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OVEREXPRESSION OF THE ATP SYNTHASE B SUBUNIT GENE ENHANCED THE ABILITY OF TOMATO (*SOLANUM LYCOPERSICUM* L.) PLANTLETS TO RESIST LOW TEMPERATURES

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Abstract

Tomato (*Solanum lycopersicum* L.) is a kind of important greenhouse vegetable in the north of China, but it is also sensitive to low temperatures. Commercial losses due to low-temperature stress are particularly serious here. The aim of this experiment is to improve the resistance of tomato plants to low-temperature stress employing genetic engineering technology. Here, the ATP synthase β subunit gene was cloned and connected with the overexpression vector CaMV35S. Then, together, they were transferred to the low-temperature-sensitive tomato variety 'Zhongshu4'. Tomato plantlets of the transgenic T1 generation and of the wild type, were placed in one or other of two artificial climate chambers. These were operated (1) at normal temperatures (25/18℃) and (2) at low temperatures (12/6℃) both under a 12-h photoperiod (350 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 7 d of low-temperature treatment, compared with low-temperature treated wild-type controls, the transgenic plantlets had increased activities of catalase, peroxidase and superoxide dismutase, but reduced contents of malondialdehyde. The transgenic plantlets also showed increased net rate of photosynthesis, transpiration rate and tomatal conductance, but reduced concentrations of intercellular CO_2. Meanwhile, the transgenic plantlets showed increases in the actual photochemical efficiency and the maximum photochemical efficiency of Photosystem II, light energy capture efficiency by open Photosystem II reaction center and photochemical quenching coefficient. These results combine to confirm that overexpression of the ATP synthase β subunit gene enhances the ability of these transgenic tomato plantlets to resist low temperatures.

Key words: ATP synthase β subunit, Overexpression, Photosynthesis, Tomato plantlets.

Introduction

Temperature is considered to be one of the most significant factors affecting the growth and development of all plant, including of vegetables (Thomashow, 1999; Diao *et al.*, 2015). If the temperature falls below the minimum for a particular species, low-temperature damage occurs (Yang *et al.*, 2017). Low-temperature damage problems are widespread and result in serious commercial loss to vegetable producers every year. Tomato is considered to be one of the most widely-cultivated vegetables in the world and it is especially sensitive to low-temperature (Spicher *et al.*, 2016; Cao *et al.*, 2015). Tomato is also a greenhouse vegetable that is grown year-round. In the north of China, greenhouse tomato crops are often affected by low-temperature stress in winter and spring (Shu *et al.*, 2016).

Gene engineering technology is potentially able to increase the resistance of tomato to low-temperature stress (Balamurugan *et al.*, 2018). The ongoing development of molecular biology enables the cloning a large number of genes that relate to the resistance of tomato to low-temperature stress. These also help us to better understand the resistance mechanisms of tomato to low-temperature stress, at the molecular level of gene expression regulation. Kumar *et al* transferred the gene encoding antifreeze protein (AFP) in carrot into tomato under the regulation of CaMV35S promoter (Kumar *et al*., 2014). The result shows that overexpression of the antifreeze gene increases the antifreeze potential of tomato. There have been a number of similar studies in tomato, these include: *SlCOR413IM1* overexpression which can alleviate chilling stress damage (Ma *et al*., 2018); overexpression of *ShCIGT* which reduces damage caused by cold stress (Yu *et al.*, 2018); overexpression of SBPase increases chilling tolerance (Ding *et al.*, 2017b); overexpression of the transcription factor *TERF2/LeERF2* enhances freezing tolerance (Zhang & Huang, 2010) and overexpression of *ShDHN* enhances tolerance to cold stress (Liu *et al.*, 2015).

To investigate the protective mechanisms of tomato plantlets against low temperature stress, the coding region of ATP synthase β subunit gene was connected to CaMV35S-GFP overexpression vector and transferred into the chill-sensitive tomato variety 'Zhongshu4'. We then measured antioxidant enzyme activities, the photosynthetic and chlorophyll fluorescence parameters of both the wild-type and transgenic T1 generation tomato seedlings under low-temperature (stress) conditions and under normal temperatures. These results showed that overexpression of the ATP synthase β subunit gene gave the modified 'Zhongshu4' significant protection against low temperature stress.

