BMC Plant Biology

RESEARCH Open Access

A tubby-like protein, MdTLP7 enhances drought and salt stresses tolerance of *Malus domestica*

Qing Wang[1], Tongtong Guo[1], Yuxiao Yi[1], Zelin Yin[1], Lili Xu[1], Fengtang Yang[1*] and Jianing Xu[1*]

Abstract

Background Drought and salt stresses as common abiotic stresses have a significant impact on the yield and quality of *Malus domestica* (apple). The tubby-like proteins (TLPs), as a family of transcription factors, play an important role in plant response to abiotic stresses, but the regulatory mechanisms involved remain unclear.

Results Here, we investigated how apple *TLP7* (*MdTLP7*) and its interacting genes regulate apple response to drought and salt stresses. The results shows that overexpression of *MdTLP7* significantly increased the drought and salt tolerance of apple through regulating gene transcription. Yeast two-hybrid screening, together with DNA affinity purification sequencing (DAP-seq), revealed that MdTLP7 interacted with MdNAC72L to regulate the expression of *MdHsp70-8* and *MdAGP*, and that the overexpression of these two genes significantly enhanced the drought and salt stresses tolerance of apple.

Conclusion The apple protein MdTLP7 could interact with MdNAC72L and promoted the expression of *MdHsp70-8* and *MdAGP*, thus improved drought and salt tolerance of apple calli. These results provide insights into the molecular mechanism of *MdTLP7* increasing the drought and salt stresses tolerance of apple as well as a theoretical basis for the expansion of apple cultivation and related research.

Keywords *Malus domestica*, MdTLP7, Drought stress, Salt stress, Transcription factors

Introduction

Apple is an important fruit resource and an integral part of the human nutritional diet in worldwide [1]. In natural environments, multiple abiotic stresses are often present simultaneously and can have serious negative effects on plants [2]. Drought plus salt are common stress combinations [3], and the simultaneous occurrence of different stresses leads to a high degree of complexity in plant responses [4]. Drought and high salt are important environmental factors that can adversely affect the growth and yield of apple [5, 6]. Therefore, the discovery and application of apple varieties resistant to drought and salt stresses can help to expand the area of apple cultivation while maintaining its yield.

Many transcription factors (TFs) involved in apple response to drought and salt stresses. For example, MdSHINE2, an AP2/ERF (APETALA2/ethylene-responsive factor) transcription factor, confers *Arabidopsis thaliana* (*A. thaliana*) drought tolerance by regulating wax biosynthesis [7]. MdDREB2A can bind to the promoter of MdNIR1 and MdSWEET12, and by regulating

*Correspondence:
Fengtang Yang
fengtangyang@163.com
Jianing Xu
xujianing1219@126.com
[1]School of Life Sciences and Medicine, Shandong University of Technology, Zibo, Shandong 255000, China

nitrogen utilisation and sucrose transport to improve drought tolerance in apple [8, 9]. MdWRKY115 improves drought and osmotic stress tolerance by directly binding to the MdRD22 promoter in apple [10]. MdMYB108L, a MYB (V-myb avian myeloblastosis viral oncogene homolog) transcription factor, positively regulates the transcription of the salt tolerance gene *MdNHX1* (Na^+/H^+ exchanger 1) and improves salt tolerance in transgenic apple [11]. MdMYB44-like positively regulates salt and drought tolerance via the MdPYL8-MdPP2CA module in apple [12]. MbDREB1 enhances low temperature, drought, and salt stress tolerance in *A. thaliana* via both ABA-dependent and ABA-independent pathways [13]. The C2H2 (Cys2/His2)-type zinc finger protein MdZAT5 increases the sensitivity of apple calli to salt stress [14]. MdZAT10 decreases the expression level of MdAPX2 and increased sensitivity to PEG6000 treatment in apple calli [15]. And *MdSAT1* of bHLH (basic helix-loop-helix) transcription factor involved in drought and salt responses [16]. MdbHLH160 can promote MdSOD1 enzyme activity and inhibiting excessive ROS levels to promotes apple drought tolerance [17]. In addition, there are also some TFs respond to drought and salt stress by acting together with other TFs. For example, the ERF (ethylene responsive factor) transcription factor MdERF38 interacts with MdMYB1 to promote anthocyanin biosynthesis under drought stress [18]. WRKY17 interacts with WRKY50 to regulate anthocyanin biosynthesis in apple to improve drought tolerance [19]. MdMYB63 and MdERF106 interact with each other to regulate anthocyanin biosynthesis to enhance salt tolerance in apple [20]. The interaction between MdWRKY55 and MdNAC17-L (NAM, ATAF and CUC) enhances salt tolerance in apple by activating *MdNHX1* expression [21].

Tubby-like proteins (TLPs) transcription factors are present as large multigene families and play very crucial role in providing tolerance to the plant against environmental stresses [22]. TLPs are widely present in eukaryotes, such as mice, human [23], rice [24], poplar [25], sorghum [26], apple [27], and wheat [28]. TLPs possess a highly conserved tubby domain, comprising 270 amino acids at the C-terminus, which form a central hydrophobic α-helix and a closed β-barrel with 12 anti-parallel strands [29]. In plants, most TLPs comprised highly conserved F-box domain in addition to TLP domain at carboxyl terminus [30]. F-box proteins mostly comprise one or more greatly varying protein-protein interaction domains like tetratricopeptide repeat (TPR), WD40, kelch repeat and leu-rich repeat (LRR) [31]. Many mammalian and plant TLPs are usually localized in the plasma membrane, TLP is capable of binding to specific phosphatidylinositol 4,5-bisphosphate of plasma membrane [29]. Under specific circumstances, TLPs can be transported from the membrane to the nucleus [32], such as, mice [33], *A. thaliana* [34], chickpea [35]. External stimuli increase the accumulation of TLPs in the nucleus of *A. thaliana* [34]. This suggests that TLPs may play an important role in transmitting external stimuli signals. In plants, TLPs have been demonstrated to play a pivotal role in abiotic stress response [36]. For instance, a previous study demonstrates that *AtTLP9* in *A. thaliana* is involved in response to drought stress and salt stresses [34]. In soybean, *GmTLP8* overexpression enhances tolerance to drought and salt stresses by regulated downstream stress-responsive genes [37]. The *GhTULP30* in cotton confers tolerance to drought stress by regulating stomatal movement [38]. Furthermore, a study on chickpea *CaTLP1* reveals that the transfer of *CaTLP1* into tobacco enhanced the tolerance of transgenic tobacco to drought and salt stresses [35]. The expression levels of some *SiTLPs* in *Setaria italica* were induced by salt stress and high temperature stress [39]. A Tubby-like protein in rice, *OsFBT4* was induced by drought stress and salt stress, and *OsFBT4* elevates salt and drought stress tolerance in *A. thaliana* plants [40].

Previously, Du et al. find that a TLP gene (*MdTLP7*) was significantly up-regulated under cold stress [41]. And the expression of MdTLP7 significantly increased the salt and temperature stress tolerance of *E. coli* cells [42]. And our previously study find the expression of *MdTLP7* leads to significant tolerance to drought, salt, cold and heat stress in *A. thaliana* [43]. But the underlying mechanism *MdTLP7* in mediating abiotic stresses tolerance is not clear. Apple "orin" calli grows rapidly and are easy to handle, making it widely used in experimental research, especially for studying the response mechanisms of apple to abiotic and biotic stresses [38, 44–46]. Here, a positive role of *MdTLP7* in regulating drought and salt stresses tolerance in apple calli was identified. The regulatory mechanisms of *MdTLP7*-mediated drought and salt tolerance in apple were elucidated by RNA-seq and DAP-seq analyses, as well as promoter binding and transcriptional regulation analyses. These results open up new horizons for studying the mechanism of action of stress tolerance in plant TLPs and may promote the development of methods to improve plant stress tolerance.

Methods and materials

Plant materials

The "orin" calli used for transformation and tolerance treatment were named OE, OE-*MdHsp70-8*, OE-*MdAGP* and OE-*MdHsp70-8* + *MdAGP* with high efficiency for transformation. The calli tissues were grown in the dark at 25℃ on Murashige and Skoog solid medium (0.4 mg/L 6-BA + 1.5 mg/L 2,4-D). Apple calli tissues were cultivated on Murashige and Skoog solid and liquid

medium (0.4 mg/L 6-BA + 1.5 mg/L 2,4-D) containing 6% PEG6000 to simulate drought stress [43], and on Murashige and Skoog solid and liquid medium (0.4 mg/L 6-BA + 1.5 mg/L 2,4-D) containing 150 mmol/L NaCl to simulate salt stress [36], and at 160 rpm for growth in the dark (25℃). Apple calli were rapidly frozen in liquid nitrogen and subsequently stored in an ultra-low temperature refrigerator at -80℃ for subsequent experimental testing.

Construction of Transgenic Apple calli

To construct *MdTLP7*, *MdHsp70-8* and *MdAGP* vectors, the coding sequences (CDS) of *MdTLP7* was inserted into Prok II plasmid, and the CDS of *MdHsp70* and *MdAGP* were inserted into pRI101 plasmid. Calli transformation was mediated by Agrobacterium tumefaciens (LBA4404), and transformants were selected on Murashige and Skoog media (50 mg/L kanamycin and 300 mg/L temetin).

Measurement of proline and malondialdehyde and hydrogen peroxide

The contents of MDA, proline and H_2O_2 in the calli of apple were determined in three biological replicates, using Malondialdehyde (MDA) Kit, Proline (Pro) Content Assay Kit, and Hydrogen peroxide (H_2O_2) content Kit follow the manufacturer's instructions (Grace Biotechnology, Suzhou, China).

RNA sequencing

Total RNA was extracted using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. An mRNA sequencing library of six different tissues was constructed on an Illumina HiSeqTM 4000 by Gene Denov O Biotechnology Co (Guangzhou, China). Sequencing data and apple genome (GDDH13_1–1; https://www.rosaceae.org/species/malus/all), and paired-end clean reads were mapped to the reference genome using HISAT2 (version 2.1.0) [47]. For each transcription region, a FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations, using RSEM software [48]. Three replications were performed for each group.

Principal component analysis

Principal component analysis (PCA) was performed with R package models (http://www.rproject.org/) in this experience. PCA is a statistical procedure that converts hundreds of thousands of correlated variables (gene expression) into a set of values of linearly uncorrelated variables called principal components. PCA is largely used to reveal the structure/relationship of the samples/data.

