Overexpression of SikRbcs2 gene promotes chilling tolerance of tomato by improving photosynthetic enzyme activity, reducing oxidative damage, and stabilizing cell membrane structure

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Abstract
Red blood cell is a small subunit encoding 1, 5-ribulose bisphosphate carboxylase/oxygenase (Rubisco). It could control the catalytic activity of Rubisco and play an important role in plant photosynthesis. SikRbcs2, a small subunit of Rubisco, is cloned from Saussurea involucrata. It has a strong low-temperature photosynthetic and photorespiration ability, but its mechanism in cold tolerance remains to be unknown. The results of quantitative PCR showed that SikRbcs2 gene could be induced by low-temperature, osmosis, and salt stress. Its expression was increased with the decrease of temperature, which was consistent with the habitat of Saussurea involucrata. Overexpression of SikRbcs2 could significantly increase the mRNA expressions of SlrbcL and SlRCA in transgenic tomato seedlings. Furthermore, the activity and content of Rubisco and Rubisco activase (RCA) in transgenic tomato seedlings were also significantly higher than those in wild-type plants. The contents of chlorophyll and carotenoids, soluble sugar, and starch in the leaves of transgenic plants were significantly higher than those in WT plants, as well as the plant height, leaf area, and dry matter weight. Moreover, compared with WT, MDA content was decreased, and activities of SOD, POD, CAT, and APX were significantly higher in transgenic lines. In conclusion, our results suggested that overexpression of SikRbcs2 can reduce the damage of low temperature on photosynthesis of tomato seedlings. It could help achieve relatively stable photosynthesis, enhance scavenging ROS ability of tomato seedlings, maintain stable membrane structure, and improve cold tolerance of tomato.

KEYWORDS
chlorophyll fluorescence, low-temperature tolerance, photosynthesis, Saussurea involucrata, SikRbcs2, tomato

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1  INTRODUCTION

1. 5-ribulose bisphosphate carboxylase/oxygenase (Rubisco) is at the circular intersection of plant photosynthetic carbon reduction and photosynthetic carbon oxidation (Portis 1990, 2003). As a key enzyme of photosynthetic carbon assimilation, it has a decisive effect on net photosynthetic rates (Andersson & Backlund, 2008; Spreitzer & Salvucci, 2002). Rubisco is made up of eight large subunits (RbcL) and eight small subunits (RbcS). RbcL plays a catalytic role while RbcS has the function of regulating Rubisco activity (Portis, Li, Wang, & Salvucci, 2007). Previous studies found that RbcS gene was suitable for the study of plant molecular evolution and phylogeny (Feller, Crafts-Brandner, & Salvucci, 1998; Sage, Way, & Kubien, 2008). RbcS gene promoter is an important tool for the specific expression of exogenous genes in the leaves of recipient plants in genetic engineering (Suzuki et al., 2007). RbcS is essential to the structure of enzyme activity, which plays an important role in the ratio of shuttle to oxidation (Whitney & Andrews, 2001). In addition, it can improve the photosynthetic capacity of recipient plants through transplanting exogenous RbcS genes into recipient plants or enhancing the gene expression of RbcS (Furbank, Chitty, von Caemmerer, & Jenkins, 1996). This exogenous RbcS gene may also be involved in the plant's antisignaling system and play an important role in growth, maturation, and senescence of plants. RbcL of spinach Rubisco has been splicing with the transporter genes of RbcS of tomato (Lam & Zechman, 2006; Van Oosten & Besford, 1994). This chimeric gene has been integrated into tobacco, so that the transgenic tobacco can grow normally. Transgenic Arabidopsis thaliana only expresses RbcS, and the expression of RbcS in the transgenic tobacco can grow normally. Transgenic Arabidopsis thaliana is significantly higher than WT. Rubisco activity and Ru-BP content would not decrease under low light intensity.

