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Full Length Article

Exogenous Calcium Treatments Improve Growth, Photosynthesis and Resistance of Tobacco Seedlings under Low Light Stress and Transcriptome Analysis

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Abstract

Many studies have shown that calcium can alleviate abiotic stress effects in plants. To determine the effect of exogenous calcium ions (Ca^{2+}) on the resistance of tobacco seedlings to low light stress, exogenous Ca^{2+} ($CaCl_2$) was applied by spraying or as a nutrient solution. The Ca^{2+} treatments increased the soluble protein content and antioxidant enzyme activities, decreased the contents of free proline and malondialdehyde (MDA), and inhibited the production of superoxide anions ($O_2^{\cdot-}$). Spraying with $CaCl_2$ at 20 m*M* had the strongest effect. Spraying with 20 m*M* $CaCl_2$ significantly increased the plant height, stem girth, main root length, leaf area, fresh weight, and SPAD value of tobacco seedlings under low light intensity. The Ca^{2+} treatments resulted in significantly increased net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate of leaves of tobacco seedlings under low light intensity, while spraying with the Ca^{2+}-specific chelating agent EGTA at 10 m*M* had the opposite effect. The Ca^{2+} treatments were able to alleviate many of the adverse effects of low light stress on tobacco seedlings. A comparative RNA-Seq transcriptome analysis between tobacco plants with and without Ca^{2+} treatment under low light revealed 13233 differentially expressed genes (DEGs), of which 7269 were up-regulated and 5964 were down-regulated by the Ca^{2+} treatment. The DEGs were divided into 15 biological processes and 14 molecular functions, and the effects of Ca^{2+} treatment of tobacco seedlings under low light stress were regulated by DEGs in several enriched pathways. More than 80 up-regulated DEGs were involved in secondary metabolite synthesis, and the most enriched pathways were ribosome biogenesis and galactose metabolism. Down-regulated genes encoded components of photosystems I and II. These results showed that Ca^{2+} affects many genes and pathways to promote the growth of tobacco under low light stress.

Keywords: Tobacco; Calcium; Low light stress; Resistance; Transcriptome

Introduction

The effect of light signal on plants is mainly reflected in photosynthesis (Vialet-Chabrand *et al.* 2017; Wei *et al.* 2018). Low light stress can increase the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in plants' antioxidant enzyme systems, and significantly affect free proline and malondialdehyde (MDA) contents (Iriti and Faoro 2009). Soluble protein and free proline contents in plants often change in response to environmental stress. Free proline is a cell osmoregulation substance that regulates a series of metabolic responses under adverse environmental conditions (Zhang *et al.* 2015). Under stress, an increase in lipid peroxidation in plant cell membranes can lead to the accumulation of MDA and cell damage. Protective enzymes such as CAT, POD, and SOD can eliminate reactive oxygen species (ROS) and free radicals, and are closely related to plant stress resistance (Sridevi and Chellamuthu 2015). In rice, the extent of decrease in the photosynthetic rate of leaves under low light stress was found to reflect differences in tolerance among varieties (Wang *et al.* 2016).

Chemical controls are often used in agricultural production. Many exogenous substances can support and/or promote physiological and biochemical processes such as photosynthesis, the transport and distribution of photosynthetic products, nutrient absorption, endogenous hormone contents, and the activity of protective enzymes (Batool *et al.* 2020). Many studies have shown that calcium ions (Ca^{2+}) contribute to the structure of plant cells, and function to stabilise and protect the structure and function of the cell plasma membrane. Calcium is a second messenger in plants, and it forms bridge between phosphate and carboxyl groups in cell membrane proteins, thereby regulating the cellular response of plants to environmental changes. Changes in environmental factors often lead to an

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increase in free Ca^{2+} activity in the cytoplasm, which changes the activity of protein kinases *in vivo*, induces the expression of related genes and/or regulates the activity of H^+-ATPases (Mclanughlin and Wimmer 1999; Romeis *et al.* 2001; Cheng *et al.* 2002). There is also evidence that Ca^{2+} can improve many kinds of resistance in plants, including resistance to cold, heat, drought, salt and disease (Kinoshita *et al.* 1995; Chen *et al.* 2004; Mahajan *et al.* 2008; Iwadate and Nakaoka 2008).

Research on the stress resistance of tobacco ranges from germplasm selection, to physiological and biochemical studies, to research at the molecular level (Schmidt and Delaney 2010; Harada *et al.* 2010). Although previous studies have explored the molecular mechanism of tobacco resistance to low temperature, drought and disease (Yin *et al.* 2015; Duan *et al.* 2016; Jin *et al.* 2017), none has focused on the mechanism by which exogenous Ca^{2+} regulates resistance to low light. Thus, the objectives of the present investigation were (1) to identify the effects of calcium on low light stress, and (2) to elucidate the transcriptome information. All these will provide important insight into plant growth under abiotic stress.

