Heterotrimeric G-protein α subunit (LeGPA1) confers cold stress tolerance in Processing tomato plants (Lycopersicon esculentum Mill)

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Abstract

Background

Tomatoes (*Lycopersicon esculentum* Mill) are key foods and are also commonly explored in studies of molecular biology and evolution. The plant originates from the tropics, thus is sensitive to cold, and its growth and development are easily affected by cold stress.

Results

In this study, *LeGPA1* was cloned from tomato leaves and used to generate *LeGPA1*-overexpressing and RNA-interference-expressing transgenic plants. The function and expression of *LeGPA1* in response to cold stress were assessed. Subcellular localization analysis identified functional LeGPA1 on the plasma membrane. Spatiotemporal expression analysis revealed that endogenous *LeGPA1* was highly expressed in the roots and leaves. Cold treatment positively induced the expression of *LeGPA1*. The overexpression of *LeGPA1* conferred tolerance to cold conditions and regulated the expression of genes related to the ICE(INDUCER OF CBF EXPRESSION)- CBF(C-REPEAT-BINDING FACTOR) pathway in tomato plants. In the *LeGPA1*-overexpressed transgenic plants, antioxidant enzyme activity and soluble sugar and proline contents increased, and the production of reactive oxygen species and membrane lipid peroxidation decreased under cold stress.

Conclusions

This suggests that better antioxidant systems can cope with oxidative damage caused by cold stress, thereby stabilizing cell membrane structures and increasing the rate of photosynthesis. These findings provide evidence for the key role of *LeGPA1* in mediating cold signal transduction in plant cells. These findings extend our knowledge of the roles of G-proteins in plants and help to clarify the mechanisms through which processing tomato plants can regulate growth and development.

Background

Abiotic stresses, such as drought, high temperature, chilling injury, salt injury, and heavy metal toxicity, seriously affect crop growth and food production, resulting in annual losses to agricultural production exceeding $100 billion [1]. In order to resist injury, plants have evolved physiological pathways for sensing and transmitting transcriptional regulation and responses to low temperature stress during long-term changes in the natural geographical environment. These include the transmembrane signal transduction pathways, in which signaling components on the cytoplasmic membrane, such as heterotrimeric G-proteins, play a crucial role [2, 3].

Heterotrimeric G-proteins are composed of a complex of three subunits, including Gα, Gβ, and Gγ. The function of G-proteins are well understood in animals [4], and more than 20 species of Gα have been found in mammals. The Gα subunits have the following functional sites: ADP-ribosylation, GTP/GDP binding, plasma membrane receptor recognition and binding, GTPase activity, and intracellular effector binding sites. These sites are closely related to heterotrimeric G-protein functions, and thus, Gα is generally considered to be a functional subunit [5]. Ma et al used modern molecular biology techniques based on mammalian G-protein α-subunit subunit sequence homologues to isolate the first *GPA1* gene from *Arabidopsis thaliana*. The protein encoded by the gene has 36% similarity to the mammalian Gα subunit and contains conserved GTP-binding regions [6, 7]. Subsequently, the cDNA sequence of the G-protein α subunit has been isolated in rice [8, 9], wild oats [10], tomatoes [11], soybeans [12, 13], peas [14], spinach [15], and lotus [16]. The predicted amino acid sequences of all the proteins encoded by these cDNAs are similar to the functional domains identified on the mammalian α subunit, suggesting that *GPA1* might a conserved gene ubiquitous to flowering plants. Furthermore, a single copy of *GPA1* occurs in plants, indicating the gene has an irreplaceable function in plant cells.

Studies have shown that heterotrimeric G-proteins are linked to several growth and development processes in plants, for example, overexpression of *GPA1* can increase the sensitivity of *Arabidopsis thaliana* to gibberellin, resulting in increased response to gibberellin during seed germination [17, 18, 19], which further affects germination. Gα can affect root development and positively regulate the growth and development of lateral roots [20]. At the same time, Gα can control the growth and development of the hypocotyl of seedlings by regulating cell differentiation [21, 22]. Compared with *Arabidopsis thaliana*, Gα-coding-gene deletion mutants of rice resulted in a wider and darker leaf color phenotype and shorter plant size [23, 24]. Studies have shown that the number of cells in *dl-l* mutants is decreased [25], therefore, Gα plays a positive regulatory role in rice cell differentiation.

Heterotrimeric G-proteins are also key signal transduction regulators in plants, controlling pathways related to abiotic stress [26]. Studies have shown that *GPA1* in *Arabidopsis* is involved in oxidative stress signal transduction and can positively regulate the abiotic stress factors upstream of reactive oxygen species (ROS) and nicotinamide adenine dinucleotide phosphate (NADPH) [27]. *Arabidopsis* Gα subunit mutations reduced the sensitivity of the plant to ozone stress [28]. In the pea (*Pisum sativum* Linn.), constitutive overexpression of Gα subunits enhanced transgenic pea resistance to salt stress [29]. Studies by Ferrero-serrano and Assmann showed that under drought stress, the *d1* mutant Gα subunit of rice had a lower leaf temperature than the wild type and, thus, stronger resistance to drought stress [30]. Chakraborty et al analysis, such as *Arabidopsis thaliana* Gα subunit mutant transcription group found that abiotic-stress-related gene expression has certainly changed, which suggests that *GAP1* may be involved in *Arabidopsis* responses to heat and cold stress [31, 32]. The expression of rice Gα, Gβ, and Gγ subunits was also strongly induced by cold stress, which suggests they have an active regulatory role in cold stress resistance in rice [33, 34]. Studies by Ma et al have shown that, in rice under cold stress, the Gα subunit RGA1(Rice G protein α subunit 1) interacts with COLD1 (CHILLING TOLERANCE DIVERGENCE

1) on the plasma membrane and endoplasmic reticulum, activating Ca^{2+} channels and enhancing resistance to low temperatures [35].

The processing tomato (*Lycopersicon esculentum* Mill.) was first grown in subtropical and tropical regions, whereas it is now cultivated worldwide. These tomato plants are highly susceptible to cold stress, which thus has the potential to incur substantial economic damage [36]. Therefore, identifying key genes for low temperature tolerance is of great theoretical and practical significance. To reveal the mechanisms behind the molecular regulation of low-temperature tolerance in tomato plants and to cultivate new low-temperature tolerant varieties, the *GPA1* gene was cloned from processing tomatoes. We constructed overexpression and RNA-interference vectors for the *LeGPA1* gene and studied the roles of *LeGPA1* in plant growth and development and the low-temperature response using transgenics. This study led to the genetic engineering of a new germplasm of low-temperature tolerant tomato plants, and it has laid the foundation for revealing the molecular regulation mechanisms of low-temperature resistance in processing tomato plants.

Results

Bioinformatics analysis of LeGPA1

Using cDNA from processing tomato leaves as a template, PCR using specific primers was used to amplicate a specific band of 1176 bp. The *LeGPA1* gene encodes 392 amino acids. DNAMAN software was used to compare LeGPA1 and other plant GPA1 protein amino acid sequences. The results show that the LeGPA1 protein from processing tomato had the highest similarity (98.72%) with the amino acid sequence of the common tomato (*Solanum lycopersicum*) (Fig. 1a). Only single amino acid point mutations exist, and there are no insertions or deletions. Phylogenetic trees of the LeGPA1 sequences from 13 species were established by ClustalX 2 and MEGA 4.1 software. The results show that LeGPA1 of processing tomato and SlGPA1 of the common tomato are on the same branch and, therefore, closely related (Fig. 1b). The Conserved Domains tool of NCBI was used to analyze the conserved domains of sequences encoded by the *LeGPA1* gene. The protein encoded by this gene has a guanine nucleotide-binding protein subunit alpha domain, which belongs to the G-alpha family (Fig. 1c).

Subcellular localization of LeGPA1

To determine the location of LeGPA1 in cells, the *LeGPA1* gene was cloned into a pCAMBIA2300-35S-GFP (green fluorescent protein) vector downstream of the 35S promoter and upstream of the GFP gene. The fusion protein expression vector p35S-*LeGPA1*-GFP constituted *LeGPA1*-GFP (Fig. 2a). The plasmids were extracted, and p35S-*LeGPA1*-GFP and pm-rk (cell membrane marker) were transformed into the protoplast of *Arabidopsis*. LeGPA1 was thereby found to localize to the cell membrane (Fig. 2b).

Expression analysis of LeGPA1 in processing tomato

We analyzed *LeGPA1* patterns of expression in different processing tomato organs using qRT-PCR. The results show detectable *LeGPA1* expression in all examined organs, with maximal expression in roots, then leaves, fruits, and the lowest expression in stems (Fig. 3a). In addition, to further verify whether the expression of *LeGPA1* is induced by stress, we also measured *LeGPA1* expression under different stress treatments. As shown in Fig. 3b, the rapid induction before 3 h was 8.01 times that before treatment at low temperature. When the treatment continued until 6 h, the value slowly decreased to 7.69 times that before treatment. The peak value at 9 h was 9.63 times that before treatment. The delay before the induction of low-temperature treatment time indicates that low-temperature treatment increased *LeGPA1* gene expression. When treated with 20% PEG-6000, the change in expression in the first 6 h was very slow. At 9 h, the induction was 4.14 times that before treatment. At 12 h the induction reached a maximum of 4.38 times that before treatment, slowly decreasing at 24 h, then falling rapidly (Fig. 3c). After treatment with 200 mm NaCl, the expression level of *LeGPA1* was 4.58 times higher than that before treatment, and there was a peak increase of about 4.84 times at 9 h. After 12 h, expression fell rapidly and leveled off (Fig. 3d). Based on these results, we concluded that *LeGPA1* is induced by a variety of stresses.

