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Full Length Article

Shade Ameliorates High Temperature-induced Inhibition of Growth in Herbaceous Peony (*Paeonia lactiflora*)

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Abstract

High temperature stress has a significant impact on plant growth and development. Herbaceous peony (*Paeonia lactiflora* Pall.) is a very important landscape plant used in greenbelt whose growth is restrained seriously by high summer temperature, but little is known about relevant solving measures. In order to find an effective measure, this paper studied the effect of black shading net with about 60% transmittance on alleviating the thermal damage of *P. lactiflora* under field conditions. The results showed that *P. lactiflora* physiological indices were higher in shaded plants than those in sun-exposured plants especially in the late stages of higher temperature, such as chlorophyll (Chl) *a*, Chl *b*, Chl *a+b*, soluble sugar, soluble protein contents; whereas the exception to the trend was in Chl *a/b* and malondialdehyde (MDA) content. Moreover, compared with sun exposure, shade increased *P. lactiflora* protective enzymes activities, made mesophyll cell ultrastructures more intact, the chloroplasts more round and the grana lamellaes arrange relatively neatly, which led to enhance its photosynthesis rate (*Pn*) and transpiration rate (*Tr*). Additionally, the full-length cDNA of a heat shock protein gene (*HSP70*) containing 2195 bp nucleotides was obtained from *P. lactiflora*, and the expression analysis of *PlHSP60*, *PlHSP70* and *PlHSP90* in four developmental stages showed that shade caused *PlHSP60* and *PlHSP70* expression levels to rise especially in the late stages. These results indicated that shade alleviated the thermal damage of high temperature stress to *P. lactiflora* through scavenging reactive oxygen species, protecting cell structures, enhancing photosynthesis and the expression levels of *HSP* under high temperature stress, which might lay a theoretical foundation for *P. lactiflora* safe over summering and cultivated form in summer.

Keywords: Herbaceous peony; High temperature stress; Heat shock protein; Thermal damage

Introduction

As one of the vital environmental signals, temperature seriously influences growth and development of plant, and non-optimum temperature will have stress on plants (Farooq *et al*., 2011; Nawaz *et al*., 2013; Lu *et al*., 2014). With the continuous development of global warming and climatic change, high temperature stress negatively affecting plant growth and development is followed (Wahid *et al*., 2007). Under the condition of high temperature, stress signal is perceived by plant cell membrane system and transmitted to the cell through the signal transmission path, which causes the changes of enzyme activity, induces related physiological and biochemical reactions, together with gene expression and regulation, thereby affects plant growth and even causes plant death (Wang *et al*., 2014). In recent years, a lot of works on high temperature stress had been reported, and rice (*Oryza sativa*) was extensively investigated. Under high temperature stress, normal physiological activities in the body of *O. sativa* were damaged, especially the changes of various resisting substances and endogenous hormones, which shortened its growth period, slowed down the speed of leaf emergence and made leaf deformity and short. Subsequently, its plants became small, the seed setting rate, head rice rate and transparency were reduced, rice chalkiness area was increased, and rice amylose content was significantly decreased (Wang *et al*., 2013). In addition, heat shock protein genes (*HSP*) are certified to significantly increase the heat resistance of the plants (Zou *et al*., 2012). And in the ornamental plants, the studies about the influence of heat stress on plant growth and its response have also been performed, such as lilac (*Syringa vulgaris*) (Jedrzejuk and Lukaszewska, 2008), chrysanthemum (*Dendranthema grandiflora*) (Janka *et al*., 2013).

Herbaceous peony (*Paeonia lactiflora* Pall.) belonging to the Paeoniaceae family is a perennial root and herbaceous flower and always symbolizes prosperity and wealth. As a traditional famous flower in China, *P. lactiflora* is regarded

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as one of the easiest and most rewarding plants to grow and mainly used for urban landscaping, garden cultivation and cut flowers, which has been widely distributed and cultivated in more than 50 countries and regions (Stevens *et al*., 1993). In terms of the growth habit, *P. lactiflora* is suitable to plant in a well drained, wet loam or sandy loam location with full sunlight, resists to slight shade and strong cold, but doesn't withstand hot summer and heat (Lv and Liu, 2008). However, high summer temperature in the middle and lower reaches of the Yangtze River basin of China is very common and lasted for a long time, and *P. lactiflora* cannot exert their excellent ornamental characteristics when growing in these areas, such as Changsha in Hunan province, Nanjing, Yangzhou and Suzhou in Jiangsu province, Hangzhou in Zhejiang province, Shanghai and so on. Obviously, these plants are not adapted to high temperature climate of the middle and lower reaches of the Yangtze River basin of China, which makes leaves become yellow and withered with dead spots, plant growth vigor reduce excessively, diseases and insect pests occur seriously, especially gray mold, rust and soft rot diseases. All of these seriously affect the landscape construction of *P. lactiflora* in garden green space after flowering, and conflict with the original intention of its configuration, consequently, hindering *P. lactiflora* further popularization and application in urban landscaping of the middle and lower reaches of the Yangtze River basin of China. But until now, relevant solving measures have not been put forward yet, therefore, how to take protective measures to reduce or alleviate the thermal damage of high temperature stress to *P. lactiflora* is still a difficulty needed to resolve quickly in front of us. Additionally, Shade can lower the environmental temperature by reducing light intensity in summer, so as to alleviate negative impact of high temperature stress on growth and development of plant including plant photosynthesis, yield and quality to some extent, which was a cultivation measure commonly used in horticultural plants (Allan and Carlson, 2003).