Materials and Methods

Experimental details and treatments

Experimental material: Tobacco (*Nicotiana tabacum* cv. Yunyan NO. 87) seedlings were grown in an artificial climate room on 16×10 point floating plates. Each treatment was repeated with three floating plates (160 seedlings per plate). The culture conditions were: 14-h day/10-h night cycle, 25°C days and 20°C nights, 80% relative humidity. Treatments started on the 16th day after cotyledon emergence.

Treatments: To determine the optimal application mode and concentration of exogenous Ca^{2+}, seedlings were treated with foliar sprays (SC) or nutrient solutions (SN) containing Ca^{2+} at different concentrations. The Ca^{2+}-specific chelating agent EGTA, which reduces the activity of endogenous Ca^{2+}, was also supplied to verify the role of Ca^{2+}. The control had a light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$, while the treatments were as follows: S (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$), SE10 (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 10 m*M* EGTA spray), SN20 (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 20 m*M* $CaCl_2$ in nutrient solution), SN30 (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 30 m*M* $CaCl_2$ in nutrient solution), SC10 (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 10 m*M* $CaCl_2$), SC20 (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ + 20 m*M* $CaCl_2$). Spraying onto the abaxial and adaxial surfaces of tobacco leaves was conducted at 8:00 AM.

Plant harvesting

Samples were collected from the control and the S, SC, and SN treatments on the 9th day after treatments to evaluate seedling growth and development, lipid peroxidation, antioxidant enzyme activity, and gene expression (by RNA-Seq transcriptome and qRT-PCR analyses). The gas exchange and transpiration rates were measured in seedlings in the control and the S, SC20, and SE10 treatments on the 9th and 12th day after treatments.

Determination of tobacco seedling growth and development

At harvest, the substrate was removed from the roots of tobacco seedlings, and then the roots were washed with water and dried on absorbent filter paper. Vernier calliper was used to measure stem girth, while straightedge was used to measure plant height and main root length. An analytical balance was used to measure fresh weight after removing the roots. The leaf area was determined by a YMJ-B scanning living leaf area meter (Zhejiang Top Instrument Co., Ltd., Zhejiang, China). The leaf SPAD value was measured with a SPAD-502 PLUS chlorophyll meter (Konica Minolta Sensing, Osaka, Japan), which provides a rapid estimate of leaf chlorophyll content. Five replicates for each treatment were measured.

Determination of lipid peroxidation and antioxidant enzyme activity

The soluble protein content was assayed following the method of Bradford (1976). Fresh leaf sample (0.2 g) was homogenized with 5 mL of buffer solution. Then the homogenate was centrifuged and 1 mL of the supernatant was mixed with 5 mL Coomassie brilliant blue solution. Soluble protein content was then detected spectrophotometrically at 595 nm. MDA content was determined by the method described by Fu and Huang (2001) with some modifications. 1.0 g of fresh leaf sample was homogenized with 4 mL of 10% trichloroacetic acid. The precipitate was removed by centrifugation, and 2 mL of supernatant extract was mixed with 2 mL of 0.6% thiobarbituric acid. The mixture was incubated in boiling water for 15 min. MDA content was detected spectrophotometrically at 532 nm. Free proline content was estimated according to Bates *et al.* (1973). Fresh leaf sample (0.5 g) was extracted in 5 mL of 3% sulphosalicylic acid. 2 mL of centrifuged supernatant was reacted with 4 mL of 2.5% acid ninhydrin reagent and 2 mL of glacial acetic acid. The mixture was incubated in boiling water for 30 min and extracted with 4 mL of toluene when chilled. Absorbance of the resulting organic layer was measured at 520 nm. Free proline content was estimated by referring to a standard curve. Fresh leaf sample (0.5 g) was homogenized in 5 mL extraction buffer (0.1 M phosphate buffer). The homogenate was then centrifuged at 12,000 × g for 15 min at 4°C, and the supernatant was used as the crude extract for antioxidants enzymes. SOD activity was measured according to the method described by Beyer and Fridovich (1987). POD and CAT activities were measured using the method of Chance and Maehly (1955). The superoxide anion production rate

was determined according to Elstner and Heupal (1976) with some modifications. Fresh leaf sample (0.2 g) was extracted in 1 mL of PBS (0.05 M phosphate buffer). 0.5 mL of centrifuged supernatant was reacted with 0.5 mL of PBS and 1 mL of hydroxylamine hydrochloride. After incubating in 25°C for 1 h, 1 mL of *p*-aminobenzenesulfonic acid and 1 mL 1-naphthylamine was added into the reaction solution. Superoxide anion production rate was detected at 530 nm by spectrophotometry, and was estimated by referring to a standard curve.

