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Exogenous strigolactones alleviate low-temperature stress in peppers seedlings by reducing the degree of photoinhibition

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Abstract

Background The growth and yield of pepper, a typical temperature-loving vegetable, are limited by low-temperature environments. Using low-temperature sensitive 'Hangjiao No. 4' (*Capsicum annuum* L.) as experimental material, this study analyzed the changes in plant growth and photosynthesis under different treatments: normal control (NT), low-temperature stress alone (LT), low-temperature stress in strigolactone pretreated plants (SL_LT), and low-temperature stress in strigolactone biosynthesis inhibitor pretreated plants (Tis_LT).

Results SL pretreatment increased the net photosynthetic rate (Pn) and PSII actual photochemical efficiency (φPSII), reducing the inhibition of LT on the growth of pepper by 17.44% (dry weight of shoot). Due to promoting the accumulation of carotenoids, such as lutein, and the de-epoxidation of the xanthophyll cycle [(Z + A)/(Z + A + V)] by strigolactone after long-term low-temperature stress (120 h), non-photochemical quenching (NPQ) of pepper was increased to reduce the excess excitation energy [(1-qP)/NPQ] and the photoinhibition degree (Fv/Fm) of pepper seedlings under long-term low-temperature stress was alleviated. Twelve cDNA libraries were constructed from pepper leaves by transcriptome sequencing. There were 8776 differentially expressed genes (DEGs), including 4473 (51.0%) upregulated and 4303 (49.0%) downregulated genes. Gene ontology pathway annotation showed that based on LT, the DEGs of SL_LT and Tis_LT were significantly enriched in the cellular component, which is mainly related to the photosystem and thylakoids. Further analysis of the porphyrin and chlorophyll biosynthesis, carotenoid biosynthesis, photosynthesis-antenna protein, and photosynthetic metabolic pathways and the Calvin cycle under low-temperature stress highlighted 18, 15, 21, 29, and 31 DEGs for further study, which were almost all highly expressed under SL_LT treatment and moderately expressed under LT treatment, whereas Tis_LT showed low expression.

Conclusion The positive regulatory effect of SLs on the low-temperature tolerance of pepper seedlings was confirmed. This study provided new insights for the development of temperature-tolerant pepper lines through breeding programs.

Keywords Pepper, Chilling stress, Strigolactones, Photosystem, Xanthophyll cycle, RNA-seq

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Background

Chilling stress is an abiotic stress that is detrimental to plant growth and agricultural productivity [1]. It limits the geographical distribution of plant species and inhibits its plant growth and development, leading to reductions in crop yield. Many crops, including rice (*Oryza sativa*), corn (*Zea mays*), soybeans (*Glycine max*), and tomatoes (*Solanum lycopersicum*), cannot adapt to low temperatures and their growth is largely limited to tropical and subtropical regions. The fruits of pepper (*Capsicum annuum* L.), of the family Solanaceae, are rich in minerals (calcium) and vitamins that are beneficial to human health. The accumulation of carotenoids, capsaicinoids, and flavonoids determines the value of peppers as natural colorants and medical products. Currently, pepper is the second most consumed vegetable worldwide, behind only tomato, and its market demand is increasing. Accordingly, the scale of cultivation of pepper in China is expanding. However, even in a solar greenhouse, the temperature in northern China is often below 10℃ during the late night to early morning during winter [2], seriously endangering the normal growth and development of peppers [3, 4]. In addition, the low-temperature environment during the flowering and fruiting periods causes serious falls in flowers and fruits in peppers, and growers often face the risk of reduced production and income damage. Therefore, exploring methods to improve the low-temperature tolerance of pepper is of great significance for improving the quality of pepper cultivation.

Strigolactones (SLs) are carotenoid-derived plant hormones named for their ability to induce seed germination of parasitic plants in Striga roots [5]. They have subsequently been identified as signaling molecules between plants and other organisms (fungi or bacteria) that regulate plant growth and development [6–8]. SLs play an important role in abiotic stress in plants. Mutants lacking SL synthesis or response are sensitive to salt and drought stress [9, 10]. Photosynthesis is an important physiological process for energy conversion in plants and is highly sensitive to low-temperature environments [11]. Low temperatures disrupt almost all the major processes of photosynthesis (electron transfer, Calvin cycle, and stomatal conductance) [12]. SLs can effectively improve the photosynthetic rate and enhance the photosynthetic capacity of various crops, such as rice [13], grapes [14], and tomatoes [15] under abiotic stress conditions; however, their specific protective mechanisms have rarely been reported. The role of SLs in temperature-induced stress has received widespread attention. Cooper et al. [16] found that low-temperature treatment led to a significant decrease in photosynthesis in pea SL synthesis and signal transduction mutants; similar phenomena were observed in SL-deficient mutant plants of *Arabidopsis* also. These results indicate that a lack of SLs induces cold sensitivity in peas and *Arabidopsis* and that SLs may have a positive regulatory effect on plant cold tolerance. Research based on RNA-Seq under low-temperature stress is beneficial for exploring plant cold-resistance genes, analyzing the regulatory mechanisms of low- temperature responses, and selecting cold-resistant varieties [17–20]. However, there have been almost no reports on the growth, photosynthetic physiology, and internal transcriptional changes of peppers under low-temperature conditions.

Therefore, using low-temperature sensitive pepper variety 'Hangjiao NO.4' as the experimental material, artificially synthesized analog (*rac*-GR24) and biosynthetic inhibitor (Tis108) of SLs were sprayed on the pepper leaves to explore the effect of exogenous SLs on the resistance of pepper seedlings to low-temperature stress. We hope to alleviate the adverse effects of low-temperature stress through a simple and environmentally friendly method and to elucidate the relevant mechanism by which SLs improve the cold resistance of pepper.

Methods

Materials

The low-temperature sensitive pepper (*Capsicum annuum* L.) variety 'Hangjiao NO.4' was used as the experimental material. The seeds obtained from Tianshui Shenzhou Lvpeng Agricultural Technology Company (Tianshui, China) were immersed in water at 55℃, stirred at a constant speed for 15 min, and then soaked in water at 25℃ for 8 h. Next, the seeds were placed on moist gauze and kept in an RDN-type artificial climate box to germinate under dark conditions at 28℃. After approximately 5 d, seeds with an embryonic root length of approximately 3 mm were selected and sown in plastic nutrient bowls (9×9 cm) filled with a seedling substrate made of peat and vermiculite (volume ratio 3:1). The seedlings were grown under conditions of 25/17℃ and a 12 h/12 h (d/night) photoperiod, with a light intensity of 24,000 lx and relative humidity of 70%. The seedlings were irrigated three times a week, with one irrigation using 1/2 Hoagland nutrient solution.

Experimental design

When the sixth true leaf of the pepper seedlings began to develop (35–40 d after planting), seedlings with similar morphologies were selected and divided into four groups:

1. Pepper grown at normal temperature (NT; 25℃, 24000 lx).
2. Pepper subjected to low-temperature stress treatment (LT; 6℃, 5000 lx).
3. Pepper pretreated with artificially synthesized analogs of SLs and low-temperature stress (SL_LT; 20 μmol *rac*-GR24, 6℃, 5000 lx).

4. Pepper pretreated with biosynthetic inhibitor of SLs and low-temperature stress (Tis_LT; 30 μmol Tis108, 6℃, 5000 lx).

NT and LT groups were foliar-sprayed with ultrapure water, whereas SL_LT and Tis_LT were foliar-sprayed with corresponding concentrations of exogenous substances. Each group was sprayed continuously for 7 d to ensure that both sides of the leaves were completely wetted each time. On the morning of 8 d, LT, SL_LT, and Tis_LT were placed in an RDN-type artificial climate box for low-temperature stress treatment. NT continued to grow in an artificial climate box under conditions of 25℃ and 24,000 lx. Samples were collected from all four treatment groups at different time points after exposure to low-temperature stress to measure the corresponding indicators.

Methods

Determination of biomass and photosynthetic pigment content in pepper plants

After 120 h of low-temperature treatment, the pepper plants were divided into shoots and roots, and their fresh weights were measured. The plants were then dried in a constant temperature oven at 115℃ for 30 min, followed by further drying at 70℃ to a constant weight (approximately 5 d). Dry weight was measured using an analytical balance.

Chlorophyll and carotenoids in the pepper leaves were extracted with 80% acetone and the absorbance values of the extraction solution at 665, 649, and 470 nm were measured using a UV-1780 spectrophotometer (Shimadzu, Japan) [21].

Determination of net photosynthetic rate and Calvin cycle enzyme activity

Functional leaves were selected after 0, 24, 72, and 120 h of low-temperature stress. The net photosynthetic rate (Pn) of the pepper leaves was measured using a photosynthetic apparatus (CIRAS-2, UK).

The activities of 1,5-ribulose diphosphate carboxylase (Rubisco), fructose-1, 6-bisphosphatase (FBPase), fructose-1,6-bisphosphate aldolase (FBA), 3-glyceraldehyde phosphate dehydrogenase (GAPDH), and transketolase (TK) were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Yanji Biotechnology Co., Ltd., Shanghai), according to the manufacturer's instructions.

Determination of chlorophyll fluorescence parameters

The chlorophyll fluorescence parameters of pepper leaves under low-temperature stress were measured using an Imaging-PAM Chlorophyll Fluorometer (Walz, Affectrich, Germany). The intensities of the modulated measuring light, photochemical light, and saturated pulse light were set to 0.1, 111, and 2700 μmol m^{-2} s^{-1}, respectively. The pulse light saturation time was set to 0.8 s and the time interval was set to 20 s. The relative electron transport rates (rETR) of the leaves were measured under different photosynthetically active radiation (PAR) intensities and a light response curve was obtained.

Lutein content and xanthophyll cycle analysis

Lutein (Lut), zeaxanthin (Zea), antheraxanthin (Ant), and violaxanthin (Vio) were extracted as described by Li et al. [22] and quantified using high-performance liquid chromatography (Waters 2695 Separation Module, USA).

The activities of zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE) were measured using ELISA kits (Yanji Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Determination of ATPase activity and ATP content in chloroplasts

Pepper leaf chloroplast suspensions were prepared according to the method described by Sun et al. [23]. Ca^{2+}-ATPase and Mg^{2+}-ATPase activities in chloroplast suspensions were measured as previously described [24].

The ATP content in the chloroplast suspension was determined using an ATP content determination kit (Suzhou Keming Biotechnology Co., Ltd., Suzhou, China) following the manufacturer's instructions.

Determination of endogenous SL content in pepper leaves

A 2.0 g sample of pepper leaves was ground in a mortar with liquid nitrogen. Next, 10 mL of pre-cooled ethyl acetate was added and the mixture was subjected to ultrasonic extraction for 30 min. After soaking overnight at 4℃, the extract was centrifuged at 8000 × *g* for 10 min. The supernatant was then dried using a rotary evaporator at 40℃. The residue was dissolved in 1 mL of an ethyl acetate and n-hexane mixture (15:85, v/v).

The dissolved substance was passed through a silica gel column activated with 3 mL of the same ethyl acetate and n-hexane mixture and then rinsed with this solution. The eluent was concentrated to dryness, redissolved in 0.2 mL of methanol, and filtered through a 0.22 μm pore size Millipore membrane.

