative transgenic plants were successfully recovered for each gene. After successfully regenerating putative transgenic plants, further validation was performed. To verify the success of the transformation, PCR was performed using gene-specific primers to detect the presence of the target gene fragments. As a result, 4–5 putative transgenic lines were identified for each construct. All steps involved in the purification and cloning of the *HtXTH* genes, including Agrobacterium-mediated transformation and plant regeneration, are illustrated in Fig. 2.

Molecular Characterization of Transgenic Tomato Plants Overexpressing *HtXTH7* and *HtXTH15*

To investigate the functions of *HtXTH7* and *HtXTH15* in tomato, putative transgenic lines overexpressing these genes were developed under the control of the CaMV 35 S promoter. Genomic DNA from T1 generation plants was extracted using the CTAB method (Schenk et al. 2023). The extracted DNA was then subjected to a PCR analysis using gene-specific primers (listed in Table S2) to confirm transgene integration. All five independent transgenic lines were PCR-positive, while no amplification was detected in the wild-type (WT) control. Among the confirmed lines, two representative lines were selected, namely: OE-1 (XTH-7.4) for *HtXTH7* and OE-2 (XTH-15.7) for *HtXTH15*. The

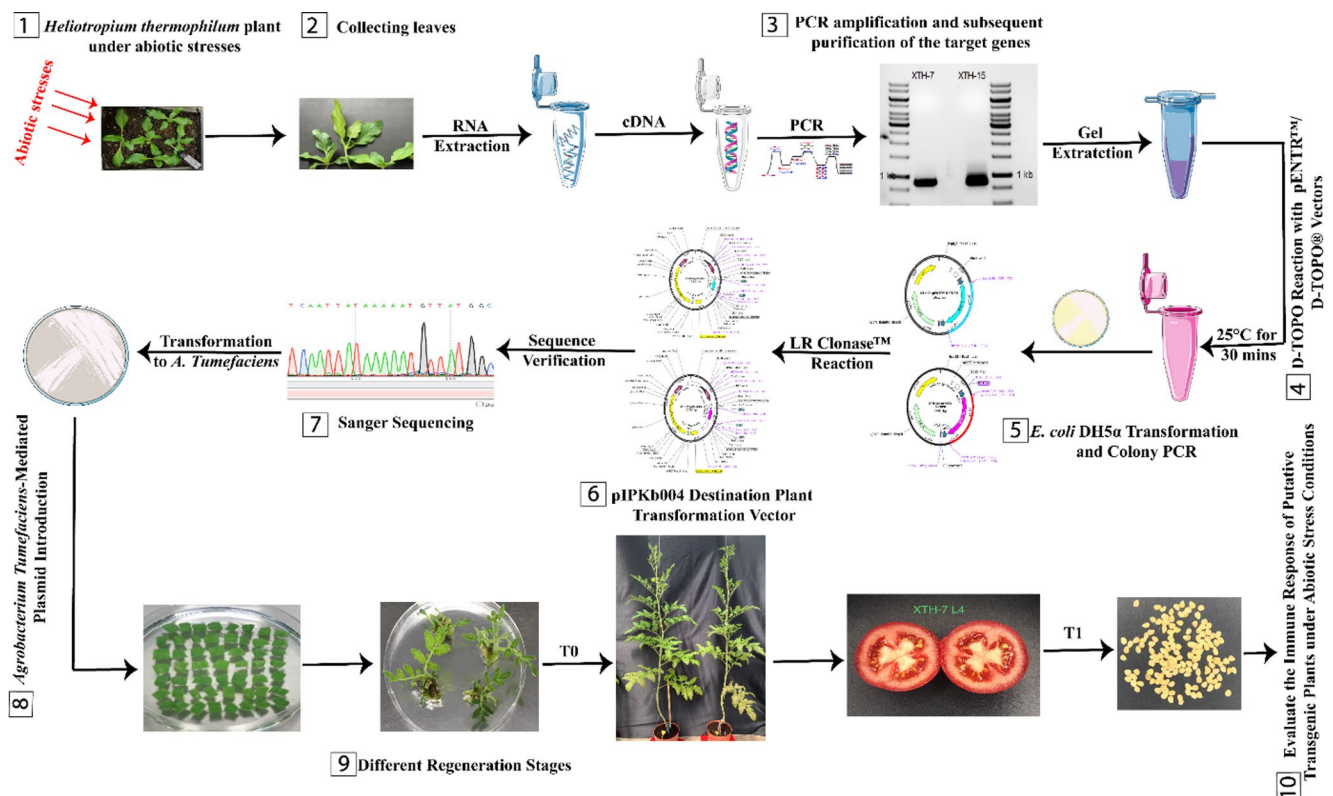


Fig. 2 Schematic overview of *HtXTH* gene purification, cloning, and plant regeneration for the development of putative transgenic tomato lines

selected lines were identified through primary morphological assessments combined with evaluation of plant health status under controlled conditions, and quantitative PCR analysis of abiotic stress-responsive marker genes under stress conditions. These lines were subsequently used in downstream physiological and biochemical assays to assess stress tolerance (Fig. 3).

Overexpressing of *HtXTH* Genes Enhanced Drought and Heat tolerance

To evaluate the protective role of *HtXTH* overexpression on the photosynthetic apparatus, leaf pigment contents were analyzed under non-stress and stress conditions. Under control conditions, WT plants exhibited higher basal levels of all measured pigments compared to the transgenic lines (OE-1 and OE-2). However, upon exposure to stress, pigment levels in WT plants underwent a drastic decline. Interestingly, the protective effect of the *HtXTH* gene varied depending on the stress type and the specific pigment. For chlorophyll a, total chlorophyll, and total carotenoids, both OE-1 and OE-2 lines successfully mitigated stress-induced degradation, maintaining significantly higher levels than WT plants across all stress conditions, including drought, heat, and combined stress (Fig. 4A, C, D). Conversely, the response of chlorophyll b was distinctly stress-dependent; under drought stress alone, WT plants maintained slightly higher or comparable levels to the transgenic lines. However,

under heat and combined stresses, the OE lines effectively prevented chlorophyll b degradation, resulting in significantly higher contents compared to the WT (Fig. 4B). The pronounced maintenance of these pigments, particularly carotenoids, which act as vital non-enzymatic photoprotectants, indicates that *HtXTH*-mediated cell wall remodeling effectively prevents stress-induced oxidative damage and premature chloroplast senescence, thereby sustaining photosynthetic capacity under severe abiotic stress conditions.