Identification of transgenic processing tomatoes

To assess the importance of *LeGPA1* in the processing tomato low-temperature stress response of processing tomato plants and to determine how it impacted transgenic plant physiology, Under aseptic conditions, tomato seeds were evenly spread on 1/2 MS medium (Fig. 4a), and tomato seedlings were obtained after about 10 d of culture (Fig. 4b). The cotyledon was cut off at both ends and about 0.5 cm of the hypocotyl was cut, placed on the differentiation medium for dark culture for 2 d (Fig. 4c). The callus grew from the explant around 20 d later (Fig. 4d) and was left for 2 months to sprout (Fig. 4e), then separated from the explant and inserted into rooting medium (Fig. 4f). After rooting (Fig. 4g, h), the cultivated seedlings were transplanted into pots containing nutrient soil for 7 d (Fig. 4i), then to the field when adaptability was strong (Fig. 4j). Regenerated plants were obtained and tested by PCR. Three *LeGPA1*-overexpressed transgenic lines from positive plants (OE-1/2/3) and three RNA-interfering transgenic lines (RI-1/2/3) were selected and used for qRT-PCR detection (Fig. 5). Relative *LeGPA* mRNA levels in OE-1, OE-2, and OE-3 plants increased 4.12, 6.13, and 7.69 times, respectively, compared with wild-type plants, and *LeGPA* mRNA levels in RI-1, RI-2, and RI-3 decreased by 0.90, 0.78 and 0.74 times, respectively. Therefore, based on the results of gene expression analysis, we selected two overexpressed lines (OE-2, OE-3) and two RNA-interfering lines (RI-1, RI-2) for further research on cold-resistance function.

Analysis of biological characteristics of transgenic tomato

As shown in Fig. 6, the biological indicators of wild-type and transgenic tomatoes at 80 d of growth were investigated. The results show that the overexpressed transgenic tomato plants grew significantly taller compared with wild-type controls, by 4–11.6%. However, the amount of RNA interference was lower compared to that of wild-type tomatoes, by 28.9–36.9%. Root length or fresh weight were comparable in wild-type and transgenic plants, whereas the Stem thick of the overexpressed transgenic plants increased

by 13.5–18.3% compared with wild-type plants, while the RNA-interference transgenic plants significantly increased by 34.1–37.4% compared with wild-type controls ($P < 0.01$).

Overexpression of LeGPA1 gene enhanced the resistance of transgenic tomato seedlings to low temperature

To test the cold stress tolerance of the transgenic tomato plants, T2-generation seeds of *LeGPA1*-overexpressed transgenic plants, RNA-interference transgenic plants, and wild-type processing plants were uniformly seeded into rectangular pots. The plants were grown under normal conditions (25℃) for 3 weeks and then transferred to a 4℃ incubator for 7 days. Phenotypes were observed and seedling fresh weight and survival were measured at 0, 3, 5, and 7 days at low temperature. As shown in Fig. 7a, both wild-type and transgenic plants grew normally at 4℃. After 3 days of 4℃ treatment, RNA interference tomato seedlings begin to show signs of wilting, i.e., slight curving of plant parts was seen. wild-type tomato seedlings were slightly wilted but not as much as the RNA-interference lines, while the overexpressed tomato plant seedlings showed better growth than the RNA-interference seedlings. After 5 days at low temperature, most of the RNA interference tomato seedlings had bent and fallen, the wild-type processing plants began to have curved tops that inhibited growth, and some of the overexpressed lines also showed wilting. After 7 days of treatment, the RNA-interference seedlings had almost all perished with only a few surviving, there were few surviving wild-type processing seedlings but more than the surviving RNA-interference lines, while the *LeGPA1* transgenic seedlings had only undergone partial wilting. After 7 days of low temperature treatment, the tomato seedlings were placed in a 4 to 25℃ incubator for 3 days under normal conditions to recover. It was found that almost all the RNA-interference tomato seedlings had died, a few wild-type plants survived, and a few overexpressed plants had died. In addition, fresh weight measurements and survival statistics were also performed and similar results were obtained. Following 7 days of cold treatment, the fresh weight of overexpressed lines had decreased by 43.4 (OE-2) and 39.6% (OE-3), that of wild-type lines decreased by 68.5%, and that of RNA-interference lines decreased by 70.7 (RI-1) and 70.1% (RI-2), compared with that before treatment (Fig. 7b). Following a 7-day low temperature treatment period, both transgenic and wild-type seedlings exhibited significantly decreased survival, but the survival rate of the overexpressed plants was increased significantly compare to the RNA-interference plants and wild-type controls, and the wild-type controls had higher survival rates compared with RNA-interfered plants (Fig. 7c). This shows that overexpression of *LeGPA1* increased transgenic tomato seedling resistance to a low temperature.

growth analysis of transgenic tomato sprouts under low temperature stress

To additionally assess transgenic tomato low temperature resistance, transgenic and wild-type plants were next grown for 6 weeks under standard conditions prior to being transferred for 5 days to 4 °C. Normal growth in wild-type and transgenic plants was observed at room temperature. Following growth at 4℃, the wild-type tomato leaves had wilted to different degrees, while all the RNAi tomato leaves had wilted and drooped, were dark brown in color with water stains on the surface and some of the leaves had

died. However, there was almost no change to the overexpressed tomatoes, which grew normally (Fig. 8). This indicated that *LeGPA1* is important mediator of low temperature resistance within these processing tomatoes.

Overexpression of LeGPA1 alleviates cell membrane damage under low temperature stress

Malondialdehyde (MDA) is a ROS-associated lipid peroxidation byproduct. Cell membrane permeability can be gauged based on MDA levels as well as relative electrolyte leakage (REL). Under low temperature stress, the MDA and REL levels of both wild-type and transgenic plants rapidly increased (Fig. 9a, b). The MDA and REL levels of RNA-interference plants were elevated compared to wild-type controls, however, wild-type plants exhibited markedly elevated MDA and REL levels relative to overexpressed transgenic plants ($P < 0.01$).

The relative water content (RWC) of plants reflects their water retention capacity and is used for measuring plant water status and osmotic regulation. The RWC of the transgenic and wild-type leaves was comparable before low temperature stress (Fig. 9c). In contrast, the RWC of both plant types decreased after stress, but the decline in the RWC of wild-type controls was significantly greater relative to overexpressed transgenic plants, RNA-interference plants were markedly heigher compared to wild-type controls ($P < 0.01$).

The maximum photochemical efficiency of the optical system II (PSII) is given as Fv/Fm. Under normal conditions, these values were comparable for wild-type and transgenic plants (Fig. 9d). However, they differed significantly at low temperature ($P < 0.01$). In all plants, the value steadily declined, but the decline was more significant in the wild-type relative to the overexpressed transgenic plants, and more obvious in the RNA-interference lines than in the wild-type plants.

Under low-temperature stress, all plants exhibited soluble sugar content and proline increases. However, the overexpressed transgenic plants had higher levels of both of these contents compared with wild-type plants, while wild-type plants exhibited higher soluble sugar and proline levels compared with RNA-interference transgenic plants (Fig. 9e, f). The results show that the overexpression of *LeGPA1* alleviated the damage caused to processing tomato plants by low temperature stress.

Overexpression of LeGPA1 alleviates the accumulation of ROS in response to low-temperature stress

Before low-temperature treatment, the H_2O_2 and O_{2-} contents were low and almost identical in the wild-type and transgenic plants (Fig. 10). However, ROS levels increased after exposure to low-temperature stress, with a more significant increase in wild-type controlls than in overexpressed transgenic plants. Moreover, the increase in RNA-interference plants was more evident than that in the wild-type. The results show that overexpression of *LeGPA1* reduced the accumulation of H_2O_2 and O_2^- under low-temperature stress.

To study the effects of overexpression of *LeGPA1* and the inhibition of *LeGPA1* on low temperature stress-related oxygen scavenging enzyme activity in transgenic plants, superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) were measured. The results show that these three activities increased in all plants follwoing low temperature exposure. However, relative to wild-type plants, there was significantly higher CAT, POD, and SOD activity in overexpressed transgenic plants ($P < 0.01$) The activity of SOD, CAT, and POD in wild-type plants was significantly higher than that in RNA-interference plants (Fig. 11a-c). The results show that overexpression of the *LeGPA1* gene increased the activity of oxygen scavenging enzymes under low temperature stress, improving the plant's ROS scavenging ability and reducing ROS toxicity. This suggests that transgenic tomato plants can degrade more ROS. To determine the cause of the high CAT, SOD, and POD activity in transgenic tomato plants, *LeSOD*, *LePOD*, and *LeCAT* transcription was detected. In standard growth conditions, the expression of *LeSOD*, *LePOD*, and *LeCAT* were similar in all lines. Following low temperature exposure, we recorded the upregulation of *LeSOD*, *LePOD*, and *LeCAT* in all plants, and the expression levels in the overexpressed lines were higher compared to wild-type lines. However, the levels in the RNA-interference transgenic tomatoes were low (Fig. 11e-f). Therefore, we concluded that the high levels of *LeSOD*, *LePOD*, and *LeCAT* expression increased the corresponding enzyme activity and cleared more H_2O_2 and O_2^- ROS in overexpressed transgenic tomatoes.

LeGPA1 **has a positive regulatory effect on cold-response gene expression in processing tomatoes**

We determined the expression levels of ICE (INDUCER OF CBF EXPRESSION) -CBF (C-REPEAT-BINDING FACTOR) genes (*LeICE1* and *LeCBF1*) and their downstream target genes (*LeTPS1*, *LeCOR413PM2*, and *LeDRCi7*) in processing tomato lines. We found that before low temperature stress, RNAi lines exhibited reduced gene expression compared to wild-type plants, and the differences in the expression levels of *LeCOR413PM2* and *LeDRCi7* were highly significant. However, the expression levels of the genes were significantly lower in the wild-type than in the overexpressed lines. Following low-temperature stress, the gene expression levels of all plants showed an overall rising trend; however, the difference in expression between wild-type and overexpressed lines further increased and showed extremely significant differences (Fig. 12).

Methods

Plants

Wild type *L. esculentum* ('Yaxin 87-5') seeds were obtained from Yaxin Seed Co. Ltd. (Shihezi City, Xinjiang, China). Plants were first grown in a 25°C tissue culture room (16/8 hour light/dark cycle) with 60-70% relative humidity and 200 μmol m^{-2} s^{-1} light intensity. Seedlings were next transplanted to pots that contained an equal mixture of soil, peat, and vermiculate, and were transferred to a 22-28°C greenhouse with natural lighting and identical humidity and light cycle conditions. While in this greenhouse, plants underwent irrigation with 500 mL of Hoagland's nutrient solution two times each week.