Differentially expressed genes (DEGs) and enrichment analysis

RNA differential expression analysis was performed by DESeq2 [49] software between two different groups (and by edgeR between two samples). KEGG enrichment analysis were performed for DEGs to further analyze their biological functions, through a pipeline on the Omicshare Bioinformatics Cloud Platform (GENEDENOVO, Guangzhou, China).

DNA affinity purification sequencing (DAP-seq)

The DAP-seq of this experiment was completed by Bluescape Scientific. Through in vitro induction, the labeled MdTLP7 transcription factor was expressed, and the total DNA of apple was extracted and fragmented to construct a genomic DNA library. Then the cell-free protein expressed in vitro was combined with the genomic DNA library in vitro, and all the DNA bound to the transcription factor was further separated. Then, the binding site of the transcription factor was found by using high-throughput sequencing technology.

Yeast Two-Hybrid screening

MdTLP7 was constructed on pGBKT7 vector, then pGBKT7-YTLP7 and pGBKT7 were transformed into AH109, coated on SD/-Trp plate, and cultured at 30℃ for 3–4 days to test whether MdTLP7 had self-activation activity. Using AH109 yeast strain containing pGBKT7-YTLP7 bait plasmid as receptor to prepare competence, the library plasmid pGADT7-Apple Nucleus cDNA was transferred into it. Clontech yeast two-hybrid system was used and coated with SD-TLH screening plate. The protein interacting with pGBKT7-YTLP7 was determined through multiple reporter gene detection, DNA sequencing and BLAST comparative analysis of positive clones.

Bimolecular fluorescence complementation assay (BiFC)

The CDS of *MdTLP7* and *MdNAC72L* were inserted into the yellow fluorescent protein (YFP) N-terminal (YFPC) and C-terminal (YFPN) constructs to generate MdTLP7-YFPC and MdNAC72L-YFPN plasmids. Agrobacterium strains carrying recombinants were genetically transformed through Agrobacterium GV3101 mediation. YFP fluorescence was observed by Laser Scanning Confocal Microscope, LSM900 (Zeiss, Oberkochen, Germany).

Quantitative Real-time PCR

Total RNA of plant materials was isolated by using Plant RNA Rapid Extract Kit (Coolaber, Beijing, China). RNA was reverse transcribed using TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen, Beijing, China). The reactions were performed using SYBR® Green Realtime PCR Master Mix (TOYOBO, osaka, Japan) as described by the manufacturer. The

process was conducted using LightCycler® 480 II (Roche, Basel, Switzerland) Real-Time PCR instrument. The apple gene *MdUBQ* was used as an internal control, and real-time PCR experiment was carried out at least three times. Primers used for qRT-PCR analysis was designed by Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), and were summarized in Table S1. The relative expression levels were calculated $2^{-\Delta\Delta Ct}$.

DNA-pull down assay

The CDS of *MdTLP7* was cloned into pET32a. The MdTLP7-His fusion plasmid was prepared and transferred into the Rosetta (DE3) Chemically Competent Cell (AngYu Biotechnologies, Shanghai, China) as described by the manufacturer. Induction of expression was achieved through the use of 0.1 M IPTG at 16℃ and 200 rpm. Purification of the target proteins was conducted using His-Tag Protein Purification Kit with TED-Ni Magnetic Agarose Beads (Beyotime Biotechnology, Shanghai, China). Gradient renaturation was performed in renaturation buffer (50 mM Tris-HCl (pH 8.5), 100 mM NaCl, 7 M/6 M/5 M/4 M/3 M/2 M/1 M/0 M urea, 1% glycine, 5% glycerol, 0.2% PEG6000, 1 mM oxidised glutathione, 1 mM reduced glutathione). Biotin-labelled probes (Tsingke, Beijing, China) containing Streptavidin (SAV) MagBeads (YEASEN, Shanghai, China) were incubated with the target proteins, which were eluted with 0.1 M glycine-HCl (pH 2.5–2.8) and then subjected to SDS-PAGE detection.

In-gel digestion

For in-gel tryptic digestion, gel pieces were destined in 50 mM NH_4HCO_3 in 50% acetonitrile (v/v) until clear. And dehydrated with 100 µl of 100% acetonitrile for 5 min, then rehydrated in 10 mM dithiothreitol and incubated at 56℃ for 60 min. Gel pieces were again dehydrated in 100% acetonitrile and rehydrated with 50 mM iodoacetamide. After incubating at room temperature, in the dark for 45 min. Gel pieces were washed with 50 mM NH_4HCO_3 and dehydrated with 100% acetonitrile. Gel pieces were rehydrated with 10 ng/µl trypsin resuspended in 50 mM NH_4HCO_3 and digested with trypsin at 37℃ overnight. Peptides were extracted with 50% acetonitrile /5% formic acid, followed by 100% acetonitrile. Peptides were dried to completion and resuspended in 2% acetonitrile/0.1% formic acid.

LC-MS/MS analysis

The tryptic peptides were dissolved in 0.1% formic acid (solvent A), directly loaded onto a home-made reversed-phase analytical column (15-cm length, 75 µm i.d.). The gradient was comprised of an increase from 5 to 34% solvent B (0.1% formic acid in 80% acetonitrile) over 40 min, 34–38% in 5 min, 38–90% in 10 min and then holding at 90% for the last 10 min, all at a constant flow rate of 300 nl/min on an EASY-nLC 1000 UPLC system. The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Thermo Scientific Orbitrap Fusion coupled online to the UPLC. The electrospray voltage applied was 2.0 kV. The m/z scan range was 350 to 1800 for full scan, and intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were then selected for MS/MS using NCE setting as 28 and the fragments were detected in the Orbitrap at a resolution of 17,500. A data-dependent procedure that alternated between one MS scan followed by 20 MS/MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set at 5E4. The resulting MS/MS data were processed using Maxquant (version 2.2.0). Tandem mass spectra were searched against Uniprot _*Malus domestica*. Trypsin/P was specified as cleavage enzyme. Mass error was set to 10 ppm for precursor ions and 0.02Da for fragment ions. Carbamidomethyl on Cys was specified as fixed modification, oxidation on Met, Deamidation (NQ), and Acetyl (Protein N-term) were specified as variable modifications.

Statistical analysis

Statistical analysis all the determinations in this study were carried out in three replicates. The statistical data were analyzed by Graphpad Prism software (version 8.3.0). The error bar indicates the standard deviation. Through one-way ANOVA analysis and Student's t test of significant difference, the * above the bar indicates significant difference ($P<0.05$), ** above the bar indicates highly significant difference ($P<0.01$).

Results

MdTLP7 improved salt and drought tolerance of Apple

To characterize the biological function of *MdTLP7* gene, overexpression calli of *MdTLP7* was constructed through agrobacterium-mediated transformation, the OE-B was selected for the follow-up experiment, named OE. The expression level of *MdTLP7* was confirmed by qRT-PCR (Figure S1). Compared with wild-type (WT) OE had higher expression level of *MdTLP7*. Based on our previously research that *MdTLP7* can improve *Arabidopsis*' tolerance to drought and salt, the performance of WT and OE calli were assessed under drought and salt stress. On Murashige and Skoog solid medium containing 6% PEG6000, the growth state of the OE was better than WT after 16 days of cultivation. However, we observed that on normal growth medium, the OE grew faster than WT (Fig. 1A). Therefore, the fresh weight of WT and OE were measured under normal growth conditions and drought conditions (Fig. 1b), and the inhibition rates of drought on WT and OE was calculated, respectively (Fig. 1C).

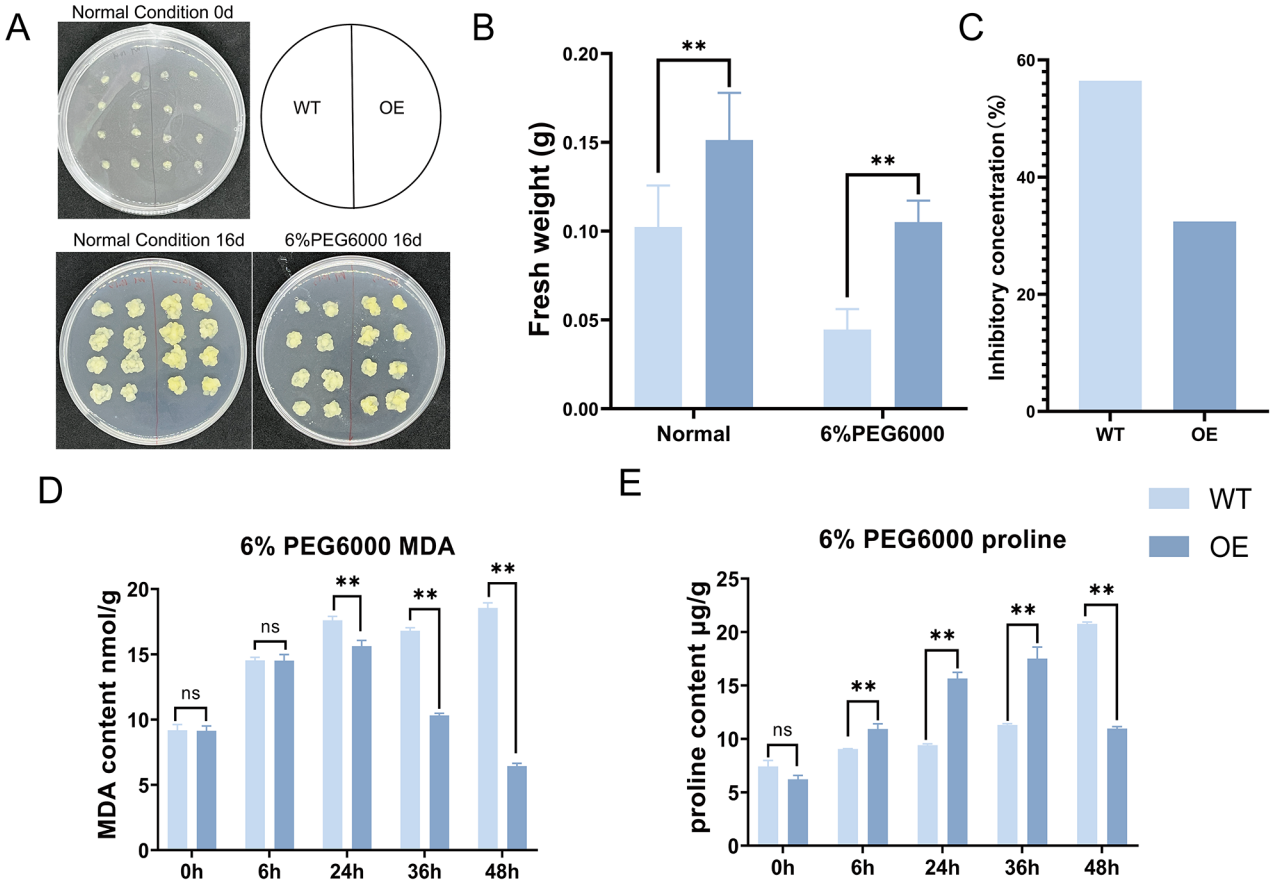

Fig. 1 *MdTLP7* enhances drought stresses tolerance of apple calli. **(A)** The growth morphology of WT and OE under normal and drought conditions. **(B)** Statistical analysis of calli weight for WT and OE under normal and drought conditions. **(C)** Growth inhibition rates of WT and OE under drought conditions. **(D)** MDA content of WT and OE under drought conditions. **(E)** Proline content of WT and OE under drought conditions. Error bars indicate the SE of three biological replicates. One-way ANOVA (Tukey's test) was performed, and significant differences are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$