Photosynthetic parameters

Photosynthetic parameters were measured of the fourth true leaf of seedlings in each treatment. Gas exchange and the transpiration rate were measured using the Li-6400 Portable Photosynthesis Measurement System (LI-COR, Lincoln, NE, United States) between 8 AM and 10 AM. The CO_2 concentration in the leaf chamber was 400 mmol·mol^{-1}. The net photosynthetic rate (Pn), stomatal conductance (gs), intercellular CO_2 concentration (Ci), and transpiration rate (Tr) were determined.

RNA-Seq transcriptome analysis

Samples from the S and SC20 treatments were designated as TBS and TBC, respectively. Leaf samples were frozen in liquid nitrogen and stored at −80°C until RNA isolation. RNA was extracted with a Total RNA Extraction Kit (Tiangen, Beijing, China) according to the manufacturer's protocol and then its integrity was assessed by 0.8% agarose gel electrophoresis. The RNA samples were reverse-transcribed into cDNAs in a reaction volume of 20 μL. The cDNA samples were sent to the Beijing Nuohe Zhiyuan Biotechnology Co., Ltd., Beijing, China) for transcriptome sequencing. Differentially expressed genes (DEGs) were identified based on the following criteria: $P < 0.05$; FDR <0.01; log2 fold change X. Differences in gene transcript levels between samples were detected using the FPKM method (Trapnell *et al.* 2012). The identified DEGs were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses.

qRT-PCR verification

Gene transcript levels were also determined by qRT-PCR using the cDNA obtained by the above method. Specific primer pairs were designed using Primer 5.0 software (Table 1). The relative mRNA levels were quantified with respect to the internal standard, L25. The qRT-PCR analyses were performed as described by Jain *et al.* (2006).

Statistical analysis

All experiments were conducted with three replicates and results are expressed as mean ± standard deviation (SD). All data were subjected to one-way analysis of variance (ANOVA) and the significance of differences was determined by LSD multiple comparison ($p<0.05$ and $p<0.01$). Excel 2007 (Microsoft, Redmond, WA, USA) and SPSS 19 software (SPSS Inc., Chicago, IL, USA) were used to process data and conduct statistical analyses.

Results

Effect of calcium on lipid peroxidation in tobacco seedlings under low light stress

First, to determine optimal application method and concentration of exogenous Ca^{2+}, solutions with different concentrations Ca^{2+} were applied as a foliar spray (SC) or as a nutrient solution (SN). Proline is an important osmoregulation substance that accumulates in plants in free form under stress. We evaluated the changes in free proline content in tobacco seedlings in the different treatments. Compared with the control, the S treatment resulted in significantly increased free proline content in tobacco leaves, while exogenous application of Ca^{2+} decreased the free proline content, especially in the SC20 treatment (Table 2). Thus, a certain concentration of exogenous Ca^{2+} applied as a foliar spray reduced the accumulation of osmotic products under low light stress.

Soluble proteins are also important osmoregulation substances that play a protective role as components of various compounds and biofilms. Therefore, the soluble protein content is often measured as an indicator of resistance. The soluble protein content in tobacco leaves was lower in the S treatment than in the control, and the application of exogenous Ca^{2+} increased the soluble protein content, especially in the SN30 and SC20 treatments. The results showed that both 30 m*M* $CaCl_2$ in nutrient solution and spraying with 20 m*M* $CaCl_2$ were able to increase the content of soluble proteins in tobacco leaves under low light stress. One of the most important products of membrane lipid peroxidation is MDA. Various environmental conditions lead to the production of ROS and free radicals in plants, and the accumulation of these substances can reflect the degree of damage under stress. Compared with the control, the S treatment increased the MDA content and $O_2^{\cdot-}$ production rate in tobacco leaves, while exogenous Ca^{2+} inhibited $O_2^{\cdot-}$ production, and the SC20 treatment resulted in a significantly reduced MDA content. The results showed that exogenous Ca^{2+} reduced the extent of increase in oxygen free radicals, MDA, and membrane lipid peroxidation products in tobacco under low light stress.

Effect of calcium on growth and development of tobacco seedlings under low light stress

As shown in Table 3, the plant height, stem girth, main root length, leaf area, fresh weight and SPAD value of tobacco seedlings were lower under low light stress than in the

Table 1: Primers in qRT-PCR

Gene	Primer sequence (5'–3')
CCaMK	FP (forward primer): AAGAACAGATGGACGCCGAGATTG
	RP (reverse primer): AAGCTGCTGCTCAAGACACTGG
CDPK9	FP: TGCGCGAAGTTGATACAGACAAGG
	RP: GCCACTGAGCACATCCTGAAGC
CAS	FP: TCACCTCTTCCTCCGCCTTCTTC
	RP:GCTGAGTGCTCTGGCTTCATGG
ACRE20	FP: AGGTGGGGATGGTTTAATTGGTG
	RP: TCTTGAAGCCCTAACAAAGCAGA
ACRE57	FP: AGATGGAAGCGAAGAAGAGAAGGC
	RP:CTCCGGCGTTATACATCCACATCC
LHCB4	FP: GGACCAGGACTGAACTTGCTGATG
	RP: AGCACCAAGTGTAGCCAACATAGC
CAB21	FP: GGCCTCTTGGTGAGGTTGTCG
	RP: ATGGTCAGCAAGGTTCTCCAATGG
rbcS	FP: AGCTGTTGCCGTTGCCACTG
	RP: GGAAGGAGGAGGCGGACTTGAG
L25	FP: GCCAAGGCTGTCAAGTCAGGATC
	RP: TTCATTGCAGACTCTGTGGTGAGG