Experimental materials and methods

Plant materials, gene and overexpression vector: The plantlets of the tomato variety 'Zhongshu4' (*Solanum lycopersicum* L.) with low temperature sensitivity, were used as experimental material. The coding sequence of the ATP synthase β subunit gene in tomato (gi/6688526) is 1380 bp. The overexpression vector is CaMV35S-GFP.

Experimental methods and procedures

Gene clone and agrobacterium mediated transformation: The total RNA extraction and cDNA synthesis respectively used the TIANGEN Trizon kit and the Superscript III first strand synthesis system (Invitrogen). PCR reaction conditions were as following: 95℃ 4 min, 94℃ 0: 40 min, 58℃ 0：40 min, 72℃ 1:30 min, 72℃ 10：00 min, 35cyclys. Gel extraction and DNA A-Tailing reaction respectively used the Gel DNA Purification Kit and DNA A-Tailing Kit (TAKARA Company). The connection between the target gene and the T vector was carried out using the pMD 18-T Vector (TAKARA company). Plasmid extraction used the TIANprep Mini Plasmid Kit. Double enzyme digestions of the target gene and expression vector were carried out using Sal I enzyme and Kpn I enzyme (TAKARA company). The connection between the target gene and overexpression vector was carried out using TIANGEN T4 DNA ligase. The recombinant plasmid was transferred into the GV3101 agrobacterium by the electric shock method. Transgenic tomato seedlings were obtained as the methods of Zhang *et al*., (2019).

Detection of transgenic tomato plantlets by laser scanning confocal microscopy: Roots of the tomato plantlets were rinsed using sterile distilled water. The 1 cm-long root was cut off with a sharp blade and placed on a clean glass slide. Then a drop of distilled water was dropped on and a glass coverslip was applied. Observation and analysis were carried out under the visual field of the laser scanning confocal microscope.

Low-temperature treatment of transgenic and wildtype tomato plantlets: When the plantlets had reached the five-leaf one heart stage, two T_1 generation strains (ATP1 and ATP2) and wildtype (WT) 'Zhongshu4' tomato plantlet were placed in artificial climate chambers. One chamber was at normal temperature (25/18℃) and contained ATP1, ATP2 and WT and the other was at low temperature (12/6℃) and contained ATP1, ATP2 and WT. Both chambers were under a 12-h photoperiod (350 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After 7 d, antioxidant enzyme activities, the photosynthetic indices and parameters of the chlorophyll fluorescence were measured.

Antioxidant enzyme activity determination: Activities of POD, CAT and SOD were assayed according to the methods of Vardhini *et al*., (2011). MDA contents were measured as the method of Hu *et al*., (2010).

Photosynthetic rate and chlorophyll fluorescence parameter analyses: The third leaves from the top of tomato plantlets were used to measured photosynthetic rate and chlorophyII fluorescence parameters. Li-6400 Photosynthesis System (LI-COR, USA) was used to measure photosynthetic rates. Parameters of Chlorophyll fluorescence were measured using a fluorescence chamber of Li-6400 system.

Statistical analyses

Antioxidant enzyme activity, photosynthetic rate and chlorophyll fluorescence parameters were all measured twice. SPSS 16.0 and Microsoft Excel 2013 were used to carry out statistical analyses.

Results

Gene clone and agrobacterium mediated transformation: The total RNA was extracted from the 'Zhongshu4' leaves. The integrity of RNA was very good. The cDNA of'Zhongshu4'leaves was used as template and a band with a fragment size of 1380 bp was obtained by RT-PCR amplification. The recombinant plasmid of the target gene and PMD-18T vector was then sent to Beijing Yingjun Company for sequencing, and the results are shown in (Fig. 1). The similarity between the sequences obtained by sequencing and the coding sequence of the ATP synthase β subunit gene was 99.9%. The results indicate the cloned sequence was the coding region of the ATP synthase β subunit of tomato.

Detection of transgenic tomato seedlings by laser scanning confocal microscopy: The roots of transgenic plantlets were observed by laser scanning confocal microscopy. As shown in (Fig. 2) transgenic-positive roots showed a green fluorescence but transgenic-negative roots did not emit green light under the vision field of the laser scanning confocal microscope.