The results showed that the inhibition rate of drought on WT was higher than on OE, which means that OE have a stronger drought tolerance compared to WT. To further assess the drought tolerance of OE, the proline content and malondialdehyde level were measured, which are typical parameters for evaluating plant tolerance to abiotic stress [47]. And to ensure the accuracy of our results, the calli were cultured in liquid medium, which allowed all cells to be exposed to the stress environment. The results showed that, compared to WT, OE produced significantly less MDA (Fig. 1D), and the proline content was significantly higher (Fig. 1E). Similar results was observed on medium containing 150 mmol/L NaCl, the growth state of the OE was better than WT after 22 days cultivation (Fig. 2A, B), and the inhibition rate of salt on WT was higher than on OE (Fig. 2C). OE had significantly less MDA and more proline than WT (Fig. 2D, E). *MdTLP7* may assist apple in maintaining normal physiological status under salt and drought conditions, thereby reducing cellular damage and conferring stronger stress tolerance. These results indicated that *MdTLP7* enhance the drought and salt stresses tolerance of apple calli.

Transcriptomic analysis of Apple calli in response to drought and salt stress

To explore which genes are affected by *MdTLP7* in apples, RNA-seq was performed on OE and WT samples. A total of 868,966,832 raw reads were generated. After filtering for sequencing contamination, adapters, and low-quality raw reads, a total of 863,051,666 clean reads with high-quality lengths were obtained, accounting for 99.32%. And a total of 92.88% of the clean reads perfectly mapped to the reference genome (Table S2). Principal component analysis (PCA) indicated a significantly separation in the transcriptome of the WT group and OE group, and their respective groups after drought or salt treatment, with variations of 66.6% and 16.7% for PC1 and PC2, respectively (Fig. 3A).

The differentially expressed genes (DEGs) were identified with the criteria of log2 fold change, |log2(FC)| >

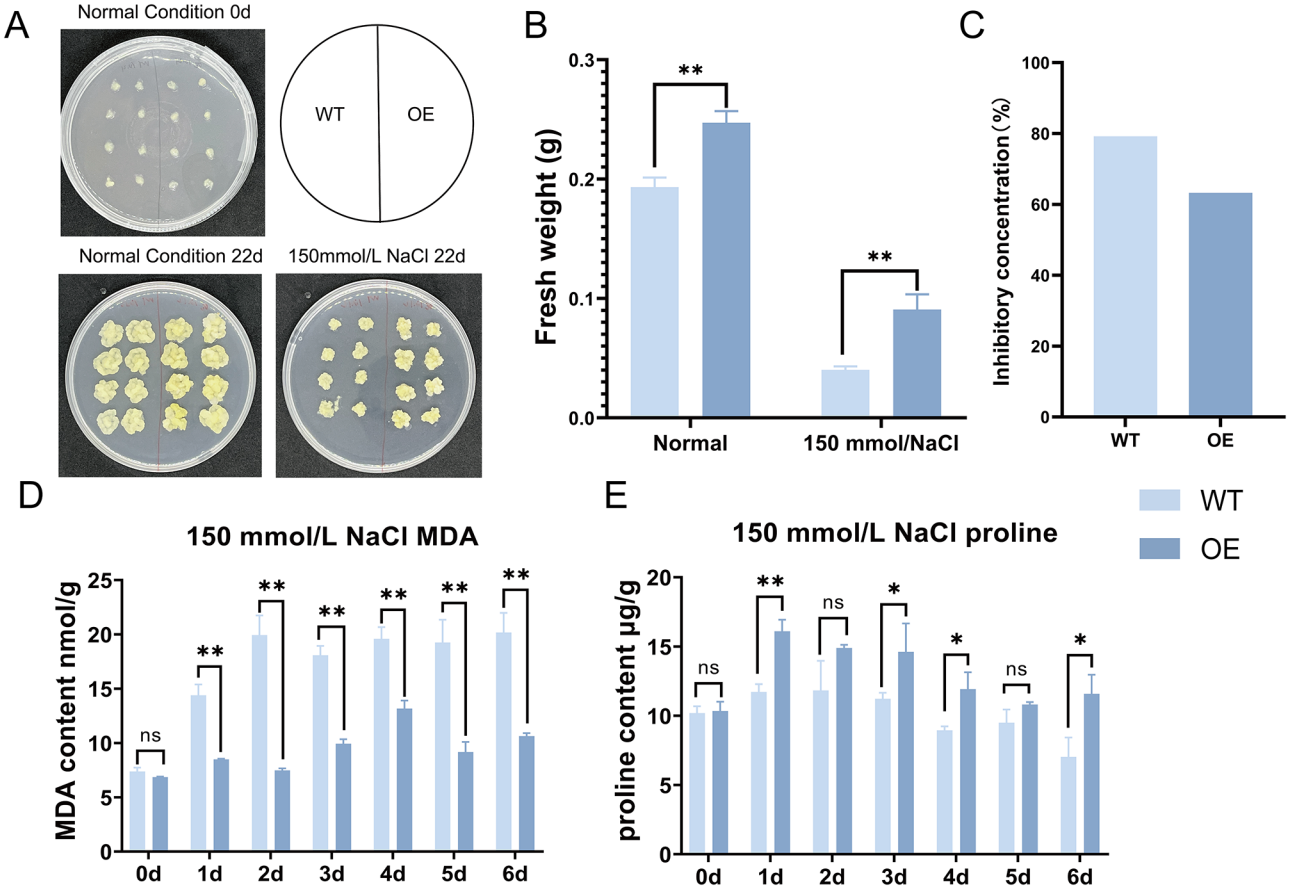

Fig. 2 *MdTLP7* enhances salt stresses tolerance of apple calli. **(A)** The growth morphology of WT and OE under normal and salt conditions. **(B)** Statistical analysis of calli weight for WT and OE under normal and salt conditions. **(C)** Growth inhibition rates of WT and OE under salt conditions. **(D)** MDA content of WT and OE under salt conditions. **(E)** Proline content of WT and OE under salt conditions. Error bars indicate the SE of three biological replicates. One-way ANOVA (Tukey's test) was performed, and significant differences are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$

1 and false discovery rate (FDR) < 0.05. Between WT and OE, there were 3,668 DEGs, of which 2,900 genes were up-regulated and 768 genes were down-regulated (Fig. 3B). There were 254 TFs in these DEGs, mainly were ARR-B (63), AP2-EREBP (33) and WRKY (23) (Table S3). To understand the functional categories of the DEGs, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was applied. Results showed that the DEGs were mainly enriched in secondary metabolite synthesis, plant hormone signal transduction and amino acid metabolism pathways (Fig. 3C). We selected some genes for qRT-PCR, the expression trend of all genes detected by qRT-PCR was similar to the trend of the results in transcriptome sequencing (Fig. 5B, C). The results demonstrated that overexpression *MdTLP7* affected gene transcription in apple calli.

Further analysis showed that under drought stress (D) 1,442 genes up-regulated and 748 genes down-regulated in WT-D-vs-OE-D (Fig. 2B). There were 142 TFs in these DEGs, mainly were ARR-B (22), AP2-EREBP (20) and WRKY (18) (Table S4). KEGG enrichment analysis showed that drought stress mainly affected plant hormone signal transduction, steroid biosynthesis and biosynthesis pathway of secondary metabolites of apple (Fig. 3D).

Under salt stress (S), there were 2,290 genes up-regulated and 3,090 genes down-regulated in WT-S-vs-OE-S (Fig. 3B). And, there were 313 TFs in these DEGs, mainly were ARR-B (62), AP2-EREBP (34) and WRKY (25) (Table S5). Under salt stress, DEGs were significantly involved in pathways included DNA replication, biosynthesis of secondary metabolites and biosynthesis pathway of amino acids (Fig. 3E). The above results indicated that *MdTLP7* affected the genes expression in apple under drought and salt stress.