The analysis was performed using an Agilent 1100 HPLC system equipped with a Kromasil C18 reverse-phase column (250 mm × 4.6 mm, 5 μm). The SL standard (5-DS, Sigma, ≥ 99.9%) was accurately weighed, dissolved in methanol, and prepared as a standard solution.

Total RNA extraction, cDNA library construction, and sequencing

Total RNA was extracted from pepper leaves (NT_1, NT_2, NT_3, LT_1, LT_2, LT_3, SL_ LT_1, SL_ LT_2, SL_ LT_3, Tis_ LT_1, Tis_ LT_2, and Tis_ LT_3) after 24 h of

low-temperature stress using a total RNA extraction kit (TRIzol Reagent, Invitrogen, USA). The quality and concentration of RNA were evaluated using 1% agarose gel electrophoresis and a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The integrity of RNA was tested using an Agilent 2100 spectrophotometer (Agilent Technologies).

Poly-A mRNA was enriched using oligo (dT) magnetic beads and randomly broken into short fragments in an NBE fragmentation buffer containing bivalent cations. Using the above mRNA as a template, first-strand cDNA synthesis was performed using random oligodeoxynucleotide hexamer primers and reverse transcriptase, and second-strand cDNA was synthesized using DNA polymerase I and RNase H. cDNA fragments of 250–300 bp after adding end-repair and nucleotide A were chosen using AMPure XP beads. Libraries were sequenced on the Illumina HiSeq 4000 platform (Illumina, USA) at NovoGene (Beijing, China).

Raw data filtering and differentially expressed genes (DEGs) screening

The raw reads from mRNA-Seq were filtered by removing adapter sequences and low-quality sequences (Qphred ≤ 20). The sequencing error rate and GC content distribution were examined, and clean reads were obtained against the *C. annuum* reference genome (https://www.ncbi.nlm.nih.gov/). Based on the localization information of reads in the reference genome, we used the feature counts tool in the subread software to compare reads from multiple regions of the genome and calculated the expression values (FPKM) of all genes in each sample. Screen differentially expressed genes (DEGs) between pairwise treatment combinations using DESeq2 software, with relative change threshold criteria of $|\log2(\text{fold change})| > 1$, and Padj ≤ 0.05.

Functional annotations of DEGs

The Gene Ontology (GO, http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/) databases were used to annotate the DEG.

Quantitative real-time PCR validation of RNA-Seq data

Sixteen DEGs were randomly selected for qRT-PCR analysis. Relative gene expression was normalized by comparing it with NT expression and analyzed using the comparative $2^{-\Delta\Delta CT}$ Method [25]. The RNA used was the sample that remained after sequencing. The primer sequences are shown in Table S1.

Data analysis

Each experiment was conducted in triplicate, and the results are presented as mean ± standard deviation (SD). Statistical analyses were performed using SPSS software (version 22.0; SPSS Institute Ltd., USA), and differences between treatments were assessed using Duncan's test. Graphs were generated using Origin Pro software (version 9.0; OriginLab Corporation, USA).

Results

Effect of exogenous application of *rac*-GR24 and Tis108 on the growth and photosynthetic pigments of peppers under chilling stress

Exogenous application of *rac*-GR24 (SL_LT) and Tis108 (Tis_LT) had no observable effects on the phenotype of NT plant seedlings at 0 h (Fig. 1A). As the duration of low-temperature stress extended (6–12 h), pepper plants continued to wilt due to dehydration, with varying degrees of wilting observed in the order of Tis_LT > LT > SL_LT. After 24 h of low-temperature treatment, water loss in pepper plants treated with LT and SL_LT began to recover, with SL_LT showing a greater degree of recovery than LT.

After 120 h of low-temperature stress, LT significantly reduced both the dry and fresh weights of pepper shoots and roots (Fig. 1B and C). Application of *rac*-GR24 (SL_LT) mitigated this reduction, whereas Tis108 (Tis_LT) exacerbated it. There were significant differences between SL_LT and Tis_LT treatments. Compared with LT treatment, SL_LT significantly increased the dry weight of shoots by 17.44%.

Furthermore, low-temperature stress significantly decreased the contents of chlorophyll a (Fig. 1D), chlorophyll b (Fig. 1E), total chlorophyll (Fig. 1F), and carotenoids (Fig. 1G) in pepper leaves. Compared with LT treatment, SL_LT increased the levels of these photosynthetic pigments by 23.7%, 31.1%, 25.4%, and 16.5%, respectively, with significant differences in chlorophyll b and total chlorophyll levels. Conversely, Tis_LT treatment reduced the content of these pigments by 10.1%, 21.5%, 12.7%, and 20.2%, respectively, compared to LT-treated plants.

Effect of exogenous application of *rac* -GR24 and Tis108 on the net photosynthetic rate and Calvin cycle enzymes of pepper leaves under chilling stress

After low-temperature stress, the net photosynthetic rate (Pn) of the pepper seedlings continued to decrease with increasing duration of stress (Fig. 2A). However, *rac*-GR24 pretreatment effectively alleviated the degree of decline in Pn in pepper seedlings, whereas the opposite was true for Tis_LT treatment. In particular, after 120 h of stress, SL_LT significantly increased Pn by 145.2% compared to LT; conversely, Tis_LT decreased Pn by 49.2%.

The activities of Rubisco (Fig. 2B), GAPDH (Fig. 2C), FBA (Fig. 2D), and FBPase (Fig. 2E) were significantly

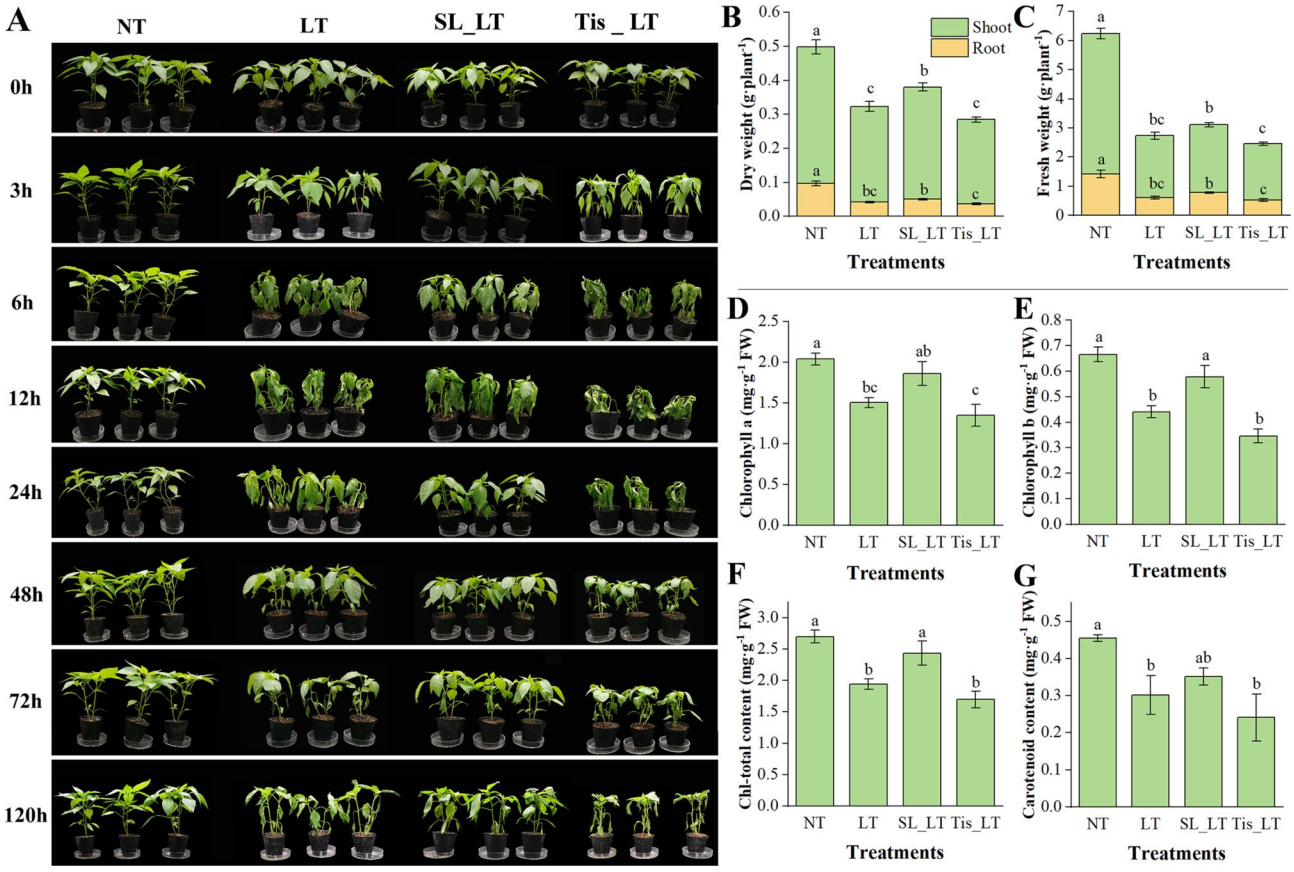

Fig. 1 Growth and photosynthesis pigments analysis of different treated pepper plants. (**A**) seedlings phenotype in different time points and (**B**) dry weight, (**C**) fresh weight, (**D**) chlorophyll a, (**E**) chlorophyll b, (**F**) total chlorophyll, (**G**) carotenoid content of seedlings after low-temperature stress 120 h. Data are means ± SD ($n = 5$), and different lowercase letters represent significant differences ($P \leq 0.05$) among treatments

lower in LT pepper seedlings than in NT pepper seedlings. The activities of Rubisco and FBPase under SL_LT were consistently higher than those under LT. The difference in Rubisco reached a significant level at 120 h and in FBPase at 24 and 72 h. Compared with LT treatment, GAPDH activity under SL_LT treatment significantly increased by 28.5%, 23.7%, and 12.5% at 24, 72, and 120 h, respectively. Although pretreatments with *rac*-GR24 and Tis108 increased and decreased the activity of FBA during low-temperature stress, respectively, the differences did not reach a significant level. In addition, low-temperature stress reduced the activity of TK in pepper seedlings (Fig. 2F); however, over time, its activity gradually increased and became significantly higher than that under NT. Compared to LT treatment, the exogenous application of *rac*-GR24 significantly increased the TK activity of plants during low-temperature stress.

Effect of exogenous application of *rac*-GR24 and Tis108 on fluorescence parameters of pepper leaves under chilling stress

At 48 h after chilling stress, the effect of SLs on PSII photoinhibition was studied (Fig. 3). The pepper leaves showed a significant decrease of Fv/Fm (Fig. 3A, B), ϕPSII (Fig. 3C), qP (Fig. 3E) and ETR (Fig. 3G) and the application of *rac*-GR24 effectively slowed down the decrease of above parameters, while the application of Tis108 further exacerbated the decrease of Fv/Fm at 24 h and ϕPSII, ETR at 48 h.

In addition, the non-photochemical quenching (NPQ) (Fig. 3D) of pepper leaves rapidly increased in the early stage of chilling stress (0–24 h) and then rapidly decreased in the later stage of stress (72–120 h). Compared with the single low-temperature treatment, *rac*-GR24 pretreatment significantly reduced NPQ in the early stage of chilling stress, and there was no significant difference in NPQ between Tis_LT and LT treatments. The excess excitation energy [(1-qP)/NPQ] (Fig. 3F) of the reaction center gradually increased with increasing low-temperature treatment time but the (1-qP)/NPQ under SL_LT was always lower than that under LT.