LeGPA1 cloning

A RNAisoPlus kit (TaKaRa) was used to extract DNA from the leaves of tomato plants, with DNase I on-column digestion being conducted based on provided instructions. PrimeScript RTase (TaKaRa) was used for first-strand cDNA synthesis, after which PCR was used to amplify full-length *LeGPA1* using the *LeGPA1* (*Kpn* I)-CF and *LeGPA1* (*Sal* I)-C*R* (Table 1) primers designed using *Solanum lycopersicum* gene sequences (GenBank ID: NM_001306055.1). PrimeSTAR Max DNA polymerase (TaKaRa) was used for PCR with thermocycler settings as follows: 95°C for 5 min; 35 cycles of 95°C for 30 s, 56°C for 30 s; 72°C for 10 min. This approach yielded a 1176-bp PCR fragment that we then cloned into the pMD19-T vector (TaKaRa). The identity of this fragment was confirmed via DNA sequencing.

Table 1 List of primers used in this study.

Name	Sequence (5′-3′)	Purpose
LeGPA1 (*Kpn* I)-F	GGTACCATGCTGTCGGTGGTTTTCGAA	Cloning
LeGPA1 (*Sal* I)-R	GTCGACTCATAATAAACCTGCTTCGAA	Cloning
LeGPA1 (*Xba* I)-R	TCTAGAAATAAACCTGCTTCGAAGAGA	Subcellular localization
LeGPA1 (*Xho* I and *Sal* I)-F	CTCGAGTCCAGATTGTGCCCATTA	RNAi upstream
LeGPA1 (*Bgl* II and *BamH* I)-R	AGATCTGACCCACTCAAAGAGTT	RNAi downstream
LeGPA1-qF	CTACAGTCAAGCCGATGATGAG	qRT-PCR
LeGPA1-qR	AAAGCCAGTTTGGAACAAGAGT	qRT-PCR
LeEF1α-qF	GGAACTTGAGAAGGAGCCTAAG	qRT-PCR
LeEF1α-qR	CAACACCAACAGCAACAGTCT	qRT-PCR
LeSOD-qF	GGCCAATCTTTGACCCTTT	qRT-PCR
LeSOD-qR	AGTCCAGGAGCAAGTCCAGT	qRT-PCR
LePOD-qF	GTCCGGGAGTTGTTTCTTGT	qRT-PCR
LePOD-qR	ATCACCATTGGCTTCTGACA	qRT-PCR
LeCAT-qF	ATTTGGTGGAGAAACTTGCC	qRT-PCR
LeCAT-qR	CTGTACACCAGGAGCTCGAA	qRT-PCR
LeDRCi7-qF	TTGTGTTTCTGTGTTGTTTTGG	qRT-PCR
LeDRCi7-qR	GCACATACATATGCACTTACATACAG	qRT-PCR
LeTPS1-qF	GGTACCTGCAGACACTGAGTGGAA	qRT-PCR
LeTPS1-qR	CTGTCGACTATACAAAGGATGCATGATTCTTAAC	qRT-PCR
*Le*lICE1-qF	GGAAGGAAAAGCGGTGAAC	qRT-PCR
*Le*lICE1-qR	AACACATCCAACACAAACCC	qRT-PCR
*Le*CBF1-qF	TTCATCGTCATCGTCGTTTTCT	qRT-PCR
*Le*CBF1-qR	TCCTCTTCCTGATTCCCCTGT	qRT-PCR
LeCOR413PM2-qF	AACTGGAGGAGCAACATA	qRT-PCR
LeCOR413PM2-qR	TCAAGCCAATCTGGAAAG	qRT-PCR
LeGPA1-qF	AGGTTCCAGATTGTGCCCATTA	RT-PCR
LeGPA1-qR	TCCTGTTGAAACTGACTGGTAATCT	RT-PCR
LeEF1α-qF	TCAGGCTGACTGTGCTGTTCTC	RT-PCR
LeEF1α-qR	CTGGGTCATCCTTGGAGTTTGAG	RT-PCR

Overexpression and RNAi plasmid construction

To overexpress *LeGPA1*, we cloned the PCR-amplified *LeGPA1* fragment into the pCAMBIA2300 binary vector using *Kpn* Ⅰ and *Sal* I restriction sites and the 35S promoter to control transcription. RNAi plasmids were constructed by cloning a 220-bp *LeGPA1-based* gene segment encoding RNAi specific for *LeGPA1* bases 340-560. This segment was amplified via PCR with appropriate primers (Table 1) prior to cloning into the pCAM2300 vector. The identities of all constructs were confirmed via sequencing.

LeGPA1 sequence assessment

DNAMAN (v8.0) was used to align the *LeGPA1* sequence. The TMHMM algorithm (http://www.cbs.dtu.dk/services/TMHMM/) was used for predicting transmembrane domains. MEGA 5.1 (http://www.megasoftware.net/) was used for phylogenetic analyses based on a Neighbor-Joining approach and 1,000 bootstrap replicates, with the deletion of bootstrap scores of <50%.

LeGPA1 subcellular localization analyses

The full-length *LeGPA1* open-reading frame (ORF) with no stop codon was amplified via PCR using appropriate primers shown in Table 1, including *LeGPA1* (*BamH* I)-SF and *LeGPA1*(*Xba* I)-SR. These primers respectively included *BamH* I and *Xba* I restriction sites, and the resultant fragment was then cloned into the pCAMBIA2300-GFP expression vector yielding a p35S-*LeGPA1*-GFP plasmid under CaMV 35S promoter control. *Arabidopsis* mesophyll protoplasts were then transfected via a PEG approach with both this p35S-*LeGPA1*-GFP plasmid as well as with the PM-rk plasmid to assess plasma membrane localization [37, 38, 39]. Following a 16 h culture at 23 ℃, protoplast fluorescence was evaluated via confocal microscopy (Leica Microsystems, Germany) with 488, 561 and 633 nm excitation wavelengths.

Assessment of the impact of stress on *LeGPA1* gene expression

Wild-type tomatoes were grown under standard greenhouse as detailed above, after which a subset of these plants that appeared phenotypically similar were selected for stress treatments that were initiated when the light cycle began. *LeGPA1* gene expression in a range of organs (fruit, flowers, stems, leaves, roots) from 80-day-old plants was assessed via qRT-PCR. In addition, *LeGPA1* expression was assessed in wild-type tomatoes grown under salt, cold, or drought stress conditions. For cold stress, plants were treated for 48 h at 4°C. For drought conditions, plants were removed from soil, and roots were submerged for 48 h 5 cm deep in 20% PEG 6000. Salt stress was induced by removing plants from their soil and immersing the roots for 48 h in 200 mM NaCl. Leaves from identical positions on these plants were collected 0, 1, 3, 6, 9, 12, 24, and 48 h after the initiation of stress conditions. Plants were grown in three separate growth chambers for replicate samples. Collected leaves were snap-frozen prior to storage at −80°C.

A RNAprep Pure Plant Kit (Tiangen, China) was used to isolate sample RNA for qRT-PCR, after which cDNA was synthesized and analyzed with SYBR Green I Master Mix using a LightCycler 480Ⅱ platform (Roche Biochemicals, Indianapolis, IN, USA). As a normalization control for cold stress-related gene

expression, the tomato *EF1* gene (GenBank ID: X53043) was utilized [40](Lovdal and Lillo, 2009). Thermocycler seting were: 2 cycles of 95°C for 30 s; 50 cycles of 95°C for 5 s, 60°C for 10 s, and 68°C for 10 s. The ΔΔCt method [41] was used for comparing relative gene expression, with triplicate samples being analyzed independently. Primers used for this assay are shown in Table 1.

Transgenic plant preparation

Transgenic plants that either overexpressed *LeGPA1* or an RNAi construct were prepared for this study. Briefly, the appropriate constructs were transformed into wild-type plants via the use of *Agrobacterium tumefaciens* strain GV3101. We then utilized 1/2 strength MS medium supplemented with 60 mg/L kanamycin to screen for transgenic plants. Isolated kanamycin-resistant T_0 plants were evaluated via semi-quantitative reverse transcription PCR using *LeGPA1* primers (Table 1), and qRT-PCR was then used to confirm the identities of different transgenic plants. Plants in the T_2-generation that retained their ability to grow on MS media supplemented with 60 mg/L kanamycin were utilized as transgenic plants in downstream assays.

Assessment of stress response-induced changes in plants

Plants (wild-type or transgenic) from the T_2-generation were grown for 3-6 weeks in a 25°C incubation chamber (16/8 h light/dark cycle; 70% humidity; 200 μmol m^{-2} s^{-1} photon flux density). After either 3 or 6 weeks, plants with uniform sizes were subjected to a 5 day cold stress exposure (4°C). Plants were grown in three separate growth chambers for replicate samples. Changes in plant phenotypes were then assessed, with imaging being conducted through the use of a Canon 80D camera. The second and third leaves from the tops of each of these plants were additionally collected, and stress response-related gene expression in these leaves was assessed via qRT-PCR. These samples were also used for antioxidant activity assays and assessments of plant physiology.

Physiological parameter analyses

Relative water content (RWC) was assessed as described in a study conducted by Lara et al [42] as follows: RWC = (FW − DW)/(TW − DW) × 100%. In this equation, FW corresponds to leaf fresh weight, TW corresponds to turgid weight (following leaves being incubated in dH2O for 24 h in the presence of light), and DW corresponds to dry weight (drying at 70 °C to a constant weight).

A modified thiobarbituric acid reaction described by Du et al [43] was used to assess MDA levels as a readout for membrane damage. Leaves were first excised, rinsed with dH2O, and discs were collected for MDA measurements with a UV-160A spectrophotometer (UV-160A, Shimadzu Scientific Instruments, Japan).

Relative electrolyte leakage (REL) was assessed via EC 215 conductivity meter (Markson Science Inc., CA, USA) based on the approach of Du et al [43] using the formula REL = (C1 − CW)/(C2 − CW) × 100, with C1

and C2 corresponding to conductivity before and after boiling, respectively, and CW corresponding to the conductivity of deionized water.

Photosystem II (PSII) maximal efficiency in tomato leaves was evaluated via portable fluorescence analyzer (DUAL-PAM-100, Walz, Germany). Briefly, leaves were placed in the dark for 30 minutes, after which they were exposed to a 1 second flash of light. Minimal fluorescence (F_0) when all PSII reaction centers are open for this assay was considered to represent the dark-adapted state, whereas maximal fluorescence (F_m) was the fluorescence intensity measured following light saturation, with all of these reaction centers being closed. Variable fluorescence (F_v) was calculated as follows: $F_v = F_m - F_0$ [44].