MdTLP7 had DNA binding activity

To clarify the molecular function of MdTLP7, Yeast One-Hybrid experiment was used to determine whether MdTLP7 can activate the expression of downstream genes. However, the results showed that MdTLP7 had no self-activation activity (Fig. 4A). Furthermore, DAP-seq

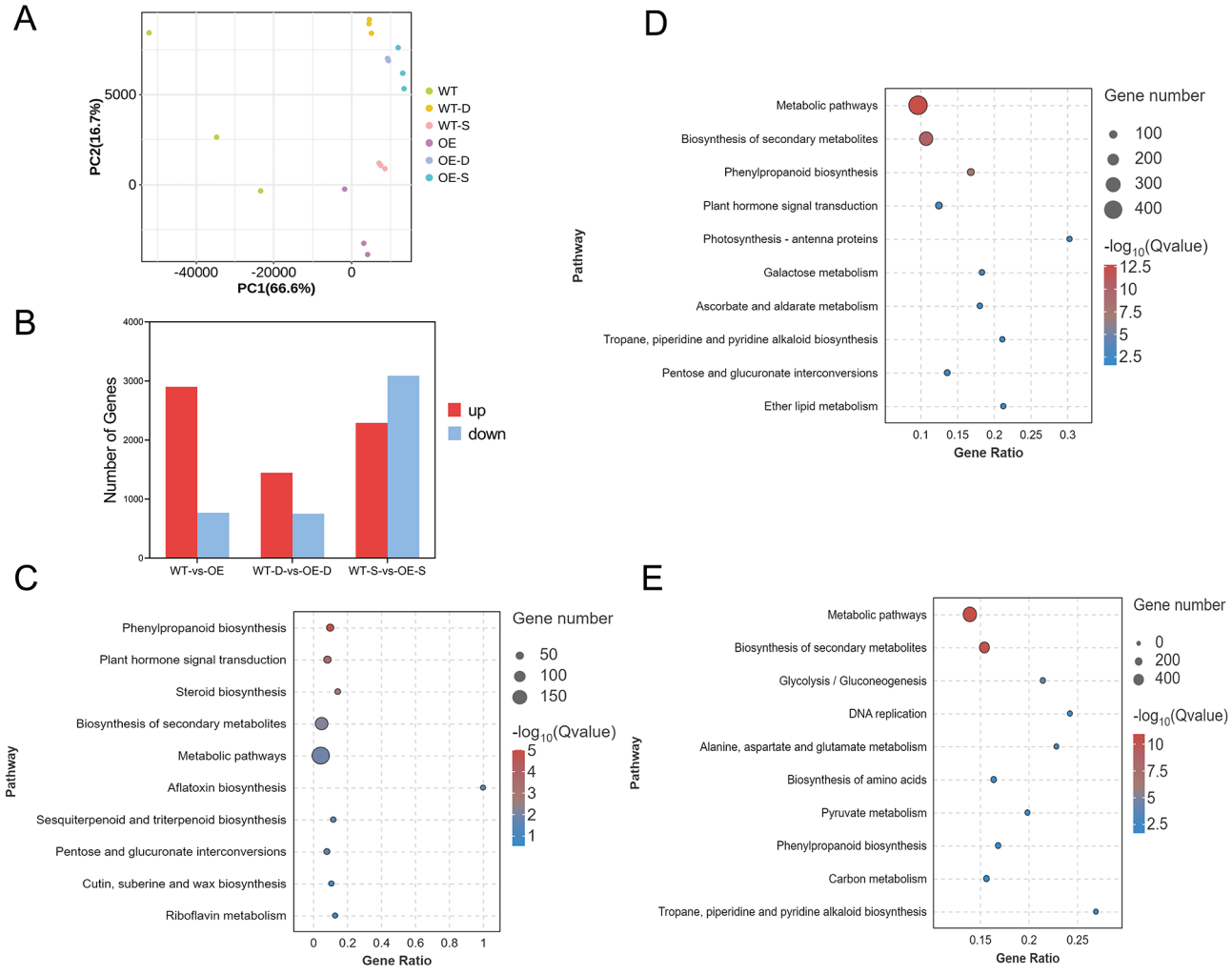

Fig. 3 *MdTLP7* affects gene expression in apple calli. **(A)** PCA analysis. **(B)** Differential expression analysis of WT and OE apple under drought and salt treatments. **(C)** KEGG enrichment analysis of differential expressed genes in WT-vs-OE. **(D)** KEGG enrichment analysis of differential expressed genes in WT-D-vs-OE-D. **(E)** KEGG enrichment analysis of differential expressed genes in WT-S-vs-OE-S

experiment was used to detect whether MdTLP7 has DNA binding activity. In total, we found MdTLP7 had 2,221 binding sites across the apple genome, and these binding sites were associated with 2,021 genes. Analysis of the binding sites discovered MdTLP7 mainly bound to the distal intergenic (55.07%) and promoter (27.42%) portions of 2,021 genes (Fig. 4B). It is also found that MdTLP7 can specifically recognize and bind to motifs that include multiple adenine (A) base sequences (Fig. 4C).

In this study, the promoter region of the binding site of MdTLP7 was mainly focused on. There were 719 promoter binding sites belonging to 704 genes. Among the 704 genes, 459 genes were detected expression in the transcriptome. KEGG enrichment analysis showed that these genes were significantly involved in Photosynthesis-antenna proteins, Phosphonate and phosphinate metabolism, and Biosynthesis of amino acids pathways (Fig. 4D). Among these 459 genes, there were 32 TFs, some of them have been shown to be associated with stress response, such as bZIP, HSF, NAC and MYB [48]. There were 320 structural genes, some structural genes have been identified as related to drought and salt stresses tolerance, such as NHX [49], PSB [50] etc. The functions of the remaining 107 genes are still unclear.

MdTLP7 interacted with MdNAC72L in nucleus

To clarify how MdTLP7 affect gene expression in apple, Yeast Two-Hybrid screening was used to detect the nucleoprotein interacting with MdTLP7. Positive yeast clones were screened in SD-TLH screening plate (Figure S2, Table S6). A total of 1,196 apple nucleoproteins that interacted with MdTLP7 were screened through sequencing. Among these genes, 25 genes were identified as TFs, and the main types were bZIP, HSF, ATHB, NAC, etc. (Table S7). Through transcriptome-wide expression

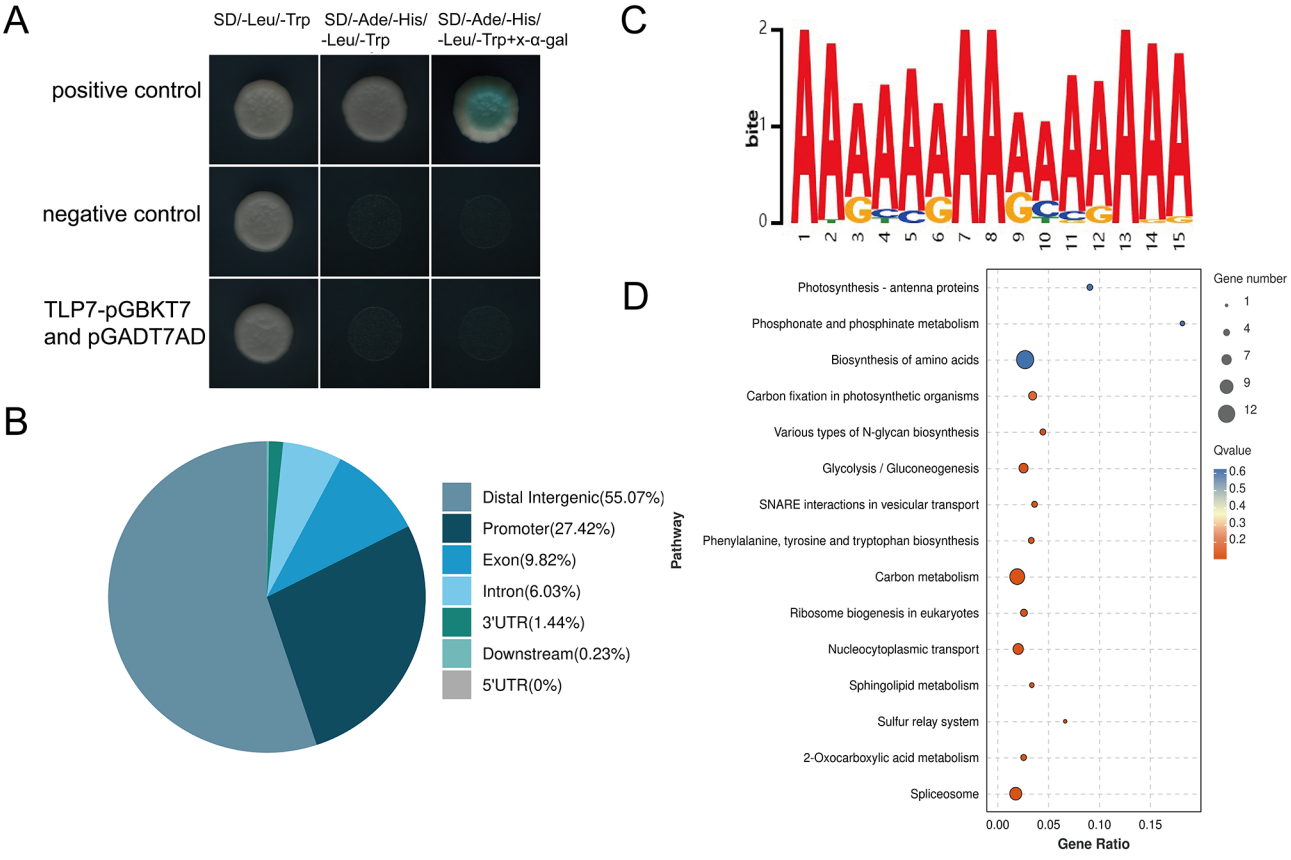

Fig. 4 MdTLP7 had DNA binding activity. **(A)** Yeast One-Hybrid experiment of MdTLP7. **(B)** Statistical analysis of the binding sites that MdTLP7 can bind to. **(C)** MdTLP7 binding specific motif. **(D)** KEGG enrichment analysis of 459 genes

profiling, we identified NAC72L (NAC domain-containing protein 72-like) as a core regulator in the NAC family, whose expression exhibited significant affected under both drought and salt stress (Figure S3). In addition, the expression level of *MdNAC72L* in untreated WT and OE strains were similar, in contrast their expression increased significantly after drought stress and salt stress treatment (Fig. 5A). Subsequently, the bimolecular fluorescence complementary experiment (BiFc) showed that when MdTLP7-CYFP and MdNAC72L-NYFP present together, yellow fluorescence was detected. However, there was no yellow fluorescence in the case of only one vector or empty vector (Fig. 5B). That indicated that MdTLP7 had physical interaction with MdNAC72L in vivo. The above results showed that MdTLP7 could interacted with MdNAC72L protein in apple nucleus.