The accumulation of H_2O_2 and the elevation of TBARS levels are primary biochemical indicators of stress-induced reactive oxygen species (ROS) generation and subsequent lipid peroxidation. Under non-stress conditions, basal levels of these oxidative markers were relatively low. Upon exposure to stress, WT plants experienced severe membrane damage, peaking particularly during the combined heat and drought treatment (Fig. 5A, B). The overexpression of the *HtXTH* gene effectively mitigated this oxidative damage, albeit in a line-specific manner. The OE-2 line exhibited a pronounced and consistent reduction in both H_2O_2 and TBARS levels across all stress conditions compared to the WT. The OE-1 line also significantly suppressed these oxidative markers under combined stress, though its levels were comparable to or slightly higher than the WT under isolated heat and drought treatments. Concurrently, the accumulation of proline, a critical osmoprotectant and cellular chaperone, displayed a highly dynamic and stress-specific response. Interestingly, under drought stress alone, WT

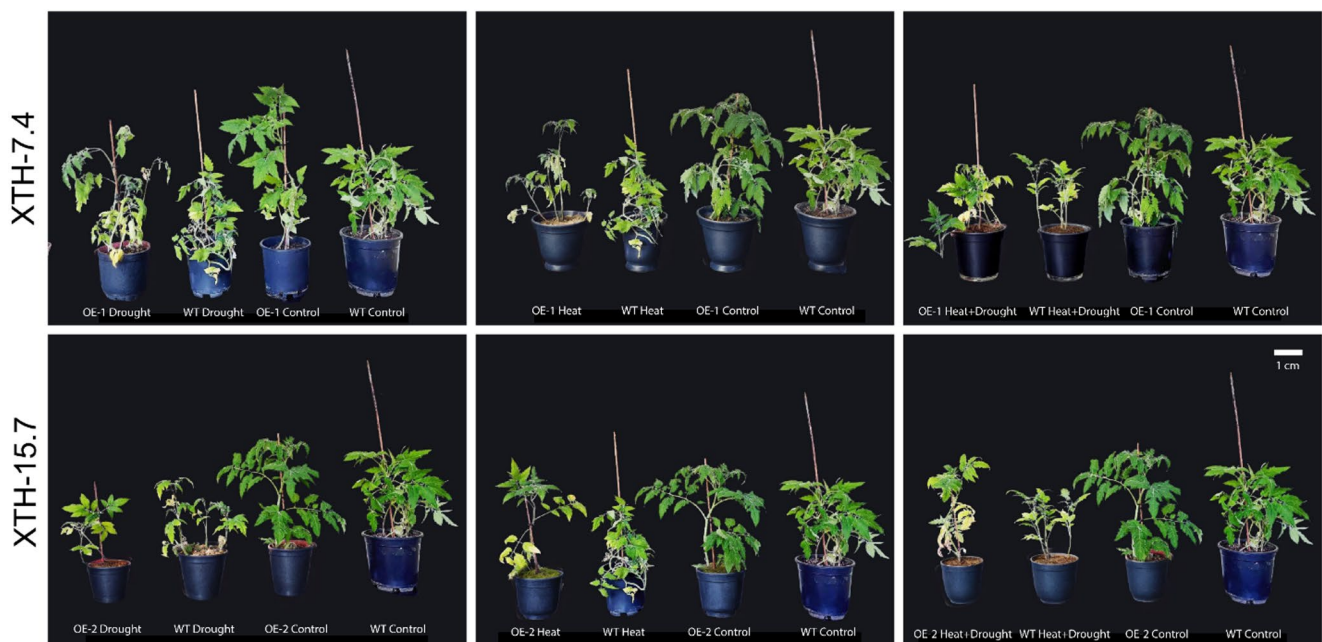


Fig. 3 Phenotypic comparison of transgenic tomato overexpressing lines (OE-1 and OE-2) under abiotic stress conditions. Tomato plants were subjected to (left to right) drought stress, heat stress, and combined heat+drought stress. Upper panels show the performance of OE-1 and wild-type (WT) plants under each condition, while lower

panels depict OE-2 and WT controls. Under drought and combined stress, OE lines displayed improved vigor, delayed wilting, and better shoot development compared to WT plants, suggesting enhanced stress tolerance conferred by *HtXTH* overexpression. All plants were imaged at the end of the stress treatment period. Scale bar = 1 cm

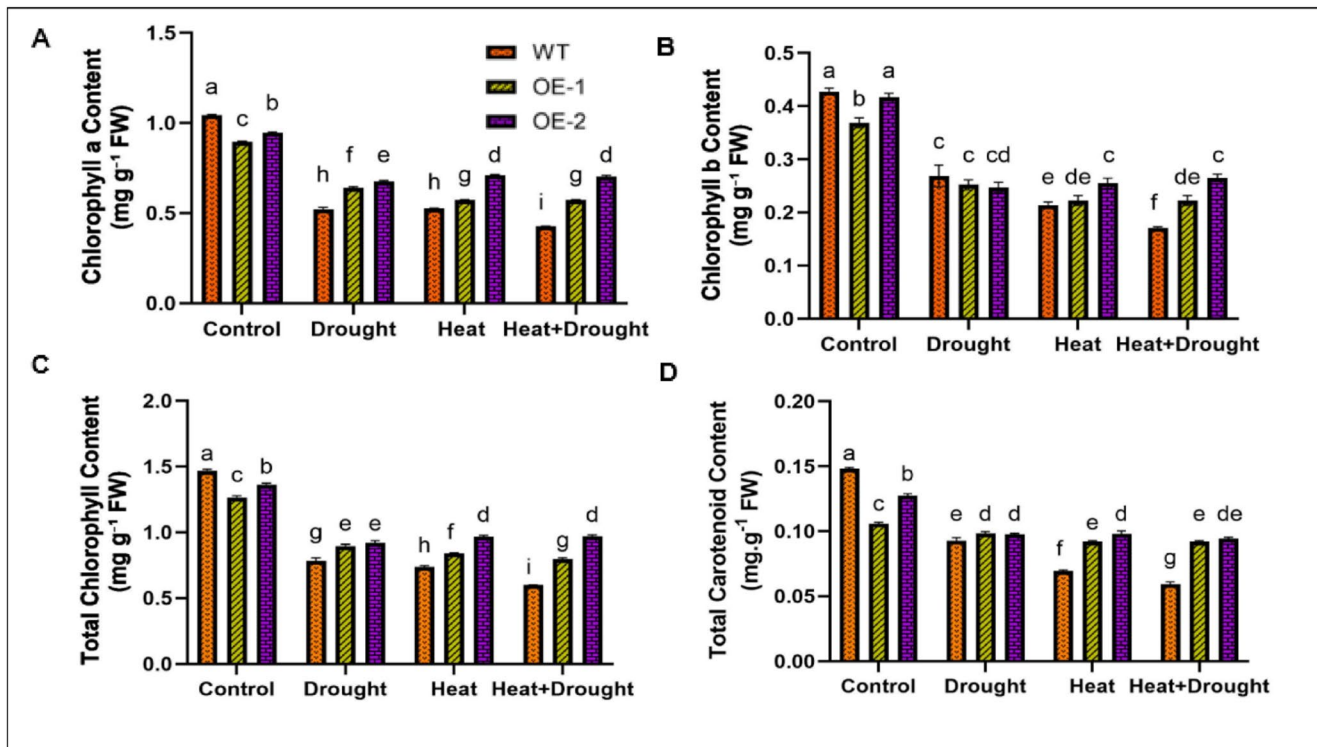


Fig. 4 Chlorophyll a (**A**), Chlorophyll b (**B**), Total chlorophyll (**C**) and Total carotenoid (**D**) content in tomato plants grown under stress and non-stress conditions. The groups are defined as WT (wild type), OE-1