Free proline was assessed as in the study of Bates et al [45]. Briefly, 4 mL of 3% sulfosalicylic acid was used to extract leaf samples (200 mg) for 10 minutes at 100°C, after which homogenates were spun for 2 minutes at 12,000 × *g*. Next, a 2 mL supernatant volume was mixed with equivalent volumes of acid-ninhydrin reagent and glacial acetic acid. This solution was then boiled for 30 minutes, prior to transfer into an ice bath. Absorbance at 520 nm was then assessed following toluene (4 mL)-mediated extraction of the organic phase. Proline concentrations were determined based upon comparisons of sample absorbance to a proline standard curve.

The Anthrone method was used for analyses of soluble sugars, using gluose as a standard [46]. Briefly, an initial leaf sample (200 mg) was ground, homogenized in a 1 mL dH2O volume, boiled for 20 minutes, spun for 10 minutes at 13,000 × *g*, and a 2 mL supernatant volume was then combined with 1.8 mL dH2O and 2.0 mL of 0.14% (w/v) Anthrone solution in 100% H_2SO_4. This solution was rested for 20 minutes in boiling water, after which it was cooled and the A_{620} was assessed. Total soluble sugar levels were assessed by comparing these A_{620} values to those derived from a glucose standard curve. Experiments were conducted in triplicate using three replicate samples.

ROS and antioxidant activity assays

A fresh sample (0.5 g) of leaves from T_2-generation wild-type or transgenic plants was collected following cold or drought stress exposure. These leaves were minced, homogenized on ice in a 4 mL volume of 50 mM sodium phosphate buffer (pH 7.8) with 1% polyvinylpyrrolidone and 10 mM β-ME, and spun at 17,426 xg for 15 minutes at 4°C. CAT activity was assessed as described by Cakmak and Marschner [47]. SOD activity was evaluated as reported by Beauchamp and Fridovich [48]. POD activity was determined based on the methods described by Doerge et al [49]. An Infinite M200 Pro microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

H_2O_2 and O_2^- levels were assessed as described by Benikhlef et al [50]. A UV-160A spectrophotometer (Shimadzu Scientific Instruments, Japan) was used to assess absorbance for these analyses.

Statistical analysis

Data were assessed using SPSS v13.0 and GraphPad Prism 7.0. Relative *LeGPA1* expression was given as means ± SD from triplicate samples, with three leaves per seedling being used as a replicate. Expression levels were normalized to baseline (0 h) levels. Dunnett's multiple comparison test was used to compare plants. *P <0.05 and **P <0.01 correspond to significance.

Discussion

The Gα subunit is an important component of the heterotrimer G-protein complex; it not only plays important roles in various plant growth and development processes but also participates in responses to abiotic stresses, such as drought and high and low temperatures [51, 52, 35]. The Gα subunit has been the subject of study in model plants, but it had not been previously identified in processing tomatoes. In this study, we identified a G-protein Gα subunit from processing tomatoes and studied its growth and development as well as the resistance to low temperature it provides.

In this study, we found the *LeGPA1* gene of processing tomatoes to be homologous with the *GPA1* gene of other nightshade species. Previous studies have found that *GPA1* is only expressed in mature seeds [53], and *GPA1* expression has been detected in all stages of development and all organs. *GPA1* expression is highest in the roots, followed by the stem tip, hypocotyl, cotyledon, and leaf [54], and levels are higher in immature than mature organs [53]. Similarly, we analyzed the expression of *LeGPA1* in different organs of processing tomato plants and found that *LeGPA1* was expressed in all the organs tested. The highest expression level was found in roots, followed by leaves, then fruits, and the lowest expression level was in stems. Therefore, we speculated that *LeGPA1* may be involved in the regulation of the growth and development of processing tomatoes. Through various techniques, the localization of G-protein at the subcellular level has been observed. Using western-blot analysis of proteins from different cells parts, GPA1 was detected in the cell membrane of *Arabidopsis thaliana* [55]. Fluorescence localization identified a *GPA1*-GFP fusion protein in the plasma membrane of suspended cells of the protoplast or pollen tube [56, 57]. The above studies indicated that GPA1 is mainly located in the plasma membranes of cells. We performed a subcellular localization experiment and found that *LeGPA1* was also localized in the cell membrane.

The G-protein Gα subunit has been identified in *Arabidopsis thaliana*, rice, and maize. The *Arabidopsis thaliana* Gα mutant *gpa1* showed reduced plant height, reduced stomatal density, and significantly inhibited lateral root growth [21]. *GPA1* was found to positively regulate cell division and proliferation in *Arabidopsis thaliana*. Similarly, the Gα subunit of rice, RGA1, positively regulates cell proliferation and growth. The rga1 mutant *d1* has a small body size, short and erect blades, and decreased root and kernel size [58]. The maize Gα mutant *ct2* showed reduced plant height, short and erect leaves, and an enlarged meristem apex [59]. The Gα subunit acts as a positive regulator of cell proliferation and growth in all three species: *Arabidopsis thaliana*, rice, and maize. Through the functional analysis of the G-protein α subunit GPA1 in cucumber, Yan et al found that overexpression of *CsGPA1* promoted seed germination and early seedling growth, while interference of *CsGPA1* inhibited seedling growth. In order to better explain the regulatory effect of *LeGPA1* on the growth and development of processing tomatoes, we determined the

biological characteristics of transgenic lines and wild-type processing tomato plants [60]. The results show that the overexpressed transgenic tomato plants were significantly taller than the wild-type plants, whereas the RNA interference transgenic plants were shorter than the wild-type. This indicates that *LeGPA1* plays an important role in the regulation of processing tomato plant height.

The G-protein α subunits also play important roles in the signal transduction pathway of abiotic stress in plants [26]. The full transcriptome microarray analysis of rice revealed that rice *RGA1* can regulate low temperature, salt, and drought stress and transmit stress signals to the small phosphorylase GTPase and corresponding effector proteins or molecules, such as ion channels [28]. In the pea, expression of the *PsGPA1* gene was significantly changed by NaCl and high temperature [29]. A study of *Brassica napus* found that the expression of *BnGA1* was induced by four abiotic stresses: 20% PEG6000, 200 mm NaCl, a low temperature of 4℃, and a high temperature of 40℃, and *BnGA1* played an important role in resisting abiotic stress [54]. We analyzed whether the expression of *LeGPA1* in processing tomatoes was induced by stress and found that *LeGPA1* is induced by drought, high salt, and low temperature, among which low temperature was very significant, suggesting that G-protein α subunits of processing tomato play a role in environmental stress.

Studies have shown that *GPA1* in *Arabidopsis thaliana* is involved in oxidative stress signal transduction. For example, it can positively regulate abiotic stress factors upstream of ROS production [27]. ROS are produced and accumulate under cold stress; they destroy cells and produce MDA [61, 62]. Antioxidant enzymes are an important part of the ROS scavenging system in plant cells and, therefore, play important roles in plant cold resistance [63, 64]. Antioxidant enzyme activities in plants have been reported to increase under low-temperature stress, which may be due to the upregulation of corresponding genes. *Arabidopsis thaliana* and rice overexpressing *GPA1* under low temperature stress (4℃) tended to have different SOD, POD, and MDA levels compared to wild-type plants, indicating that *GPA1* is involved in the low temperature stress response [65, 66]. Yan et al analyzed the function of cucumber *GPA1* and found it participated in adaptation to low temperatures by adjusting the contents of proline, soluble protein, and the activities of antioxidant enzymes (SOD, POD, and CAT) [60]. In this study, expression of *LeGPA1* in processing tomatoes was up-regulated under low temperature stress. Transgenic studies have shown that overexpression of *LeGPA1* improves antioxidant capacity and decreases the degree of membrane lipid peroxidation under low temperature stress, indicating that *LeGPA1* positively regulates the response of processing tomatoes to low temperature.

ICE1-activation of the CBF pathway is an important way for plants to cope with cold stress [67, 68, 69]. The overexpression of *SlICE1* improved the cold tolerance of tomato plants by up-regulating *SlCBF1* and its downstream dehydration regulatory gene *SlDRCi7* [70]. Trehalose 6-phosphatase (TPS) is a key enzyme in trehalose synthesis. Trehalose phosphatase (TPP) dephosphorylates trehalose 6-phosphate to trehalose. One of the TPS-related genes in tomatoes, *SlTPS 1*, is induced under low temperature and salt stress. *OsTPP 1* showed an obvious induction effect under low temperature stress [71], and trehalose treatment can reduce the chilling injury of rice. These observations indicate that the synthesis of trehalose is a key step in the development of cold tolerance in tomatoes and rice [72]. *LeCOR413* is a

homolog of the *Arabidopsis thaliana* cold resistance gene 413 (*AtCOR413*), which is important for frost resistance [73]. This study analyzed the expression levels of genes related to low temperature stress in processing tomatoes. *LeGPA1* increased *LeICE1* gene expression in processing tomatoes, so as to regulate the transcription of the *LeCBF1* gene. Thus, the expression of *LeCOR413PM2* and *LeTPS1* and its downstream regulatory gene *LeDRCi7* was induced. This indicates that the overexpression of *LeGPA1* in processing tomato plants benefits their tolerance to low temperatures. However, interference of the gene reduces the plant's resistance to low temperature.

Conclusion

Together, these findings suggest that *LeGPA1* overexpression may be a viable approach to improving tomato resistance to cold stress. We found that *LeGPA1* overexpression was associated with increased cell membrane integrity and stability in response to cold stress while also reducing membrane lipid peroxidation product accumulation, decreasing rates of ion leakage, increasing antioxidant enzyme gene expression. These changes were associated with improved photochemical electron transport efficiency and antioxidant enzyme activity such that *LeGPA1* overexpressing plants exhibited reduced ROS levels compared with wild-type controls. Low temperature tolerance was improved in these plants via the maintenance of elevated permeable proline and soluble sugar levels to protect against cellular damage.

Abbreviations

APX	Ascorbate peroxidase
CAT	Catalase
CBF	C-repeat-binding factor
cDNA	Complementary DNA
DNA	deoxy ribonucLEAse tetrahydrochloride
F0	The minimal fluorescence
Fm	The maximal fluorescence
Fv	The variable fluorescence
Fv/Fm	Maximal photochemical efficiency of PSII
GFP	Green fluorescent protein
GPA1	G-protein α subunit
H_2O_2	Hydrogen peroxide
ICE	Inducer of CBF expression
MDA	Malondialdehyde
O_2^-	Superoxide anions
OE	Overexpression
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
POD	Peroxidase
qRT-PCR	Quantitative Real-time PCR
REL	Relative electrolyte leakage
RI	RNA interference
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptative PCR
RWC	Relative water content
SOD	Superoxide dismutase
TPP	Trehalose phosphatase
TPS	Trehalose 6-phosphatase

Declarations

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

LZ Conceptualization, Writing- Original draft preparation. JJL and XYG Methodology, Data curation, Visualization. zwz Supervision. PH Software. WWW Validation. MW Investigation. JBZ Writing- Reviewing and Editing. All Authors read and approved the manuscript.