Table 2: Effect of calcium on proline, soluble protein, MDA content, and superoxide anion production rate of tobacco seedlings under low light stress

Treatments	Free proline (μg•g^{-1}FW)	Soluble protein (mg•g^{-1}FW)	$O_2^{\cdot-}$ production rate (μmol•min^{-1}•g^{-1}FW)	MDA (μmol•g^{-1}FW)
Control	62.2±1.9cB	14.5±0.37aA	3.23±0.03cC	1.70±0.18cB
S	79.6±4.5aA	9.0±0.65dC	4.71±0.14aA	2.90±0.31aA
SN20	80.0±3.9aA	10.2±0.14cdC	4.19±0.27bAB	2.32±0.10bAB
SN30	72.2±1.9abAB	11.5±0.33bcBC	4.15±0.05bAB	1.98±0.28bcB
SC10	77.1±2.3aA	10.0±0.19cdC	3.96±0.22bB	2.03±0.08bcB
SC20	66.7±4.5bcAB	12.9±0.80bAB	3.91±0.05bB	1.73±0.07cB

Note: Lower-case letters represent 0.05 level and upper-case letters represent $P < 0.01$ level

Table 3: Effect of calcium on tobacco agronomic traits under low light stress

Treatments	Plant height (cm)	Stem girth (cm)	Main root length {cm)	Leaf area (cm^2)	Fresh weight (g)	SPAD value
Control	0.49±0.02aA	0.83±0.03aA	7.60±0.17aA	11.91±0.36aA	1.33±0.05aA	30.52±1.20aA
S	0.34±0.03dC	0.51±0.03eD	5.26±0.21dD	7.32±0.31fE	0.79±0.06eE	21.76±1.11dD
SN20	0.43±0.02bcB	0.68±0.04cB	6.32±0.14cC	8.69±0.10dD	1.07±0.08cC	26.00±0.34cC
SN30	0.45±0.04bAB	0.71±0.03cB	6.96±0.24bB	10.20±0.22cC	1.20±0.04bB	27.94±0.19bB
SC10	0.39±0.02cdBC	0.61±0.02dC	6.21±0.43cC	8.17±0.26eD	0.93±0.04dD	25.19±1.26cC
SC20	0.45±0.05bAB	0.78±0.03bA	7.30±0.27abAB	10.82±0.28bB	1.20±0.05bAB	29.26±0.50aAB

Note: Lower-case letters represent 0.05 level and upper-case letters represent $P < 0.01$ level

control and the SN20 and SN30 treatments resulted in increased values of the agronomic indexes of tobacco seedlings under low light stress (Table 3). Compared with the SN20 treatment, the SN30 treatment significantly increased the main root length, leaf area, fresh weight, and leaf SPAD value of tobacco seedlings under low light stress. The SC10 and SC20 treatments also significantly increased some agronomic indexes of tobacco seedlings under low light stress. Compared with the SC10 treatment, the SC20 treatment had a more obvious effect. Compared with the SN30 and S treatments, the SC20 treatment resulted in significantly greater stem girth, leaf area, and leaf SPAD under low light stress (increased by 29.4, 57.5, and 36.2%, respectively, compared with the S treatment; and by 9.9, 8.4, and 6.1%, respectively, compared with the SN30 treatment). The results showed that Ca^{2+} treatment can effectively promote the growth and development of tobacco seedlings, and that spraying was the most effective application method.

Effect of calcium on antioxidant enzyme system in leaves of tobacco under low light stress

The antioxidant enzyme system can eliminate reactive oxygen species and free radicals, maintain the balance of plant metabolism and participate in resistance to external stress. Compared with the control, the S treatment significantly increased SOD activity, and the SN30 and SC20 treatments significantly increased SOD activity by 6% and 19%, respectively. These results indicated that exogenous Ca^{2+} can improve the ability of tobacco seedling leaves to scavenge $O_2^{\cdot-}$ under low light stress (Table 4). The POD activity in the SC10 and SC20 treatments was 35% higher and 46% higher, respectively, than that in the control. Another main scavenger of H_2O_2 is CAT, which is abundant in mesophyll cells. The SC20 treatment significantly increased CAT activity under low light stress by 15%. These results showed that exogenous Ca^{2+} increased the H_2O_2 scavenging capacity in tobacco seedlings under low light stress.