(XTH-7.4), and OE-2 (XTH-15.7). Data are presented as means $\pm$ SE ($n=3$). Different letters indicate statistically significant differences among treatments according to Duncan's test ($P<0.05$)

plants accumulated more proline than the transgenic lines, likely reflecting a severe physiological distress signal due to rapid dehydration. However, under heat and combined stresses, both transgenic lines (particularly OE-2) mounted a massive and statistically significant proline surge, far exceeding the WT capacity (Fig. 5C). Collectively, these findings indicate that *HtXTH*-mediated cell wall remodeling not only preserves plasma membrane integrity by restricting lipid peroxidation but also synergistically reprograms osmolyte biosynthesis, thereby conferring robust biochemical tolerance against severe abiotic stresses.

To further elucidate the ROS scavenging capacity of the transgenic lines, the activities of SOD and APX, two pivotal metalloenzymes that sequentially coordinate the detoxification of superoxide radicals into water, were quantified. Under non-stress conditions, the transgenic lines exhibited inherently higher basal APX activities compared to the WT. Upon stress induction, APX activity, which is directly responsible for H_2O_2 detoxification, was significantly and consistently upregulated in both OE-1 and OE-2 lines across all stress treatments, reaching its maximum under combined heat and drought stress (Fig. 6B). The SOD activity profile, however, revealed a highly dynamic and stress-specific response. While the OE lines demonstrated superior SOD activity under drought and combined stress conditions, the

WT exhibited a massive increase in SOD activity under isolated heat stress, matching or slightly exceeding the transgenic lines (Fig. 6A). This specific WT response provides a crucial mechanistic insight: the disproportionately high SOD activity in WT under heat stress rapidly converts superoxide radicals into H_2O_2 , but due to its concurrently lower APX capacity, the WT fails to effectively scavenge the resulting H_2O_2 accumulation. In contrast, the *HtXTH*-overexpressing lines, particularly OE-2, maintain a tightly synchronized upregulation of both SOD and APX. This precise enzymatic coordination ensures that the ROS generated during severe abiotic stress is efficiently detoxified, thereby explaining the reduced lipid peroxidation, lower H_2O_2 levels, and enhanced membrane stability observed in the transgenic plants.

Molecular Responses in *HtXTH7* and *HtXTH15*-Overexpressing Transgenic Lines Under Drought, Heat, and Combined Stress Conditions

To confirm the successful integration and expression of the transgenes, the expression patterns of *HtXTH7* and *HtXTH15* were initially evaluated using qRT-PCR (Table S4). In the *HtXTH7* overexpression lines, transcript abundance showed massive increases ranging from 5- to 50-fold

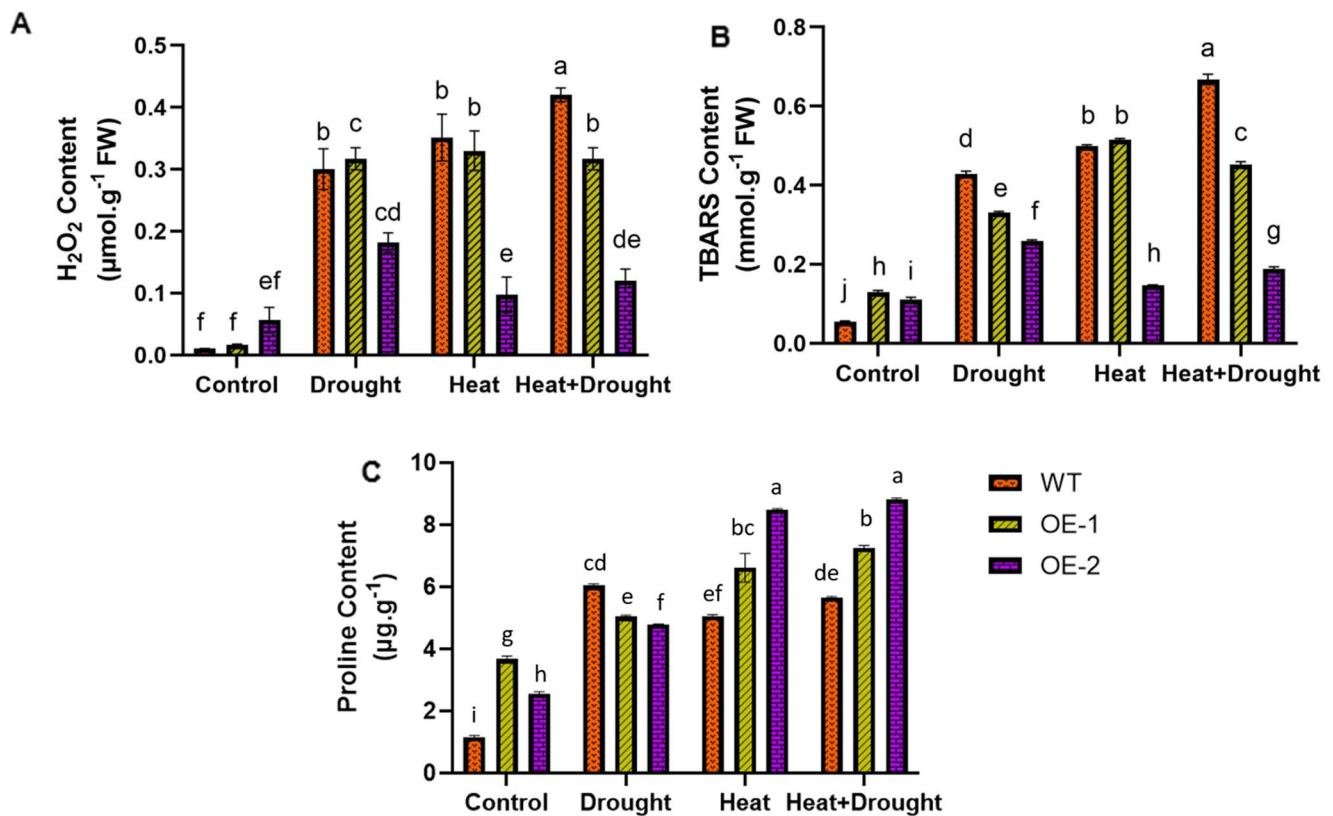


Fig. 5 H₂O₂ (A), TBARS (B), and Proline (C) contents in tomato plants under stress and non-stress conditions. Groups include WT, OE-1 (XTH-7.4), and OE-2 (XTH-15.7). Data are expressed as means±SE