Availability of data and materials

The datasets supporting the conclusions of this article are included within

the article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

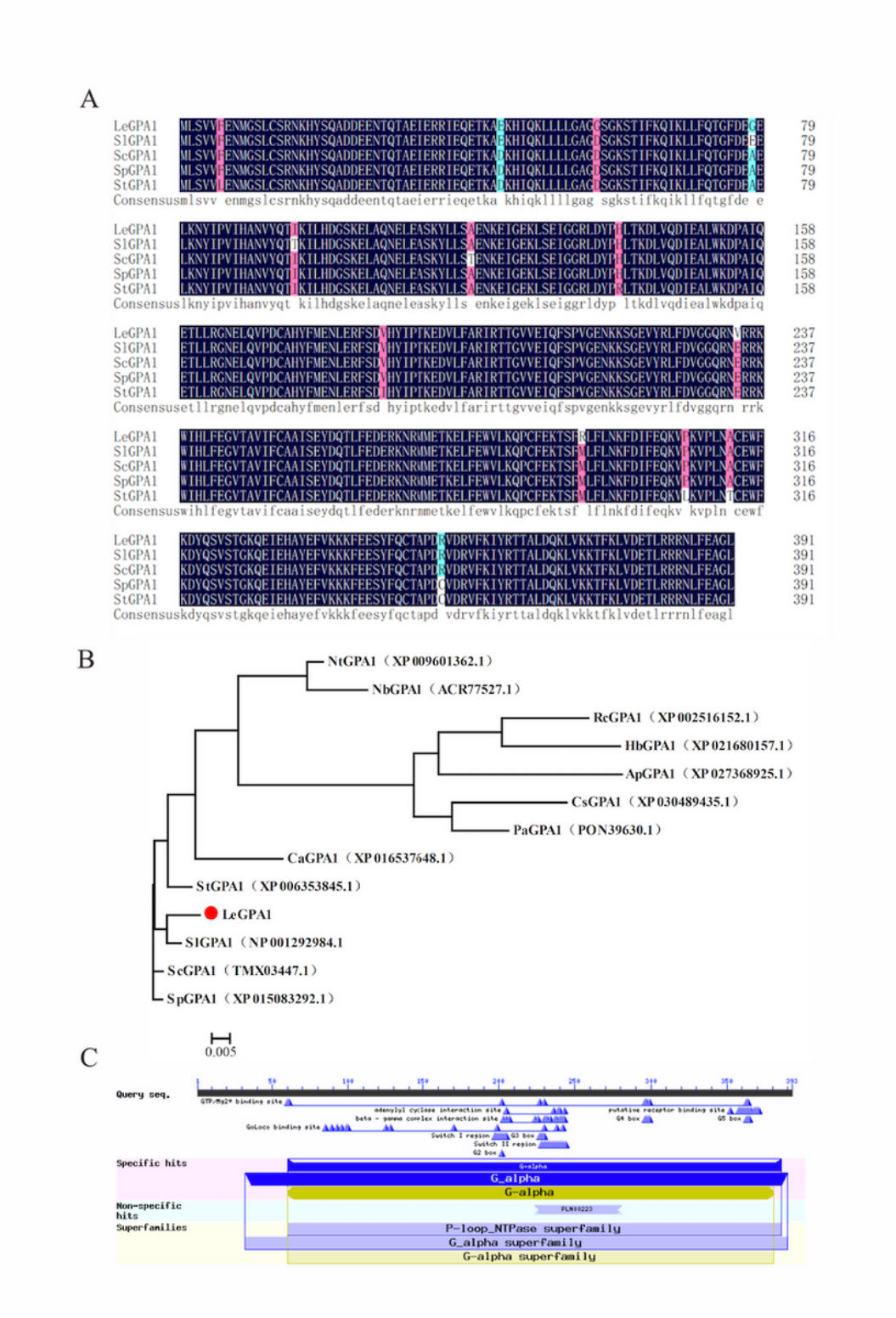

Figure 1

Sequence analysis of from Lycopersicon esculentum LeGPA1 sequence analysis. a LeGPA1 amino acid sequence alignment with other plant species. b Phylogenetic relationship between LeGPA1 protein and GPA1 proteins from other plant species. MEGA 5.1 was used for phylogenetic tree consrruction, with protein sequences used for such construction being from GenBank as follows: Nicotiana tomentosiformis (XP_009601362.1); Nicotiana benthamiana (ACR77527.1); Ricinus communis

(XP_002516152.1); Hevea brasiliensis (XP_021680157.1); Abrus precatorius (XP_027368925.1); Cannabis sativa (XP_030489435.1); Trema orientale (PON39630.1); Capsicum annuum (XP_016537648.1); Solanum tuberosum (NP_001275141.1); Solanum lycopersicum (NP_001292984.1); Solanum chilense (TMX03447.1); Solanum pennellii (XP_015083292.1). 1000x bootstrap replicate values were obtained. c Prediction of conserved domain of LeGPA1.

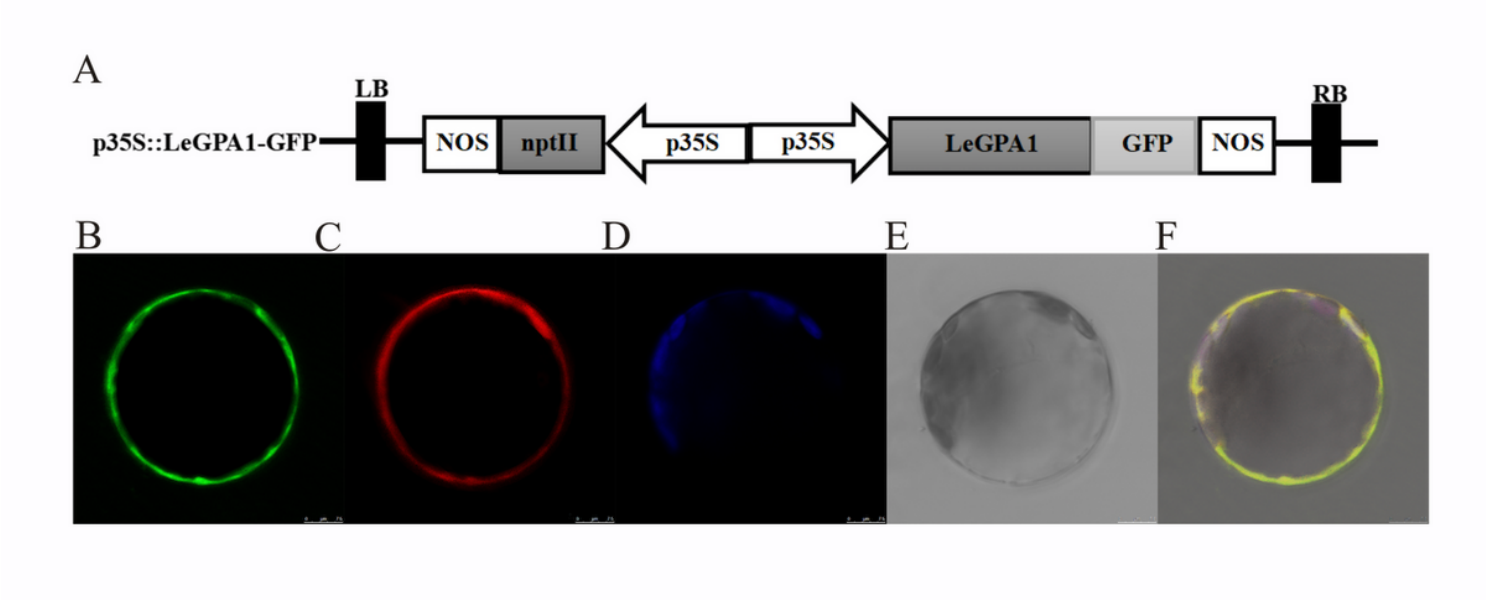

Figure 2

LeGPA1 localization within cells. Arabidopsis mesophyll protoplasts co-transformed with p35S- LeGPA1-GFP and a plasma membrane marker PM-rk. We co-transformed Arabidopsis mesophyll protoplasts using p35S- LeGPA1-GFP and the PM-rk plasma membrane marker. a The p35S-LeGPA1-GFP plasmid. b Arabidopsis mesophyll protoplast images following LeGPA1-GFP fusion protein expression. c Arabidopsis mesophyll protoplast images following Pm-rk expression. d Chloroplasts within Arabidopsis protoplasts. e Arabidopsis mesophyll protoplasts in a bright-field image. f Fluorescent images collected from LeGPA1-GFP and PM-rk expressing protoplasts were merged. Scale bars = 7.5 μm.