Table 4: Effect of calcium on SOD, POD and CAT activity of tobacco leaves under low light stress

Treatments	SOD activity (U•min^{-1}•g^{-1}FW)	POD activity (U•min^{-1}•g^{-1}FW)	CAT activity (U•min^{-1}•g^{-1}FW)
Control	5.44±0.12dC	10.1±0.32cC	45.9±2.12bB
S	5.67±0.01cC	14.6±1.77bBC	49.5±4.67bAB
SN20	5.66±0.09cC	14.3±0.95bBC	47.6±1.91bAB
SN30	6.02±0.12bB	14.6±2.30bBC	47.1±1.27bB
SC10	5.71±0.03cC	19.7±1.70aAB	51.5±0.64abAB
SC20	6.75±0.01aA	21.4±0.39aA	57.0±1.70aA

Note: Lower-case letters represent 0.05 level and upper-case letters represent $P < 0.01$ level

Table 5: Effect of calcium regulation on the photosynthesis parameters of tobacco leaves under low light intensity environment (the 9st day after treatments)

Treatments	Pn (μmol·m^{-2}·s^{-1})	gs (mol·m^{-2}·s^{-1})	Ci (μmol·mol^{-1})	Tr (mmol·m^{-2}·s^{-1})
Control	8.7±0.36aA	0.15±0.01aA	169±3.51aA	1.62±0.17aA
S	6.3±0.45cC	0.06±0.01cC	136±6.56cC	0.62±0.04cC
SC20	7.6±0.35bB	0.09±0.01bB	154±3.21bB	0.95±0.04bB
SE10	4.1±0.25dD	0.04±0.01dC	108±4.16dD	0.43±0.03dC

Note: Lower-case letters represent 0.05 level and upper-case letters represent $P < 0.01$ level

Table 6: Effect of calcium regulation on the photosynthesis parameters of tobacco leaves under low light intensity environment (the 12th day after treatments)

Treatments	Pn (μmol·m^{-2}·s^{-1})	gs (mol·m^{-2}·s^{-1})	Ci (μmol·mol^{-1})	Tr (mmol·m^{-2}·s^{-1})
Control	10.7±0.51aA	0.27±0.02aA	207±5.03aA	2.45±0.08aA
S	7.9±0.20cC	0.09±0.00cC	159±3.06cB	1.04±0.09cC
SC20	8.9±0.20bB	0.13±0.01bB	171±4.72bB	1.43±0.09bB
SE10	6.7±0.40dD	0.08±0.01dC	137±3.51dC	0.68±0.04dD

Note: Lower-case letters represent 0.05 level and upper-case letters represent 0.01 level

Table 7: Reads quality statistics after filtering

Sample	Raw reads	Clean reads	Q20 (%)	GC content (%)
TBS_1	59303848	58596638	96.67	43.32
TBS_2	51983730	51373534	96.64	43.27
TBC_1	46005596	45340932	96.59	42.51
TBC_2	51324516	50658704	96.51	42.72

Effect of calcium on gas exchange and transpiration rate of tobacco leaves under low light stress

On the 9th day after treatments, the Pn was decreased significantly in the S treatment, to 72.41% of that in the control (Table 5). The SC20 treatment increased the Pn in tobacco leaves by 20.63% under low light, but it was still lower than that in the control. The SE10 treatment significantly reduced the Pn of tobacco leaves by 34.92%. On the 12th day after treatments, the Pn and gs had increased (Table 6). The gs of tobacco leaves in the S treatment was only 37.5% of that in the control on the 12th day after treatments. Exogenous Ca^{2+} treatments increased gs, while the SE10 treatment significantly decreased the gs in leaves of tobacco under low light stress.

The Ci of tobacco leaves in the S treatment continued to decrease during the experimental period. Compared with the control, the S treatment resulted in 19.53% lower Ci on the 9th day after treatments, and 23.19% lower Ci on the 12th day. Compared with the S treatment, the SC20 treatment increased the Ci by 13.24% and 7.54% on the 9th and 12th day, respectively. In contrast, the Ci in the SE10 treatment decreased sharply under low light. The Tr of leaves increased during seedling growth. On the 12th day after treatments, the Tr in the S treatment was only 42.45% of that in the control, but was 37.5% higher in the SC20 treatment than in the S treatment. The Tr was significantly reduced by the SE10 treatment. These results show that exogenous Ca^{2+} helped to maintain a higher photosynthetic capacity in tobacco under low light stress.

Quality control of transcriptome data and screening of DEGs

Tobacco seedlings grown under low light intensity (TBS_1, TBS_2) and under low light intensity with foliar application of Ca^{2+} (TBC_1, TBC_2) were used to prepare the sample libraries for RNA-Seq. We obtained 5933848, 51983730, 46005596 and 51324516 raw reads for the TBS_1, TBS_2, TBC_1, and TBC_2 libraries, respectively (Table 7). After removing adapter-related reads, reads containing more than 10% N, and low-quality reads, the number of reads in the TBS_1, TBS_2, TBC_1, and TBC_2 was 58596638, 51373534, 45340932, and 50658704, respectively. After removing base fragments with phred values of less than 20, 96.67, 96.64, 96.59, and 96.51% of the libraries remained, with GC contents of 43.32, 43.27, 42.51, and 42.72%, respectively.