The response curve of the relative electron transfer rate (rETR) to photosynthetic effective radiation (PAR) is plotted (Fig. 3H-L). The analysis showed that the rETR of each treatment gradually increased with increasing PAR intensity and tended to stabilize upon reaching a light

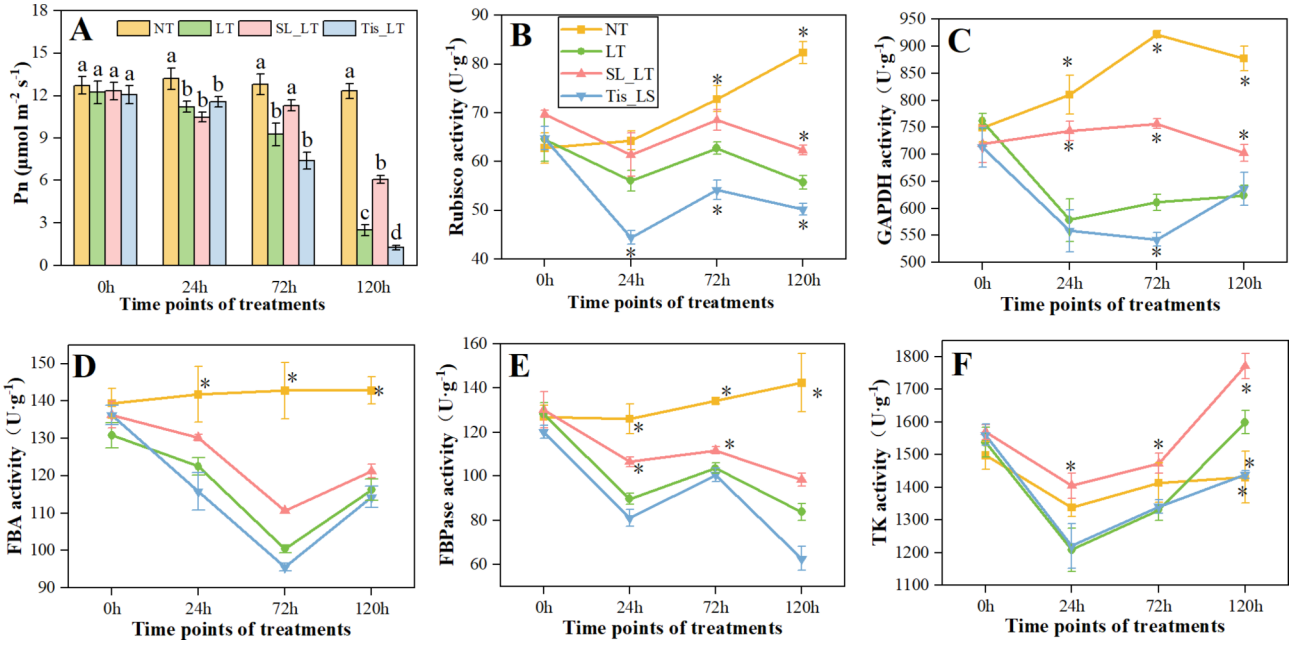

Fig. 2 Net photosynthetic rate and Calvin cycle enzymes analysis of different treated pepper plants. (**A**) Net photosynthetic rate, Pn. (**B**) Ketosaccharide-1, 5-diphosphate carboxylase, Rubisco. (**C**) glyceraldehyde-3-phosphate dehydrogenase, GAPDH. (**D**) Fructose-1, 6-diphosphate aldolase, FBA. (**E**) Fructose-1,6-bisphosphatase, FBPase. (**F**) Transketolase, TK. Data are mean ± SD ($n = 5$), and different lowercase letters indicate significant differences among treatments ($P \leq 0.05$). * indicate significant differences at $P \leq 0.05$ relative to LT pepper plants

saturation state. After chilling stress, the increase in rETR was inhibited and the longer the duration of stress, the greater the degree of inhibition. However, the response curve under SL_LT was consistently higher than those under LT and Tis_LT, and the differentiation trend was most obvious at 24 h.

Further fitting was performed on the response curve of low-temperature stress at 24 h (Table 1). Applying *rac*-GR24 increased the maximum electron transfer rate (rETRmax), light energy utilization efficiency (α), and ability to resist strong light (Ik) of plants under low-temperature conditions and increased α by 16.3%, a significant increase. Application of Tis108 reduced the rETRmax and Ik of plants under low-temperature conditions, with a 44.0% difference in Ik. These results indicate that SLs can improve the light energy utilization efficiency of chili seedlings under low-temperature stress, whereas Tis108 reduces the ability of chili seedlings to resist strong light.

Effect of exogenous application of *rac*-GR24 and Tis108 on xanthophyll cycle in pepper seedlings under chilling stress

The lutein [26], zeaxanthin [27, 28], and xanthophyll cycle [29] are beneficial for protecting the stability of PSII. For plants treated with LT and Tis_LT, the content of lutein (Fig. 4A) and zeaxanthin (Fig. 4B) showed a trend of first increasing and then decreasing with the persistence of low-temperature treatment. SL_LT treatment resulted in a sustained increase in lutein levels during stress, which was significantly lower and higher than that under LT at 72 and 120 h, respectively. The zeaxanthin content showed a fluctuating trend, with a significant increase of 0.8 and 1.7 times compared to LT-treated plants at 12 and 120 h, respectively, but a significant decrease at 24 and 72 h.

The antheraxanthin (Fig. 4C) and violaxanthin (Fig. 4D) contents under LT and Tis_LT showed a trend of first decreasing, increasing, and then decreasing with the extension of the low temperature. The antheraxanthin content under SL_LT continued to decrease during the stress period but remained higher than that under LT. Except at 72 h, the violaxanthin content under SL_LT was significantly higher than that under LT.

In addition, LT significantly increased the de-epoxidation degree of the xanthophyll cycle [(Z+A)/(Z+A+V)] in pepper leaves compared to NT and there was no significant difference between Tis_LT and LT treatments (Fig. 4E). In the early stage of stress (12–72 h), the (Z+A)/(Z+A+V) under LT was significantly higher than that under SL_LT, especially at 24 h, with 9.0-fold difference; in the late stage of stress (120 h), the (Z+A)/(Z+A+V) under SL_LT was 1.9 and 2.0 times higher than those under LT and Tis_LT, respectively.

Enzyme activity in the xanthophyll cycle revealed that low temperatures significantly reduced the activity of ZEP, but its activity gradually increased with prolonged stress time and returned to normal levels after 120 h (Fig. 4F). Compared to LT, Tis108 pretreatment had no

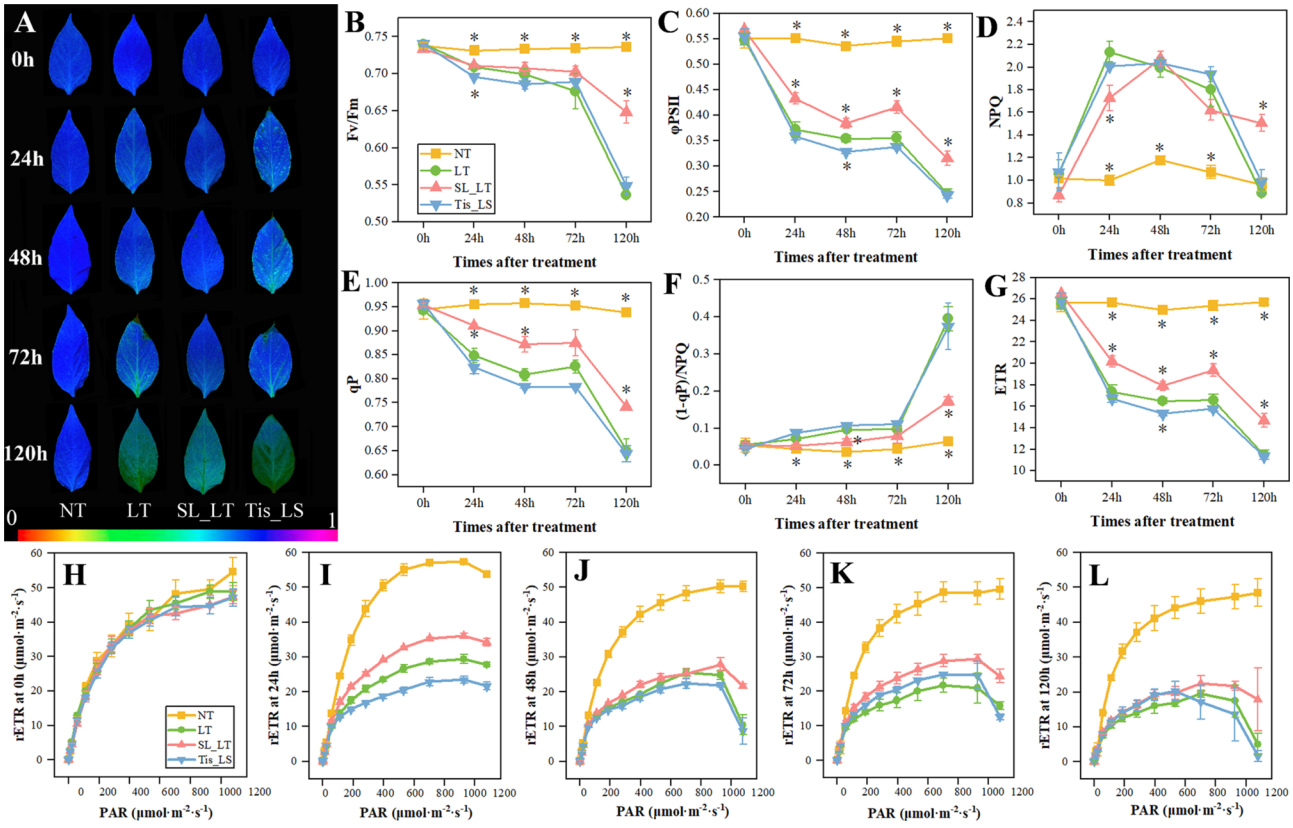

Fig. 3 Chlorophyll fluorescence parameters and light response curve of different treated pepper plants. (**A**) Images of Fv/Fm. False colors represent values of the parameter ranging from 0 (black) to 1.0 (purple). (**B**) Maximum photochemical efficiencies of PSΠ, Fv/Fm. (**C**) Actual photochemical efficiency of PSII, φPSII. (**D**) Nonphotochemical quenching, NPQ. (**E**) Photochemical quenching, qP. (**F**) Excess excitation energy, (1-qP)/NPQ. (**G**) Photosynthetic electron transport rate, ETR. (**H**-**L**) Light response curves of pepper leaves under chilling stress for 0, 24, 48, 72, and 120 h. Data are mean ± SD ($n = 3$). * indicate significant differences at $P \leq 0.05$ relative to LT pepper plants

Table 1 Fitting parameters of light-curves of pepper leaves under chilling stress for 24 h

Treatment	rETRmax	α	Ik
NT	74.77 ± 7.065 a	0.27 ± 0.010 a	275.31 ± 19.865 a
LT	24.05 ± 2.041 bc	0.18 ± 0.008 c	135.06 ± 7.616 b
SL_LT	30.34 ± 1.318 b	0.21 ± 0.009 b	147.34 ± 9.794 b
Tis_LT	16.70 ± 0.779 c	0.22 ± 0.002 b	75.70 ± 2.924 c

Note Data are mean ± SD ($n = 3$). Different lowercase letters represent significant differences ($P \leq 0.05$) among treatments, according to Duncan's multiple comparison tests

significant effect on ZEP and VDE activities. Pretreatment with *rac*-GR24 significantly increased ZEP activity (at 0 h), promoting the conversion of zeaxanthin to violaxanthin. During the low-temperature treatment period, SL_LT accelerated and increased the speed and degree of ZEP activity reduction and recovery. LT caused VDE activity to show a trend of first increasing and then decreasing with the prolongation of stress time (Fig. 4G). VDE activity under SL_LT continued to increase with prolonged stress time.