(*n*=3). Different letters denote significant differences among treatments (Duncan's test, *P*<0.05)

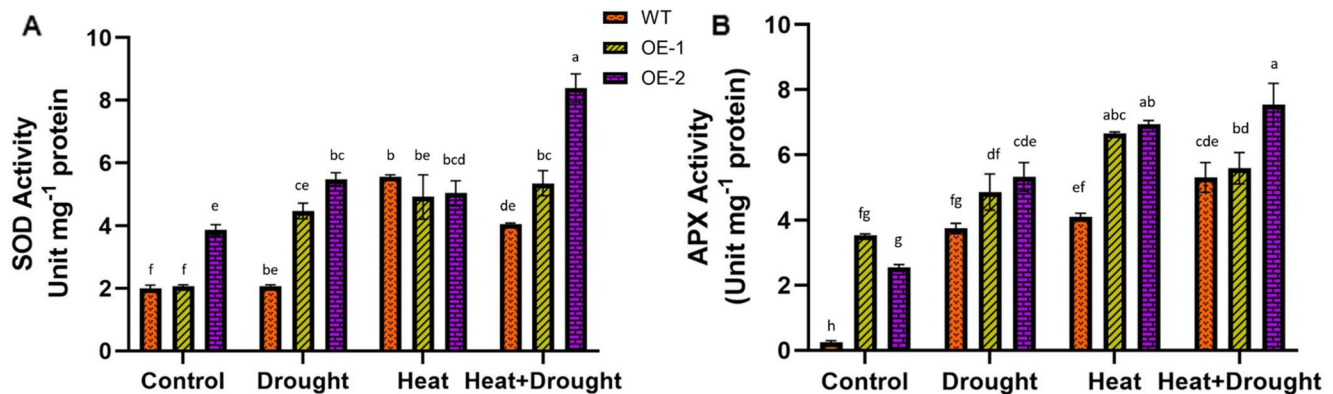


Fig. 6 Superoxide dismutase (SOD, A) and ascorbate peroxidase (APX, B) activities in tomato plants under stress and non-stress conditions. Groups include WT, OE-1 (XTH-7.4), and OE-2 (XTH-15.7).

Results are presented as means±SE (*n*=3). Different letters indicate significant differences among treatments (Duncan's test, *P*<0.05)

relative to the empty vector (EV) control, with the highest expression observed in line #7 (50-fold), followed by line #16 (40-fold) (Fig. S4). Similarly, in the *HtXTH15* overexpression lines, expression levels exhibited an 8- to 65-fold increase, with the highest expression detected in line #7 and the lowest in line #4 (Fig. S5). To explore the functional consequences of *HtXTH7* and *HtXTH15* overexpression on the endogenous abiotic stress response networks,

we selected three independent, stably expressing transgenic lines for each construct: *HtXTH7* lines 7-2, 7-5, and 7-8 (where 7-2 corresponds to OE-1 in previous assays), and *HtXTH15* lines 15-3, 15-4, and 15-7 (where 15-7 corresponds to OE-2). These lines were subjected to drought, heat, and combined heat and drought stress, and the expression profiles of key stress marker genes, *RD22*, *NCED*,

HSP70, *HSP90*, and *HSEF*, were quantified relative to wild-type (WT) plants (Fig. 7A–K).

In *HtXTH7* tomato lines, the drought-responsive marker gene *SIRD22* showed substantial upregulation in all lines under heat ($\text{Log}_2\text{FC} \sim 1.0\text{--}1.2$) and combined stress conditions ($\text{Log}_2\text{FC} \sim 1.4\text{--}1.5$), with statistically significant increases compared to control (Fig. 7A). A detailed assessment of the individual drought treatment confirmed a robust molecular response across both transgenic lines. In *HtXTH7* lines, *SIRD22* expression increased significantly under drought stress, with Log_2FC values ranging between 1.0 and 1.3. Similarly, *HtXTH15* lines exhibited a strong induction of *SIRD22* during individual drought treatment, with Log_2FC values reaching 1.1 to 1.3 (Fig. 7B). Under heat stress and combined stress, *SIRD22* levels remained elevated in both backgrounds, peaking at 1.5 in *XTH7* and 1.4 in *XTH15* lines. Similarly, *HtXTH15* transgenic lines also exhibited consistent upregulation of *SIRD22* across all stress treatments, with positive Log_2FC values indicating a robust induction compared to control plants (Fig. 7B). The expression levels in *XTH15* lines were particularly pronounced under drought and combined stress, mirroring the pattern observed in *XTH7* lines and suggesting a shared regulatory mechanism for enhancing tolerance to these stressors. *SINCE*D, a key enzyme involved in abscisic acid (ABA) biosynthesis, showed moderate expression in *HtXTH7* lines under drought conditions ($\text{Log}_2\text{FC} \sim 0.5\text{--}0.7$) and heat stress ($\sim 0.2\text{--}0.3$), but was significantly upregulated under combined stress ($\text{Log}_2\text{FC} \sim 1.3\text{--}1.6$) across all lines (Fig. 7C). In *HtXTH15* lines, *SINCE*D displayed a more complex, treatment-specific regulatory pattern. While the basal expression was lower than WT, *SINCE*D was significantly upregulated in response to individual heat stress ($\text{Log}_2\text{FC} 0.1\text{--}0.3$) and drought stress ($\text{Log}_2\text{FC} 0.3\text{--}0.5$) compared to non-stressed *XTH15* plants. However, this induction was not sustained under combined heat and drought stress, where *SINCE*D expression returned to levels similar to the non-stressed transgenic control (Fig. 7D). These results suggest that while *HtXTH15* plants are capable of activating ABA biosynthesis under individual drought or heat stress, this hormonal response is specifically suppressed or prioritized differently during the more severe transition to multifactorial combined stress.