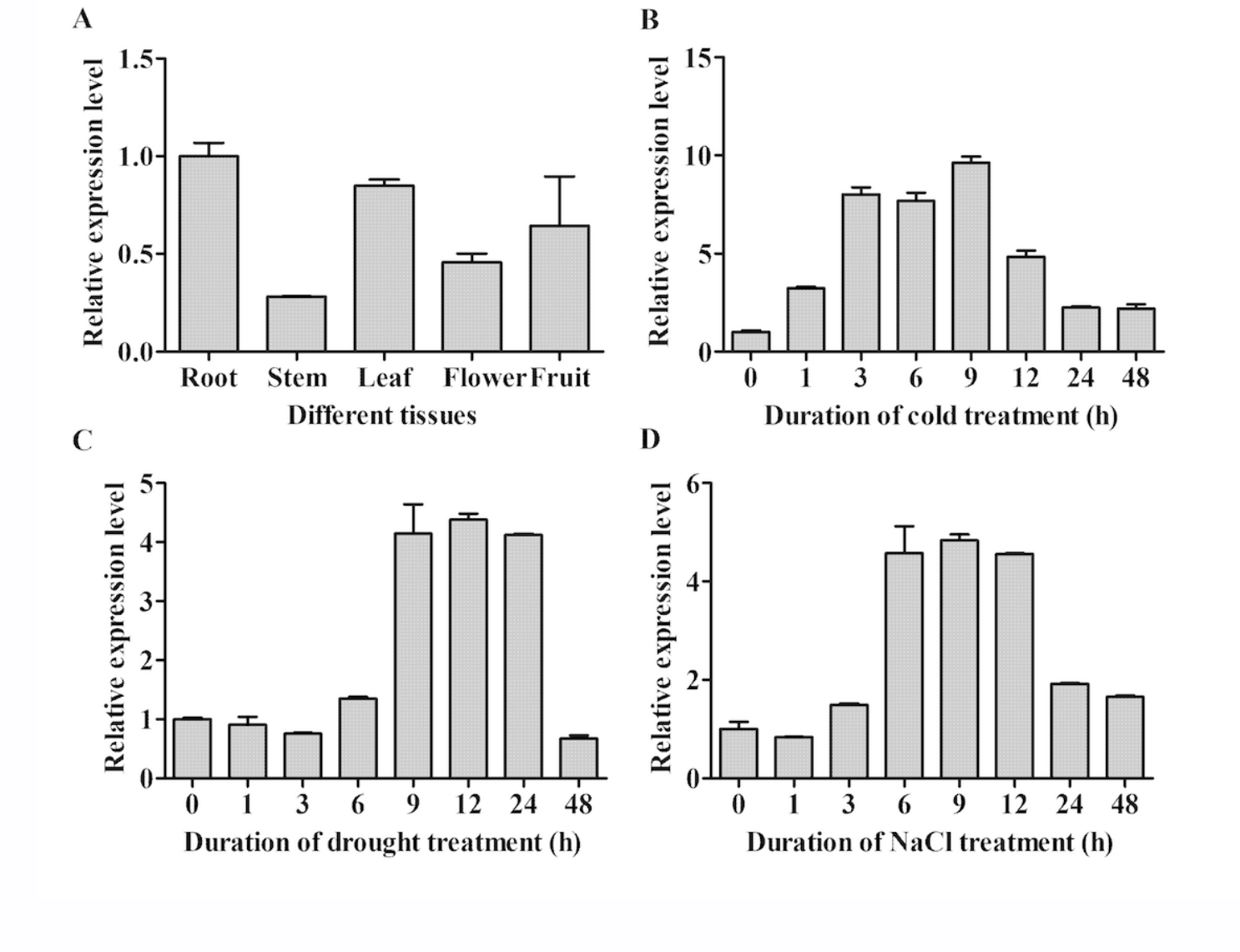

Figure 3

Assessment of LeGPA1 mRNA expression in wild-type plants a LeGPA1 expression was evaluated in the fruits, stems, leaves, flowers, and roots of L. esculentum following growth at 25 oC. b LeGPA1 gene expression was evaluated after 0, 1, 3, 6, 9, 12, 24, and 48 h at 4°C in the leaves of these plants. c Expression of LeGPA1 gene in L. esculentum leaves following drought stress generated using 20% PEG-6000 for 0, 1, 3, 6, 9, 12, 24, and 48 h. d Expression of LeGPA1 gene in leaves of L. esculentum treated with salt stress generated using 200 mM NaCl for 0, 1, 3, 6, 9, 12, 24, and 48 h. Reported data are derived from triplicate analyses.

Figure 4

The acquirements of LeGPA1-OE and LeGPA1-RNAi lines a Planting of tomato seeds. b Tomato seedlings. c Tomato explants. d The explants form a callus. e The tissue culture bud differentiation

period adventitions. f-h Rooting screening culture period. i Transplanting of transgenic tomato to flowerpot. j Transgenic tomatoes transplanted into to the field.

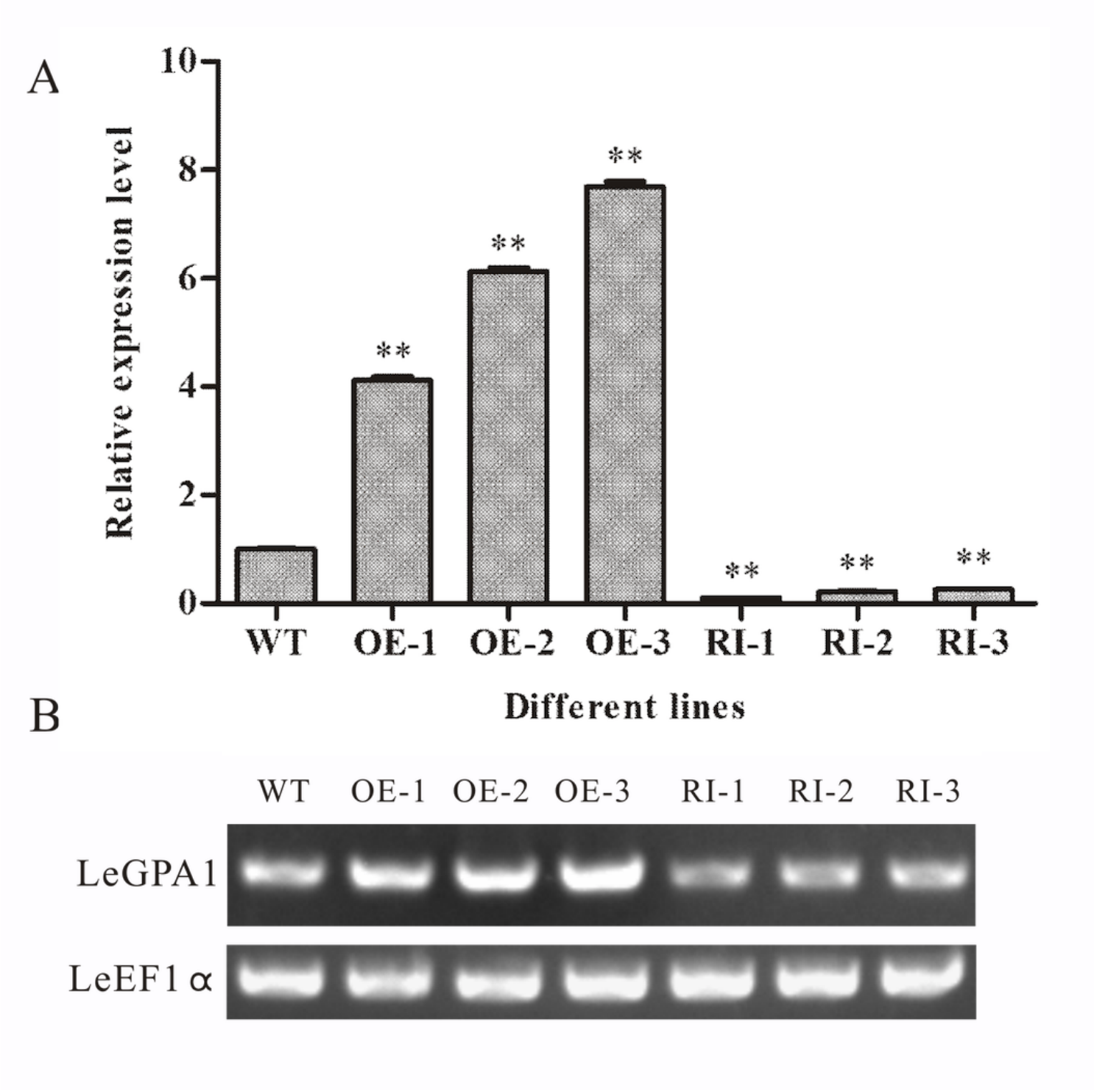

Figure 5

LeGPA1 expression in wild-type (WT), LeGPA1–overexpressing transgenic tomato plants (OE1-OE3), and RNAi-expressing transgenic tomato plant lines (RI1-RI3). a qRT-PCR was used to assess gene expression. b Semi-quantitative PCR was used to assess expression in leaves collected from plants grown under

standard conditions. Experiments were repeated twice, with results all being representative of triplicate samples. Bars represent SDs. *P < 0.05 and **P < 0.01.