Effect of exogenous application of *rac*-GR24 and Tis108 on ATPase activity and ATP content in chloroplasts of pepper seedlings under chilling stress

Under LT, the contents of ATP (Fig. 5A), Ca^{2+}- ATPase (Fig. 5B), and Mg^{2+}- ATPase (Fig. 5C) in the chloroplasts were significantly lower than those under NT and the longer the stress time, the greater the degree of decrease. *Rac*-GR24 pretreatment (SL_LT) effectively inhibited the decrease in ATPase activity and ATP content in chloroplasts: compared with LT, the Ca^{2+}-ATPase activity under SL_LT was significantly increased by 35.6% and 68.7% at 6 and 12 h of stress, respectively. Mg^{2+}- ATPase activity and ATP content in the chloroplasts of plants under SL_LT significantly increased by 57.5% and 94.5%, respectively, compared to those of LT-treated plants at 12 h of stress. Tis_LT treatment further exacerbated the decrease in ATPase activity and ATP content in chloroplasts under low temperatures, and the Mg^{2+}-ATPase activity under Tis_LT was significantly reduced by 26.4% after 6 h of stress. These results indicate that chloroplast energy production in pepper was maintained by SL pretreatment under chilling stress.

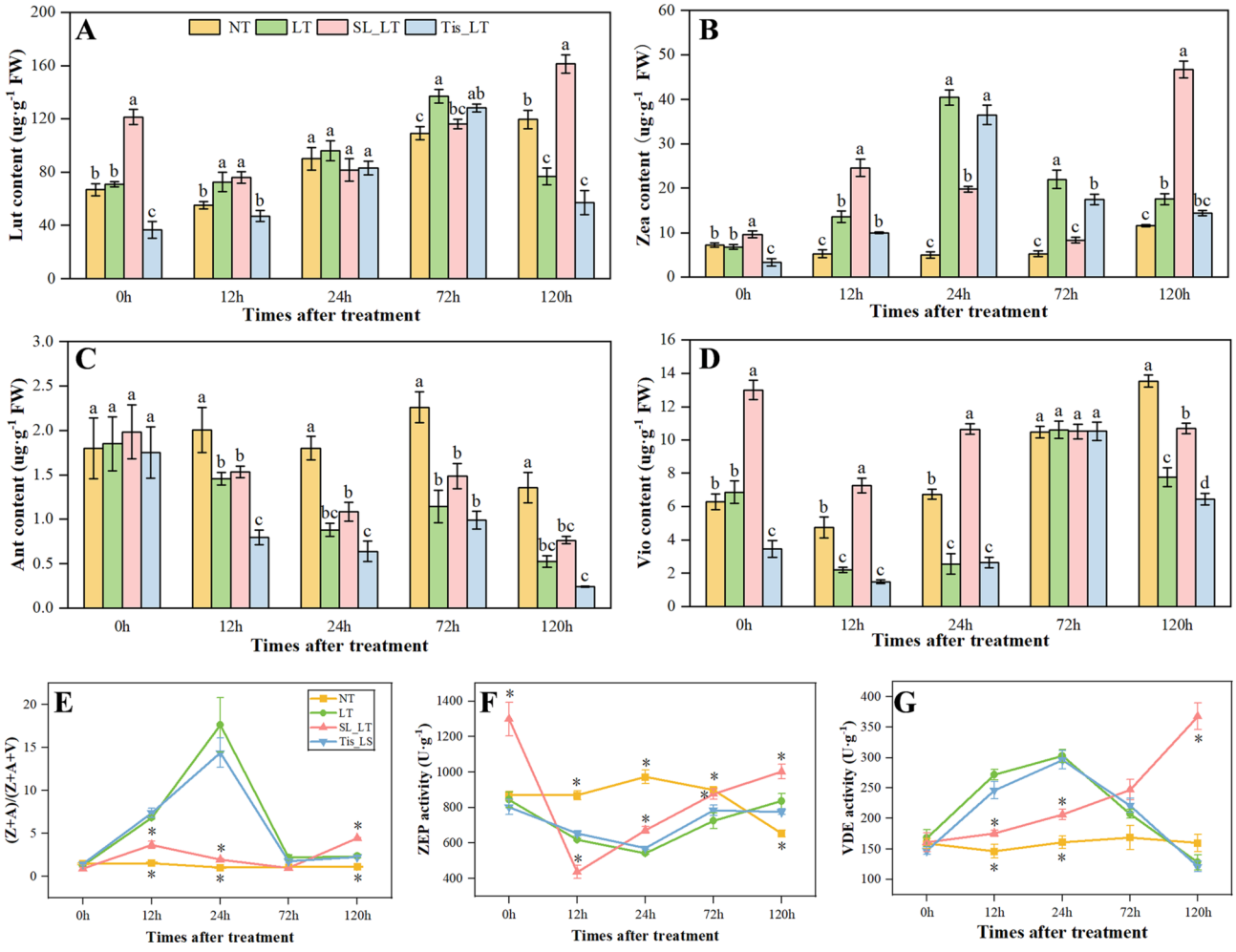

Fig. 4 Photoprotective carotenoid content and the activity of xanthophyll cycle enzymes of different treated pepper plants. (**A**) lutein content, (**B**) zeaxanthin content, (**C**) antheraxanthin content, (**D**) vioxanthin content, (**E**) de-epoxidation degree of xanthophyll cycle, (**F**) zeaxanthin epoxidase (ZEP), and (**G**) violaxanthin de-epoxidase (VDE). Data are mean ± SD ($n = 3$). Different lowercase letters indicate significant differences among treatments ($P \leq 0.05$). * indicate significant differences at $P \leq 0.05$ relative to LT pepper plants

Content of endogenous SL in pepper leaves

The endogenous 5-Deoxystrogol (5-DS) in each treatment group was measured (Fig. 5D). Compared with the plants without exogenous treatment (NT and LT), continuous application of *rac*-GR24 for 7 d significantly increased the content of endogenous 5-DS in pepper leaves (by 14.5 times), whereas the content of endogenous 5-DS in plants treated with Tis108 (Tis_LT) was significantly reduced by 75.4% and 76.9%, respectively, compared with NT and LT. After 24 h of low-temperature treatment, compared with NT plants, the content of 5-DS in pepper leaves treated with LT increased by 29.1%, the content of 5-DS under SL_LT significantly increased by 32.0 times, and that under Tis_LT decreased by 83.3%.

Transcriptome sequencing data and alignment analysis

cDNA libraries of pepper at normal temperature (three samples) and three treatments under chilling stress for 24 h (nine samples) were constructed using 40,275,892–45,159,956 original reads. After filtering, a total of 77.19 Gb of clean reads were obtained, with a single sample containing clean reads over 5.96 Gb. The average Q30 quality score for the transcriptomes was greater than 92%. After alignment with the reference genome sequence of *Capsicum annuum* L, the total matching rates of the localized reads were all above 93.38%, with unique matching efficiencies ranging from 88.02 to 90.21% (Table 2). These data indicate that transcriptome sequencing could be used for subsequent analyses.

DEGs analysis

Four treatments were paired to screen for DEGs (Fig. 5E). The results showed that there were 11,608 DEGs between

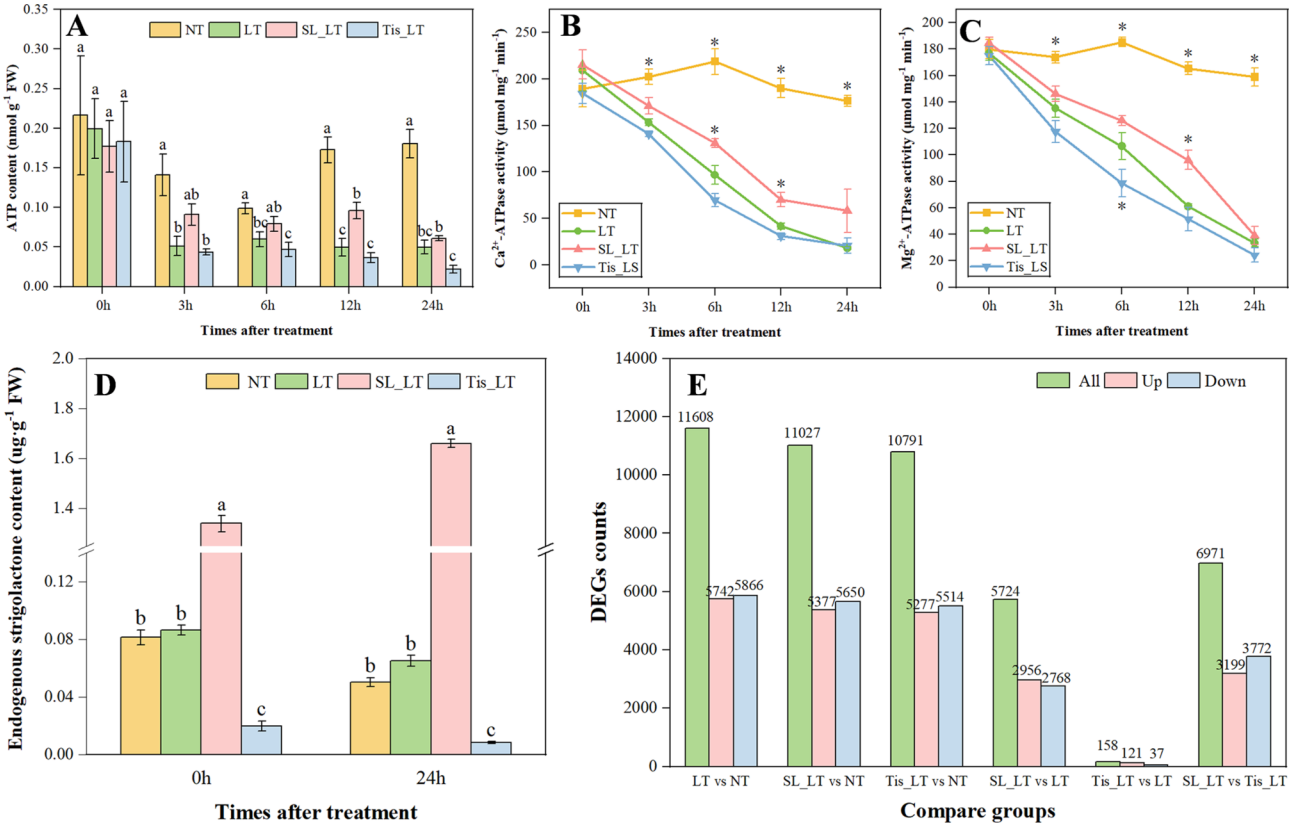

Fig. 5 ATP, endogenous strigolactone, and counts of differentially expressed genes. (**A**) ATP content, (**B**) Ca^{2+}-ATPase activity, (**C**) Mg^{2+}-ATPase activity, (**D**) endogenous strigolactone content, and (**E**) counts of differentially expressed genes under different treatments. Data are mean ± SD ($n = 3$). Different lowercase letters indicate significant differences among treatments ($P \leq 0.05$). * indicate significant differences at $P \leq 0.05$ relative to LT pepper plants