MdTLP7 and MdNAC72L positively regulated *MdHsp70-8* and *MdAGP*

To find out MdTLP7 regulates which downstream genes to response to drought and salt stresses, DAP-seq experimental results and RNA-seq results were analyzed in combination (Fig. 5C). Among these genes two genes were noticed that showed a upregulated after the overexpression of *MdTLP7*, and also upregulated after drought stress and salt stress treatments: *MdHsp70-8* (MD13G1191900, Heat Shock Proteins) and *MdAGP* (MD16G113100, Arabinogalactan Proteins). The expression levels of *MdHsp70-8* and *MdAGP* were further verified by qRT-PCR under the conditions of drought and salt treatments for 36 h and 48 h, respectively. *MdHsp70-8* expression increased significantly after stress treatments and was significantly higher in OE than in WT (Fig. 5D), similarly, *MdAGP* had the same expression trend (Fig. 5E). Consequently, we postulated that MdTLP7 exerts a positive regulatory effect on the expression of *MdHsp70-8* and *MdAGP*. To further detect the specificity of MdTLP7 binding to the promoters regions of *MdHsp70-8* and *MdAGP*, DNA-pull down experiments were designed. The biotin-labeled probe was designed based on the binding element (Fig. 4c). The probe was incubated with streptavidin magnetic beads, and then incubated with the His-MdTLP7 fusion protein before the eluate was tested. The results indicated that His-MdTLP7 was able to interact with the motif (Fig. 4c) in the promoter (Fig. 5F). The eluted protein was subjected to mass spectrometry sequencing and the results indicate that it was MdTLP7, the target protein. The above results

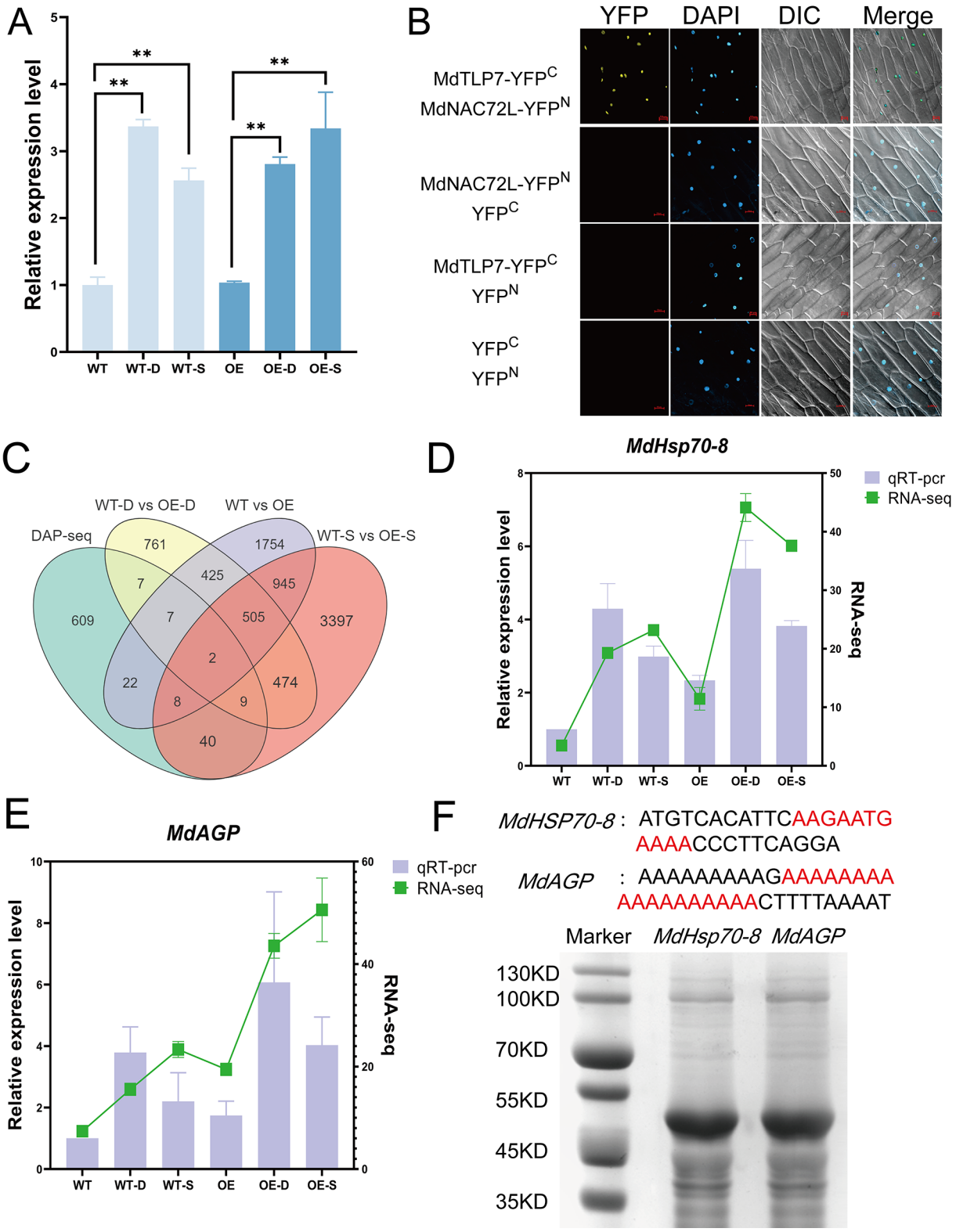

Fig. 5 MdTLP7 interacted with MdNAC72L and regulated *MdHsp70-8* and *MdAGP*. **(A)** The expression of *MdNAC72L* in response to drought and salt stresses. **(B)** Bimolecular fluorescence complementation assays to confirm the interaction between MdTLP7 and MdNAC72L. Bars = 50 μm. MdTLP7. **(C)** Venn diagram analysis of the DEGs and DAP-seq. **(D)** Transcriptome expression and qRT-PCR expression of *MdHsp70-8*. **(E)** Transcriptome expression and qRT-PCR expression of *MdAGP*. **(F)** DNA-pull down validation of MdTLP7 binding to *MdHsp70-8* and *MdAGP* promoter region. Error bars indicate the SE of three biological replicates. One-way ANOVA (Tukey's test) was performed, and significant differences are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$

confirmed that MdTLP7 can combine with the promoters of *MdHsp70-8* and *MdAGP*, and regulate the expression of them.

Overexpression of *MdHsp70-8* and *MdAGP* enhanced salt and drought stress tolerance of Apple calli

To detect whether *MdHsp70-8* and *MdAGP* played positive regulatory roles in apple stress tolerance, three overexpressing apple calli were constructed, OE-*MdHsp70-8*, OE-*MdAGP*, OE-*MdHsp70-8* + *MdAGP*, respectively. The transformation were confirmed by qRT-PCR detection of target genes (Fig. 6A, B). On Murashige and Skoog solid medium containing 6% PEG6000, the growth state of the three overexpressing apple calli were better than WT after 33 days of cultivation, and there have similar results on medium containing 150 mmol/L NaCl (Fig. 6C, D). Subsequently, the MDA, proline and H_2O_2 content of the transgenic lines were measured under drought stress for 48 h and salt stress for 36 h in liquid medium. Compared with the WT, OE-*MdHsp70-8* produced significantly lower content of MDA and H_2O_2 under drought and salt stresses (Fig. 6E, F). Compared to normal growth conditions, OE-*MdHsp70-8* had no significantly higher content of MDA and H_2O_2 after stress, and there were no significant changes in proline content (Fig. 6E, F, G). It indicated that apples OE-*MdHsp70-8* exhibited enhanced tolerance to drought and salt stresses, reduced adverse effects of these stresses. Similarly, apple calli OE-*MdAGP*, OE-*MdHsp70-8* + *MdAGP* had similar results under drought and salt stresses (Fig. 6E, F, G). The above results showed that *MdHsp70-8* and *MdAGP* enhanced drought and salt tolerance of apple calli.

Discussion

TLPs are widely present in plants and their expression are induced by abiotic stresses [22]. Previous studies have found that TLPs enhance tolerance to drought and salt stresses in *Arabidopsis* [36], cotton [38], chickpea [35], and other plants. TLP transcription factors play important roles in abiotic stresses, but the mechanisms of their responses are unclear, especially in fruit crops. Therefore, elucidating the molecular mechanism of *MdTLP7* in response to drought and salt stresses in apple is important for understanding the function of TLP in plants. Based on resulted obtained in the current study, we propose a model for the molecular mechanisms regulating *MdTLP7* in response to drought and salt stresses (Fig. 7). MdTLP7 received drought or salt stresses signals and transferred to the nucleus, where it interacted with the nuclear protein MdNAC72L. MdTLP7 bound to the promoter regions of *MdHsp70-8* and *MdAGP*, enhancing the expression of these two genes and improving the tolerance of apple to drought and salt stresses.

In this study, we found *MdTLP7* can enhanced the tolerance to drought and salt stresses in apple (Fig. 1). The results showed that under drought stress, OE produced significantly less MDA compared to WT after 24 h (Fig. 1B). The proline content of OE was significantly higher than WT at 6 h. It is noteworthy that at 48 h when the proline content of WT was elevated, the proline content of OE had already recovered to the normal level, which suggests that it may have been acclimatised to the drought environment (Fig. 1C). MdTLP7 may regulate some genes in apples to respond to drought and salt stress, in order to identify which genes it affects we sequenced the transcriptome of apple tissues overexpressing *MdTLP7*. We found 2,900 genes were up-regulated and 768 genes were down-regulated compared with WT apples (Fig. 2B). Furthermore, these DEGs were partially associated with plant hormone signal transduction (Fig. 2C). In *Arabidopsis*, overexpression and repression of *AtTLP9* affects ABA sensitivity during the processes of seed germination and early seedling development [30]. Another study has also demonstrate that the *AtTLP2* mutant is insensitive to ABA [36]. Some of the DEGs were associated with the photosynthesis pathway. A study in chickpea find higher photosynthesis and higher chlorophyll retention in *CaTLP1* transgenic plants, which supported to our results. Furthermore, *CaTLP1* transgenic plants exhibit enhanced growth rates, branch height and biomass also increase [35]. Additionally, *MdTLP7* was observed to influence the expression of genes involved in secondary metabolite synthesis and the pathway of pentose and glucuronate interconversions. There have few research about the effects of TLPs on plant gene expression. Our data address this gap in this field. Additionally, we conducted a transcriptome analysis of *MdTLP7* transgenic apples under drought and salt stresses (Fig. 2D, E), providing data support and references for the study of TLP-related genes.