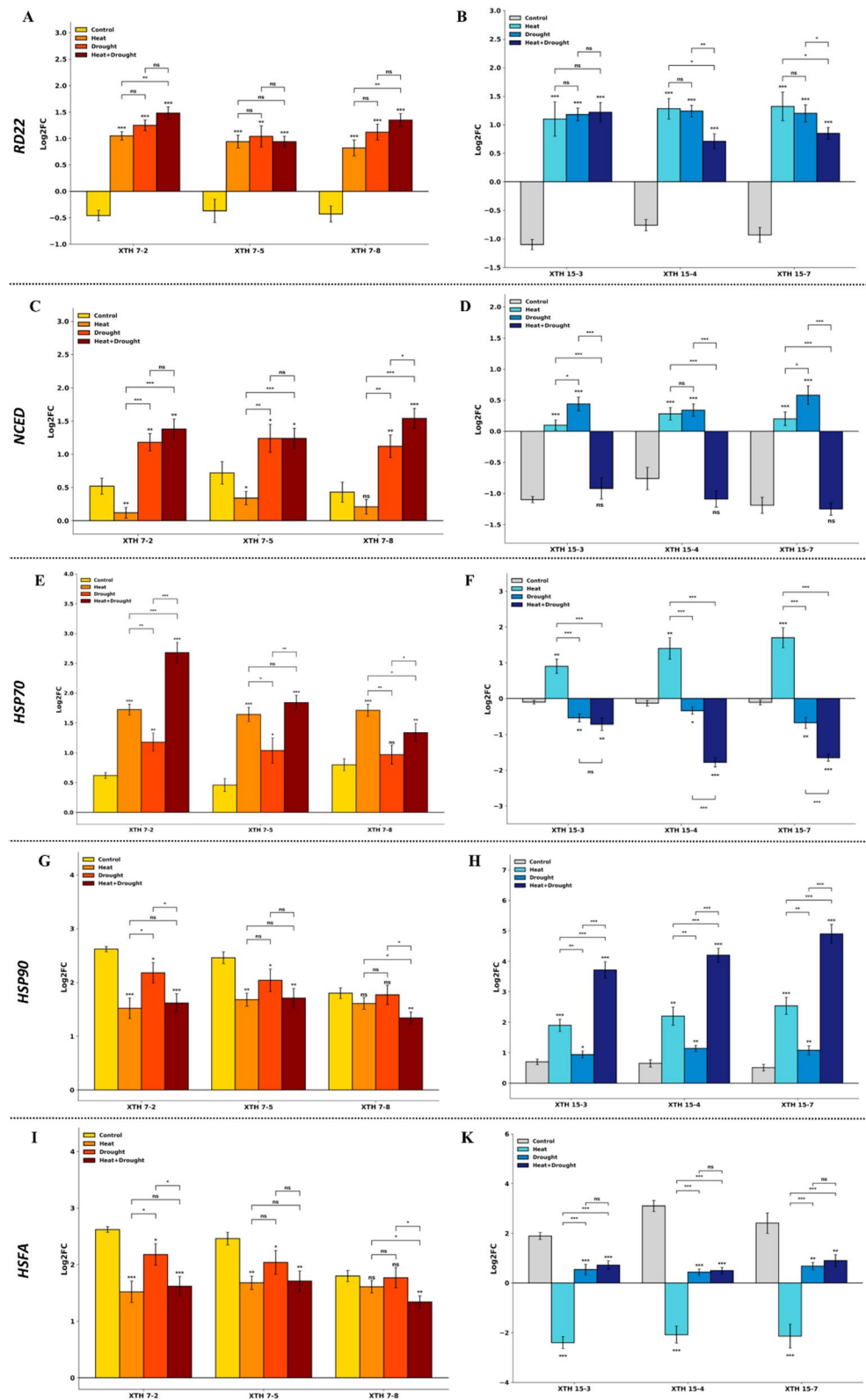
SIHSP90 and *SIHSP70*, molecular chaperones critical for protein stabilization during heat stress, were significantly induced under stress conditions in *HtXTH7* and *HtXTH15* overexpression lines. *SIHSP70* expression under combined stress peaked at $\text{Log}_2\text{FC} \sim 2.8$ in line *HtXTH7-2* (Fig. 7E). Conversely, *SIHSP70* expression was repressed in *XTH15*

lines under combined stress (Fig. 7F). *SIHSP90* showed moderate positive expression ($\text{Log}_2\text{FC} \sim 1.3\text{--}1.9$) with statistically significant differences between control and *HtXTH7* transgenic stress groups (Fig. 7G). In *HtXTH15* transgenic lines, *SIHSP90* exhibited a strong increase in stress-responsive, with the highest induction observed under combined stress ($\text{Log}_2\text{FC} \sim 3.8\text{--}5.0$ across lines) and heat-only conditions ($\text{Log}_2\text{FC} \sim 2.0\text{--}2.6$) (Fig. 7H). In *HtXTH7* overexpression lines, *SIHSEF*, a key transcriptional regulator of the heat response (Gu et al. 2019), exhibited high basal expression in control plants ($\text{Log}_2\text{FC} \sim 4.2\text{--}5.2$), but was highly downregulated under both heat ($\text{Log}_2\text{FC} \sim -4.5$) and combined stress conditions ($\text{Log}_2\text{FC} \sim -2.0$) (Fig. 7I). Similarly, *SIHSEF* expression in *HtXTH15* lines was strongly expressed under control conditions ($\text{Log}_2\text{FC} \sim 2.5\text{--}2.8$), but was significantly downregulated under heat stress ($\text{Log}_2\text{FC} \sim -2.0$ to -2.5), and to a lesser extent under combined stress ($\text{Log}_2\text{FC} \sim -0.5$ to -0.8) (Fig. 7K).

Unraveling Biological Pathways through PPI and GO Enrichment

To further explore the downstream molecular effect of genes associated with *HtXTH7* and *HtXTH15* overexpression in tomato, candidate genes were selected based on the protein–protein interaction (PPI) network analysis (Fig. 8A, E). For *HtXTH7*, the stress-related genes *A0A3Q7FSD1* and *EXP18* were identified, while for *HtXTH15*, *A0A3Q7F2G9* and *A0A3Q7FXZ3* were found as high-confidence interactors. In *HtXTH7* lines, *EXP18*, a protein involved in cell wall loosening, was highly upregulated under drought stress ($\text{Log}_2\text{FC} \sim 2.0\text{--}2.3$), downregulated under heat stress ($\text{Log}_2\text{FC} \sim -2.0$), and showed mild expression under combined heat and drought stress ($\text{Log}_2\text{FC} \sim 0.2\text{--}0.3$) (Fig. 8C). Conversely, *A0A3Q7FSD1*, a stress-inducible antioxidant enzyme, was markedly downregulated under all stress conditions, with Log_2FC values as low as ~ -7.5 to -8.2 (Fig. 8D). This significant downregulation suggests a potential downregulation of ROS scavenging pathways in these lines. In *HtXTH15*, *A0A3Q7F2G9* and *A0A3Q7FXZ3* were identified, both proteins exhibited mild upregulation under drought stress ($\text{Log}_2\text{FC} \sim +0.2$), but were significantly downregulated under heat stress ($\text{Log}_2\text{FC} \sim -1.0$ to -1.4) and combined heat plus drought stress ($\text{Log}_2\text{FC} \sim -0.8$ to -1.1) (Fig. 8G, H). These expression patterns indicate that *A0A3Q7F2G9* and *A0A3Q7FXZ3* play role in stress-sensitive regulation in the *XTH15* overexpressing background, further supporting their potential roles in modulating stress responses.