Tomatoes (Lycopersicon esculentum) belong to cold sensitive plant. In the winter, they often encounter low-temperature stress, resulting in the decrease of photosynthesis, and the decrease of yield and quality (Zhang et al., 2011). Rubisco is a speed-limiting enzyme for photosynthesis (Ngemprasirtsri, Kobayashi, & Akazawa, 1988). RbcS, which regulates the expression of RbcL, is an essential part of enzyme activity structure (Fritz, Wolter, Schenkemeyer, Herget, & Schreier, 1993). Therefore, cloning and genetic transformation of RbcS gene are of great significances to explore the effect of RbcS overexpression on growth, development, and photosynthesis of tomato at molecular level. Saussurea involucrata has response and adaptation mechanism to low-temperature stress and improves photosynthetic capacity at low temperature (Wang et al., 2007). However, studies on the role of Rubisco in Saussurea involucrata and its regulation mechanism of photosynthesis have not been reported. Therefore, the purpose of this study was to investigate the adaption mechanism of tomatoes to low-temperature stress with the assistance of SikRbcs2.

2  MATERIALS AND METHODS

2.1  Materials

Saussurea involucrata plants were cultured in our laboratory at 21°C/19°C, with a photoperiod of 16 hr light/8 hr dark and a light intensity of 13 K lux. Tomato (Lycopersicon esculentum) variety was Yaxin 87-5. Genetic transformation receptor in this study was provided by Yaxin Seed Co. Ltd.

S. involucratas seedlings were kept in a 20°C light incubator for 3 days and kept in a 20°C dark incubator for another 3 days. Then, S. involucratas seedlings were transferred to the dark for 24 hr. S. involucratas seedlings, which continued to be dark for 3 days, were also transferred to the light for 24 hr. For cold treatment, the S. involucratas seedlings were treated at 20°C were used as control. S. involucratas seedlings were treated at different temperatures in the order of 10°C, 4°C, and −2°C for 6 hr each time. S. involucratas tissue culture seedlings were immersed in 20% PEG and 150 mmol NaCl for 24 hr. The leaves of S. involucratas seedlings were treated at different times, frozen with liquid nitrogen for 5 min, and then stored at −70°C.

WT tomato and those two transgenic tomatoes growing at the age of 2 months were transferred into an artificial climate chamber (light intensity, 70 µmol/m²/s; temperature, 25°C/25°C; photoperiod, 12 hr/12 hr; relative humidity, 60%-70%). After 2 days of adaptation, they were treated by low-temperature stress with temperature gradient of 16°C, 8°C, and 4°C for 5 days and treated at 4°C for 7 days. The experiment was repeated 3 times, with 20 nontransgenic and transgenic tomato plants each time.

2.2  Isolation of full-length SikRbcS2 cDNA

A full-length cDNA library from S. involucrata was constructed using the Creator™ SMART™ cDNA Library Construction Kit (Clontech). Ninety-six single clones were randomly selected and sequenced. One cDNA sequence showed homology to a gene which encoded the Rubisco RbcS gene. This cDNA sequence was named SikRbcS2. The 5′ end, CDS region, and 3′ end of SikRbcS2 gene by prokaryotic expression (data not shown). A phylogenetic tree was plotted using DNAMAN and MEGA4.1 software based on amino acid sequences from the NCBI database.

2.3  RNA extraction and RT-qPCR analysis

Total RNA was isolated from different treatment and control samples separately using RNAiso Plus kit (TaKaRa) with on-column DNase I treatment. According to the S. involucrata GAPDH (accession no. KF563904.1), the reference gene, primers GAPDHF1...
and GAPDH R1. Primers SikRbcS2F1 and SikRbcS2R2 were designed based on the SikRbcS2 gene sequence (Table 1). Using reverse-transcribed cDNA as a template, GAPDH F1, GAPDH R1 and SikRbcS2F1, and SikRbcS2R2 were used as primers following the instructions of the SYBR Green I Master Mix kit. Amplification was performed using a Light Cycler® 480 I I PCR machine (Roche). Each sample was repeated 3 times, and the data were analyzed by the $2^{-\Delta \Delta \text{CT}}$ method.