Antioxidant enzyme activity determination: Compared with normal temperature conditions, it can be seen in (Fig. 3) that the levels of POD, CAT and SOD were all decreased, but the levels of MDA were increased under low-temperature conditions. After 7 d of low temperature, the SOD levels in the two T_1 generation strains (ATP1 and ATP2) were 17.6 and 25.4% higher, respectively, than in the WT lines. The POD levels in ATP1 and ATP2 were 29.5 and 37.5% higher, respectively, than in the WT lines. The CAT levels in ATP1 and ATP2 were 14.5 and 38.7% higher, respectively, than in the WT lines. The MDA levels in ATP1 and ATP2 were 20.0 and 22.1% lower, respectively, than in the WT lines.

Photosynthetic rate and chlorophyll fluorescence parameter analyses: It can be seen in (Fig. 4) that compared to under normal temperatures, the values of Tr, Pn and Gs were all lower but the value of Ci was higher, under low-temperature conditions.

After 7 d at low temperature, the Pn values in ATP1 and ATP2 were 42.6 and 54.9% higher, respectively, than in the WT lines. The Gs values in ATP1 and ATP2 were 10.2 and 7.7% higher, respectively, than in the WT lines. The Tr values in ATP1 and ATP2 were 9.1 and 13.0% higher, respectively, than in the WT lines. The Ci values in ATP1 and ATP2 were 5.3 and 8.1% lower, respectively, than in the WT lines.

It can be seen in (Fig. 5) that, compared with under normal temperatures, the values of Fv′/Fm′, Fv/Fm, Qp and ΦPSII all decreased under low temperatures. After 7 d of low temperature, the Fv/Fm values of ATP1 and ATP2 were 11.9 and 16.6% higher, respectively, than in the WT lines. The Qp values of ATP1 and ATP2 were 6.1 and 8.9% higher, respectively, than in the WT lines. The Fv′/Fm′ values of ATP1 and ATP2 were 7.6 and 11.6% higher, respectively, than in the WT lines. The ΦPSII values of ATP1 and ATP2 were 11.8 and 13.3% higher, respectively, than in the WT lines.