Table 2 Overview of sequencing data and mapping

Sample name	Raw reads	Clean reads	Q30 (%)	Total reads	Total mapped reads	Uniquely mapped reads
NT_1	40,275,892	39,708,954	92.75	39,708,954	94.93%	88.53%
NT_2	43,169,268	42,505,608	93.09	42,505,608	94.50%	89.58%
NT_3	47,621,016	46,714,904	92.98	46,714,904	93.38%	87.85%
LT_1	41,271,746	40,565,834	92.58	40,565,834	94.46%	88.71%
LT_2	43,812,484	42,998,488	92.59	42,998,488	94.35%	89.30%
LT_3	43,773,628	42,811,902	92.46	42,811,902	94.59%	88.65%
SL_LT_1	43,821,724	43,103,844	93.24	43,103,844	94.12%	88.02%
SL_LT_2	45,159,956	44,506,368	93.03	44,506,368	94.36%	88.22%
SL_ LT_3	44,856,912	44,078,938	93.21	44,078,938	94.56%	88.56%
Tis_ LT_1	43,772,434	42,917,330	93.01	42,917,330	94.21%	90.21%
Tis_ LT_2	44,627,270	43,552,334	92.69	43,552,334	94.17%	89.01%
Tis_ LT_3	41,969,418	41,086,786	93.27	41,086,786	94.11%	90.14%

LT and NT, of which 5866 were upregulated and 5742 were downregulated. Application of *rac*-GR24 and Tis108 reduced the number of DEGs between LT and NT treatments. There were 5724 DEGs between SL_LT and LT treatments, of which 2956 were downregulated and 2768 were upregulated. Only 158 DEGs, including 37 upregulated and 121 downregulated genes, were identified between Tis_LT and LT treatment groups. Compared with Tis_LT treatment, SL_LT treatment resulted in 3772 upregulated and 3199 downregulated genes. Compared with the other two comparison combinations at low temperatures, SL_LT vs. Tis_LT had the highest number of DEGs and an increased ratio of upregulated to downregulated genes.

GO functional enrichment analysis of DEGs

GO functional enrichment analysis of DEGs revealed several significant findings. In the comparison between

SL_LT and LT treatments, DEGs were notably enriched in nine cellular component (CC) terms. These included photosystem II (GO:0009523), photosystem (GO:0009521), photosynthetic membrane (GO:0034357), and thylakoid (GO:0009579), all of which were up-regulated (Fig. 6A). Additionally, DEGs were enriched in 11 molecular function (MF) terms, including N-acetyltransferase activity (GO:0008080), calcium ion binding (GO:0005509), and UDP glucose transfer activity (GO:0046527).

In contrast, the DEGs in the Tis_LT vs. LT comparison group showed significant enrichment in 22 GO terms, including six CC and 16 MF terms. These terms predominantly exhibited downregulation or a higher proportion of downregulated genes than upregulated genes (Fig. 6B). This suggests that the application of SLs inhibitor may significantly impair cellular components in pepper plants, particularly those involved in photosynthesis, such as photosystem II, photosystem II, photosynthetic membrane, and thylakoid, along with related molecular functions, including endopeptidase inhibitor activity (GO:0004866), enzyme inhibitor activity (GO:0004857), and proton-transporting ATP synthase activity (GO:0046933).

Furthermore, the DEGs in the SL_LT vs. Tis_LT comparison group were enriched in biological process (BP) and MF terms, with 2 and 10 terms, respectively, with almost all DEGs upregulated (Fig. 6C). This comparison highlights that under chilling stress conditions, pepper plants rich in SLs exhibited enhanced biological processes, such as thylakoid (GO:0009579), photosynthesis (GO:0015979), and cellular amino acid metabolic processes (GO:0006520). Additionally, components critical for photosynthesis, such as the photosynthetic membrane (GO:0034357), photosystem (GO:0009521), and photosystem II oxygen-evolving complex (GO:0009654), were effectively protected compared to under Tis_LT.

KEGG pathway analysis of DEGs

The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment results for the DEGs between the low-temperature treatments are shown in Fig. 7. There were no significantly enriched KEGG pathways in the DEGs of SL_LT vs. LT, whereas plant hormone signal transduction (sly04075) and ascorbate and aldarate metabolism (sly00053) were more enriched than other metabolic pathways (Fig. 7A). DEGs were significantly enriched in the photosynthesis-antenna protein pathway (sly00196) under Tis_LT vs. LT. Compared with SL_LT vs. LT, SLs inhibitor (Tis) increased the enrichment of thiamine metabolism (sly00730), photosynthesis (sly00195), nitrogen metabolism (sly00910), glyoxylate, and dicarboxylic acid metabolism pathways (sly00630) under chilling stress (Fig. 7B). In addition, DEGs under SL_LT vs. Tis_LT were significantly enriched in 7 metabolic pathways, including photosynthesis antenna protein (sly00196), photosynthesis (sly00195), carbon fixation in photosynthetic organisms (sly00710), glyoxylate and dicarboxylic acid metabolism (sly00630), porphyrin and chlorophyll metabolism (sly00860), carbon metabolism (sly01200), and nitrogen metabolism (sly00910) (Fig. 7C).

DEGs in porphyrin and chlorophyll metabolism pathway

Further analysis was conducted on the DEGs of the porphyrin and chlorophyll metabolic pathways under chilling stress (Fig. 8A). Glutamyl-tRNA reductase (EC: 1.2.1.70) was the first limiting enzyme in chlorophyll synthesis. Subsequently, glutamate-1-semialdehyde, the reduced product of L-glutamyl tRNA, was converted into an important precursor for chlorophyll synthesis (5-aminolevulinate), through the action of Glutamate-1-semialdehyde 2,1-aminomutase (EC: 5.4.3.8). After a series of enzymatic reactions, 5-aminolevulinate was converted into protoporphyrin IX. *Rac*-GR24 and Tis108 pretreatment upregulated and downregulated the expression of genes encoding these enzymes, respectively, under chilling stress (Gene ID: 107840125, 107867325, 107863035. etc.). Subsequently, Protoporphyrin IX Mg-chelatase subunit D (EC: 6.6.1.1) catalyzed the binding of magnesium ions to protoporphyrin IX, which was upregulated or downregulated (including 107870073, 107870054, and 107845383) by *rac*-GR24 and Tis 108, respectively, in pepper leaves. Subsequently, the chelating product, Mg-protoporphyrin IX, was sequentially catalyzed by Mg-protoporphyrin O-methyltransferase (EC 2.1.1.11), Mg protoporphyrin IX monomethyl ester oxidative cyclase (EC: 1.14.13.81), and divinyl protochlorophyllide 8-vinyl-reductase (EC: 1.3.1.75) to generate the direct precursor of chlorophyll. The application of *rac*-GR24 and Tis108 also upregulated and downregulated the expression of 107,862,118, 107,844,048, and 107,842,746, respectively, which encode the above three enzymes, at low temperatures. In addition, the expressions of a gene (107842693) encoding chlorophyll a synthase ChlG (EC: 2.5.1.62) and two genes (107868082, 107872994) encoding chlorophyllide a oxygenase (EC: 1.14.13.122) were upregulated by *rac*-GR24 and downregulated by Tis108, respectively. Although the expression of 107,872,996 (chlorophyllide a oxygenase) under SL_LT was not upregulated compared to that under LT, it was significantly downregulated under Tis_LT.

DEGs in the carotenoid biosynthesis pathway

A total of 15 DEGs were annotated in the carotenoid biosynthesis pathway (Fig. 8B). Among these, 107,859,651 encodes phytoene synthase (EC: 2.5.1.32), which catalyzes the formation of the first carotenoid phytoene. Lycopene generates ε-carotene under the action of lycopene epsilon cyclase (CrtL e, EC: 5.5.1.18). Lycopene

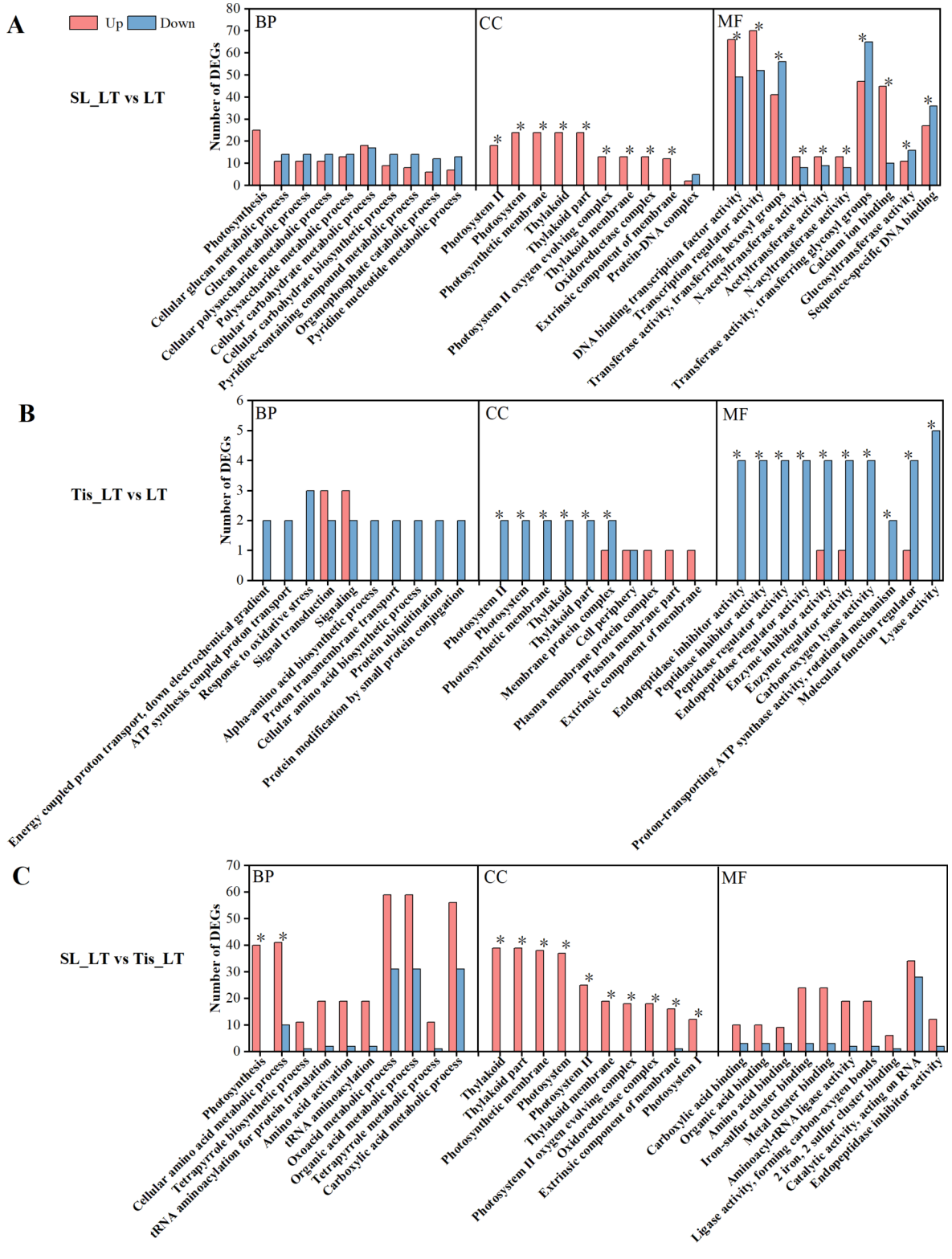

Fig. 6 GO enrichment analysis of DEGs among different treatment combinations under chilling stress