Previous studies showed that TLPs do not have self-activating activity in most species, such as tomato [51], strawberry [27], cotton [52], chickpea [35] and rice [53], but Li et al. reported that CsTLP8 in cucumber has self-activating activity [54]. Similarly, we found that MdTLP7 does not have self-activating activity (Fig. 3A). In plants, TLPs can bind specific Skp1-like (SK) proteins to form SCF-type E3 ligases [22]. However, there are few studies showing interactions between TLP proteins and other TFs in plants. In our study, we found that MdTLP7 was able to interact with MdNAC72L in the nucleus (Fig. 4C). There are no relevant studies showing the interaction between TLP and NAC. Notably, there have been no studies about the DNA-binding activity of TLPs, our study firstly identified a DNA motif which MdTLP7 is able to bind (Fig. 3C), providing information for the study of other TLP genes.

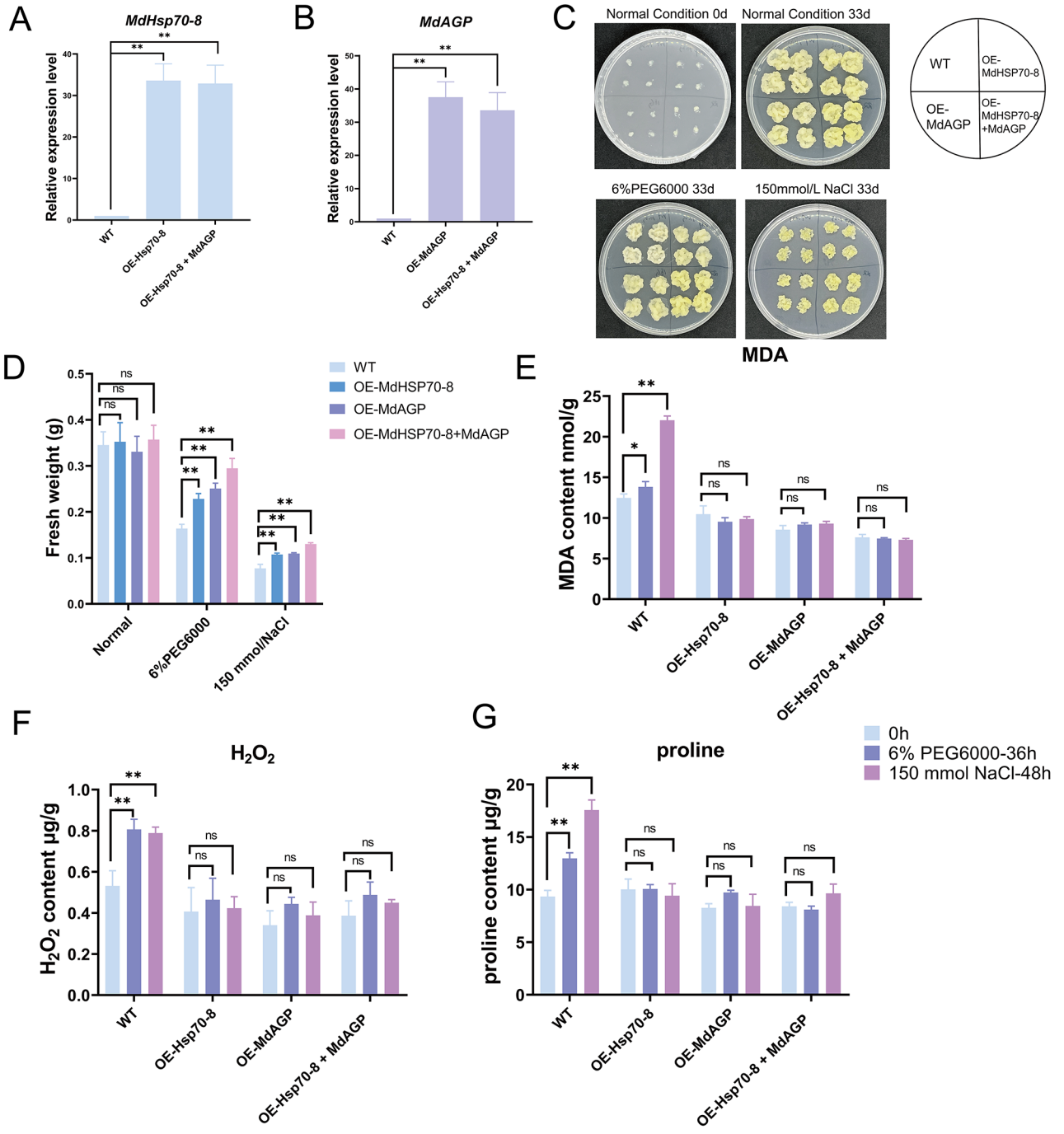

Fig. 6 Overexpression of *MdHsp70-8* and *MdAGP* improves drought and salt stresses tolerance in apple calli. **(A)** Identification of apple overexpressing *MdHsp70-8*. **(B)** Identification of apple with overexpressing *MdAGP*. **(C)** The growth morphology of WT *MdHsp70-8*, *MdAGP* and *MdHsp70-8* + *MdAGP* under normal, drought and salt conditions. **(D)** Statistical analysis of callus weight for WT and *MdHsp70-8*, *MdAGP* and *MdHsp70-8* + *MdAGP* under normal, drought and salt conditions. **(E)** MDA content of WT and three overexpression apple under drought and salt stresses. **(F)** Proline content of WT and three overexpression apple under drought and salt stresses. **(G)** H_2O_2 content of WT and three overexpression apple under drought and salt stresses. Error bars indicate the SE of three biological replicates. One-way ANOVA (Tukey's test) was performed, and significant differences are indicated by: $^{*}P < 0.05$; $^{**}P < 0.01$

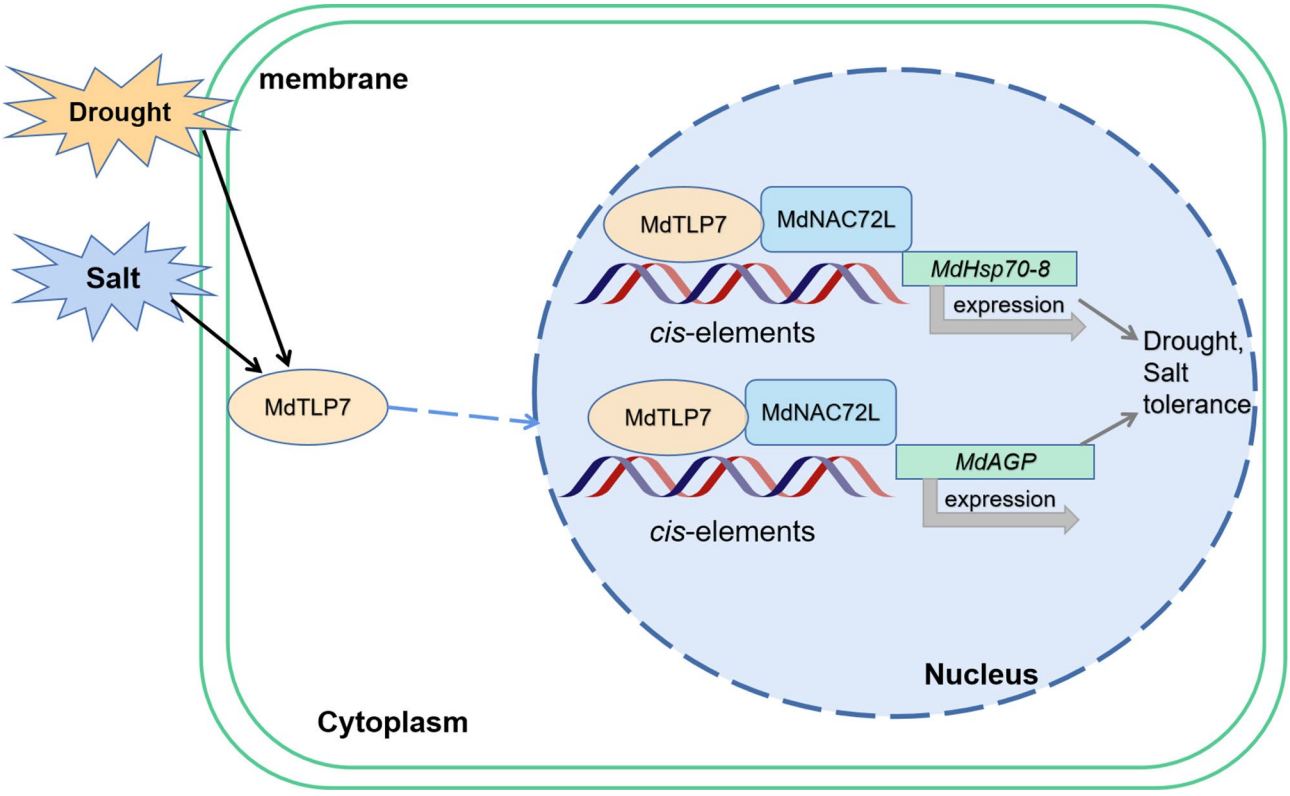

Fig. 7 Molecular mechanisms of MdTLP7 in response to drought and salt stresses

HSPs typically express in response to stress conditions [55]. They act as molecular chaperones, which are crucial regulators of proteins that play a role in maintaining intracellular stability. Previous studies have demonstrated that Hsp70 plays a significant role in plant disease tolerance and in mediating abiotic stress tolerance in plants [56]. For instance, there is a notable positive correlation between Hsp70 gene expression and the acquisition of heat tolerance [57]. Nevertheless, only a few studies have demonstrated that Hsp70 also exerts a beneficial influence in the context of drought and salt stresses. In the present study, we observed that the expression of *MdHsp70-8* was activated by drought and salt treatments (Fig. 5B), and its overexpression enhanced the tolerance of apple to drought and salt stresses (Fig. 6C, D, E). It is conceivable that *MdHsp70-8* may function as a drought and salt stresses tolerance gene in apple.

Furthermore, while analyzing the data, we found some other notable genes, such as *MD15G1177100*, a gene with significantly decreased expression, which is a possible receptor-like kinase. It has been demonstrated that receptor kinase are responsible for the direct perception of the cellular environment and that they can receive a diverse range of ligands that regulate various aspects of plant growth, development, reproduction, and response to adversity [58]. Therefore, MdTLP7 likely increase the tolerance of apple by inhibiting the expression of this receptor kinase, which makes apple less sensitive to adversity signals. In the future, we will further study others genes to mature the molecular mechanism of MdTLP7 in response to drought and salt stresses.