On the thermal damage of *P. lactiflora*, only Lv and Liu (2008) had preliminary clarified its physiological mechanism under high temperature stress until now, and he considered that with the temperature rising, malondialdehyde (MDA) content, conductivity, praline content and soluble protein content went up; soluble sugar and peroxidase (POD, EC 1.11.1.7) activity went down; superoxide dismutase (SOD, EC 1.15.1.1) activity was not stable and the synthesis of chlorophyll (Chl) was obstructed. Moreover, the effects of shade on *P. lactiflora* plant growth under optimum temperature had been clarified by us (Zhao *et al*., 2012a). Nevertheless, the effect of shade-induced thermotolerance was still far from being complete. In order to elucidate the impact of shade on *P. lactiflora* thermotolerance, 'Dafugui' that was a major *P. lactiflora* cultivar in Yangzhou also had been selected in this study (Fig. 1), and its life after flowering could be divided into four different developmental stages including S1 (the stage of May, average temperature 28.21°C), S2 (the stage of June, average temperature 32.64°C), S3 (the stage of July, average temperature 34.11°C) and S4 (the stage of August, average temperature 35.90°C). Firstly, physiological indices, protective enzyme activities and photosynthetic characteristics were determined; secondly, cell ultrastructure was observed; lastly, isolation and expression analysis of related *HSP* were studied. This study was aimed at providing a beneficial reference for *P. lactiflora* cultivated form in summer.

Materials and Methods

Plant Materials

P. lactiflora cv 'Dafugui' was used as the material, which grew in the germplasm repository of Horticulture and Plant Protection College, Yangzhou University, Jiangsu Province, China (32°30′ N, 119°25′ E). Under field conditions, one part of plants growing in the same conditions were shaded using black shading net with about 60% transmittance and 1.2 meters height when their buds exposed to the ground in March, while other plants grew in the open ground. From the end of flowering in May15 to plant death in August, four different developmental stages (S1, May, average temperature 28.21°C; S2, June, average temperature 32.64°C; S3, July, average temperature 34.11°C; S4, August, average temperature 35.90°C) were divided to study whether shade could alleviate the thermal damage of high temperature in *P. lactiflora*. In each stage, photosynthetic characteristics were measured firstly, and then the leaves were taken for cell ultrastructure observation, physiological indices, protective enzymes activities, gene isolation and expression. Additionally, all leaves were the fully expanded and from the fourth apical node.

Physiological Indices Determinations

Chl *a*, Chl *b* and Chl *a*+*b* contents were assayed according to Zou (2000) in alcohol extract and the absorbance was read at 665 nm and 649 nm on a spectrophotometer UV BlueStar A (LabTech, Inc., China). Soluble protein content was performed by Total protein quantitative assay kit (Nanjing Jiancheng Bioengineering Institute, China), and soluble sugar content was assayed using the anthrone method (Zou, 2000). MDA content was measured according to the thiobarbituric acid (TBA) method described by Zou (2000). 1 g fresh leaves were ground with 2 mL of 10% trichloroacetic acid (TCA), washed using 8 mL of 10% TCA and centrifuged at 4,000 $r \cdot min^{-1}$ for 10 min. 2 mL of 0.6% TBA was added to 2 mL supernatant, heated in a boiling water bath for 15 min, and allowed to cool in an ice bath quickly. The supernatant was centrifuged and the resulting supernatant was used for spectrophotometric determination at 532 nm, 600 nm and 450 nm.

Protective Enzyme Activities Measurements

Firstly, the extracts which was from 0.5 g leaf powders extracting by ice-cold 50 mM phosphate buffer (pH 7.8) were centrifuged at 4°C and 10,000 × g for 15 min, this isolated supernatants called crude extracts could be used for enzyme activities assay (Zou, 2000). Thereafter, SOD, POD and catalase (CAT: EC 1.11.1.6) activities were evaluated using the photochemical nitroblue tetrazolium (NBT) method (Zou, 2000), guaiacol oxidation method (Maehly and Chance, 1954) and a reagent kit (Nanjing Jiancheng Bioengineering Institute, China), respectively.

Photosynthetic Characteristics Determinations

Portable photosynthesis system (LI-6400, Li-Cor, Lincoln, NE, USA) was used to determinate photosynthetic characteristics from 07:00 to 9:00 am at a cloudless day. Standard leaf chamber was 2 cm × 3 cm, photosynthetic photon quanta flux density (*PPFD*) was set at 1000 $\mu mo1 \cdot m^{-2} \cdot s^{-1}$ using a self-taking red and blue LED source. Net photosynthesis rate (*Pn*) and transpiration rate (*Tr*) were also recorded in the system.