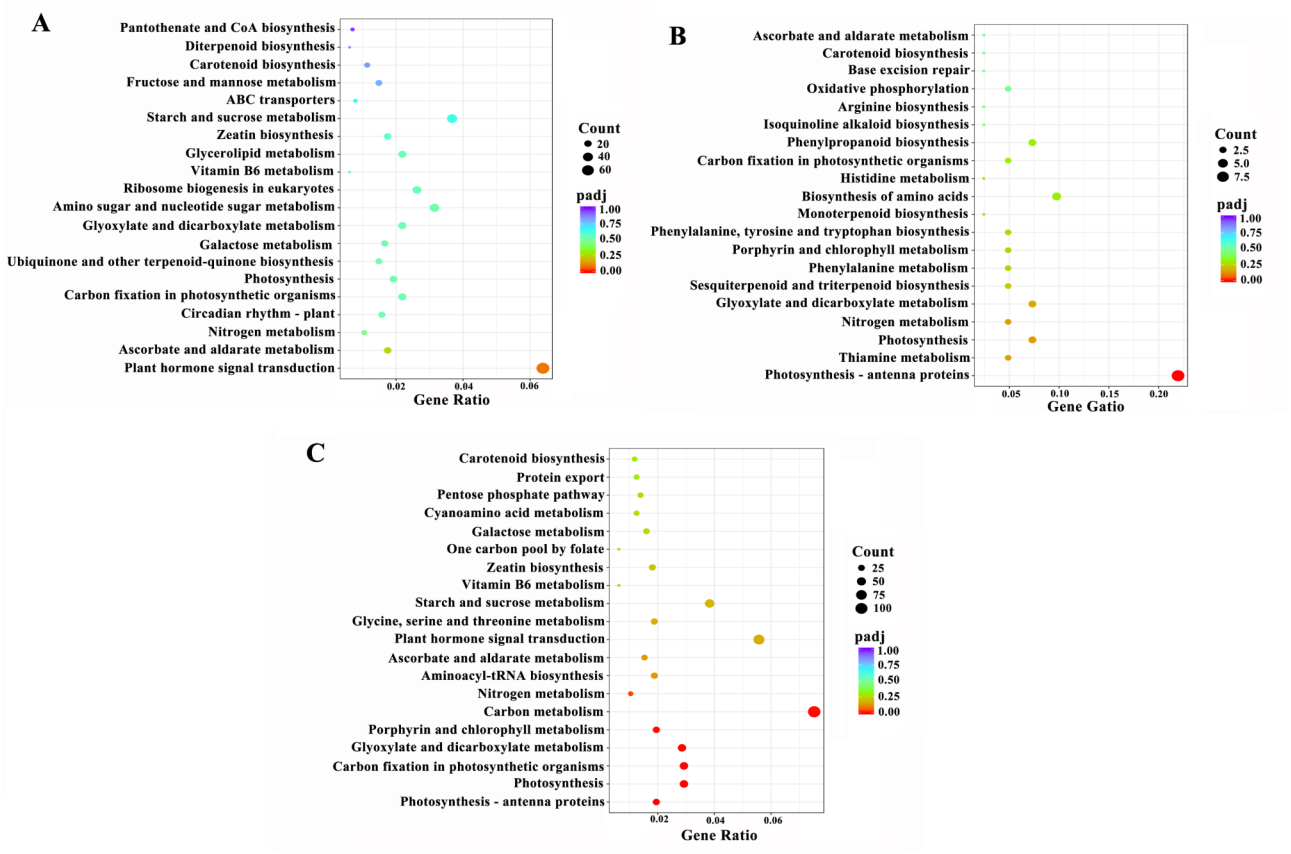

Fig. 7 KEGG enrichment analysis of DEGs among different treatment combinations under chilling stress. (**A**) SL_LT vs. LT, (**B**) Tis _LT vs. LT, and (**C**) SL_LT vs. Tis_LT

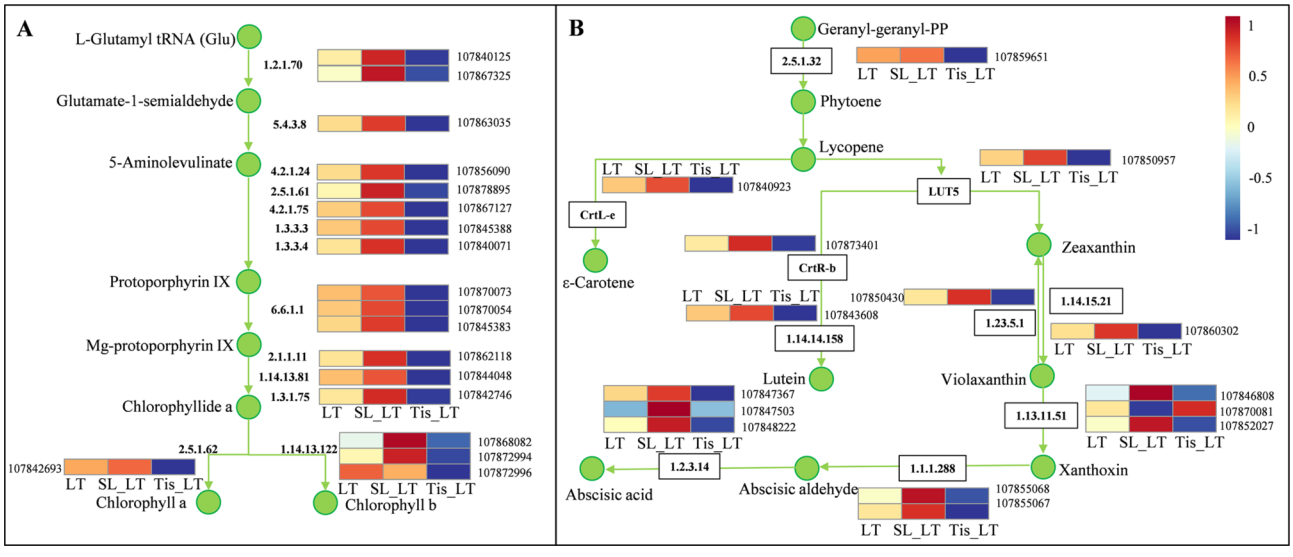

Fig. 8 Analysis of DGEs in (**A**) porphyrin and chlorophyll metabolism and (**B**) carotenoid biosynthesis of pepper. Colors indicate the expression values of the genes, which are presented as FPKM-normalized $\log_2$ transformed counts

also generates zeaxanthin and lutein through the action of beta-ring hydroxylase (LUT5) and beta-carotene hydroxylase (CrtR-b, EC: 1.14.15.24). The expression levels of the DEGs encoding the above enzymes in different treatments under low-temperature stress were SL_LT > LT > Tis_LT.

In addition, the application of *rac*-GR24 significantly upregulated the transcriptional expression of 107,860,302

(encoding ZEP) and 107,850,430 (encoding VDE) in low-temperature environments, whereas the application of Tis108 significantly downregulated the transcriptional expression of these two genes. Subsequently, abscisic acid (ABA) was formed through the catalysis of 9-cis-epoxycarotenoid dioxygenase (NCED, EC: 1.13.11.51), xanthine dehydrogenase reductase (EC: 1.1.1.288), and ABA aldehyde oxidase (EC: 1.2.3.14). During this process, seven DEGs were highly expressed under SL_LT conditions and low under Tis_LT conditions, whereas the expression of the 107,870,081 gene was the opposite.

DEGs in photosynthetic metabolic pathways

There were 21 DEGs in the photosynthesis antenna proteins under LT, SL_LT, and Tis_LT (Fig. 9A). Among these, 17 genes were annotated as light-harvesting chlorophyll complexes of PSII (LHCII), including 12 encoding light-harvesting complex II chlorophyll a/b binding protein 1 (Lhcb1), two encoding Lhcb2, two encoding Lhcb3, and one encoding Lhcb6. The remaining four DEGs were annotated in the light-harvesting chlorophyll complexes of PSI (LHCI): 107,872,234 (encoding chlorophyll a-b binding protein 6 A, Lhca1), 107,838,850 (encoding Lhca2), and 107,867,983 and 107,862,252 (encoding Lhca4). Compared to LT treatment, the expression of all the genes mentioned above was upregulated under SL_LT and downregulated under Tis_LT.

Further analysis revealed that 29 DEGs were involved in the photosynthetic pathway (Fig. 9A). Among them, 13 DEGs were annotated as PSII (including 107860359, 107858690, and 107843581) and eight were annotated as Photosystem I (including 107875316, 107866881, and 107846125). SL_LT and Tis_LT upregulated and downregulated the expression of these genes, respectively.

Seven DEGs encoded Ferredoxin (Fd) and were annotated for photosynthetic electron transfer. Expression of these genes at low temperatures was upregulated by *rac*-GR24 and downregulated by Tis108. This may be because SLs can activate the expression of genes encoding the PSI-reducing side electron acceptor ferredoxin under low-temperature stress, thus improving the photosynthetic electron transfer rate of plants. In addition, one DEG (107851209) encodes the ATP synthase delta chain in the chloroplast thylakoid membrane. Compared to LT, Tis_LT significantly downregulated the expression of this gene.

DEGs in the Calvin cycle

A total of 31 DEGs in the Calvin cycle were identified in pepper seedlings under chilling stress (Fig. 9B). Among these, seven DEGs coded genes for ribose 1,5-diphosphate carboxylase (Rubisco, EC: 4.1.1.39), including 107,860,049, 107,860,048, and 107,860,051, used to complete CO_2 fixation. Compared with LT, *rac*-GR24 pretreatment upregulated the expression of these genes, while Tis108 pretreatment downregulated them.

The reduction of CO_2 is the second most important stage of the Calvin cycle, during which energy is stored. DEGs encoding phosphoglycerate kinase (107878881 and 107878882), glyceraldehyde-3-phosphate dehydrogenase (107878927), and NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (107866774, 107870368, 107851146, and 107877699) were identified.