Our findings indicate that MdTLP7 can interact with 15 transcription factors and that MdTLP7 has binding sites for 2021 genes in apple. This suggests that MdTLP7 may be located further upstream in the signaling pathway and is an important transcription factor for stress tolerance. Given that TLP genes are not self-activating in many plants but are responsive to abiotic stresses and can enhance plant tolerance to abiotic stresses, it can be hypothesized that the "TLP-transcription factor-downstream gene" network may be a style for TLP genes to respond to abiotic stresses in plants. In summary, our data provide a molecular mechanism for MdTLP7 respond to drought and salt stresses, and provide new insights into the molecular function of TLP in tolerance to abiotic stresses.

Conclusion

In this study, we investigated of the role of TLP transcription factor in apple response to drought and salt stress. The result showed that overexpression of *MdTLP7* in transgenic calli resulted in reduced accumulation of MDA and increased accumulation of proline under PEG or salt induced stress. We found MdTLP have no

self-activating activity. Through the combined analysis of RNA-seq and DAP-seq analysis and the Y2H and BiFC experiments, we revealed that TLP7 interacted with NAC72L to activate the expression of a heat shock protein and a gene of unknown function. This investigation provide a molecular mechanism for MdTLP7 respond to drought and salt stresses, and provide new insights into the molecular function of TLP in tolerance to abiotic stresses.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06643-2.

Supplementary Material 1: Figure S1. Identification of apple with overexpressing ***MdTLP7***.

Supplementary Material 2: Figure S2. Partial MdTLP7 and partial yeast two-hybrid validation of apple cytosolic proteins.

Supplementary Material 3: Figure S3. Expression of 25 transcription factors after drought stress and salt stress.

Supplementary Material 4: Table S1. Primer pairs used for qRT-PCR. Table S2. Summary statistics of quality preprocessing of RNA-Sequencing reads and comparison of reference genome mappin. Table S3. Statistics of transcription factor types in WT-vs-OE differential expressed genes. Table S4. Statistics of transcription factor types in WT-D-vs-OE-D differential expressed genes. Table S5. Statistics of transcription factor types in WT-S-vs-OE-S differential expressed genes. Table S6. Sequencing results of two-hybrid sequencing of MdTLP7 with the yeast apple nucleoprotein. Table S7. Transcription factor type statistics for proteins interacting with MdTLP7.

Acknowledgements
Not applicable.

Author contributions
The study was conceived by Q W under the surpervision of F Y and J X. Q W designed the experiments, wrote the manuscript. T G and Y Y prepared figures and tables, Z Y and L X performed the experiments. All authors have read and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (Grant Nos. 12105163, 32001987) and the Natural Science Foundation of Shandong Province (Grant Nos. ZR2020QD133, ZR2020MC142).

Data availability
Apple sequence data can be found in the Genome Database for Rosaceae (GDR; https://www.rosaceae.org; Malus 9 domestica GDDH13 v1.1) with accession nos. MdTLP7 (MD15G1259500), MdNAC72L (MD03G1222700), MdHsp70-8 (MD13G1191900), MdAGP (MD16G1131300). The transcriptome data have been deposited at NCBI with the project ID PRJNA1143688.

Declarations

Ethics approval and consent to participate
No specific permits were required for the described field studies. All experiments were performed according to institutional guidelines of Shandong University of technology, China. This study does not contain any research requiring ethical consent or approval.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 8 March 2025 / Accepted: 28 April 2025
Published online: 10 May 2025

References

1. Chen K, Song M, Guo Y, Liu L, Xue H, Dai H, Zhang Z. MdMYB46 could enhance salt and osmotic stress tolerance in Apple by directly activating stress-responsive signals. Plant Biotechnol J. 2019;17(12):2341–55. https://doi.org/10.1111/pbi.13151.
2. Sales CRG, Ribeiro RV, Silveira JAG, Machado EC, Martins MO, Lagôa AMMA. Superoxide dismutase and ascorbate peroxidase improve the recovery of photosynthesis in sugarcane plants subjected to water deficit and low substrate temperature. Plant Physiol Biochem. 2013;73:326–36. https://doi.org/10.1016/j.plaphy.2013.10.012.
3. Ahmed IM, Dai H, Zheng W, Cao F, Zhang G, Sun D, Wu F. Genotypic differences in physiological characteristics in the tolerance to drought and salinity combined stress between Tibetan wild and cultivated barley. Plant Physiol Biochem. 2013;63:49–60. https://doi.org/10.1016/j.plaphy.2012.11.004.
4. Suzuki N, Rivero RM, Shulaev V, Blumwald E, Mittler R. Abiotic and biotic stress combinations. New Phytol. 2014;203(1):32–43. https://doi.org/10.1111/nph.12797.
5. Chaves MM, Flexas J, Pinheiro C. Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot. 2009;103(4):551–60. https://doi.org/10.1093/aob/mcn125.
6. Ponnu J. Shouldering the stress: ubiquitin-mediated degradation of a B-box protein regulates drought responses in Apple. Plant Physiol. 2022;188(1):32–4. https://doi.org/10.1093/plphys/kiab506.
7. Zhang YL, Zhang CL, Wang GL, Wang YX, Qi CH, You CX, Li YY, Hao YJ. Apple AP2/EREBP transcription factor MdSHINE2 confers drought resistance by regulating wax biosynthesis. Planta 2019, 249(5):1627–43. https://doi.org/10.1007/s00425-019-03115-4
8. Zhang TT, Lin YJ, Liu HF, Liu YQ, Zeng ZF, Lu XY, Li XW, Zhang ZL, Zhang S, You CX. The AP2/ERF transcription factor MdDREB2A regulates nitrogen utilisation and sucrose transport under drought stress. Plant Cell Environ 2024, 47(5):1668–84. https://doi.org/10.1111/pce.14834
9. Lian X, Zhao X, Zhao Q, Wang G, Li Y, Hao Y. MdDREB2A in Apple is involved in the regulation of multiple abiotic stress responses. Hortic Plant J. 2021;7(3):197–208. https://doi.org/10.1016/j.hpj.2021.03.006.
10. Dong Q, Tian Y, Zhang X, Duan D, Zhang H, Yang K, Jia P, Luan H, Guo S, Qi G. Overexpression of the transcription factor MdWRKY115 improves drought and osmotic stress tolerance by directly binding to the MdRD22 promoter in Apple. Hortic Plant J. 2024;10(3):629–40. https://doi.org/10.1016/j.hpj.2023.05.005.
11. Du B, Liu H, Dong K, Wang Y, Zhang Y. Over-expression of an R2R3 MYB gene, MdMYB108L, enhances tolerance to salt stress in Transgenic plants. Int J Mol Sci. 2022;23(16). https://doi.org/10.3390/ijms23169428.
12. Chen C, Zhang Z, Lei YY, Chen WJ, Zhang ZH, Li XM, Dai HY. MdMYB44-like positively regulates salt and drought tolerance via the MdPYL8-MdPP2CA module in Apple. Plant J. 2024;118(1):24–41. https://doi.org/10.1111/tpj.16584.
13. Yang W, Liu XD, Chi XJ, Wu CA, Li YZ, Song LL, Liu XM, Wang YF, Wang FW, Zhang C. Dwarf Apple MbDREB1 enhances plant tolerance to low temperature, drought, and salt stress via both ABA-dependent and ABA-independent pathways. Planta. 2011;233(2):219–29. https://doi.org/10.1007/s00425-010-1279-6.
14. Wang DR, Yang K, Wang X, Lin XL, Rui L, Liu HF, Liu DD, You CX. Overexpression of MdZAT5, an C2H2-type zinc finger protein, regulates anthocyanin accumulation and salt stress response in Apple calli and *Arabidopsis*. Int J Mol Sci 2022, 23(3). https://doi.org/10.3390/ijms23031897
15. Yang K, Li CY, An JP, Wang DR, Wang X, Wang CK, You CX. The C2H2-type zinc finger transcription factor MdZAT10 negatively regulates drought tolerance in Apple. Plant Physiol Biochem. 2021;167:390–9. https://doi.org/10.1016/j.plaphy.2021.08.014.
16. Yang YY, Zheng PF, Ren YR, Yao YX, You CX, Wang XF, Hao YJ. Apple MdSAT1 encodes a bHLHm1 transcription factor involved in salinity and drought responses. Planta 2021, 253(2). https://doi.org/10.1007/s00425-020-03528-6
17. Mao K, Yang J, Sun Y, Guo X, Qiu L, Mei Q, Li N, Ma F. MdbHLH160 is stabilized via reduced MdBT2-mediated degradation to promote MdSOD1 and MdDREB2A-like expression for Apple drought tolerance. Plant Physiol. 2024;194(2):1181–203. https://doi.org/10.1093/plphys/kiad579.