Fig. 7 Differential expression of stress-responsive genes in HtXTH7- and HtXTH15-overexpressing tomato lines under control, drought, heat, and combined (heat+drought) stress conditions. **A–K** Bar plots showing mean Log₂ fold-change (Log₂FC) values of selected stress-responsive genes from qRT-PCR analysis across three XTH7-OE lines (XTH7-2, XTH7-5, XTH7-8; left panels) and three XTH15-OE lines (XTH15-3, XTH15-4, XTH15-7; right panels). Target genes include: **A–B** RD22 (*Solyc08g068150*), a drought marker gene. **C–D** NCED (*Solyc07g056570*), involved in ABA biosynthesis. **E–F** HSP70 (*Solyc11g020040*), a molecular chaperone. **G–H** HSP90 (*Solyc06g036290*), another heat-responsive chaperone. **I–K** HSFA (*Solyc08g062960*), a key heat stress transcription factor. Statistical significance was determined using one-way ANOVA followed by Tukey's post-hoc test ($p < 0.05^*$, $p < 0.01^{**}$, $p < 0.001^{***}$; ns = not significant). Asterisks placed directly above the bars indicate statistical significance compared to the control group, while asterisks on brackets (horizontal bars) denote significant differences between specific stress treatment groups. Error bars represent $\pm$ standard error (SE) from three biological replicates



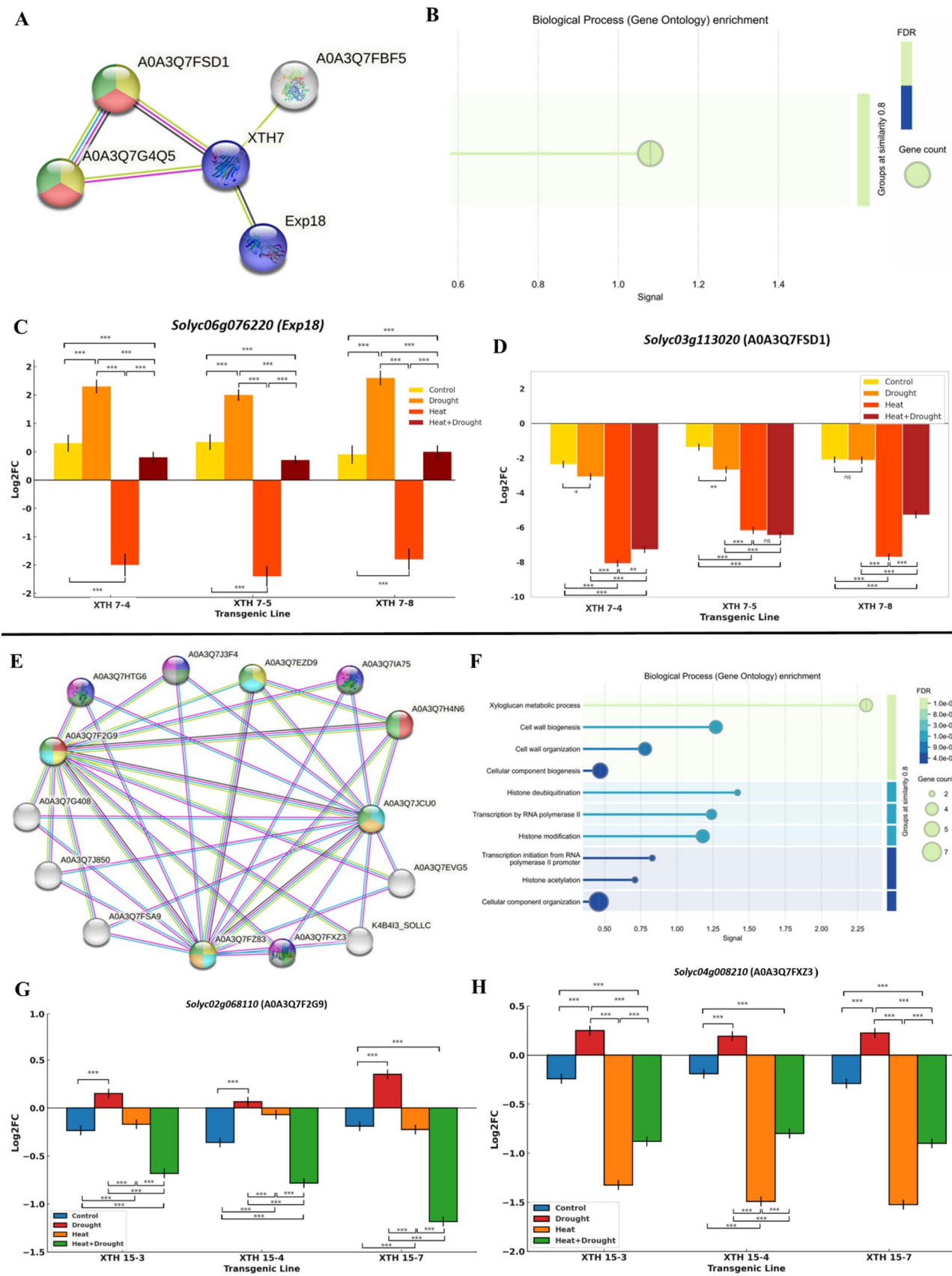


Fig. 8 Protein–protein interaction (PPI) network analysis and expression profiles of stress-related genes in *HtXTH7*- and *HtXTH15*-overexpressing tomato lines under abiotic stress. In the PPI networks, proteins are identified by their UniProt accession numbers (e.g., *HtXTH15* is represented by A0A3Q7IA75) and ITAG locus identifiers. The thickness of the lines (edges) corresponds to the strength of the interaction based on the combined score, while the colors represent the specific types of biological evidence as described in the methodology. **(A)** Predicted PPI network for *HtXTH7* and its interacting partners (A0A3Q7FSD1, A0A3Q7F2G9, A0A3Q7FXZ3, Exp18) generated using STRING database. **(B)** Gene Ontology (GO) enrichment analysis of *HtXTH7* PPI network, highlighting biological processes with significant enrichment (FDR < 0.05). **(C–D)** qRT-PCR analysis of *Exp18* (*Solyc06g076220*) and *A0A3Q7FSD1* (*Solyc03g113020*) expression in *HtXTH7*-overexpressing lines (XTH7-2, XTH7-5, XTH7-8) under control, drought, heat, and combined stress. **(E)** PPI network of *XTH15* and its candidate interactors, including *A0A3Q7F2G9* and *A0A3Q7FXZ3*. **(F)** GO enrichment of *HtXTH15* PPI network showing involvement in cell wall organization, transcriptional regulation, and stress signaling pathways. **(G–H)** Expression profiles of *Solyc02g068110/A0A3Q7F2G9* and *Solyc04g008210/A0A3Q7FXZ3* in *HtXTH15*-overexpressing lines (XTH15-3, XTH15-4, XTH15-7) under various stress treatments. Bars represent mean Log₂ fold-change (Log₂FC) from three biological replicates. Statistical significance was determined by one-way ANOVA followed by Tukey's HSD test ($p < 0.05$, $p < 0.01$, $p < 0.001$). Error bars indicate $\pm$ SE