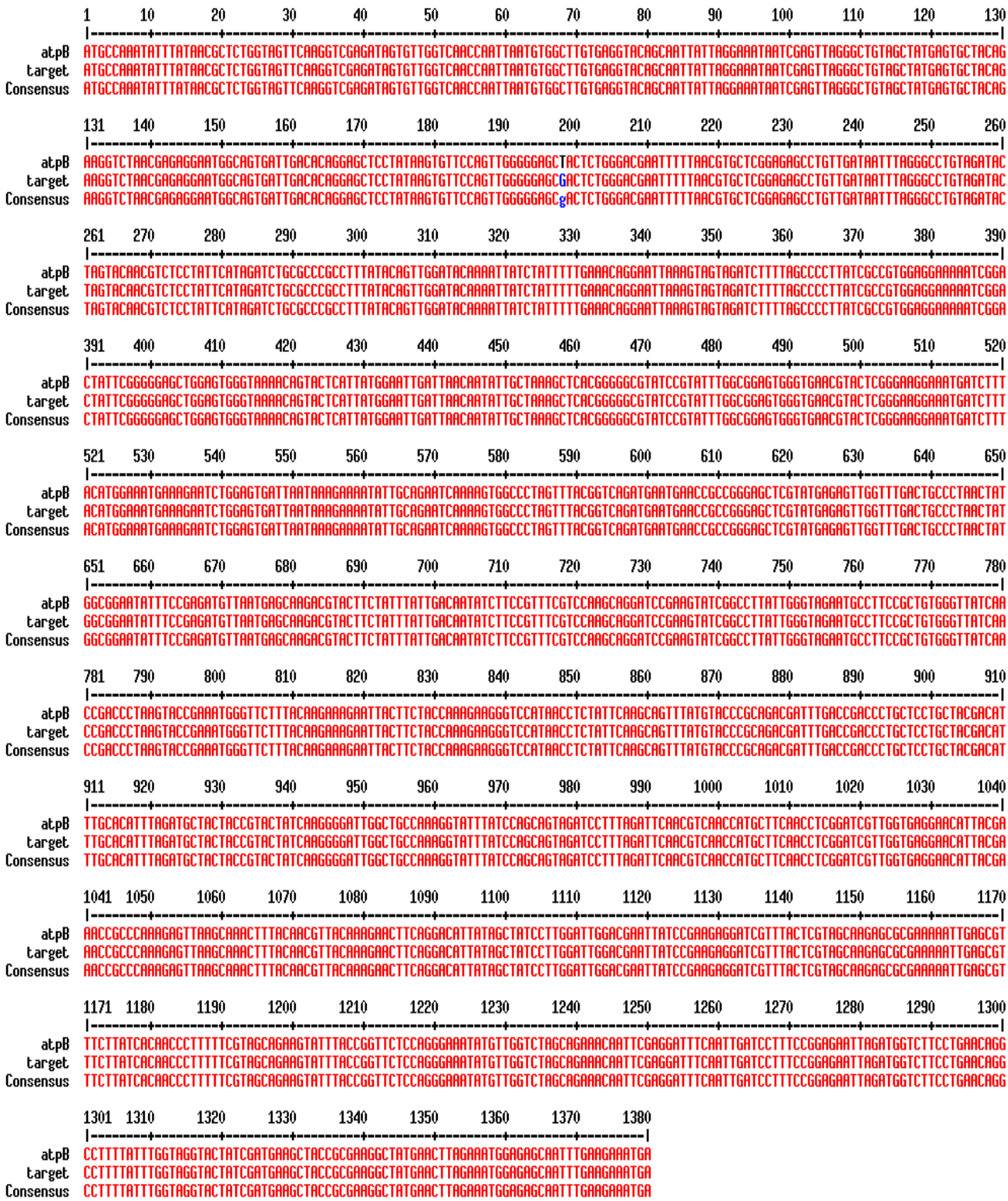

Fig. 1. The sequence alignment result of ATP synthase β subunit.

Discussion

When tomato seedlings are exposed to stressful low temperatures, reactive oxygen species (ROS) begin to accumulate. ROS can destroy structure and function of cell and may even cause death of cell. POD, CAT and SOD are three key enzymes that scavenge ROS *In vivo*. Therefore, antioxidant enzyme activities are considered important indices of plant stress tolerance. In this experiment, the activities of CAT, POD and SOD were higher in the transgenic tomato plantlets than in the WT ones. This demonstrates our transgenic plantlets are likely more tolerant of low-temperatures. MDA content is considered to be an indicator of membrane lipid peroxidation, and it can reflect the extent of plant injury under stress conditions. Here, compared with WT controls, transgenic plantlets had lower MDA contents after 7 d of low temperature treatment.