2.4 | Plasmid construction and tomato transformation

To obtain the Pro35S: SikRbcS2 construct, SikRbcS2 cDNA was amplified using the forward primer 5′-CCATGGAGTTATCAGTCGAC GGTAACCCGCAAGTGTTCTAAGAC-3′ (underline indicates NcoI) and the reverse primer 5′-GGTAACCCGCAAGTGTTCTAAGAC-3′ (underline indicates BstEII). The PCR product was digested with NcoI and BstEII and ligated into a pCAMBIA1301 vector double digested with the same enzymes. To obtain the ProRD29A: SikRbcS2 construct, the RD29A promoter was first amplified using the forward primer 5′-AAGCTTCGACTCAAAACAA ACTTACGAA-3′ (underline indicates HindIII site) and the reverse primer 5′-CCATGGGAATCCACCTTATTCTGA-3′ (underline indicates NcoI site), and was cloned into the pCAMBIA1301 vector. The construct was then digested with NcoI and BstEII and ligated to the SikRbcS2 PCR product digested with the same enzymes. The identity of clone insert was confirmed by sequencing. These two constructs were introduced into Lycopersicon esculentum WT plants (Yaxin 87-5) via Agrobacterium tumefaciens-mediated (strain GV3101) T-DNA transformation. Transfomers were selected on MS medium (Sigma-Aldrich) containing 80 µg/l of kanamycin and then transferred to soil to set seeds. Kanamycin-resistant T1 seedlings were confirmed by RT-PCR using primers of SikRbcS2. Almost all seedlings from independent transgenic T1 lines were survived with MS medium containing 80 mg/L kanamycin. These were then transferred to soil to set seed. The T2 generation transgenic seeds were germinated in flower pots at 25°C in 60%-70% humidity, with a light intensity of 70 µmol/m²/s and a 12 hr light/12 hr dark cycle.

2.6 | Measurement of chlorophyll pigment content

Chlorophyll pigment content was measured according to the following procedure. Leaves of WT tobacco and transgenic tobacco (0.1 g) were obtained after treating by different temperatures, respectively. Leaves were cut into filaments of 1 mm wide and added to test tubes. 15 ml of acetone ethanol mixture (V: V = 1:1) was then added, and the extraction was performed in dark until the filaments were completely white at room temperature. The supernatant was mixed and used to test absorbance values at 470, 645, and 663 nm, respectively. The contents of chlorophyll a (Chla), chlorophyll b (Chlb), and carotenoids (Car) were calculated according to those values.

2.7 | Determination of growth index of tomato overexpressing SikRbcs2 gene

Leaf growth index (PI) was calculated by PI = n + (In n-ln R)/ (In Ln-ln Ln + 1). The reference leaf length R is 180 mm, n is the number of leaves whose blade length is longer than the reference leaf length, and In Ln and ln Ln + 1 are the lengths of the nth and n + 1 leaves, respectively. Plant height was measured with a ruler, stem was measured with a vernier caliper, and fresh weight and dry weight were measured with an electronic balance. They were measured before treatment, after 12 days of continuous treatment at low-temperature gradient, and after 14 days of recovery.

2.8 | Measurement of physiological indices

The malondialdehyde (MDA) content was determined by the modified thiobarbituric acid reaction outlined by Du et al. using a spectrophotometer (UV-160A, Shimadzu Scientific Instruments). Briefly, leaves excised from the tomato plants were washed in deionized water. Leaf disks were punched out, and membrane damage was quantified by measuring MDA concentration. Relative electrolyte leakage (REL) was determined by using an EC 215 Conductivity Meter (Markson Science Inc.) with the method of Du et al. The youngest fully expanded leaves were randomly selected and subjected to electrolyte leakage analysis using conductivity meter. The relative
conductance was calculated using the formula: \( \text{REL} = \frac{(C1 - CW)}{(C2 - CW)} \times 100 \). \( C1 \) is the electrical conductivity value during the first measurement, \( C2 \) is the conductivity value after boiling, and \( CW \) is the conductivity of deionized water.

2.9 | Antioxidant enzymes and reactive oxygen species

After exposure to cold and drought treatments, 0.5 g samples of fresh \( T_{2} \) WT and transgenic tomato plant leaves were collected. These leaves were cut into pieces and homogenized in an ice bath with 4 ml of 50 mmol/L sodium phosphate buffer (pH 7.8) containing 1% polyvinylpyrrolidone and 10 mmol/L \( \beta \)-mercaptoethanol. The homogenate was transferred to a tube and centrifuged (18,894.2 \( g \)) for 15 min at 4°C. The supernatant fluid was then used for the determination of enzyme activity. Ascorbate peroxidase (APX) activity was determined as the decrease in absorbance of ascorbate at 290 nm. The activity of catalase (CAT) was determined. We assessed the activity of superoxide dismutase (SOD) with the light absorption value at 560 nm. Peroxidase (POX) activity was determined. Absorbance was recorded with an Infinite M200 Pro microplate reader (Tecan Group Ltd., Männedorf).