Discussion

Drought and heat are undoubtedly critical abiotic constraints that significantly impair crop yield and pose a substantial threat to global food security, a challenge further intensified by the escalating impacts of climate change and the rising frequency and severity of these stress conditions (Lamaoui et al. 2018). Plants have evolved dynamic morphological, physiological, and biochemical strategies that enable them to escape or adapt to adverse environmental conditions. Although plants exhibit diverse stress-associated phenotypes, a set of common responses known as stress-induced morphogenic responses (SIMRs) has been identified. These responses involve modifications in cell division and elongation, which are thought to serve as central regulatory hubs during stress adaptation (Potters et al. 2007, 2009). *XTHs* are key cell wall-modifying enzymes known to play critical roles in plant growth, development, and responses to abiotic stress (De Caroli et al. 2021). *Heliotropium thermophilum*, a species restricted to geothermal habitats with exceptionally high soil temperatures, provides a valuable model for investigating plant responses to heat stress (Sezgin Muslu et al. 2025). In this study, we explored two *XTH* genes from *H. thermophilum*. *HtXTH7* and *HtXTH15* were selected from our transcriptome studies published earlier (Ozturk et al. 2020; Sezgin Muslu et al. 2025) and assessed their structural features, phylogenetic relationships, and functional roles through overexpression in transgenic tomato under drought, heat and combination of both stress conditions.

HtXTH7 and *HtXTH15* proteins exhibited conserved motif organization and structural features; however, motif 7 was uniquely detected in *HtXTH7*, whereas motif 9 was exclusive to *HtXTH15*. According to Pfam annotation, motifs 1–3 and 5 were assigned to the glycosyl hydrolase family 16 (GH16) domains, whereas motif 4 corresponded to the xyloglucan endo-transglycosylase (XET) C-terminal domain. Domain architecture analysis further revealed that most *XTH* proteins contained the GH16_XET, Glyco_hydro_16, and BglS domains. Our results are consistent with those of Yang et al., who demonstrated that several Glycoside Hydrolase Family genes associated with lignin, cellulose, hemicellulose, and pectin metabolic pathways were co-expressed with *MtBGAL1* and *MtBGAL23*. Furthermore, subcellular localization analysis revealed that both *MtBGAL1* and *MtBGAL23* are localized to the cell wall, suggesting their involvement in the modification and remodeling of cell wall components in *Medicago truncatula* (Yang et al. 2021). In contrast, *HtXTH15* and *Solyc07g055990* lacked the GH16_XET domain and retained only the Glyco_hydro_16 and BglS domains (Fig. 1). This aligns with canonical observations in other species: the conserved motif patterns and domain architecture of *XTH* genes in *Brassica*, poplar, wheat, and soybean reflect consistent evolutionary constraints (Bi et al. 2024; Han et al. 2013; Wu et al. 2020). Moreover, *Os4bglu12* β -glucosidase was functionally characterized and shown to exhibit strong exoglucanase activity, supporting its proposed involvement in cell wall metabolism and remodeling processes (Opassiri et al. 2006). The majority of plant glycoside hydrolases are involved in the metabolism of cell wall polysaccharides, which supports the hypothesis proposed in this study. In addition to this primary role, these enzymes participate in glycan biosynthesis and remodeling, energy mobilization, plant defense responses, symbiotic interactions, cellular signaling, secondary metabolism, and glycolipid metabolism (Feng et al. 2021; Minic 2008). Our results are consistent with those reported by Strohmeier et al. 2004; raising the possibility that *XTHs* from higher plants may be active not only on cell wall xyloglucans but also on (1,3;1,4)- β -D-glucans and arabinoxylans, which are major structural components of grass cell walls (Strohmeier et al. 2004). Moreover, phylogenetic grouping into clades corresponds with previously defined groups I/II, III-A/III-B, and early-diverging categories in *Arabidopsis* and *Brassica* *XTH* families (Chen et al. 2024a, b; De Caroli et al. 2021). Notably, the clustering of *HtXTH7* proteins with tomato and *Arabidopsis* members in one sub-clade and *HtXTH15* with rice and another tomato cluster supports their assignment to distinguish functional modules of the *XTH* family. These studies collectively reveal that gene structure, phylogenetic placement, and catalytic motif composition strongly influence whether a given *XTH*

overexpression confers growth stimulation, developmental modification, or stress tolerance phenotypes.

XTH genes play a significant role in plant morphological development by controlling the remodeling of xyloglucan cross-links within the primary cell wall. XTH proteins regulate cell wall extensibility, thereby influencing cell expansion, organ growth, and overall plant architecture. *AtXTH18*, *AtXTH19*, and *AtXTH20* overexpression in *Arabidopsis* stimulated hypocotyl elongation through enhanced cell wall extensibility, characterized by more linear creep and reduced yield threshold, in contrast to the lack of growth response observed when overexpressing the hybrid aspen PttXET16-34 in *Arabidopsis* hypocotyls (Miedes et al. 2013). Zhang et al., demonstrated that overexpression of *GmXTH1* significantly enhanced root system architecture, as evidenced by increases in total root length, surface area, projected area, root volume, average root diameter, total crossing number, and root tip number. These morphological improvements likely promoted greater water uptake efficiency while limiting leaf water loss through reduced transpiration, thereby alleviating cell membrane damage and ultimately enhancing drought tolerance in soybean (Song et al. 2018; Zhang et al. 2021). *DkXTH1* transgenic plants exhibited larger and more irregularly shaped cells, accompanied by increased cell wall density and expanded intercellular spaces (Han et al. 2017). These structural alterations are likely attributable to enhanced XET activity, which promotes extensive cell wall remodeling during cell wall assembly. Moreover, the elevated frequency of stomatal closure observed in 35 S: *CaXTH3* plants is likely attributable to enhanced *CaXTH3*-mediated cell wall remodeling in guard cells, which may contribute to reduced transpirational water loss under dehydration stress (Choi et al. 2011). In durum wheat, drought stress triggered a notable increase in XTH expression and XET activity, particularly in the root apex, while heat stress generally led to repression of XTH activity, suggesting tissue- and stress-specific regulation (Iurlaro et al. 2016). These findings underscore the functional diversity of *XTH* genes and their distinct effects on plant morphology, which are likely mediated by differential cell wall remodeling.