18. JP A, Zhang XW, Bi SQ, You CX, Wang XF, Hao YJ. The ERF transcription factor MdERF38 promotes drought stress-induced anthocyanin biosynthesis in Apple. Plant J. 2019;101(3):573–89. https://doi.org/10.1111/tpj.14555.
19. Bai Y, Shi K, Shan D, Wang C, Yan T, Hu Z, Zheng X, Zhang T, Song H, Li R. The WRKY17-WRKY50 complex modulates anthocyanin biosynthesis to improve drought tolerance in Apple. Plant Sci. 2024;340. https://doi.org/10.1016/j.plantsci.2023.111965.
20. Yu L, Liu W, Guo Z, Li Z, Jiang H, Zou Q, Mao Z, Fang H, Zhang Z, Wang N. Interaction between MdMYB63 and MdERF106 enhances salt tolerance in Apple by mediating Na+/H + transport. Plant Physiol Biochem. 2020;155:464–71. https://doi.org/10.1016/j.plaphy.2020.08.017.
21. Su M, Wang S, Liu W, Yang M, Zhang Z, Wang N, Chen X. Interaction between MdWRKY55 and MdNAC17-L enhances salt tolerance in Apple by activating MdNHX1 expression. Plant Sci. 2022;320. https://doi.org/10.1016/j.plantsci.2022.111282.
22. Bano N, Aalam S, Bag SK. Tubby-like proteins (TLPs) transcription factor in different regulatory mechanism in plants: a review. Plant Mol Biol. 2022;110(6):455–68. https://doi.org/10.1007/s11103-022-01301-9.
23. North MA, Naggert JK, Yan Y, Noben-Trauth K, Nishina PM. Molecular characterization of TUB, TULP1, and TULP2, members of the novel tubby gene family and their possible relation to ocular diseases. Proc Natl Acad Sci U S A. 1997;94(7):3128–33. https://doi.org/10.1073/pnas.94.7.3128.
24. Kou Y, Qiu D, Wang L, Li X, Wang S. Molecular analyses of the rice tubby-like protein gene family and their response to bacterial infection. Plant Cell Rep. 2009;28(1):113–21. https://doi.org/10.1007/s00299-008-0620-z.
25. Yang Z, Zhou Y, Wang X, Gu S, Yu J, Liang G, Yan C, Xu C. Genomewide comparative phylogenetic and molecular evolutionary analysis of tubby-like protein family in arabidopsis, rice, and Poplar. Genomics 2008, 92(4):246–53. https://doi.org/10.1016/j.ygeno.2008.06.001
26. Yulong C, Wei D, Baoming S, Yang Z, Qing M. Genome-wide identification and comparative analysis of the TUBBY-like protein gene family in maize. Genes Genomics. 2015;38(1):25–36. https://doi.org/10.1007/s13258-015-0338-6.
27. Xu JN, Xing SS, Zhang ZR, Chen XS, Wang XY. Genome-Wide identification and expression analysis of the Tubby-Like protein family in the *Malus domestica* genome. Front Plant Sci 2016, 7:1693. https://doi.org/10.3389/fpls.2016.01693
28. Hong MJ, Kim DY, Seo YW. Interactions between wheat Tubby-like and SKP1-like proteins. Genes genet syst 2015, 90(5):293–304. https://doi.org/10.1266/ggs.14-00084
29. Mukhopadhyay S, Jackson PK. The tubby family proteins. Genome Biol. 2011;12(6):225. https://doi.org/10.1186/gb-2011-12-6-225.
30. Lai CP, Lee CL, Chen PH, Wu SH, Yang CC, Shaw JF. Molecular analyses of the *Arabidopsis* TUBBY-Like protein gene family. Plant Physiol. 2004;134(4):1586–97. https://doi.org/10.1104/pp.103.037820.
31. Jain M, Nijhawan A, Arora R, Agarwal P, Ray S, Sharma P, Kapoor S, Tyagi AK, Khurana JP. F-Box proteins in rice. Genome-wide analysis, classification, Temporal and Spatial gene expression during panicle and seed development, and regulation by light and abiotic stress. Plant Physiol. 2007;143(4):1467–83. https://doi.org/10.1104/pp.106.091900.
32. Wang M, Xu Z, Kong Y. The tubby-like proteins Kingdom in animals and plants. Gene. 2018;642:16–25. https://doi.org/10.1016/j.gene.2017.10.077.
33. Carroll K, Gomez C, Shapiro L. Tubby proteins: the plot thickens. Nat Rev Mol Cell Biol. 2004;5(1):55–64. https://doi.org/10.1038/nrm1278.
34. Bao Y, Song WM, Jin YL, Jiang CM, Yang Y, Li B, Huang WJ, Liu H, Zhang HX. Characterization of *Arabidopsis* Tubby-like proteins and redundant function of AtTLP3 and AtTLP9 in plant response to ABA and osmotic stress. Plant Mol Biol. 2014;86(4–5):471–83. https://doi.org/10.1007/s11103-014-0241-6.
35. Wardhan V, Jahan K, Gupta S, Chennareddy S, Datta A, Chakraborty S, Chakraborty N. Overexpression of CaTLP1, a putative transcription factor in Chickpea (*Cicer arietinum* L.), promotes stress tolerance. Plant Mol Biol. 2012;79(4–5):479–93. https://doi.org/10.1007/s11103-012-9925-y.
36. Jain N, Khurana P, Khurana JP. AtTLP2, a Tubby-like protein, plays intricate roles in abiotic stress signalling. Plant Cell Rep. 2023;42(2):235–52. https://doi.org/10.1007/s00299-022-02953-z.
37. Xu HR, Liu Y, Yu TF, Hou ZH, Zheng JC, Chen J, Zhou YB, Chen M, Fu JD, Ma YZ, Wei WL, Xu ZS. Comprehensive profiling of Tubby-Like proteins in soybean and roles of the GmTLP8 gene in abiotic stress responses. Front Plant Sci. 2022;13. https://doi.org/10.3389/fpls.2022.844545.
38. Li Z, Liu J, Kuang M, Zhang C, Ma Q, Huang L, Wang H, Fan S, Peng J. Improvement of plant tolerance to drought stress by cotton tubby-like protein 30 through stomatal movement regulation. J Adv Res. 2022;42:55–67. https://doi.org/10.1016/j.jare.2022.06.007.
39. Jiang Z, Chen X, Ruan L, Xu Y, Li K. Molecular analyses of the tubby-like protein gene family and their response to salt and high temperature in the Foxtail millet (Setaria italica). Funct Integr Genomics 2024, 24(5):170. https://doi.org/10.1007/s10142-024-01458-0
40. Jain N, Khurana P, Khurana JP. Overexpression of a rice Tubby-like protein-encoding gene, OsFBT4, confers tolerance to abiotic stresses. Protoplasma. 2023;41063–79. https://doi.org/10.1007/s00709-022-01831-5.
41. Du F, Xu JN, Li D, Wang XY. The identification of novel and differentially expressed apple-tree genes under low-temperature stress using high-throughput illumina sequencing. Mol Biol Rep. 2015;42(3):569–80. https://doi.org/10.1007/s11033-014-3802-5.
42. Du F, Xu JN, Zhan CY, Yu ZB, Wang XY. An obesity-like gene MdTLP7 from Apple (*Malus* × *domestica*) enhances abiotic stress tolerance. Biochem Biophys Res Commun. 2014;445(2):394–7. https://doi.org/10.1016/j.bbrc.2014.02.005.
43. Xu J, Xing S, Sun Q, Zhan C, Liu X, Zhang S, Wang X. The expression of a tubby-like protein from Malus domestica (MdTLP7) enhances abiotic stress tolerance in Arabidopsis. BMC Plant Biol. 2019;19(1). https://doi.org/10.1186/s12870-019-1662-9.
44. Gao M, Sun Q, Zhai L, Zhao D, Lv J, Han Z, Wu T, Zhang X, Xu X, Wang Y. Genome-wide identification of Apple PPI genes and a functional analysis of the response of MxPPI1 to Fe deficiency stress. Plant Physiol Biochem. 2022;189:94–103. https://doi.org/10.1016/j.plaphy.2022.08.017.
45. Liu JH, Nada K, Honda C, Kitashiba H, Wen XP, Pang XM, Moriguchi T. Polyamine biosynthesis of Apple callus under salt stress: importance of the arginine decarboxylase pathway in stress response. J Exp Bot 57(11):2589–99. https://doi.org/10.1093/jxb/erl018
46. Zheng J, Liu L, Tao H, An Y, Wang L. Transcriptomic profiling of Apple calli with a focus on the key genes for ALA-induced anthocyanin accumulation. Front Plant Sci. 2021;12(640606). https://doi.org/10.3389/fpls.2021.640606.
47. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015;12(4):357–60. https://doi.org/10.1038/nmeth.3317.
48. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12:323. https://doi.org/10.1186/1471-2105-12-323.
49. Love MI, Huber W, Anders S. Moderated Estimation of fold change and dispersion for RNA-seq data with DESeq2.Genome biol 2014, 15(12):550. https://doi.org/10.1186/s13059-014-0550-8
50. Alvarez ME, Savouré A, Szabados L. Proline metabolism as regulatory hub. Trends Plant Sci. 2022;27(1):39–55. https://doi.org/10.1016/j.tplants.2021.07.009.
51. Zhang J, Wang X, Dong X, Wang F, Cao L, Li S, Liu Z, Zhang X, Guo YD, Zhao B. Expression analysis and functional characterization of tomato Tubby-like protein family. Plant Sci. 2022;324:111454. https://doi.org/10.1016/j.plantsci.2022.111454.
52. Li S, Wang Z, Wang F, Lv H, Cao M, Zhang N, Li F, Wang H, Li X, Yuan X. A tubby-like protein CsTLP8 acts in the ABA signaling pathway and negatively regulates osmotic stresses tolerance during seed germination. BMC Plant Biol. 2021;21(1):340. https://doi.org/10.1186/s12870-021-03126-y.
53. Jain N, Khurana P, Khurana JP. Overexpression of a rice Tubby–like protein-encoding gene, OsFBT4, confers tolerance to abiotic stresses. Protoplasma. 2022;260(4):1063–79. https://doi.org/10.1007/s00709-022-01831-5.
54. Li Z, Wang X, Cao X, Chen B, Ma C, Lv J, Sun Z, Qiao K, Zhu L, Zhang C. GhTULP34, a member of tubby-like proteins, interacts with GhSKP1A to negatively regulate plant osmotic stress. Genomics. 2021;113(1 Pt 2):462–74. https://doi.org/10.1016/j.ygeno.2020.09.024.
55. Guo M, Zhai YF, Lu JP, Chai L, Chai WG, Gong ZH, Lu MH. Characterization of CaHsp70-1, a pepper heat-shock protein gene in response to heat stress and some regulation exogenous substances in *Capsicum annuum* L. Int J Mol Sci. 2014;15(11):19741–59. https://doi.org/10.3390/ijms151119741.
56. Nakashima K, Takasaki H, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. NAC transcription factors in plant abiotic stress responses. Biochim Biophys Acta. 2012;1819(2):97–103. https://doi.org/10.1016/j.bbagrm.2011.10.005.
57. Lee S, Lee DW, Lee Y, Mayer U, Stierhof YD, Lee S, Jürgens G, Hwang I. Heat shock protein cognate 70–4 and an E3 ubiquitin ligase, CHIP, mediate plastid-destined precursor degradation through the ubiquitin-26S proteasome system in *Arabidopsis*. Plant Cell. 2009;21(12):3984–4001. https://doi.org/10.1105/tpc.109.071548.

58. Dievart A, Gottin C, Périn C, Ranwez V, Chantret N. Origin and diversity of plant receptor-like kinases. Annu Rev Plant Biol. 2020;71:131–56. https://doi.org/10.1146/annurev-arplant-073019-025927.

Publisher's note