Transmission Electron Microscope Observation

Firstly, fifteen-minute wash were performed 3 times for fixed leaves using 0.1 mol/L phosphate buffer, and post-fixed with 1% osmium tetroxide for 4 h at room temperature. After 3 times fifteen-minute wash again, the leaves were dehydrated using 50%, 70%, 85%, 95% and 100% gradient ethanol for 15 min each. Moreover, they were treated with 100% acetone solution (15 min) and acetone solution containing anhydrous sodium sulfate (15 min), infiltrated in Spurr resin and then hardened at 70°C for 24 h. seventy-nm-thick sections were cut using a Leica EM UC6 ultramicrotome (Leica Co., Austria) with a diamond knife and stained using 1% uranyl acetate in 70% methanol, and 1% lead citrate before examination. After these, the samples were observed and imaged with a Tecnai 12 transmission electron microscope (Philips Co., Holland).

Isolation of *HSP70*

Total RNA of *P. lactiflora* leaves was extracted by a modified CTAB method (Zhao *et al*., 2011). After testing its quality and integrity (Eppendorf, Germany), it was used for isolation of *HSP70* cDNA according to 3' and 5' full Rapid Amplification of cDNA Ends (RACE) Core Set Ver. 2.0 (TaKaRa, Japan). According to the *HSP70* sequence of other plants and the above sequenced 3'-region of *PlHSP70*, the primers of 3' RACE (outer primer: 5'-GACCTCGGGACGACCTAC-3', inner primer: 5'-CCTGCTTACTTCAACGATTC-3') and 5' RACE (outer primer: 5'-TTGTTGTCTCGGGTCCTT-3'; inner primer: 5'-TCTCAACAGGGTCCATACA-3') were designed. And the sequences of 3' and 5' ends could be obtained combining with universal primers in kits. PCR amplification products were purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 (TaKaRa, Japan) after 1% agarose gel electrophoresis, connected with p*EASY*™-T5 Zero vector (Trans, China) and transformed into competent Escherichia coli *Trans*1-T1 cells (Trans, China). The extracted plasmids were sequenced by Shanghai Sangon Biological Engg. Technology & Services Co., Ltd. (Shanghai, China).

Gene Expression Analysis

Gene-specific primers used for real-time quantitative polymerase chain reaction (Q-PCR) were designed by Primer Premier 5.0 (Table 1). *P. lactiflora Actin* (JN105299) had been used as internal control (Zhao *et al*., 2012b). Gene expression levels were analyzed using a BIO-RAD CFX96™ Real-Time System (C1000™ Thermal Cycler) (Bio-Rad, USA). Q-PCR was performed according to the SYBR® Premix Ex Taq™ (Perfect Real Time) (TaKaRa, Japan), and the amplification condition was 50°C for 2 min, 95°C for 5 min, 40 cycles at 95°C for 15 s, 51°C for 15 s, and 72°C for 40 s. And the expression level of *HSP60* in S1 of sun exposure was used as the control.

Sequence and Statistical Analysis

Sequence splicing and analysis were performed by DNAMAN 5.0 software (Lynnon Corporation, Canada). Physical and chemical parameters of proteins were detected using ProtParam tool (http://us.expasy.org/tools/protparam.html). Conserved domains search was performed using NCBI Conserved Domains Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Homology analysis was carried out using the GenBank BLAST (http://www.ncbi.nlm.nih.gov/blast/). Phylogenetic tree were constructed by MEGA 5.05. All data were means of three replicates at least with standard deviations. The results were analyzed for variance using the SAS/STAT statistical analysis package (version 6.12, SAS Institute, Cary, NC, USA).

Results

Physiological Indices

Shade treatment effectively affected the contents of photosynthetic pigments in *P. lactiflora* (Table 2). Regardless of Chl *a*, Chl *b* or Chl *a+b*, their contents all showed a constant decline with the environmental temperature increasing. Especially in the last two stages when the ambient temperature was higher, and the decrease in the sun-exposed plants was more remarkable than the shaded plants. In addition, during the development of the plants, the contents of Chl *a*, Chl *b* and Chl *a* + *b* in plants grew in shade were always higher than those grew with sun

Table 1: Primers sequence for detection by real-time quantitative polymerase chain reaction

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Actin	GCAGTGTTCCCCAGTATT	TCTTTTCCATGTCATCCC
HSP60	GAAGGGGCTGTTGTTGTA	GCCTCAGTCGTAGTCATCA
HSP70	CGTATTATCAATGAGCCAAC	GAAGGGAGACATCAAAAG
HSP90	CTAAAAGTGGTGATGAGATGAC	CCTCAATACCCTTCTTCTTT

HSP60 = heat shock protein 60 gene, *HSP70* = heat shock protein 70 gene, *HSP90* = heat shock protein 90 gene

Table 2: Effects of shade treatment on the physiological indices in *P. lactiflora*