In our study, both OE-1 and OE-2 lines showed significantly higher levels of chlorophyll a, total chlorophyll, and total carotenoids compared with wild-type plants under all stress treatments. A notable increase in chlorophyll b was detected only in the OE-1 line under combined stress, and in the OE-2 line under both heat stress and combined stress conditions. This finding is consistent with the results of Han et al. (2025), who reported that overexpression of *RsXTH25* of *N. benthamiana* transgenic seedlings maintained significantly higher levels of chlorophyll a, chlorophyll b, and total chlorophyll under Pb stress compared with wild-type

plants, thereby indicating enhanced photosynthetic performance (Han et al. 2025). Moreover, in tomato, expression of *CaXTH3* cDNA under the 35 S promoter resulted in transgenic plants that, when exposed to 100 mM NaCl, retained leaf chlorophyll content and maintained normal root elongation (Choi et al. 2011). The reduced accumulation of TBARS and H₂O₂ in transgenic plants points toward a decrease in oxidative membrane damage, highlighting enhanced ROS management. This response was accompanied by increased antioxidant enzyme activities (SOD and APX), particularly under combined stress in OE-2, aligning with improved detoxification capacity. Proline levels increased markedly in all transgenic lines when exposed to abiotic stress compared with their corresponding plants maintained under control conditions. Among the tested lines, OE-2 showed the greatest proline accumulation, particularly under heat stress and the combined drought–heat treatment, exceeding the levels recorded in the other transgenic lines under the same conditions., further supporting the activation of osmoprotective and ROS-scavenging mechanisms. Song et al. (2022) demonstrated that, under drought stress, transgenic lines overexpressing the *GmXTH1* gene exhibited significantly elevated activities of SOD, POD, and CAT (Song et al. 2022). Meng et al. 2025 showed that, following drought stress, wild-type tobacco exhibited a greater increase in SOD, POD, CAT activities and proline content compared with *StXTH9* overexpression lines. In contrast, transgenic plants displayed a more pronounced accumulation of MDA and H₂O₂ than wild-type plants (Meng et al. 2025). These results underscore the functional importance of *HtXTH7* and *HtXTH15* in improving stress tolerance through structural and biochemical pathways.

In our study, the consistent and robust upregulation of *RD22* in both *HtXTH7*-OE and *HtXTH15*-OE lines under all stress conditions provides strong evidence for a common stress-priming mechanism facilitated by XTH activity. This induction is particularly significant given that *RD22* is a hallmark of the ABA-dependent signaling pathway, essential for mitigating the effects of water deficit and thermal stress (Goh et al. 2003; Yamaguchi-Shinozaki and Shinozaki 1993). The mechanistic link between XTH-mediated cell wall remodeling and the activation of *RD22* can be explained through the Cell Wall Integrity monitoring system. As proposed by Bacete et al., plants possess sophisticated sensing mechanisms, such as receptor-like kinases, that constantly monitor the structural state of the cellulose-xyloglucan network (Bacete et al. 2018). Modifications in the cell wall matrix, such as those induced by the overexpression of *HtXTH7* and *HtXTH15*, likely trigger a ‘wall-to-nucleus’ retrograde signaling cascade. This process informs the cell of structural changes, which in turn leads to the rapid synthesis of ABA and the subsequent induction of dehydration-responsive

genes like *RD22* and *NCED*. Our results align with the findings of Choi et al., where the overexpression of a *Capsicum annuum* *XTH* in tomato was shown to enhance tolerance by specifically priming these downstream stress-responsive networks (Choi et al. 2011). The explicit interpretation of individual drought stress further reinforces this model; the significant upregulation of *SIRD22* ($\text{Log}_2\text{FC} > 1.0$) in both lines during standalone water deficit confirms that XTH-mediated wall modifications serve as a primary trigger for the dehydration-responsive machinery.

A fascinating and nuanced divergent regulation was observed between the ABA-related *NCED* pathway and the heat-shock chaperone machinery. While *HtXTH7* overexpression appears to follow a classic hormonal resilience strategy, *HtXTH15* seemingly adopts a distinct proteostatic defense mechanism. Specifically, *NCED*—the rate-limiting enzyme in ABA biosynthesis—was significantly upregulated in *XTH7-OE* lines, a finding consistent with recent evidence from Cai et al., who demonstrated that *XTH7* is positively regulated to maintain cellular homeostasis and osmotic balance under salt stress (Cai et al. 2023). This suggests that *HtXTH7* enhances tolerance by reinforcing ABA-dependent pathways, which in turn regulate stomatal closure and the expression of dehydration-responsive genes like *RD22*. In contrast, *HtXTH15-OE* lines exhibited a massive induction of *HSP90* ($\text{Log}_2\text{FC} > 4.5$) alongside a simultaneous downregulation of *NCED*. It is noteworthy that *SINCE*D was actually induced in *HtXTH15* lines under individual heat or drought treatments, suggesting that the ABA-dependent pathway is functional but selectively bypassed when stresses are combined. This divergence suggests that *HtXTH15* might prioritize a ‘proteostatic’ survival tactic over traditional hormonal signaling under extreme combined stress conditions. The heavy investment in heat shock proteins indicates a strategic shift toward preventing protein denaturation and maintaining folding integrity, which is vital during the ‘proteotoxicity’ phase of heat stress (Fragkostefanakis et al. 2015). Such a trade-off—suppressing the energy-expensive ABA pathway to prioritize the chaperone machinery—has been documented as a specialized survival strategy in plants facing multi-factorial abiotic stress (Zhu 2016). Consequently, our findings imply that while *HtXTH7* and *HtXTH15* belong to the same family, they have undergone functional sub-specialization: *HtXTH7* acts primarily as a regulator of structural and hormonal (ABA) responses, whereas *HtXTH15* functions as a facilitator of thermal protein stability. This dual-strategy model highlights the complex regulatory roles of XTH genes in coordinating the plant’s molecular response to heat and drought.

Conclusion

In this study, the heterologous overexpression of *Heliotropium thermophilum* genes *HtXTH7* and *HtXTH15* in tomato significantly enhanced plant tolerance to individual and combined heat and drought stresses. The transgenic lines successfully preserved photosynthetic capacity by maintaining significantly higher levels of chlorophylls and carotenoids compared to wild-type plants. Furthermore, *XTH* overexpression bolstered the plant’s antioxidant defense system (elevated SOD and APX activities), which effectively scavenged H_2O_2 and minimized stress-induced lipid peroxidation (TBARS). This biochemical resilience was coupled with a robust reprogramming of stress-responsive molecular networks, highlighted by the pronounced accumulation of proline and the strong, synergistic induction of key marker genes, including *RD22* and specific molecular chaperones. Collectively, these core findings demonstrate that *HtXTH7* and *HtXTH15* mediate highly effective biochemical and structural defense strategies, providing valuable genetic targets for breeding climate-resilient crops.

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Data Availability All data is provided within the article.

Declarations

Conflict of interest The authors declare no conflict of interest.

Generative AI and AI-assisted technologies During the preparation of this work, the authors used AI to assist with text editing. After using this tool or service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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