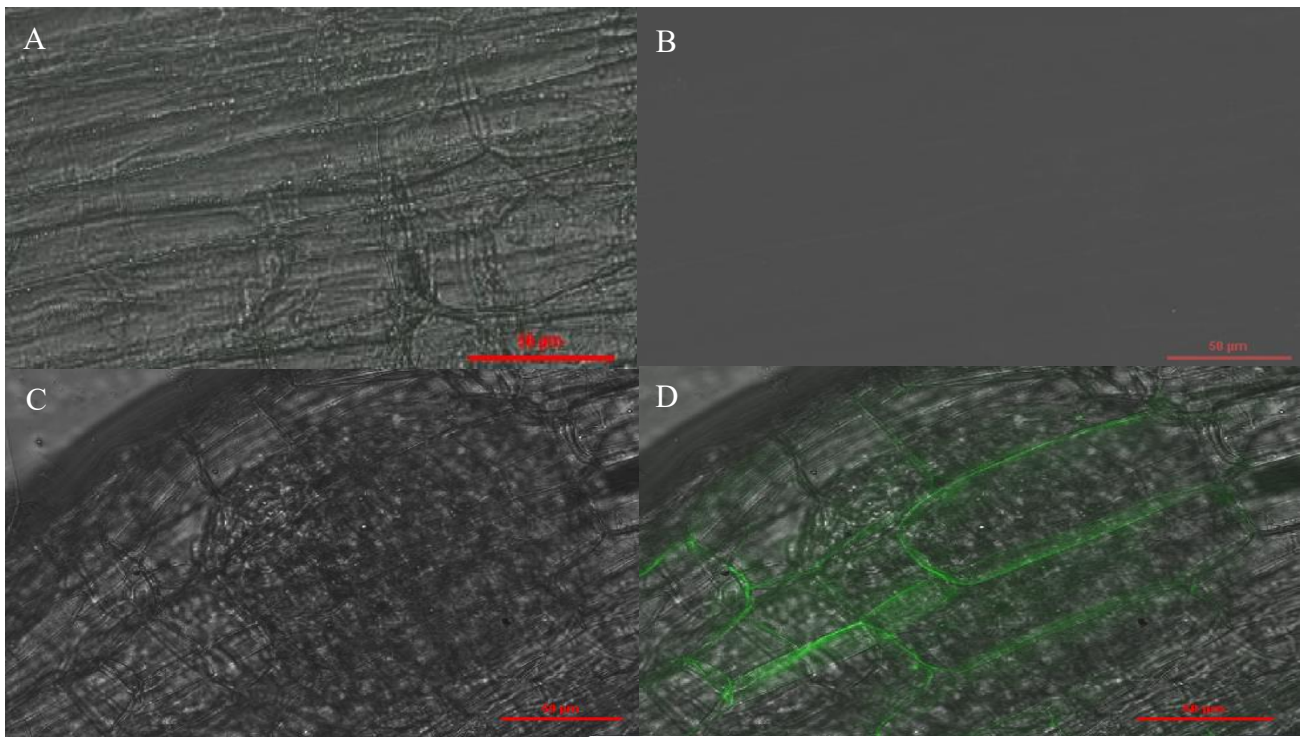

Fig. 2. Detection result of transgenic tomato plantlets by laser scanning confocal microscopy (a) The root image of wildtype under white light (b) The root image of wildtype under blue fluorescent light (c) The root image of transgene-positive plants under white light (d) The root image of transgene-positive plants under blue fluorescent light.