Content	Treatment	Developmental stages			
		S1	S2	S3	S4
Chl *a*	Sun exposure	1.22±0.11abc	1.20±0.05bc	0.63±0.01e	0.29±0.06f
($mg \cdot g^{-1}$)	Shade	1.37±0.04a	1.31±0.03ab	1.13±0.06c	0.95±0.03d
Chl *b*	Sun exposure	0.78±0.08b	0.39±0.02d	0.24±0.08e	0.12±0.04f
($mg \cdot g^{-1}$)	Shade	0.96±0.02a	0.49±0.01c	0.44±0.06cd	0.39±0.04d
Chl *a/b*	Sun exposure	1.56±0.01c	3.04±0.05a	2.61±0.15b	2.45±0.35b
	Shade	1.42±0.01c	2.66±0.03b	2.55±0.15b	2.42±0.06b
Chl *a+b*	Sun exposure	2.00±0.06b	1.59±0.07c	0.87±0.04e	0.42±0.09f
($mg \cdot g^{-1}$)	Shade	2.33±0.02a	1.80±0.03bc	1.57±0.06cd	1.35±0.07d
MDA	Sun exposure	17.42±4.21f	22.08±2.37c	31.15±5.33b	40.13±3.93a
($nmol \cdot g^{-1}$)	Shade	8.33±0.50h	16.10±0.28g	19.99±0.39e	21.54±0.35d
Soluble sugar ($ug \cdot g^{-1}$)	Sun exposure	14.66±1.43b	13.16±0.87b	17.73±0.48a	17.98±2.30a
	Shade	13.40±1.24b	11.80±0.64b	17.97±1.86a	18.67±2.79a
Soluble protein ($mg \cdot g^{-1}$)	Sun exposure	5.45±0.51a	4.84±0.33ab	3.87±0.25c	4.95±0.23ab
	Shade	4.68±0.81ab	4.88±0.14ab	4.30±0.57bc	5.26±0.24a

Chl *a* = Chlorophyll *a*, Chl *b* = Chlorophyll *b*, Chl *a/b* = Chlorophyll a/b, Chl *a+b* = Chlorophyll *a+b*, MDA = Malondialdehyde, S1 = May, S2 = June, S3 = July, S4 = August, different letters indicate significant differences ($P < 0.05$)

expose, especially in the last two stages with significant difference. While Chl a/b continued to decline since S2, and it was lower in plants grew in shade than those grew with sun expose throughout the entire process. As far as MDA was concerned, its contents in the plants under shade and sun expose treatments all gradually increased, and the MDA contents of shaded plants increased relatively slowly from S1 to S4, which was 0.58 times that of the increase by sun-exposed plants. Moreover, compared with the sun-exposed plants, the MDA contents of shaded plants were relatively lower, as well as the greatest difference between the two was 2.09 fold. Besides, the effect of shade treatment on the soluble sugar and soluble protein contents in *P. lactiflora* was insignificant, they basically showed an initial downward trend and then increased for the remaining process, and their contents of shaded plants were lower compared to sun-exposed plants in S3 and S4 without significant difference.

Activities of the Protective Enzymes

The overall activities of SOD, POD and CAT in *P. lactiflora* under high temperature were enhanced by shade treatment (Fig. 2). When SOD was concerned, its activity in both shaded and sun-exposed plants gradually increased but declined in S4, and the SOD activity in plants with shade treatment was significantly higher than that of sun expose treatment, together with the greatest difference between the two was 1.62 fold. As the SOD activity, POD activity of plants under shade and sun expose treatments also basically

Sun exposure Shade

Fig. 1: Plants of 'Dafugui' in S4 under sun exposure and shade treatments

presented an upward trend with increasing ambient temperature, and in the last two stages, the POD activity were higher in plants grew in shade than those grew with sun expose. Moreover, the activity of CAT in both shaded and sun-exposed plants reached its maximum in S2 and then decreased gradually. Only in S4, the activity of CAT in shaded plants was lower than that in sun-exposed plants, whose difference did not reach the significant level.

Photosynthetic Characteristics

Shade treatment also influenced the photosynthetic characteristics in *P. lactiflora* (Fig. 3). Firstly, *Pn* in both shaded and sun-exposed plants showed the downtrend during the development, especially the reduction from S1