\( H_{2}O_{2} \) and \( O_{2}^- \) contents were determined according to a standard curve. Absorption values were recorded at 415 and 530 nm using a spectrophotometer (UV-160A, Shimadzu Scientific Instruments).

2.10 | Determination of Rubisco and Rubisco Activase (RCA)

Plant activating enzyme ELISA kit (Ta Ka Ra) and plant ribulose-1,5-bisphosphate carboxylase/oxygenase ELISA kit (Ta Ka Ra) adopted following the instructions. The absorbance was measured at 450 nm using a microplate reader (M200pro). Sample RCA and Rubisco concentration were calculated by standard curve.

3 | RESULTS

3.1 | Isolation and characterization of full-length SikRbcS2 cDNA in \textit{S. involucrata}

Full-length libraries were constructed using the Switching Mechanism at the 5' end of RNA Transcript (SMART) method. Facilitate the preliminary mapping of transcription start sites due to the high percentage of full-length clones. Previously, we selected ten clones for sequencing, all of which were revealed to be full-length cDNAs. To further evaluate the quality of our libraries and identify interest genes, ninety-six randomly selected single clones were sequenced, from which the full-length SikRbcS2 gene was obtained and subsequently confirmed by sequencing. According to DNAMAN (http://us.expasy.org/tools/protparam.html), the full-length cDNA of the SikRbcS2 gene is 904 bp in length and it possesses a 420 bp open reading frame (ORF). Analysis using transmembrane helix prediction (TMpred) revealed no transmembrane helices in the deduced SikRbcS2 protein sequence, suggesting that the SikRbcS2 protein may have no significant role in the membrane and it must play a role in other places (e.g., in the cytosol or nucleus). Homology analysis of the deduced protein sequence was then performed using Phyre (http://www.sbg.bio.ic.ac.uk/phyre/). Next, we discovered that 139 amino acid residues of the SikRbcS2 protein were highly similar (61.45% identity) with a protein from \textit{Cynara cardunculus var. scolymus} by using NCBI BLASTP (Figure 1a). As shown in the phylogenetic tree (constructed using MEGA), the SikRbcS2 protein shared the closest genetic relationship with an RbcS protein from \textit{Cynara cardunculus var. scolymus} (Figure 1b).

3.2 | Analysis of expression pattern of SikRbcS2 in \textit{S. involucrata} under different conditions

In order to study the function of promoter, the \textit{S. involucrata} seedlings were treated with light, low-temperature, simulated drought, and salt stress according to the relevant functional elements of the sequence. And tissue expression specificity of SikRbcS2 in the seedlings was detected. Materials treated under different conditions were collected to extract their total RNA, and the relative expression levels of SikRbcS2 in different treatments were analyzed by RT-qPCR. The results showed that the expression of SikRbcS2 in \textit{S. involucrata} seedlings growing for 3d under continuous white light was significantly higher than that growing for 3d under continuous dark by 6.28 times (Figure 2a), which suggested that light may regulate SikRbcS2 expression. SikRbcS2 expression increased rapidly and continuously after seedlings which grow in darkness transferring to light. After 3 hr of illumination, the expression of \textit{SikRbcS2} was increased approximately 1.79 times as much as that in darkness. After 24 hr of illumination, the expression of \textit{SikRbcS2} was increased approximately 4 times as much as that in darkness (Figure 2b). However, the expression of SikRbcS2 was decreased rapidly after seedlings transferred to dark condition. After 3 hr of dark treatment, the expression of SikRbcS2 was reduced to about 0.4 under light condition. During the whole process of white light turning into darkness, the expression of SikRbcS2 showed an overall downward trend. After 24 hr of dark treatment, its expression was approximately reduced to 0.13 under light conditions (Figure 2c). These results suggested that light promoted the transcription of SikRbcS2, while darkness inhibited its transcription and was sensitive to light condition transformation.