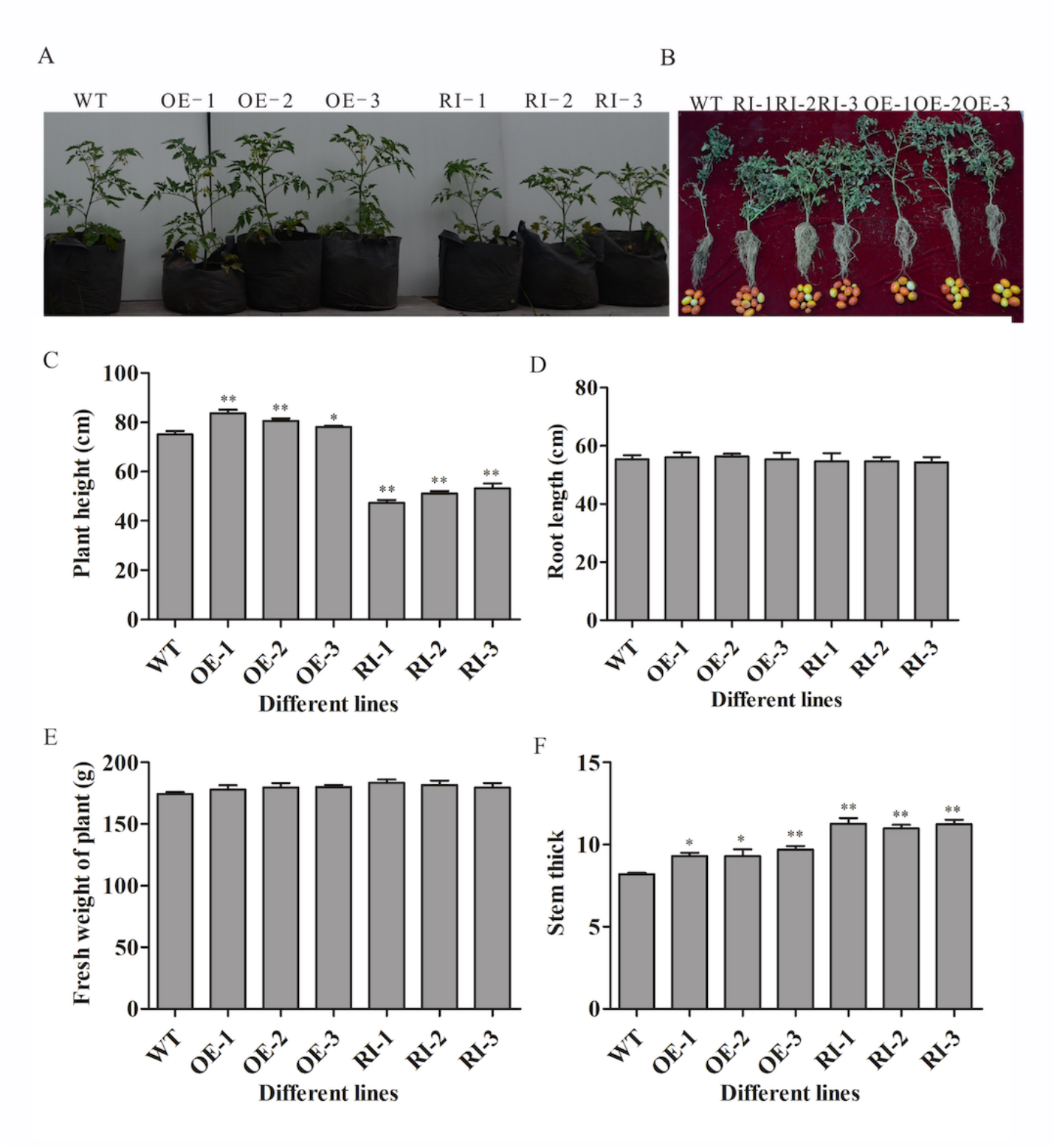

Figure 6

Assessment of the characteristics of wild-type and transgenic plants. a The growth of wild-type plants and transgenic plants expressing LeGPA1 gene in field. b Plant fruiting. c Plant height. d Root length. e

Plant fresh weight. f Stem thick. Data are means and SDs from triplicate samples. *P < 0.05 and **P < 0.01 vs. WT.

Figure 7

Assessment of young wild-type (WT) and transgenic tomato plant cold resistance a Phenotypes of 3-week-old WT and transgenic tomato plants treated or untreated with cold stress for 5 d. b Fresh weight. c Survival rate. Data are means and SDs from triplicate samples. *P < 0.05 and **P < 0.01.

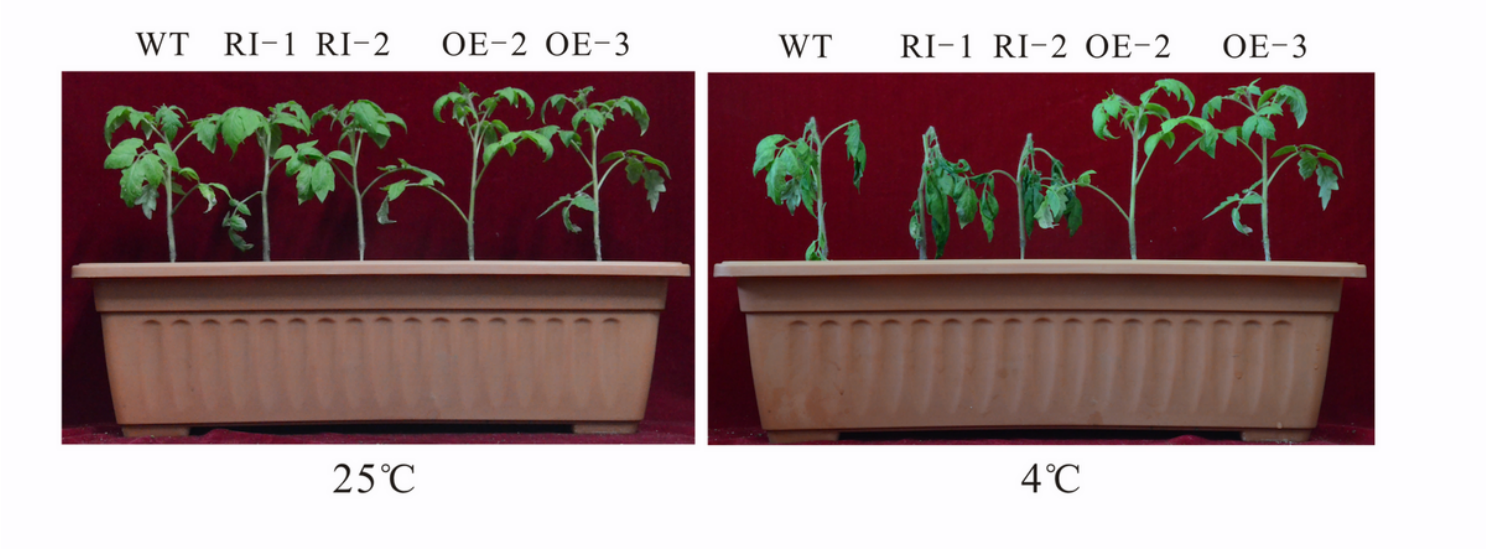

Figure 8

The growth of 6-week-old WT and transgenic plants for 0 or 5 days under 4 °C.

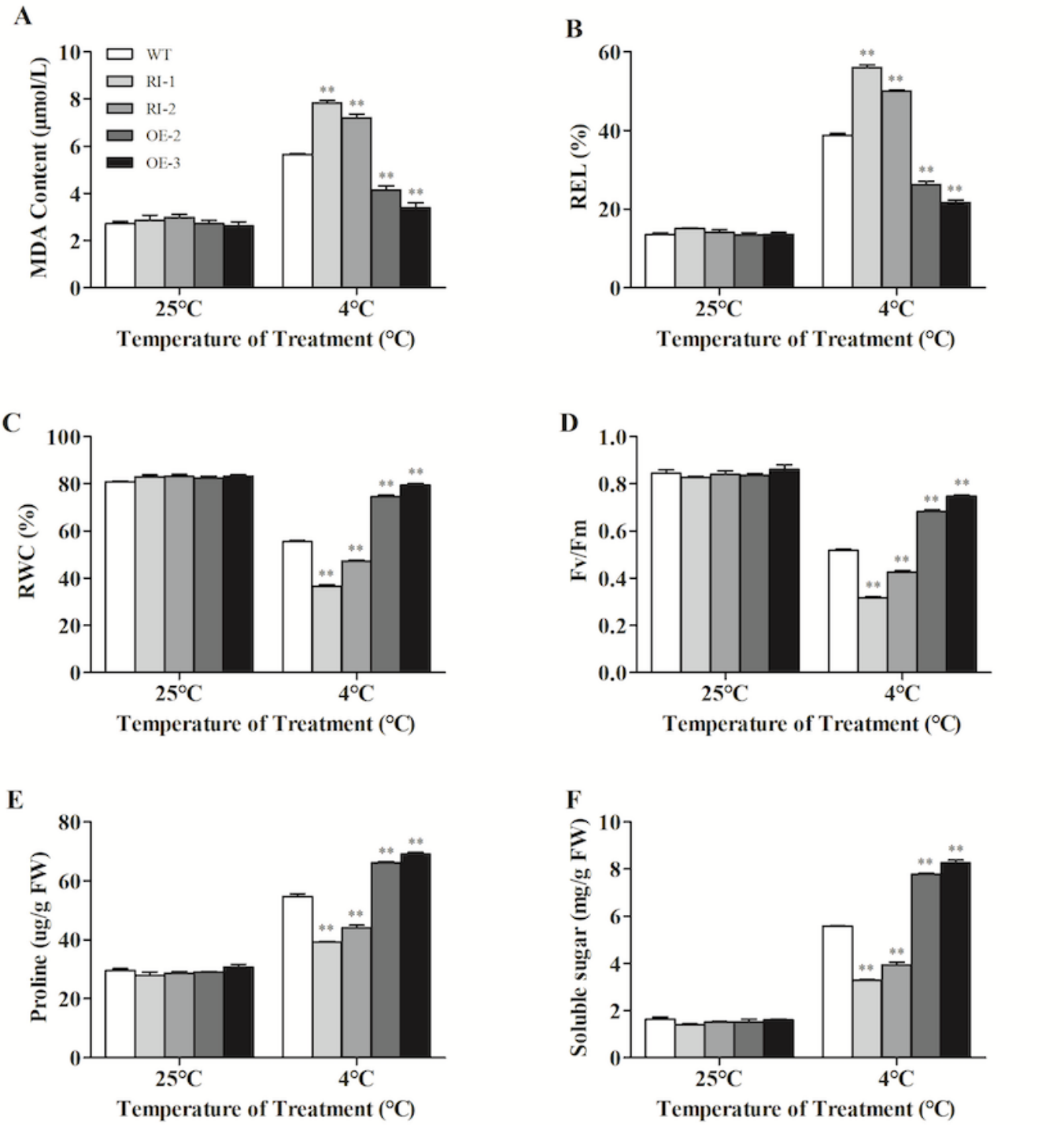

Figure 9

Changes in WT and transgenic plant responses to cold stress in 6-week-old plants. a MDA contents. b REL. c RWC. d Fv/Fm values. e Proline contents. f Solute sugar levels. Uniformly sized 6-week-old tomato plants were subjected to a 5 day cold stress exposure at 4°C. Leaves that were the second and third from the top were assessed. Data are means and SDs from triplicate samples.*P < 0.05 and **P < 0.01.