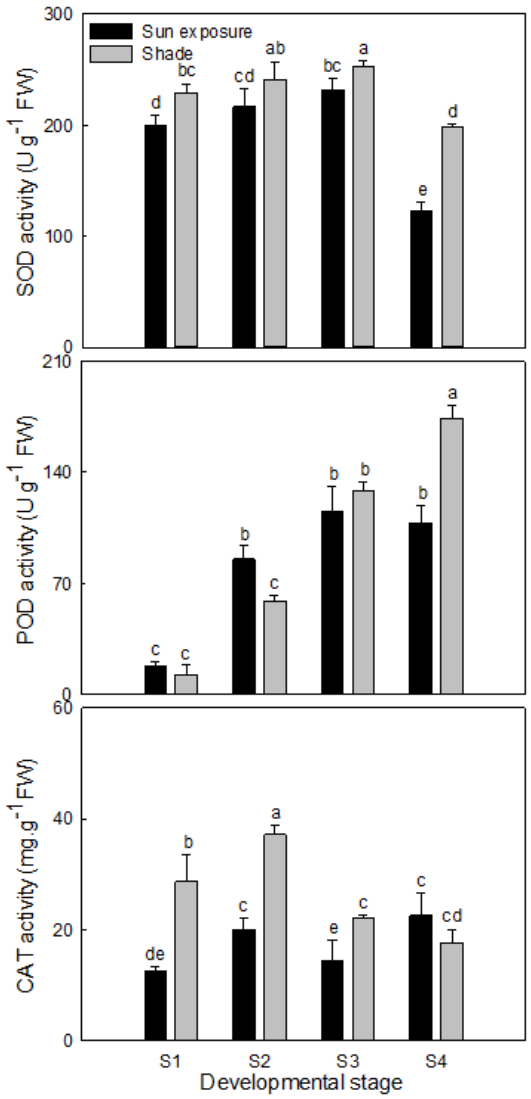

Fig. 2: Effect of shade treatment on the protective enzymes activities in *P. lactiflora*, different letters indicate significant differences ($P < 0.05$)

Note: SOD = superoxide dismutase, POD = peroxidase, CAT = catalase, S1 = May, S2 = June, S3 = July, S4 = August

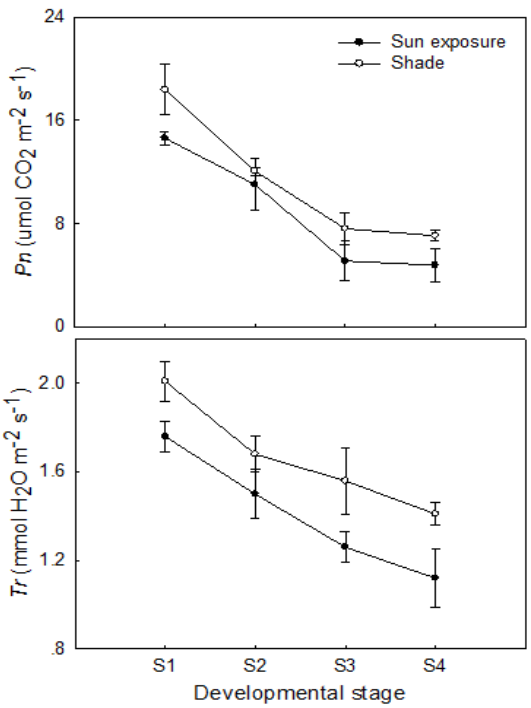

Fig. 3: Effect of shade treatment on the photosynthetic characteristics in *P. lactiflora*

Note: *Pn* = net photosynthesis rate, *Tr* = transpiration rate, S1 = May, S2 = June, S3 = July, S4 = August

to S3 was relatively greater, *Pn* in sun-exposed plants was significantly decreased by 65.00%, while shaded plants with 58.52%. When shade treatment was concerned, its *Pn* was always higher than that under sun expose treatment during the development of plants, with the greatest difference (1.49 times) occurring in S3.

Secondly, the trend of *Tr* was similar for plants grew in shade and sun expose, which declined linearly with plant development, and the value of *Tr* in shaded plants had a significantly higher average proportion with 18.98%.

Ultrastructure

Through observation and analysis of *P. lactiflora* mesophyll cell ultrastructures from S1 to S4, we found that the mesophyll cell ultrastructures of shaded and sun-exposed plants in S1 were similar to each other (Fig. 4A1, A2). Chloroplasts were the more prominent cell organelles, which were arranged close to the cell membrane in greater quantities; the chloroplasts were mostly oval in shape and the grana lamellaes displayed in neat rows inside (Fig. 4B2); but in sun-exposed plants, some chloroplasts showed swelling, and contained white starch grains together with some bore small lipid spheres (Fig. 4B1).

In S4, the mesophyll cell ultrastructures in plants under shade treatment were more intact than under sun expose treatment, and the chloroplasts had a more rounded shape than previously observed emerging with large starch grains (Fig. 4C2); the grana lamellaes were arranged relatively neatly and coupled with an increased number of lipid spheres, which was obviously less than in sun-exposed plants (Fig. 4D2). But for sun-exposed plants in S4, the chloroplasts appeared round (Fig. 4C1) and were distributed with a large number of lipid spheres (Fig. 4D1); the grana lamellaes were bent, swollen and disorganized with numerous cavities; as well as the chloroplast membrane was blurred in some cells (Fig. 4D1).

Isolation and Sequence Analysis of Heat Shock Protein Genes

HSP plays an essential role in plant stress resistance. In this study, *HSP70*, one of the most extensively researched HSP,

was isolated using 3'- and 5'-RACE strategies. The splicing result displayed that the full-length of cDNA was 2195 bp, and encoded 650 amino acids (AA) (Fig. 5). Homology search revealed that this cDNA shared 98% similarity with *P. suffruticosa HSP70* (JN639533), 86% similarity with *Vitis vinifera HSP70* (XM_002283972), 85% similarities with *Nicotiana tabacum HSP70* (AB689673), *Solanum tuberosum HSP70* (XM_006340980) and *Solanum lycopersicum HSP70* (XM_004246354). Therefore, this cDNA sequence was named *PlHSP70* with registration serial number JN180465 registered in GenBank.