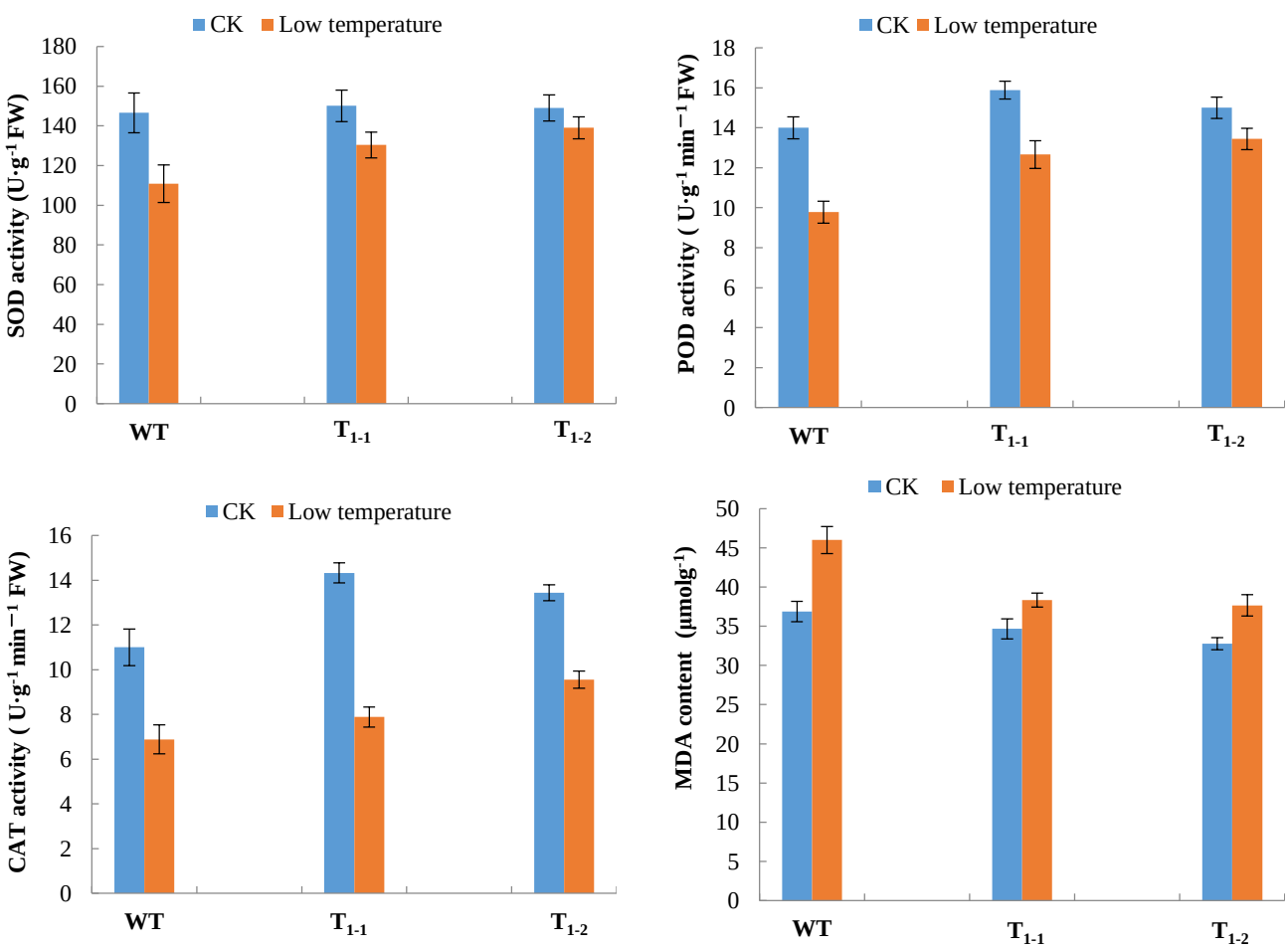

Fig. 3. Effect of low temperatures on antioxidant enzyme activity in transgenic and wild type tomato plantlets. Each line represents the mean ± standard error of six independent experiments (n=6).