Under long sunshine, the expression of SikRbcS2 was increased rapidly after illumination, while that was decreased rapidly after darkness. In 24 hr, the expression of SikRbcS2 in daytime was generally higher than that in night (Figure 2d). Light intensity often changes under natural light conditions, so changes in SikRbcS2 expression levels under different light intensity were further detected. The results showed that in the selected light intensity ranges, the expression levels of SikRbcS2 were increased significantly with the increase
of light intensity (Figure 2e). In addition, SikRbcS2 expression had tissue expression specificity. The expression of SikRbcS2 in the over ground part of S. involucrata was significantly higher than that in the underground part. In addition, the expression of SikRbcS2 in above ground organs under long sunlight was also significantly higher than that in darkness (Figure 2f). The above results showed that SikRbcS2 was highly expressed by light and could change with the physiological rhythm of plants, the tissue expression was specific.

The S. involucrata was treated at different temperatures. The results showed that, with the decrease of stress temperature, the expression of SikrbcS2 in seedling leaves of S. involucrata was increased gradually, which was 3.5 times higher than the control level at 8°C, and reached the highest level at 4°C with 5.2 times higher than the control level. Subsequently, the expression of SikrbcS2 gene was decreased at 0°C, but it was still higher than the control level (Figure 2g). Under the cold treatment condition of 4°C, the
expression of SikRbcS2 gene was increased rapidly, reaching the highest level at 12 hr with 6.4 times higher than the control group (Figure 2h). This indicated that the expression of SikRbcS2 gene was induced by low temperature. After soaking S. involucrata in 20% PEG6000 and 150 mmol NaCl for 24 hr, it was found that the expression of SikRbcS2 gene was increased rapidly under simulated drought conditions. However, it was decreased after 3 hr and increased slowly after 12 hr (Figure 2i). Furthermore, the response to salt stress was different. Its expression reached the highest level at 3 hr and then was decreased rapidly (Figure 2j). These results indicated that the expression of SikRbcS2 gene responded to low temperature, drought, and salt stress. This was consistent with the relevant functional elements of the promoter sequence.

3.3 | Generation of transgenic tomato plants expressing SikRbcS2

15 strains of 35S::SikRbcS2 and 12 strains of RD29A::SikRbcS2 independent kanamycin-resistant transgenic plants (T0 generation) were established, and PCR analysis confirmed that SikRbcs2 gene was successfully introduced into tomato plants. 8 transgenic plants transfected with 35S::SikRbcs2 and 7 RD29A::SikRbcs2 were obtained by PCR amplification using SikRbcs2 primers. Among these transgenic plants, 4 strains had kanamycin resistance, respectively, with 3:1 of separation ratio in the T1 generation (Figure 3a). These 8 strains were selected for RT-PCR and qRT-PCR analysis. Compared with WT tomato, the relative mRNA levels of SikRbcs2 of transgenic plants S3 and R4 were 2.3 and 2.1 times than selected these two functional analyses, respectively (Figure 3b).

3.4 | Effect of Rubisco and Rubisco activate on SikRbcS2-overexpression tomato and WT tomato at low temperature

Before low-temperature stress, the relative mRNA expression level of SikRbcs in 35s transgenic tomato was 52% higher than that in rd29a transgenic tomato (Figure 4a). With the decrease of temperature, the mRNA expression level of 35s transgenic changed little, but the expression level of rd29a transgenic gene was increased significantly, this indicated that the obtained transgenic lines were stable and reliable. Rubisco, a key enzyme in the Calvin cycle, consists of two subunits, RbcL and RbcS. It can be seen from Figure 4b and c that the overexpression of SikRbcs can significantly increase the mRNA expressions of RbcL in transgenic plants of 35s and rd29a. The mRNA expression levels of RbcL in 35s and rd29a were increased by 17% and 5%, respectively, in comparison with WT. Before low-temperature stress and after low-temperature stress, the mRNA expression levels of RbcL in each treatment were significantly decreased.
mRNA expression of 35s and rd29a was increased by 57% and 0.7%, respectively, before low-temperature stress. After low-temperature stress, mRNA expression levels of RCA in all treatments were significantly decreased. However, the mRNA expression levels of 35s and rd29a were still significantly higher than WT. As shown in Figure 4h and i, the changes in the activity of transgenic RCA were consistent with the changes in the relative mRNA expression levels of the corresponding RCA.