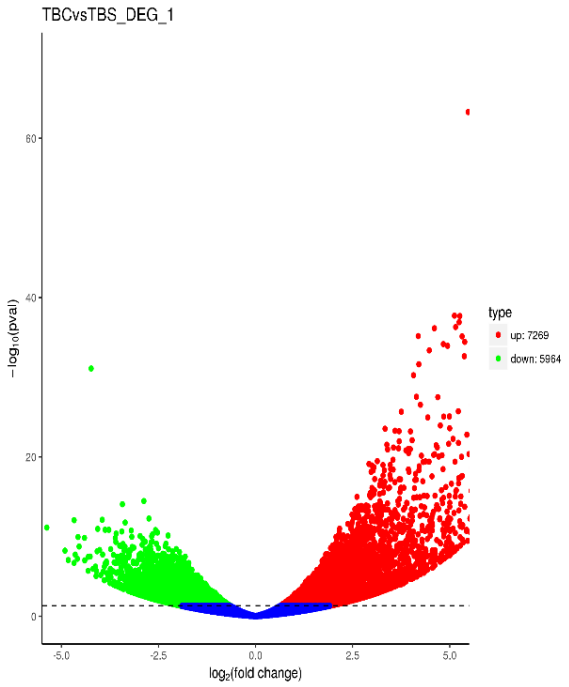

Fig. 1: Scatter-plot Distribution of DEG
Note: X and Y axis represents the logarithmic value of gene expression

The results confirmed that the quality of the sequencing data was sufficient for further biological analyses. The genes significantly up-regulated, down-regulated, and without significant differences in expression are shown in red, green, and blue, respectively Fig. 1. A total of 13233 DEGs were identified, of which 5964 were down-regulated and 7269 were up-regulated in the TBC group compared with the TBS group. There were more up-regulated genes than down-regulated genes. Many genes related to growth and development were activated and only a few genes were inhibited in the TBC treatment.

GO and KEGG analyses for DEGs

The DEGs were mainly divided into two categories in the GO enrichment analysis: biological process with 15 DEGs) and molecular function with 14 DEGs (Fig. 2). The DEGs in the biological process category were in the subcategories of regulation of transcription, DNA-template, regulation of nucleic acid template, regulation of RNA biosynthetic process, regulation of nucleobase-containing compounds, regulation of biosynthetic process, and regulation of RNA metabolic process. In the molecular function category, the transporter activity subcategory contained the most DEGs, followed by nucleic acid binding transcription factor activity, transcription factor activity, and RNA polymerase activity. The effect of Ca^{2+} on tobacco seedlings under low light stress was regulated by DEGs in these enriched pathways.

The top 20 pathways with the lowest qvalues are shown in Fig. 3. In the Figure, the number of DEGs is

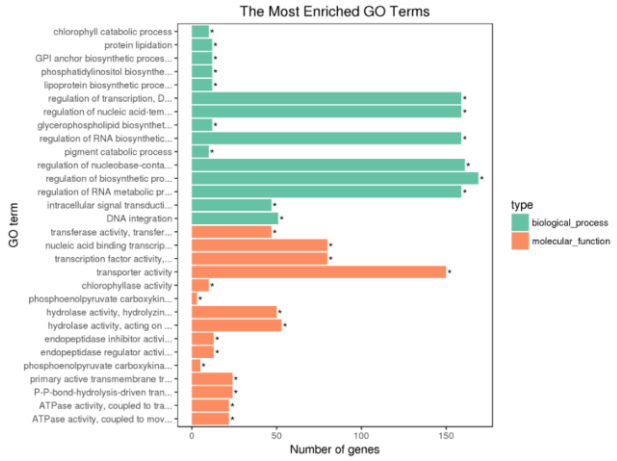

Fig. 2: Go enriched analysis of differentially expressed genes.
Note: * representing padj<0.05 significant enrichment.

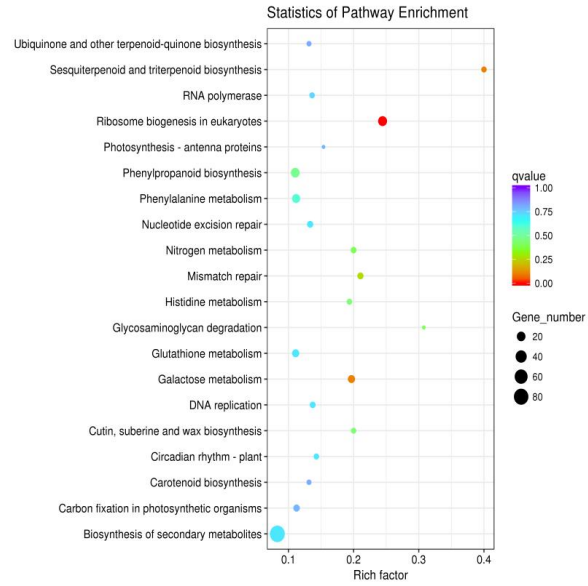

Fig. 3: Scatter plot of differential gene KEGG enrichment.
Note: The number of differentially expressed genes was represented by the size of the point, and the different qvalue ranges were distinguished by the color of the point.