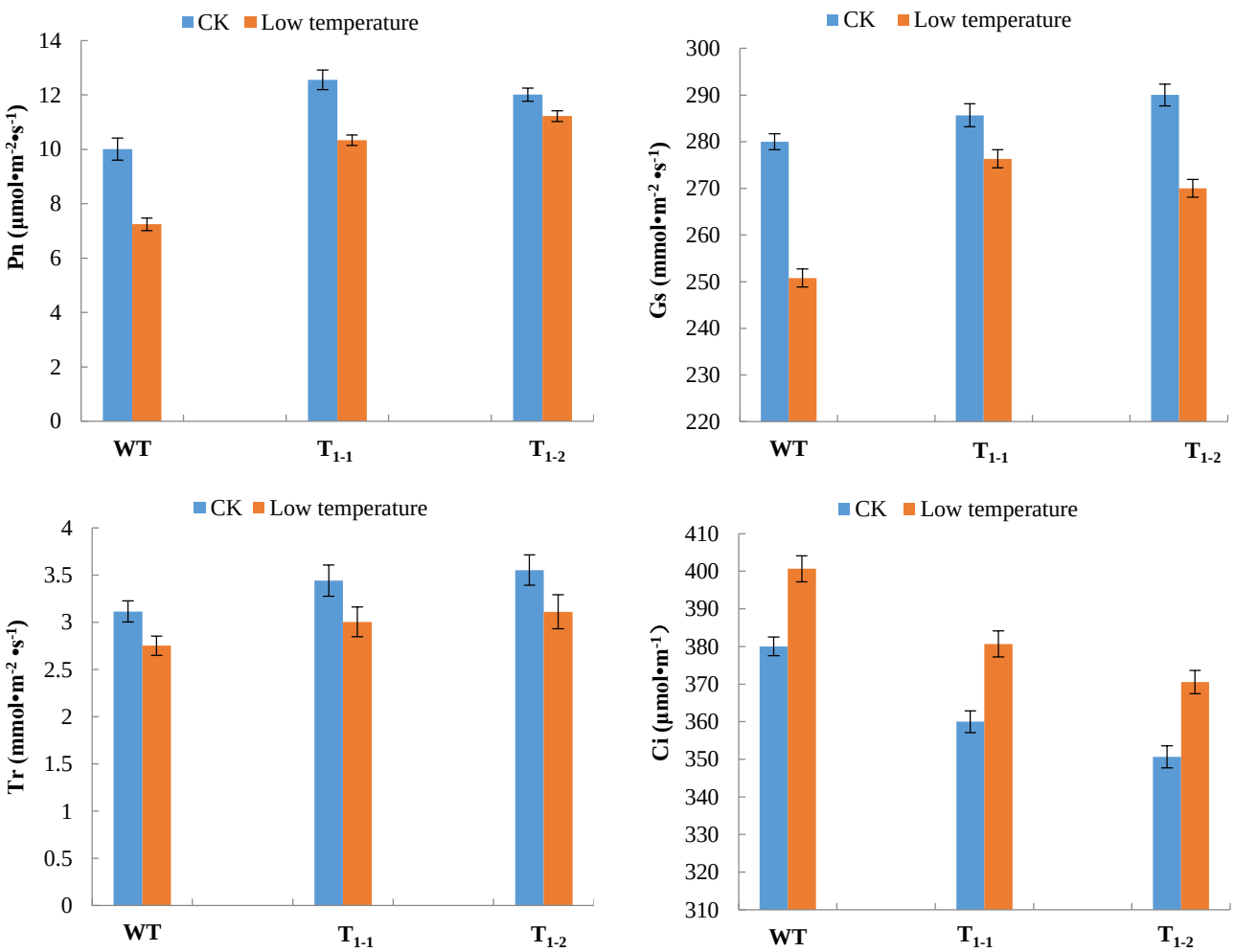

Fig. 4. Effect of low temperatures on photosynthetic parameters in transgenic and wild type tomato plantlets. Each line represents the mean ± standard error of six independent experiments (n=6).

Low temperature stress not only inhibits the growth and development of tomato plantlets (Ding *et al.*, 2017), but it also damages tomato seedlings (Dong *et al.*, 2019). The nature of this low temperature damage is varied but the effects on photosynthesis are particularly important (Powles, 1984). Obviously, photosynthesis is the most important component of a plant's energy metabolism. Therefore, its activity can reflect the growth and development status of a plant; hence photosynthesis is an important indicator of plant stress.

The photosynthetic process contains many interactive factors; these include: Pn, Gs, Tr, and Ci (Gao *et al.*, 2016). As the most representative parameter, the value of Pn directly reflects the photosynthetic capability of a plant (Sicher and Bunce 2001). Under low temperature conditions, Pn of our tomato plantlets decreased markedly. The value of Pn was reduced because stomatal and non-stomatal factors are determined by changes in Ci and Gs (Farquhar & Sharkey, 1982). In our study, compared with under normal temperatures, the values of Gs, Tr and Pn, were all decreased, but that of Ci was increased, after 7 d of low temperatures. These findings suggest the limitation to Pn was non-stomatal in nature. We also found that after 7 d of low temperatures, the values of Pn, Gs and Tr in the transgenic plantlets, with overexpression of the ATP synthase β subunit gene, increased compared with the WT. These suggest that the photosynthetic organs of the transgenic plantlets suffered relatively less damage under low temperatures. These results could also be because the ATP synthase increase allowed the plantlets to absorb more light energy and so produce larger amounts of ATP, which ultimately increased photosynthesis in the transgenic plantlets.

The chlorophyll fluorescence parameter is an intrinsic indicator that can reflect the strength or weakness of the photosynthesis processes of a plant and, hence, it can serve as a stress-tolerance index for plants under adverse conditions (Li *et al.*, 2021). In this experiment, the values of ΦPSII, Fv'/Fm', Qp and Fv/Fm were all reduced after 7 d of low temperatures, indicating that the low temperatures had inhibited the photochemical activity of PSII, which is known to be very sensitive to low temperature stress. When the photochemical activity of PSII is inhibited by low temperatures, photosynthesis in tomato is reduced. We also found that after 7 d of low temperatures, the values of ΦPSII, Fv'/Fm', Qp and Fv/Fm of plantlets overexpressing the ATP synthase β subunit gene increased compared with WT ones. This is likely because under low-temperature stress, the plantlets overexpressing the ATP synthase β subunit gene were better able to protect photosystem II in the chloroplasts, and so reduce the damage.

Under low-temperature stress conditions, the capacity to harvest and dissipate light energy is imbalanced (Cheng *et al*., 2016; Miura & Furumoto, 2013). Moreover, the utilization of absorbed light energy and CO_2 assimilation rate are all reduced. This is because low temperatures reduce enzyme activities in the Calvin cycle (Mignolet-Spruyt *et al*., 2016; Gururani *et al*., 2015). In our study, overexpression of the ATP synthase β subunit increased photosynthesis in the plantlets under low-temperature conditions. This may because the β is the main subunit of ATP synthase, which is the main enzyme for ATP production in photosynthesis. Thus, our transgenic tomato plantlets can produce larger amounts of ATP and, ultimately, this increases photosynthesis under conditions of low-temperature stress.