3.5 | Effect of low temperature on chlorophyll fluorescence and photosynthetic parameters in WT and transgenic seedlings

Low-temperature stress was found to lead to significant decreases in Pn, but the extent of decrease varied among treatments. After treatment at 4°C for 5d, Pn was decreased by 79% and 75% in 35S:SikRbcS2 and RD29A:SikRbcS2, respectively, and decreased by 89% in WT plants. Afterward, Pn was continually decreased in both transgenic and WT lines when the duration of the exposure to low-temperature stress was increased, but the decrease in Pn was more obvious in WT than in transgenic plants (Figure 5a). The Gs and Tr values of transgenic and WT leaves were found to both decrease in response to longer and more severe low-temperature stress, whereas Ci values were increased as the time under stress increased. This suggested that the decrease in Pn under low-temperature stress may be associated with nonstomatal limitation. Compared to WT plants, the transgenic plants showed greater Gs and Tr values, but lower Ci values during low-temperature stress. These results suggested that the overexpression of SikRbcS2 played a significant role in alleviating injuries to the photosynthetic apparatus caused by low temperatures, ostensibly by maintaining higher photosynthetic activity in tomato seedling mesophyll cells (Figure 5b–d).

3.6 | The effect of low temperature on chlorophyll pigment content in WT and transgenic seedlings

Next, we measured the content of chlorophyll a, chlorophyll b, and carotenoids, three major pigments involved in plant photosynthesis. We found that relative to WT seedlings, seedlings from the two transgenic lines of tomato showed lower reductions in chlorophyll content in response to low-temperature stress treatments (Figure 6a–c). This finding was consistent with the morphology of these plants. Taken together, this finding suggested that transgenic tomato plants expressing SikRbcS2 had better low-temperature stress resistance than WT tomato plants. We found no significant changes in the chlorophyll a/b (Chla/Chlb) ratio among the WT and transgenic seedlings (Figure 6d). Moreover, the carotenoid (Car) content of WT tomato seedlings also fell sharply (i.e., at 4°C it had fallen by 64% compared to pretreatment levels). In contrast, the carotenoid levels of 35S:SikRbcS2 tomatoes fell slowly from 25°C to 8°C and then rose again at 4°C. A similar pattern was found with RD29A:SikRbcS2 seedlings. The decline in photosynthetic pigments...
contents meant that at low temperatures, the photosynthetic capacity of the transgenic tomato lines was higher than that of WT tomato seedlings (Figure 6e).

### 3.7 Physiological analysis and accumulation of ROS in wild-type and SikRbcS2-overexpressing transgenic tomato plant lines under cold stress

Under normal conditions, there was no significant difference in REL and MDA contents between transgenic and WT plants. With the decrease of temperature, the REL and MDA contents of leaves of both WT and transgenic plants were gradually increased, but the content of transgenic plants was smaller than that of WT plants (Figure 7a and b). These results showed that the overexpression of SikRbcS2 reduced the damage of low-temperature stress to plant cell membrane. We examined the levels of H$_2$O$_2$ and O$_2^-$ in the leaves of plants. Under normal growth conditions, transgenic plants were the same as WT plants. There was very little accumulation of H$_2$O$_2$ and O$_2^-$ in the leaves. As the treatment temperature decreased, the accumulation of H$_2$O$_2$ and O$_2^-$ in the leaves of transgenic plants and WT plants were increased. Compared with WT, the accumulation of H$_2$O$_2$ and O$_2^-$ in the leaves of transgenic lines was significantly less (Figure 7c and d). Excessive accumulation of ROS in the leaves of WT plants will cause oxidative stress, cell damage, and even death.

### 4 DISCUSSION

Our analysis of chloroplast pigment content found that the contents of Chla, Chlb, and Chla + Chlb of the transgenic SikRbcS2-carrying tomato lines were significantly higher than those of WT tomato, while the Chla/Chlb values of all lines did not significantly differ. These results showed that transgenic tomato was more tolerant to low-temperature stress. At the same time, the carotene content of the transgenic tomato lines was significantly higher than that of WT tomato when all plants were subjected to low-temperature stress treatments. This pigment is related to heat dissipation and can help plants to dissipate extra light energy to resist damage caused by light. The presence of higher carotene content in the transgenic tomato lines indicated that these lines had better photosynthetic protection and maintained higher linear electron transport capacity at low temperatures.