represented by the size of the point, and different colors indicate different qvalue ranges. More than 80 DEGs were related to the biosynthesis of secondary metabolites. Ribosome biogenesis in eukaryotes and galactose metabolism had the lowest qvalues, indicating that they were the pathways most enriched with DEGs. These results highlighted which pathways were most significantly enriched with up-regulated DEGs.

DEGs and qRT-PCR verification

We conducted qRT-PCR analyses for some of the DEGs identified from the RNA-Seq data. The results were consistent with those of the RNA-Seq analysis,

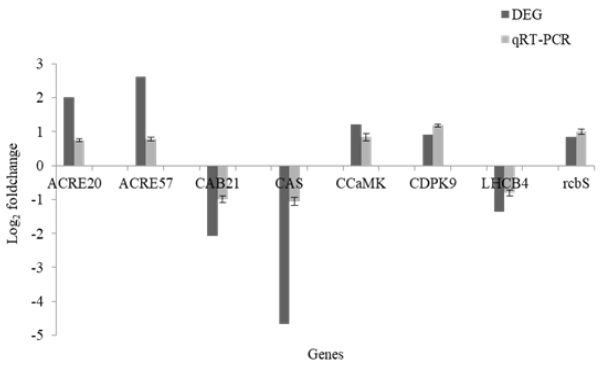

Fig. 4: Quantitative real-time PCR validations of gene expression levels from digital gene expression (DGE) analysis. Primers used are listed in Supplementary Table 1.

confirming that the results of RNA-seq were reliable (Fig. 4). The *CCaMK* gene, which encodes a calcium/calmodulin dependent protein kinase, was up-regulated in the TBC treatment, as were *CDPK9* (encoding calcium-dependent protein kinase), *ACRE20* and *57* (encoding calmodulin-like proteins) and *rcbS* (encoding the RuBisCo small subunit). The significantly down-regulated genes included *CAS* (encoding a calcium receptor), *CAB21* (encoding the light harvesting chlorophyll A/B binding protein) and *LHCB4* (encoding a secondary light harvesting pigment protein).

Discussion

Several previous studies have shown that Ca^{2+} can have a protective effect on plants under stress conditions. For example, Zoysia grass pre-treated with 10 m*M* $CaCl_2$ showed higher Pn and antioxidant enzyme activities than those of the control under drought stress, and the drought tolerance of Zoysia grass was improved by applying a moderate concentration of Ca^{2+} (Xu *et al.* 2013). Under all kinds of stress, various metabolic pathways in plants become disordered and unbalanced, and the accumulation of ROS results in oxidative stress and cell membrane damage. Proline, when accumulated in free form, is an important osmolyte in plants to combat stress conditions. Soluble proteins are also involved in osmoregulation, and are also components of important nutrients. Therefore, the soluble protein content is often determined as an indicator of resistance (Suzuki *et al.* 2012). In another study, treatment with 27 m*M* $CaCl_2$ significantly decreased the MDA content, increased the activities of protective enzymes, and increased the soluble protein content in the leaves of tomato seedlings under low light stress (Li and Li 2008). In general, compared with the SN treatments, the SC treatments had greater effects on membrane lipid peroxidation. Among others, 20 m*M* $CaCl_2$ spray treatment had the strongest protective effect: increased soluble protein content by 30.2%, decreased content of free proline and MDA by 16.2 and 40.3% respectively, and lowered production of $O_2^{\cdot-}$ by 17% in tobacco seedlings under low light stress. This result is basically consistent with those of previous studies, and shows that spraying with Ca^{2+} can improve the tolerance of tobacco seedlings to low light stress (Xu *et al.* 2013; Li and Li 2008).

In practical production, factors such as the medium and long-term rainy weather lead to low light conditions in the greenhouses. This results in slower growth and development of tobacco plants, a prolonged growth period, and reduced dry matter accumulation, which negatively affect the yield and quality of tobacco. Therefore, it is of theoretical and practical significance to improve the tolerance of tobacco plants to low light stress via various agronomic measures (Wang *et al.* 2014). Previous studies have shown that Ca^{2+} pretreatment (spraying with 3 mmol·L^{-1} $CaCl_2$ every 4 days, four times in total) improved the dry weight and total chlorophyll content of tobacco seedlings, and enhance their overall quality (Yang *et al.* 2018). In this study, spraying with 20 m*M* $CaCl_2$ significantly increased the plant stem girth, leaf area, and SPAD value by 29.4, 57.5 and 36.2%, respectively under low light stress. The SC20 treatment was more effective than the SN20 or SN30 treatments of tobacco seedlings under low light stress, suggesting that this treatment may be used to support and promote the growth of tobacco under adverse weather conditions at the early stage of seedling growth and development.