Sequence analysis of *PlHSP70* amino acid showed that the putative molecular weight was 71.28 kDa, theoretical isoelectric point (pI) was 5.06. In amino acid composition, alanine (Ala), glutamate (Glu), glycine (Gly), lysine (Lys) and leucine (Leu) were the most abundant with 8.9%, 8.0%, 8.0%, 8.0% and 8.0%, respectively. And a typical domain of heat shock 70 kDa protein with an accession number of conserved domain PTZ00009 was found in *PlHSP70* using NCBI Conserved Domains Database. Meanwhile, homology analysis revealed that this protein shared 94–99% identity and 97–99% similarity with *HSP70* from *P. suffruticosa* (AFA51946), *Vitis vinifera* (XP_002284008), *Theobroma cacao* (XP_007027052), *Gossypium hirsutum* (ACJ11741), *Cucumis sativus* (XP_004142749) and *Solanum lycopersicum* (XP_004250958), and contained three highly conserved *HSP70* family signatures, namely, IDLGTTYS (12-19 AA), IFDLGGGTFDVSLL (203-216 AA) and VVLVGGSTRIPKVQQ (340-354 AA). Phylogenetic tree analysis of *HSP70* amino acid sequences from *P. lactiflora* and some other plants showed that the phylogenetics of *HSP70* was in line with the traditional plant taxonomy (Fig. 6). *HSP70* in these genes was divided in two categories, namely, dicots (*P. lactiflora*, *P. suffruticosa*, *Cucurbita maxima*, *Cucumis sativus*, *Solanum tuberosum*, *Solanum lycopersicum* and *Cyclamen persicum*) and monocotyledons (*Dendrobium officinale*, *Zea mays*, *Oryza sativa Japonica Group*, *Oryza sativa Indica Group*, *Triticum aestivum* and *Lilium longiflorum*). Moreover, *HSP70* in the same and allied plants were put together, and *P. suffruticosa* was the one most similar to *P. lactiflora*.

Expression Analysis of Heat Shock Protein Genes

In order to make clear whether heat shock protein genes could regulate the heat resistance in *P. lactiflora*, the expression patterns of *PlHSP70* and two other genes (*PlHSP60* and *PlHSP90*), which previously registered in NCBI with accession numbers KC985245 and KC985246 were analyzed by Q-PCR, and the expression levels of the different types of *HSP* responded differently to shade treatment (Fig. 7). The expression level of *PlHSP60* in sun-exposed plants increased gradually and peaked in S4, whereas that in shaded plants increased gradually in the early stages, peaked in S3 and then decreased in S4

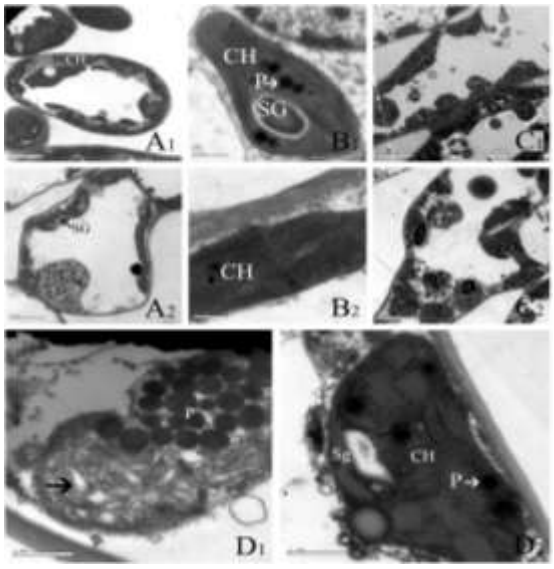

Fig. 4: Effect of shade treatment on the cell ultrastructure in *P. lactiflora*, A1: The mesophyll cell of *P. lactiflora* under Sun exposure in S1 (Bar=5um); A2: The mesophyll cell of *P. lactiflora* under shade treatment in S1 (Bar=5um); B1: The chloroplast of *P. lactiflora* under Sun exposure in S1 (Bar=0.5um); B2: The chloroplast of *P. lactiflora* under shade treatment in S1 (Bar=0.5um); C1: The mesophyll cell of *P. lactiflora* under Sun exposure in S4 (Bar=5um); C2: The mesophyll cell of *P. lactiflora* under shade treatment in S4 (Bar=5um); D1: The chloroplast of *P. lactiflora* under Sun exposure (Bar=1um); D2: The chloroplast of *P. lactiflora* under shade treatment in S4 (Bar=1um)

Note: CH = chloroplast, SG = starch grain, P = plastoglobuli, M = mitochondria

which was only 47% of that in sun-exposed plants. In terms of *PlHSP70*, its expression levels in the shaded and sun-exposed plants trended similarly, they all increased in the first two stages (peaked in S2) and then declined. Besides, the expression levels of *PlHSP70* in the shaded and sun-exposed plants were almost the same in S1 and S2, but the former was significantly higher than that of sun-exposed plants in the high temperatures of S3 and S4 with 168% and 263% increase, respectively. In four stages of plant development, the expression level of *PlHSP90* in sun-exposed plants fluctuated minimally, which was lower than that of shaded plants all the time. In addition, the expression level of *PlHSP90* in shaded plants was significantly enhanced in S3 and reached its maximum, which was 212% that of sun-exposed plants however, its expression level decreased by 43.8% in S4 compared with the levels observed in S3.