Chlorophyll fluorescence parameters reflect light energy absorption and the light utilization of plant leaves (Maxwell & Johnson, 2000). The photosynthetic pigments in the optical
A system can be destroyed during exposure to low temperatures, resulting in decreased photo-absorption capacity of the optical system (PSI and PSII) and eventually to decreased photosynthetic capacity (Kooten & Snel, 1990). The light energy absorbed by chlorophyll is converted into chlorophyll fluorescence. The measurement of chlorophyll fluorescence is an effective alternative.
to the measurement of light absorption capacity of plant leaves under adversity (Krause & Weis, 1984). The effects of low-temperature stress on photosynthesis in plants are multifaceted and involve not only directly causing damage to the photosynthetic apparatus, but also affecting the enzymes involved in photosynthetic electron transport, photosynthetic phosphorylation, and dark reactions (Schreiber, Bilger, & Neubauer, 1995). In this study, we found that under low-temperature stress, the fixed fluorescence F0 of chlorophyll was increased. We also found that ERF and the ratios of Fv/Fo and Fv/Fm were significantly reduced. Taken together, these data suggested that the PSII reaction center was inactivated. Low-temperature stress reduced the ability of plants to utilize light energy. The analysis of chlorophyll fluorescence parameters showed that, in response to low-temperature stress conditions, SikRbcS2-carrying transgenic lines of tomato showed greater leaf PSII photochemical efficiency (Fv/Fm) and PSII activity (Fv/Fo) than WT tomato plants. We also found that transgenic tomato plants showed gradual decreases in qP and ETR values, and at each temperature point, the transgenic lines’ values were significantly higher than those of WT tomatoes. Taken together, these results indicated that transgenic tomatoes can maintain higher photosynthetic electron transfer efficiency and generate a greater isomerization force to meet the demand of dark reaction processes. The reduction in Pn caused by abiotic stress is attributed to an inhibition of Rubisco activity. All tomato lines showed that reduced Pn as the low-temperature stress treatments became more extreme. At 16°C and 4°C, we found that the Pn values of the transgenic tomatoes were significantly higher than those of the WT tomatoes. These results suggested that the photosynthetic mechanisms of the transgenic tomatoes were less damaged by exposure to low-temperature treatments than those of the WT tomatoes.

During photosynthesis, stomata are windows for the exchange of gases, including H₂O and CO₂ (Farquhar & Sharkey, 1982; Wong,

| Regulatory sequence | Sequence | Biological function |
|---------------------|----------|---------------------|
| A-box               | CCGTCC   | cis-acting regulatory element |
| AE-box              | AGAAACAA | Part of a module for light response |
| ATCT-motif          | AATCTAATCT | Part of a conserved DNA module involved in light responsiveness |
| AuxRE               | TGCTCTAATAAG | Part of an auxin-responsive element |
| CAT-box             | GCCACT   | cis-acting regulatory element related to meristem expression |
| CCGTCC-box          | CCGTCC   | cis-acting regulatory element related to meristem specific activation |
| GA-motif            | AAAGATGA | Part of a light responsive element |
| GAG-motif           | AGAGAG/T | Part of a light responsive element |
| HSE                 | AGAAAAATTG | cis-acting element involved in heat stress responsiveness |
| I-box               | GATATGG  | Part of a light responsive element |
| MBS                 | T/CAACTG | MYB binding site involved in drought-inducibility |
| O2-site             | GATGATGTTG | cis-acting regulatory element involved in zein metabolism regulation |
| P-box               | GCATTTTGAT | Gibberellin-responsive element |
| Skn_1_motif         | GTCAT    | cis-acting regulatory element required for endosperm expression |
| TATA-box            | TATA     | Core promoter element around -30 of transcription start |
| TC-rich repeats     | ATTCTCTAAC | cis-acting element involved in defense and stress responsiveness |
| TGACG-motif         | TGACG    | cis-acting regulatory element involved in the MeJA-responsiveness |
| ABRE                | CCTACGTGTC | cis-acting element involved in the abscisic acid responsiveness |
| ARE                 | TGGTTT   | cis-acting regulatory element essential for the anaerobic induction |
| LER                 | CCGAAA   | cis-acting element involved in low-temperature responsiveness |
| ELI-box3            | AAACCAATT | Elicitor-responsive element |
| G-Box               | CACGTT   | cis-acting regulatory element involved in light responsiveness |
| GARE-motif          | AAACAAGA | Gibberellin-responsive element |
| GC-motif            | CCCCCCG | Enhancer-like element involved in anoxic specific inducibility |
| GCN4_motif          | TGTGTCA  | cis-regulatory element involved in endosperm expression |
| Sp1                 | GGGGGG   | Light responsive element |
| TCT-motif           | TCTTAC   | Part of a light responsive element |
| TGA-element         | AACGAC   | Auxin-responsive element |
| Circadian           | CAANNNNATC | cis-acting regulatory element involved in circadian control |
The change of stomatal conductance to CO\textsubscript{2} will cause changes in intercellular CO\textsubscript{2} concentration (Ci), which can thereby affect the photosynthetic rate (Ainsworth & Rogers, 2007). Low temperatures may decrease the photosynthetic rate by reducing stomatal conductance (Gs) and restricting the entry of external CO\textsubscript{2} into cellular spaces via stomata (Delucia, 1986). We found that all tomato lines showed reduced GS as the low-temperature stress treatments became more extreme. Between 25°C and 8°C, we found no significant differences between WT and transgenic tomato lines with respecting to Ci. However, at 4°C, the intercellular CO\textsubscript{2} concentration fell sharply. This indicated that under low-temperature stress, inhibition of stomatal system affected the plant absorption of CO\textsubscript{2} and thus inhibited photosynthesis. We showed that the transpiration rate of transgenic tomatoes decreased as the stress treatments reached lower temperatures.