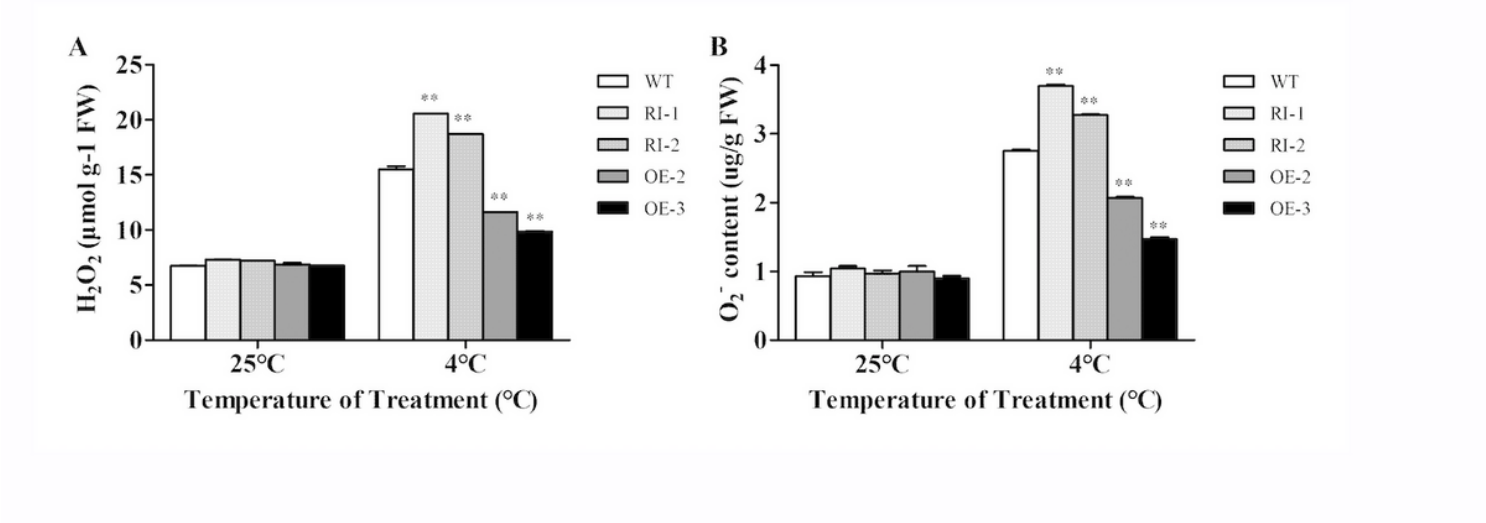

Figure 10

ROS (H2O2 and O2–) accumulation in WT and transgenic tomato plants exposed to cold stress. a H2O2 levels. b O2- content. Uniformly sized 6-week-old tomato plants were subjected to a 5 day cold stress exposure at 4°C. Leaves that were the second and third from the top were assessed. Data are means and SDs from triplicate samples. *P < 0.05 and **P < 0.01.

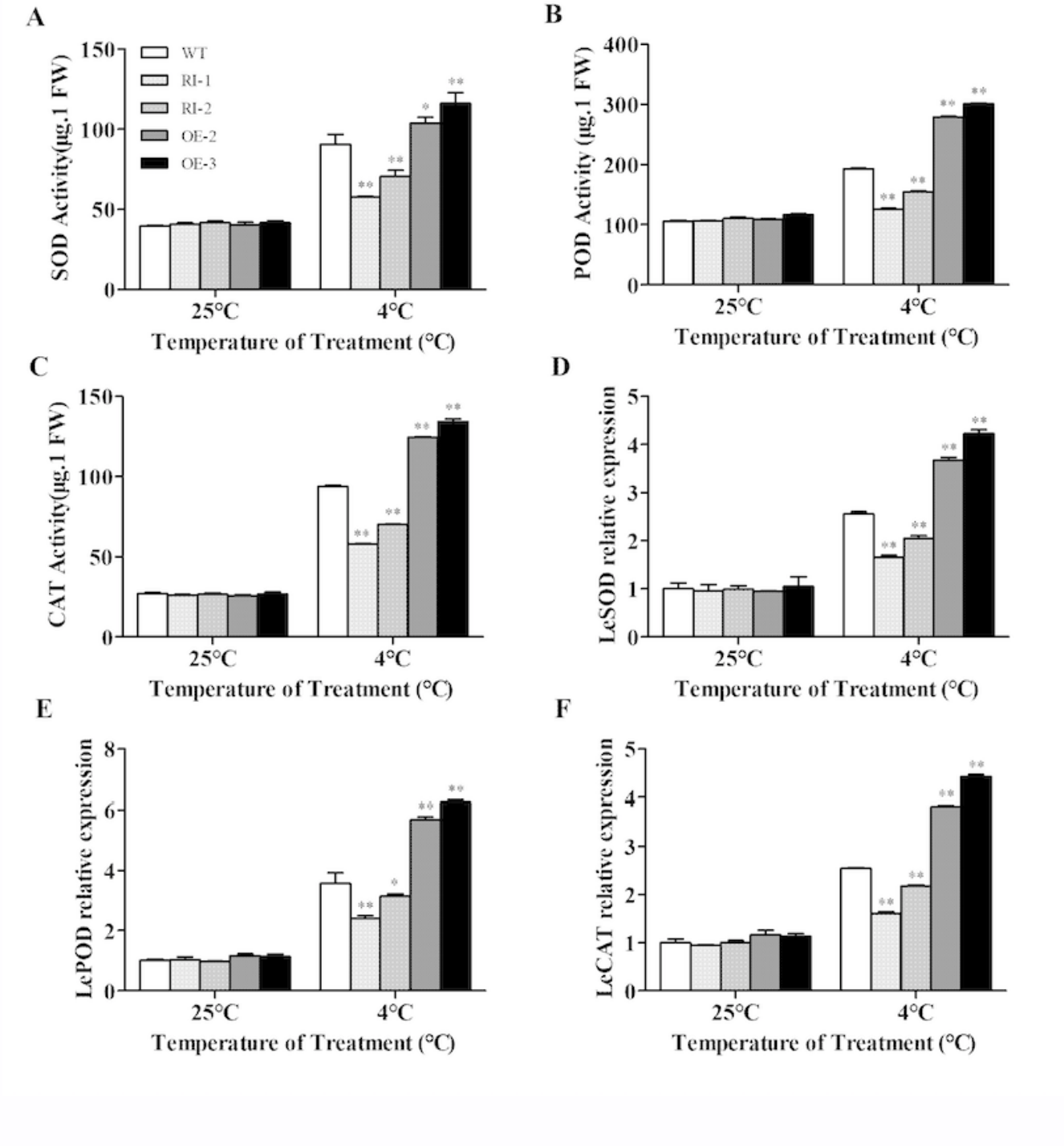

Figure 11

Antioxidant enzyme (SOD, POD, and CAT) activity and expression of ROS-scavenging genes in WT and transgenic tomato plants exposed to cold stress. a SOD activity. b POD activity. c CAT activity. d Expression level of LeSOD. e Expression level of LePOD. f Expression level of LeCAT. Uniformly sized 6-week-old tomato plants were subjected to a 5 day cold stress exposure at 4°C. Leaves that were the

second and third from the top were assessed. Data are means and SDs from triplicate samples.*P < 0.05 and **P < 0.01.

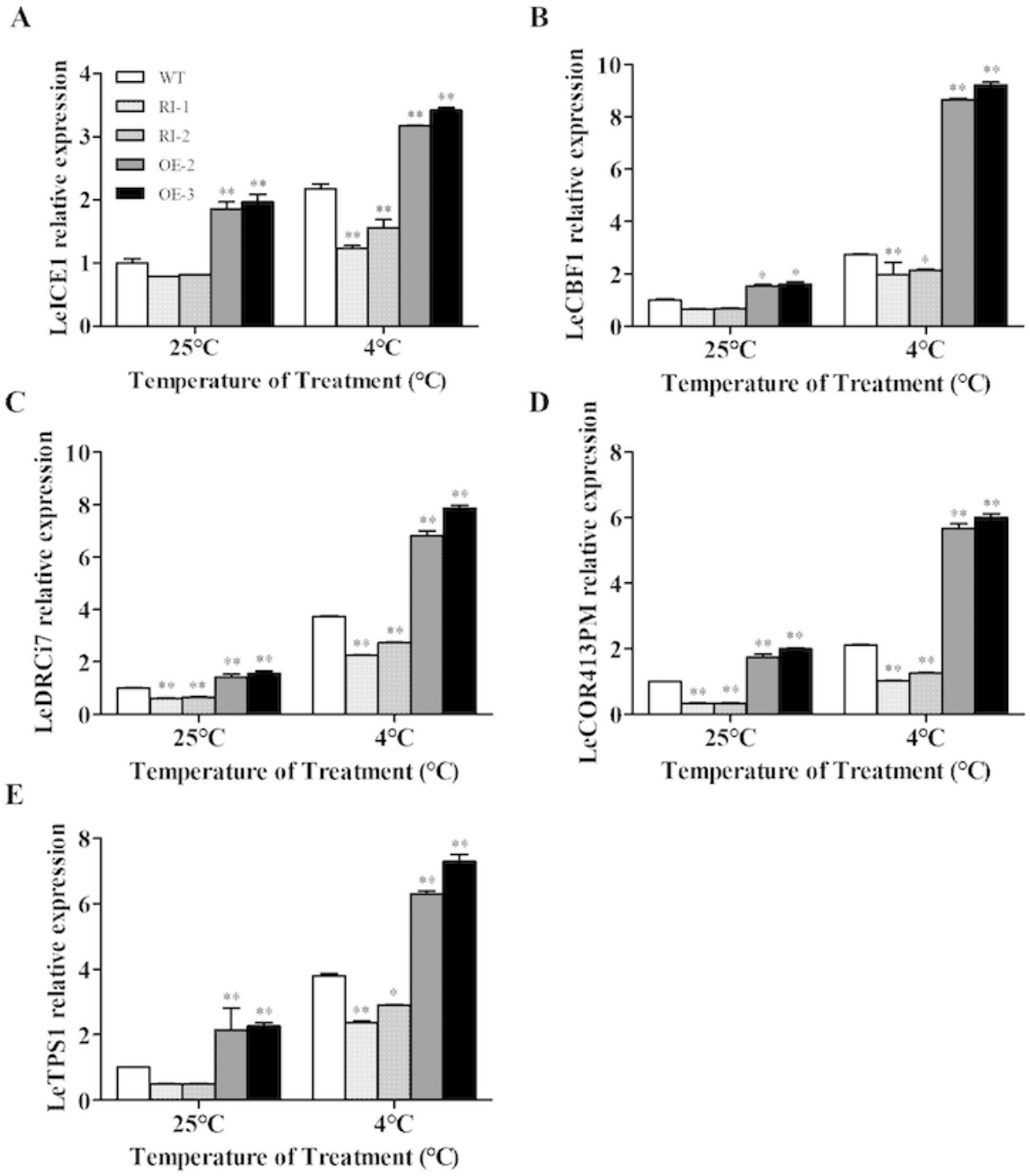

Figure 12

Relative expression of stress-related genes in WT and transgenic tomato plants exposed to cold stress. a Expression level of LeICE1. b Expression level of LeCBF1. c Expression level of LeDRCi7. d Expression level of LeCOR413PM. e Expression level of LeTPS1. Total RNA was extracted from untreated and 4◦C-

treated 6-week-old WT and transgenic tomato plants. The expression levels of stress-related genes were analyzed by qRT-PCR. Data are means of three replicate samples. Bars represent SDs. *P < 0.05 and **P < 0.01.

Supplementary Files

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- SupplementaryMaterial.docx