In addition, the remaining 17 DEGs were annotated into three branches of the RuBP update stage. Among them, 107,862,261, which was annotated as Ribulose-phosphate 3-epimerase (EC 5.1.3.1), was highly expressed under SL_LT. The expression of 107,869,777, annotated

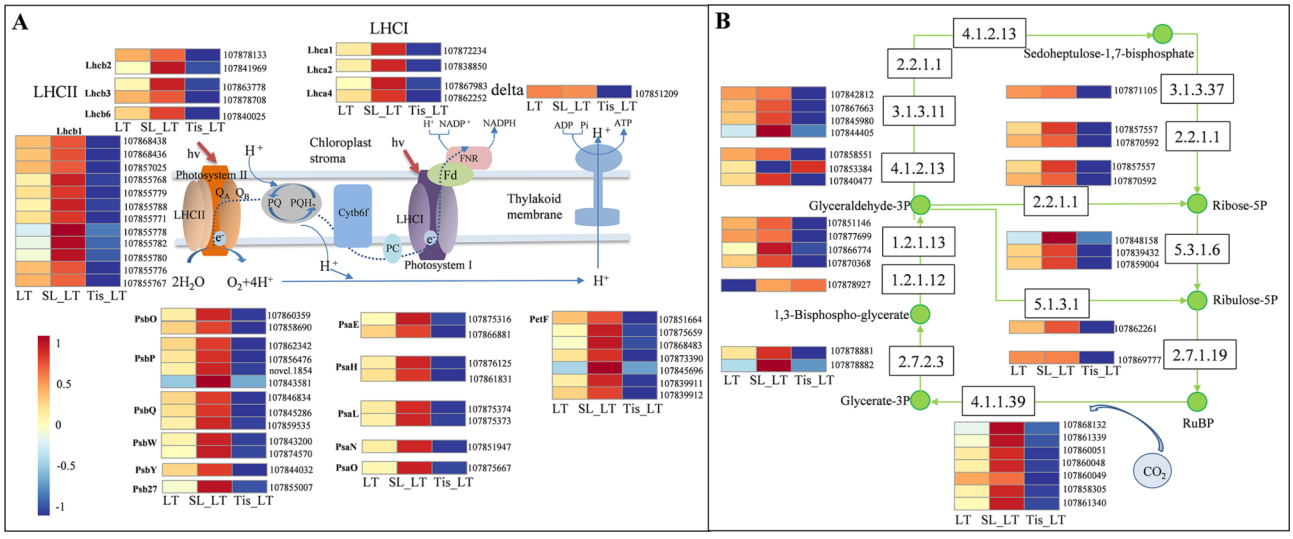

Fig. 9 Analysis of DGEs in (**A**) photosynthesis - antenna proteins, photosynthesis pathways and (**B**) Calvin cycle of pepper. Colors indicate the expression values of the genes, which are presented as FPKM-normalized log_2 transformed counts

as Phosphoribulokinase (EC: 2.7.1.19), was significantly downregulated under Tis_LT. The transcriptional expression levels of the two genes annotated as transketolase (EC 2.2.1.1) and three genes annotated as ribose 5-phosphate isomerase (EC 5.3.1.6) in the second branch were SL_LT > LT > Tis_LT. In the third branch of RuBP regeneration, four genes were annotated as fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11). Except for 107,842,812, the other three genes were highly expressed under SL_LT and were significantly different from LT. Three DEGs annotated to fructose-bisphosphate aldolase (FBA, EC 4.1.2.13), among them, 107,858,551 and 107,840,477 were all lowly expressed under Tis-LT treatment and highly expressed under SL_LT; the expression of 107,871,105, which was annotated in Sedoheptulose-1,7-bisphosphatase (SBP, EC 3.1.3.37), was at a lower level under Tis_LT. Overall, the presence of SLs can improve the efficiency of carboxylation, reduction, and regeneration in the Calvin cycle of pepper seedlings under a low-temperature stress environment.

qRT-PCR validation

Sixteen DEGs were randomly selected for qRT-PCR validation and the results are shown in Fig. 10. Among them, the quantitative validation and transcriptome sequencing results of one gene, LOC107844032, did not match,

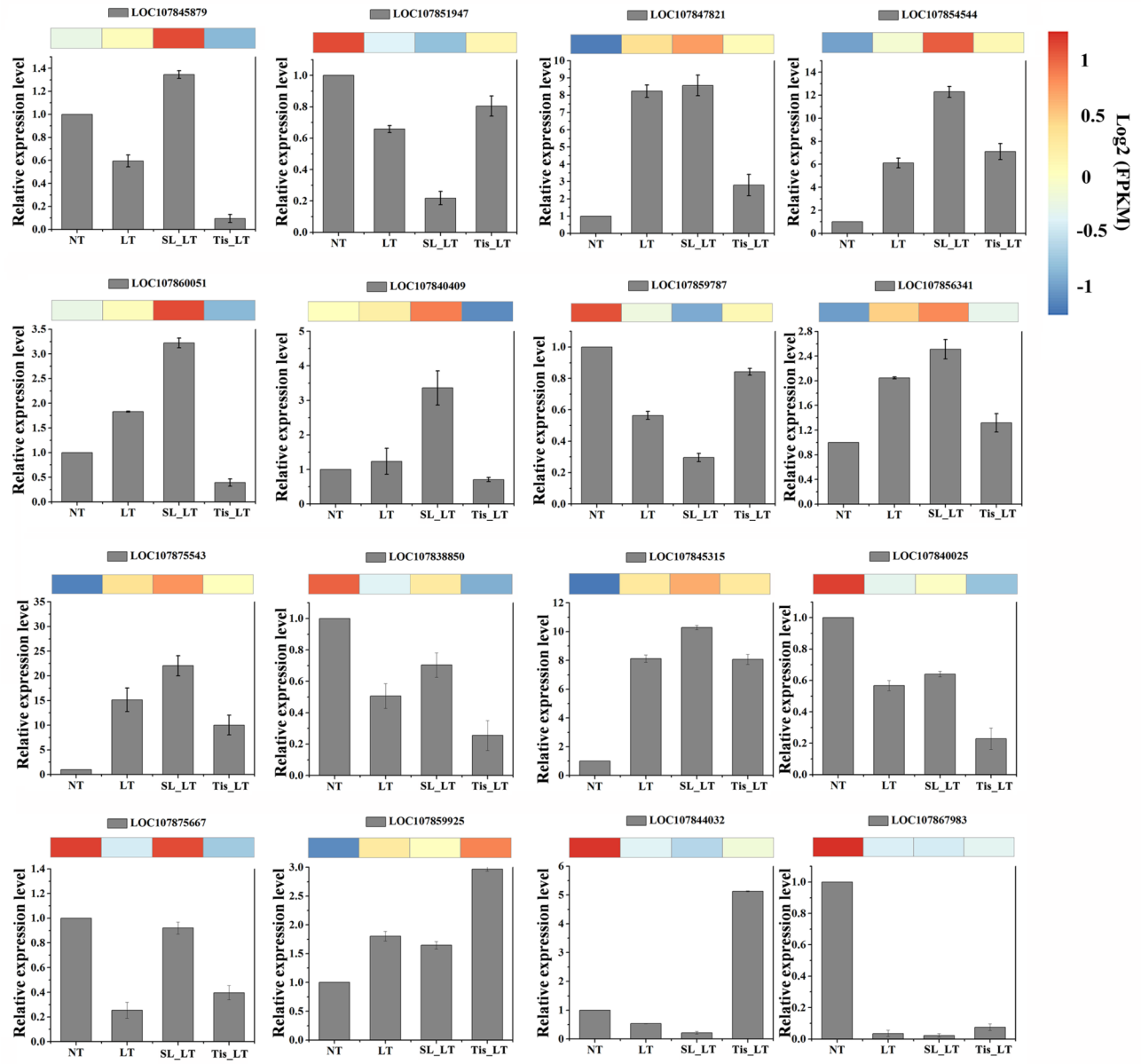

Fig. 10 qRT-PCR validation of transcriptome data. The gray bar represents the qRT-PCR results and the different colored blocks represent the transcriptome gene expression results shown as Log_2(FPKM)

whereas the results of the remaining 15 DEGs under LT, SL_LT, and Tis_LT showed a consistency of 93.5%, indicating the reliability of the sequencing data.

Discussion

SLs effectively alleviate the damage of low temperatures to chili seedlings and the degradation of photosynthetic pigments

Tis108, a triazole-type inhibitor of the biosynthesis of SLs, can reduce 2'epi-5-deoxyresveratrol (epi-5-DS) in rice, but GR24 treatment can restore the *Arabidopsis* SL-deficient phenotype induced by Tis108 to the wild-type [30]. After continuous application of *rac*-GR24 and Tis108 for 7 d, compared with the control (application of water), *rac*-GR24 significantly increased the level of 5-DS in pepper leaves, whereas Tis108 reduced the level of 5-DS. Moreover, after 24 h of low-temperature stress, the changes of pepper 5-DS content in the three treatments became more pronounced. These results indicate that *rac*-GR24 and Tis108 are effectively absorbed by plants to exert their effects.

Plants exposed to low-temperature environments typically exhibit wilting and leaf necrosis [3]. In this study, the exogenous application of *rac*-GR24 effectively alleviated the damage caused by low-temperature stress on the growth of pepper seedlings and reduced the loss of dry and fresh weight of plants, whereas Tis108 aggravated the loss of the low-temperature damage phenotype and biomass of plants. This may be attributed to the promotion of photosynthesis by SL, which enhanced the accumulation of photosynthetic products. In Cooper et al. [16], exposure to dark chilling caused a visible decrease in the biomass of SL synthesis (*max4-1*) and signaling (*max2-1*) mutants compared to that of the *Arabidopsis* wild-type when plants were grown in pots. This was similar to the results for pepper pretreated with a synthetic inhibitor of SLs in our study. However, when grown on agar plates, a chilling-induced decrease in leaf area was observed in all lines in the presence of GR24. One possible explanation for this contradictory finding is that the growth media contained high levels of sucrose, and SL may be involved in sugar metabolism and sugar signaling pathways that modulate seedling development [31].

Besides, when plants are exposed to low-temperature stress, it is often accompanied by the degradation of photosynthetic pigments (including chlorophyll a, chlorophyll b, total chlorophyll, and carotenoids). In this study, exogenous *rac*-GR24 pretreatment restored the levels of photosynthetic pigments under low-temperature stress, whereas Tis108 pretreatment intensified the degradation of photosynthetic pigments under low-temperature conditions. These results are consistent with the results of Lu et al. and Min et al. [14, 15].

Chlorophyll biosynthesis is mediated by more than 17 enzymes [32], among which the formation of ALA and the binding of Mg^{2+} to protoporphyrin IX, which are mainly regulated by glutamyl-TrNA reductase and magnesium ion chelatase, are the main control points limiting chlorophyll yield [32, 33]. Mazlish-Gati et al. [34] found that compared to wild-type tomato plants, the chlorophyll (Chl) content in the *SI-ORT1* mutant plant was reduced. In our study, the application of *rac*-GR24 and Tis108 significantly upregulated and downregulated genes that encode glutamyl-tRNA reductase and magnesium ion chelatase, respectively, as well as genes that control the direct synthetases of Chl-a and Chl-b. These results indicate that SLs may enhance ALA formation, Mg^{2+} binding to protoporphyrin IX under low-temperature stress, increase chlorophyll content in pepper leaves, and improve photosynthetic performance by activating gene expression.