Discussion

In the middle and lower reaches of the Yangtze River basin

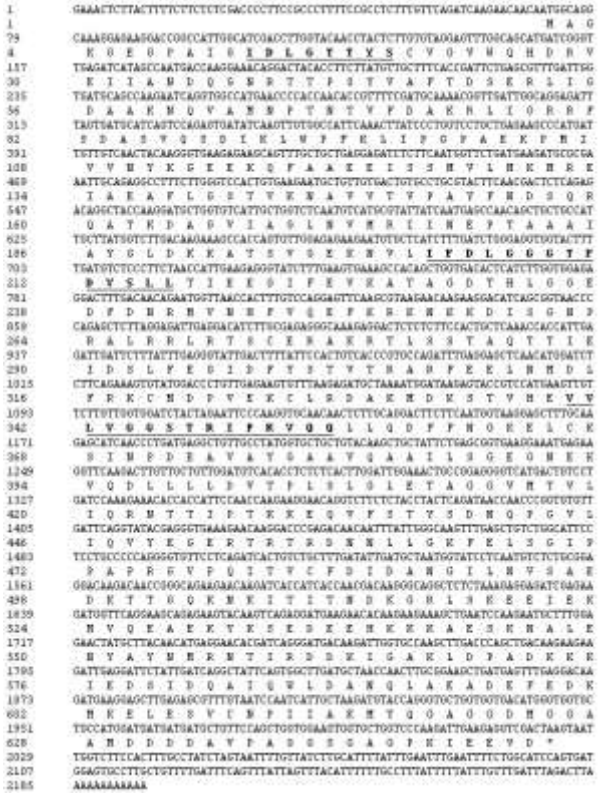

Fig. 5: Nucleotide sequence and the deduced amino acid sequence of *PlHSP70*, the deduced amino acid sequence was showed underneath the corresponding nucleotide sequence, others was un-coding region, the deduced amino acid sequence with bold type and underline indicated three highly conserved *HSP70* family signatures and stop code was indicated with *"Underlined amino acids were three highly conserved HSP70 family signatures

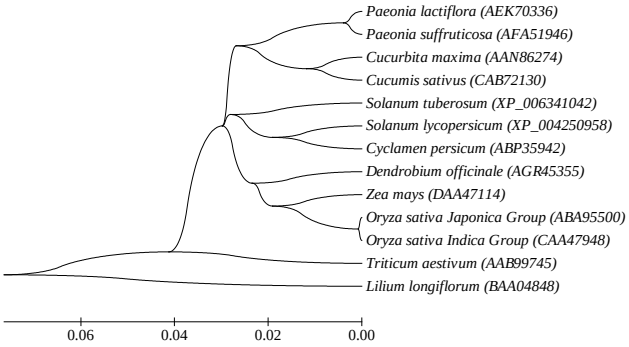

Fig. 6: Phylogenetic tree of *HSP70* amino acid sequences from *P. lactiflora* and some other plants

of China, summer is characterized by clear weather, intense solar radiation, abnormal continuous or intermittent high temperature and intense heat owe to the western pacific subtropical anticyclone control. Summer high temperature, an extreme weather condition, is very bad for the normal

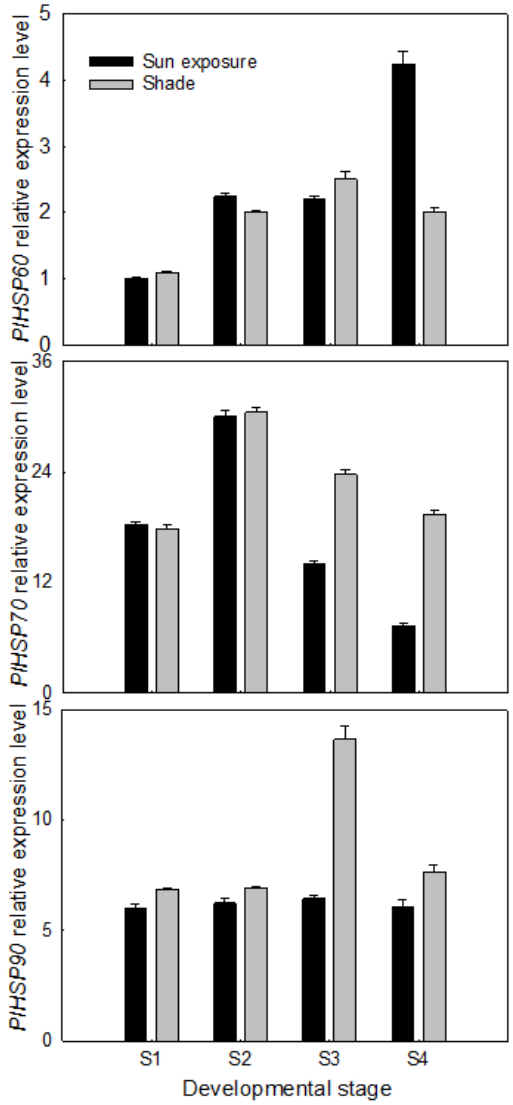

Fig. 7: Effect of shade treatment on the expression patterns of *PlHSP60*, *PlHSP70* and *PlHSP90*