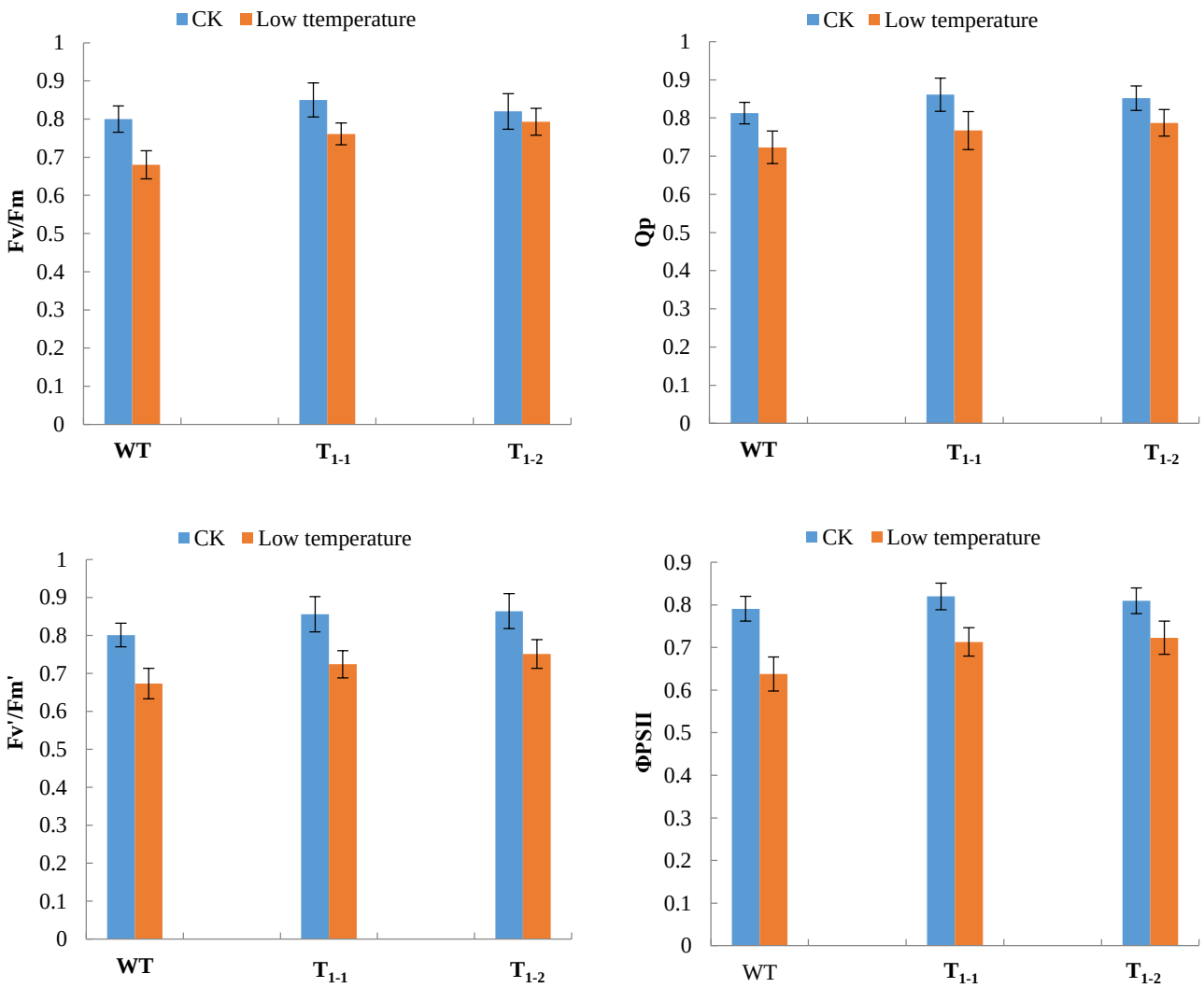

Fig. 5. Effect of low temperatures on chlorophyll fluorescence in transgenic and wild type tomato plantlets. Each line represents the mean ± standard error of six independent experiments (n=6).

Conclusions

Low-temperature stress conditions decreased the activities of CAT, POD and SOD, but increased the content of MDA in WT 'Zhongshu4' tomato plantlets. It also decreased the values of Tr, Gs and Pn, but increased Ci. It further decreased the values of ΦPSII, Fv'/ Fm', Qp and Fv/Fm. However, overexpression of the ATP synthase β subunit gene increased the ability of the transgenic 'Zhongshu4' tomato plantlets to resist low temperatures.

Acknowledgments

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