SLs significantly improved the photosynthetic performance of pepper seedlings at low temperature

Photosynthesis directly contributes to plant growth and yield [21]. Many studies have shown that exogenous application of GR24 can effectively improve the photosynthetic capacity of various plant species, such as rape, wheat, rice, grape, and sunflower, under abiotic stress [13, 14, 35–37]. Although the application of *rac*-GR24 reduced the photosynthetic capacity of pepper seedlings at the initial stage of low-temperature stress (24 h), it did not reach a significant level, which may be due to a reduction in stomatal conductance by SLs by inducing stomatal closure. With the extension of the low-temperature stress time, *rac*-GR24 and Tis108 pretreatment significantly increased and decreased the photosynthetic capacity of the pepper seedlings, respectively. In addition, low-temperature stress inhibits the photosynthetic carbon fixation process and ultimately photosynthesis by restricting the activities of Rubisco, FBPase, and ribulokinase phosphate [12]. Our results showed that the application of *rac*-GR24 and Tis108 significantly up-regulated and down-regulated the expression of genes annotated to these enzymes, respectively, accompanied by an increase in and inhibition of the corresponding enzyme activity. It has been speculated that SLs enhance the activity of response enzymes by activating gene expression during the Calvin cycle, thereby promoting photosynthetic carbon fixation. Additionally, SLs could maintain chloroplast energy production under low-temperature stress to a certain extent. This facilitates the reduction of triose phosphate and regeneration of the CO_2 receptor RuBP (ribulose-1, 5-diphosphate) in the Calvin cycle.

Based on the gene expression levels under LT, SL_LT and Tis_LT were significantly enriched in the CC and MF. In CC, these DEGs were mostly related to the

components of photosynthesis; however, they were upregulated under SL_LT and downregulated under Tis_LT. Moreover, DEGs significantly enriched in photosynthesis-related components and photosynthetic biological processes were upregulated under SL_LT compared to Tis_LT. PsbO, PsbP, and PsbQ proteins of photosystem II play crucial roles in maintaining optimal Mn, Ca, and Cl concentrations at the active site of photosystem II [38]. Yi et al. [39] found that in transgenic *Arabidopsis thaliana* plants with medium and low PsbP expression, Fv/Fm decreased with decreasing PsbP protein expression. In higher plants, PsbQ stabilizes the functional binding of PsbP to photosystem II [40]. Our results showed that after low-temperature stress, the gene expression of PsbP and PsbQ was high under SL_LT, whereas the gene expression of PsbP and PsbQ was low under Tis_LT. It was speculated that SLs could protect PsbQ and stabilize the functional binding between PsbP and PSII. Additionally, PsbW and PsbY assist in the assembly and insertion of the PSII complex into the thylakoid membrane [41]. Loss of PsbW destroys the supramolecular structure of PSII in plants and is accompanied by a decrease in Fv/Fm [42]. Our results showed that, compared with the gene expression levels annotated to PsbW and PsbY under LT, the expression of these genes under SL_LT and Tis_LT was upregulated and downregulated, respectively. This indicates that SL helps maintain the assembly and stability of the PSII complex in pepper plants under low-temperature stress. Psb27 has been identified as a PSII-associated intracavitary protein that contributes to the effective recovery of the photodamaged PSII complex [43]. In this study, the genes related to Psb27 were highly expressed under SL_LT, moderately expressed under LT, and showed low expression under Tis_LT. These results indicate that SLs may improve the damage recovery efficiency of PSII under low-temperature stress by stabilizing Psb27 and enhancing the photosynthetic capacity of pepper.

The DEGs identified in PSI were mainly concentrated in the PsaE, PsaH, PsaL, PsaN, and PsaO subunits and were upregulated and downregulated by SL and SL inhibitor, respectively, compared with the low-temperature treatment alone. These results indicate that SLs can maintain the circulating electron flow around PSI in pepper at low temperatures, the stability of PSI trimers, and the energy balance between PSII and PSI [44–46]. Ferredoxin (PetF) was highly expressed under SL_LT, moderately expressed under LT, and showed low expression under Tis_LT. Although the expression level of ATPase was not significantly different between SL_LT and LT treatments, it was lower under Tis_LT. These results suggest that SLs alleviates the low-temperature restriction of carbon fixation by protecting the stability of ferredoxin and ATP proton pumps at low temperatures.

SLs reduce the degree of PSII photoinhibition in pepper seedlings by increasing the consumption and dissipation of light energy absorbed at low temperatures

Low temperatures limit the transfer of photosynthetic electrons in plants, resulting in the excess accumulation of excitation energy in the photosystem [2, 47]. In addition, during chilling stress, the decrease in Calvin cycle enzyme activity leads to excess energy in the photosystem and ultimately leads to photoinhibition and even photo oxidative damage [48, 49]. Fv/Fm represents the maximum photochemical efficiency of photosystem II and its decrease is often used to measure the degree of plant photoinhibition [50]. In this study, low-temperature stress significantly reduced the Fv/Fm of pepper seedlings, accompanied by a linear increase in excess excitation energy [(1-qP)/NPQ] and excitation pressure (1-qP). *Rac*-GR24 and Tis108 inhibited and intensified the decrease in Fv/Fm, respectively, while reducing and increasing the excitation pressure faced by PSII to a certain extent. This is similar to the results reported by Lu et al. for tomatoes [15].

Plants have evolved a series of adaptive mechanisms to alleviate or avoid the harm caused by photoinhibition and photooxidation. For example, dissipating excess energy through NPQ is the main method used to prevent excess excitation energy from damaging the photosystem [29, 51]. During the initial low-temperature stress period (24 h), the NPQ value of the pepper leaves sharply increased. During this period, the NPQ and [(1-qP)/NPQ] of SL_LT treatment were significantly lower than those of LT treatment, indicating that there may be other pathways that reduce the accumulation of excess excitation energy in the photosynthetic system of chili leaves under initial low-temperature stress. The fitting of the photoresponse curve of low-temperature stress for 24 h showed that the light use efficiency (α) of plants increased under low-temperature conditions after the application of *rac*-GR24. Therefore, the higher electron transfer rate of *rac*-GR24 pretreated plants at low temperatures increased their consumption of absorbed light energy, thereby reducing the production of excess excitation energy. After prolonged low-temperature stress, the NPQ under LT and Tis_LT returned to the level under normal growth conditions, indicating that long-term low-temperature stress may have disrupted the occurrence of NPQ, whereas SLs may increase the degree of de-epoxidation by regulating the xanthophyll cycle components, promoting the dissipation of excess excitation energy through the NPQ pathway in pepper [29].

Lutein and zeaxanthin are synthesized in large quantities under stressful conditions to maintain the stability of the light-harvesting pigment complex (LHCII) and reaction centers in the photosystem. The de-epoxidation state [(Z+A)/(Z+A+V)] of the xanthophyll cycle (composed

of Zea, Ant, and Vio) is the main contributor to plant heat dissipation. The recovery of total carotenoid levels in pepper leaves may be due to the significant accumulation of lutein and zeaxanthin induced by SLs at low temperatures. Therefore, maintaining normal levels of chlorophyll and carotenoids may be an effective strategy for plants to adapt to low-temperature stress with the help of SLs. Further analysis of DEGs annotated to the carotenoid biosynthesis pathway revealed that SL activated the expression levels of genes associated with the beta-carotene, lutein, zeaxanthin, and violaxanthin synthesis pathways as well as downstream genes associated with ABA synthesis, and the expression of these genes was substantially downregulated by SLs inhibitor. This contributes to the accumulation of photoprotectants or antioxidants (β-carotene, lutein, zeaxanthin), as well as ABA in peppers to quench excess activation energy, protect photosynthesis, remove ROS, and ultimately improve low-temperature tolerance.

The light-harvesting chlorophyll protein complex (LHC) captures and transfers the energy required for plant photosynthesis, and regulates its distribution in PSII and PSI. This is an important mechanism for dissipating excess excitation energy and providing light protection. Havaux and Tardy found that under different environmental conditions, LHCII could stabilize PSII complexes and maintain the functional state of the water-oxidizing system [52]. Studies have also reported that the *ch1-3* mutant (lacking LHCII: Lhcb1-6) enhances the sensitivity of PSII to photoinactivation and increases non-functional PSII complexes in plants [53]. Our results showed that, compared to low-temperature treatment alone, SL significantly upregulated the expression levels of genes annotated as LHCII (Lhcb1, Lhcb2, Lhcb3, and Lhcb6), whereas SL inhibitor significantly downregulated the expression of these genes. In addition, four LHCI genes were annotated as highly expressed under SL_LT, moderately expressed under LT, and weakly expressed under Tis_LT. This helps increase the absorption cross-section of PSI under low-temperature stress, resulting in photochemical charge separation and providing energy for the PSI core complex [54]. These results indicated that the protective effect of SLs against LHC may be an important strategy for pepper seedlings to cope with low-temperature stress.

Conclusion

The positive regulatory effect of SLs on the low-temperature tolerance of pepper seedlings was confirmed by applying the synthetic analog *rac*-GR24 and the biosynthesis inhibitor Tis108 of SLs. By improving linear electron transport, SLs promoted the metabolic consumption of energy absorbed by the photosystem in the early stage of stress, increased thermal energy dissipation based on the xanthophyll cycle in the later stage of stress, and reduced the accumulation of excess excitation energy in the photosystem, thus alleviating the degree of low-temperature-induced photoinhibition. The transcriptome results indicated that genes related to the photosynthetic system and thylakoid cell components were regulated by SLs and their inhibitor Tis108. The next step should be to consider the specific effects of SLs in field production and explore the molecular mechanisms mediating the response of pepper seedlings to low-temperature stress. In addition, this study provided new insights for the development of temperature-tolerant pepper lines through breeding programs.

Abbreviations

Ant	Antheraxanthin
BP	Biological process
CC	Cellular component
DEGs	Differentially expressed genes
ETR	Photosynthetic electron transport rate
FBA	Fructose-1,6-bisphosphate aldolase
FBPase	Fructose-1, 6-bisphosphatase
Fv/Fm	The maximum photochemical efficiency of PSΠ
GAPDH	3-glyceraldehyde phosphate dehydrogenase
Lut	Lutein
MF	Molecular function
NPQ	Non-photochemical quenching
PAR	Photosynthetically active radiation
Pn	Net photosynthetic rate
qP	Photochemical quenching
rac-GR24	The artificially synthesized analogs of strigolactones
Rubisco	1,5-ribulose diphosphate carboxylase
SLs	Strigolactones
TK	Transketolase
VDE	Violaxanthin de-epoxidase
Vio	Violaxanthin
ZEP	Zeaxanthin epoxidase
φPSII	Actual photochemical efficiency of PSII
(1-qP)/NPQ	The excess excitation energy of PSII

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-024-05622-3.

Supplementary Material 1

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Author contributions

J. Z. Writing- original draft, Funding acquisition; C. T. Data Curation, Writing–original draft; J. X. Supervision, Funding acquisition, Writing- review & editing; J. L. Resources, Methodology; C. W. Formal analysis, Visualization; X. Z. Formal analysis, Software. All authors read and approved the final manuscript.

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Data availability
All data are available upon request to the corresponding author, Jianming Xie (xiejianminggs@126.com).Sequence data generated for this study is available publicly in the NCBI Sequence Read Archive under BioProject ID: PRJNA1135320.

Declarations

Ethics approval and consent to participate
This study does not include human or animal subjects.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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