Chlorophyll is responsible for light energy conversion and transmission during plant photosynthesis. In this study, exogenous Ca^{2+} not only improved the SPAD value of leaves, but also enhanced their photosynthetic rate. The Pn, gs, Ci, and Tr of tobacco leaves were significantly increased by spraying with 20 m*M* $CaCl_2$ under low light intensity, while spraying with 10 m*M* EGTA had the opposite effect. On the 12th day after treatments, SC20 treatment increased Pn, gs, Ci and Tr by 12.7, 44.4, 7.54 and 37.5%, respectively. In another study, cucumber seedlings pretreated with 10 m*M* $CaCl_2$ maintained high Pn because of increased activities of photosynthesis-related enzymes under low light, while EGTA resulted in lower Pn because of decreased enzyme activities (Liang *et al.* 2009). The differences in the optimal concentration of Ca^{2+} between our study and other studies may be related to differences in plant species or treatment processes.

Many of the genes that were up-regulated by Ca^{2+} treatment in tobacco plants under low light stress encode enzymes involved in growth, development, and stress responses. For example, CCaMKs can instantly respond to changes in Ca^{2+} concentrations and induce gene expression, as well as participating in plant growth, leaf development, signal transduction, and responses to stress (Tirichine *et al.* 2006). CDPKs have a similar function, and their activity is related to the Ca^{2+} concentration. They are involved in the abiotic stress response of plants and the regulation of stomatal movement. Previous studies have shown that CDPKs are also related to responses to the light

environment (Giammaria *et al.* 2011; Saijo *et al.* 2011). ACREs have a wide range of biological functions, such as regulating seedling morphogenesis and stress responses, and participating in light and hormone signalling during plant growth and development (Friml *et al.* 2004; Bender *et al.* 2013). Many ACREs are involved in primary defence responses (Rowland *et al.* 2005). A previous microarray study showed that expression of *ACRE* genes responds to abiotic stress signals to varying degrees (Chinpongpanich *et al.* 2012). Rubisco, which is composed of eight large subunits (rbcL) and eight small subunits (rbcS), is the key enzyme for carbon fixation in photosynthesis. Decreased Rubisco activity is a non-stomatal limiting factor of the photosynthetic rate in plants (Xu *et al.* 2012). In this study, genes encoding all of these proteins were up-regulated by Ca^{2+} treatments in tobacco under low light stress, indicated that they may be involved in the process of Ca^{2+}-mediated regulation of light tolerance.

Among 5964 down-regulated genes in Ca^{2+} treatments under low light stress, some were also involved in photosynthesis. CAB is a component of photosystem I, and is a part of the photopigment protein complex. It participates in the transformation and transmission of light energy and in balancing the distribution of energy between the two light systems. Thus, it plays roles in light protection and adaptability to various environments (Labate *et al.* 2004). LHCB4, a secondary light harvesting pigment protein, is a component of the chlorophyll-a/b-binding sub-complex in the internal antenna of photosystem II, and plays an important role in plant light protection (Mozzo *et al.* 2008; Bianchi *et al.* 2011; Miloslavina *et al.* 2011). Genes encoding this protein have been cloned from a variety of crops, and the expression of *LHCB4* under various environmental conditions has been studied (Klimmek *et al.* 2006; Wehner *et al.* 2006; Pan *et al.* 2011). Previous studies have shown that strong light stress can induce the phosphorylation of LHCB4 (Kargul *et al.* 2005). These results indicate that some light-harvesting proteins in photosystems I and II are affected by Ca^{2+} during the regulation of light tolerance in tobacco seedlings.

Conclusion

Spraying with 20 m*M* $CaCl_2$ can promote the growth of tobacco seedlings under low light stress by increasing their antioxidant capacity, enhancing osmotic regulation, reducing the extent of oxidative damage, improving photosynthetic performance, and facilitating normal cellular function. Spraying with 20 m*M* $CaCl_2$ can alleviate and restore the damage to tobacco seedlings under low light stress. A transcriptome sequencing analysis revealed 13233 DEGs between tobacco seedlings treated with and without Ca^{2+} under low light stress. These DEGs were grouped into 15 biological process and 14 molecular function categories, and the most enriched pathways were secondary metabolite biosynthesis, ribosome biogenesis, and galactose metabolism.

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Author Contributions

Xiaoxi Guan and Changling Sui planned the experiments and made the write up, Zhifeng Chen and Chaoyan Lv interpreted the results, Kailian Luo, Jihong Chen and Wei Wu statistically analyzed the data and made illustrations.

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