When plants are under stress, reductions in the net photosynthetic rate are often caused by two main factors, stomatal and nonstomatal factors (Augé, Toler, & Saxton, 2015; Nilsen & Orcutt, 1996). When the closure of stoma causes reduction of intercellular CO\textsubscript{2} concentration, the result is a reduced net photosynthetic rate (Radin, Parker, & Guinn, 1982). However, when the net photosynthetic rate and stomatal conductance are decreased together, the intercellular CO\textsubscript{2} concentration is increased, that is, the reduction of net photosynthetic rate was likely caused by nonstomatal factors. In this experiment, the net photosynthetic rate was decreased due to stomatal closure, which led to reductions in intercellular CO\textsubscript{2} concentration, thereby reducing the photosynthetic capacity. Thus, we concluded that the reduced photosynthetic capacity was caused by stomatal factors.

The RD29A promoter is cold-induced. In this experiment, cold-induced expression of SikRbcS2 genes in tomato plants subjected to low-temperature stress treatments (Kasuga, Miura, Shinozaki, & Yamaguchi-Shinozaki, 2004). It resulted in the values of Fv/Fm, ETR, qP, and qN being similar to those of the transgenic lines which constitutively expressed SikRbcS2. Here, we observed that the SikRbcS2 gene notably improved the low-temperature stress response of tomato plants. However, whether this effect is due to changes in the structure or function of Rubisco is not clear, and it should be a focal point of future studies of SikRbcS2.

**TABLE 3** The effect of low-temperature stress on the fresh (FW) and dry weights (DW) of shoots and roots of WT and SikRbcS2 tomato plants

| Treatment | 25°C | 16°C | 8°C | 4°C |
|-----------|------|------|-----|-----|
| Shoot     |      |      |     |     |
| WT        | 28.96 ± 2.78 | 29.76 ± 1.32 | 28.06 ± 3.12 | 23.26 ± 2.83 |
| 3SS:SikRbcS2 | 34.42 ± 1.79 | 35.82 ± 2.39 | 35.87 ± 1.81 | 35.87 ± 3.12 |
| RD29A:SikRbcS2 | 31.89 ± 3.0 | 35.69 ± 2.95 | 33.71 ± 2.92 | 31.51 ± 2.09 |
| Root      |      |      |     |     |
| WT        | 6.28 ± 1.33  | 7.18 ± 0.65  | 7.14 ± 0.24  | 4.84 ± 0.29  |
| 3SS:SikRbcS2 | 6.7 ± 0.37    | 7.9 ± 0.73    | 7.92 ± 0.34  | 6.02 ± 0.24  |
| RD29A:SikRbcS2 | 6.6 ± 0.16    | 8.11 ± 0.30   | 8.1 ± 0.35   | 6.1 ± 0.23   |

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