Note: *PlHSP60* = heat shock protein 60 gene in *P. lactiflora*, *PlHSP70* = heat shock protein 70 gene in *P. lactiflora*, *PlHSP90* = heat shock protein 90 gene in *P. lactiflora*, S1 = May, S2 = June, S3 = July, S4 = August

growth of plants, resulting in a series of changes in plant morphology, anatomy, physiology, biochemistry and phenology drastically (Wahid *et al*., 2007; Snider *et al*., 2009). Therefore, it is important and urgent to take protective measures to reduce or alleviate the thermal damage of high temperature stress to plants. At present, the measures of alleviating high temperature damage in plants are mainly spraying exogenous substances, such as salicylic acid, abscisic acid, 24-epibrassinolide and other plant growth regulators, which play a key role in alleviating oxidative damage and photosynthesis decrease, together with inducing HSP synthesis (Kumar *et al*., 2012; Zhang *et al*., 2014). Besides these measures, as a simple cultivating measure involved in controlling the summer high temperature, shade

has drawn much attention over the past several decades. Armson *et al.* (2012) found that the surface temperatures of grass in shade can be 4–7°C cooler than the surrounding air, and our previous study also revealed shade reduced leaf temperature in *P. lactiflora* (Zhao *et al*., 2012a). Therefore, shade is widely applied in horticultural plants in summer.

Formation of plant resistance is often closely related to increased antioxidant system activity (Yuan *et al*., 2011). Many adversity stresses can lead to active oxygen metabolism disorder, the accumulation of free radical and further damage of cell membrane structure, and yet plant can maintain the membrane stability by means of resisting and eliminating reactive oxygen with antioxidant enzymes and antioxidants and inhibiting the membrane lipid peroxidation (Ye *et al*., 2000; Davis and Swanson, 2001). For example, MDA content of *P. lactiflora* under pH4.0 irrigation water treatment was significantly increased, but its damage was alleviated by increased SOD, POD and CAT activities (Zhao *et al*., 2013). And under the high temperature stress, the MDA content and the activities of SOD and POD of *P. lactiflora* in this study also increased significantly, whereas the CAT activity declined. Meanwhile, these three protective enzyme activities all enhanced under shade which resulted in reducing the accumulation of active oxygen in plant cells and decreasing the level of membrane lipid peroxidation so as to make the *P. lactiflora* grow well at a high temperature.

Morphological structure and physiological function of the plant organs were closely to get with its growing environment, among which the chlorophyll content and proportion was an important indicator for its adaptation and use of environmental factors (Sharma *et al*., 2012).

Under the high temperature stress, the chlorophyll content of tall fescue (*Festuca elata*) was decreased, cell membrane structure was damaged, and the growth rate was inhibited (Wang *et al*., 2009), which was similar with *P. lactiflora* growth status. But after shade treatment, the chlorophyll content of *P. lactiflora* reduced slowly, photosynthesis enhanced significantly, and soluble sugar content in the late stages was also higher than that of control. All of these were consistent with the performance of poinsettia under shade in the summer (Pan and Jiang, 2006), indicating that *P. lactiflora* could accumulate more nutrients for plant normal growth through its own adaptive adjustment. In addition, *Tr* of shaded *P. lactiflora* was relatively higher, which might be attributed to considerably larger stomatal conductance of leaf, comparatively stronger water metabolic activity, and so as to keep photosynthesis operating smoothly and promote the synthesis of organic compounds.

Leaf is the vital organ of plants, and the variation and plasticity of its shape and internal structure was maximum under shade treatment (Dias *et al*., 2007). In microstructure, high temperature destroyed the chloroplast and thylakoid structures of poor heat resistance blueberry (*Vaccinium uliginosum*), even caused more lipid spheres appearing and affecting the plant growth (Chen *et al*., 2012). Similarly, high temperature also destroyed chloroplast, stroma lamella and grana lamella structures of *P. lactiflora*, increased the number of lipid spheres in cells. However, shaded *P. lactiflora* had intact chloroplasts, neatly arranged stroma lamellas and grana lamellas, significantly fewer number of lipid spheres in cells than control, which was beneficial to the plant photosynthesis. This result was in agreement with higher chlorophyll content and photosynthesis in shaded *P. lactiflora*.

HSP was stress protein produced by organism in response to high temperatures or other stress conditions, which could synthesize and accumulate largely in a short time and improve the tolerance of organism to adverse environment including high temperature (Schoffl *et al*., 1999). Based on the approximate molecular weight, HSP of plants have been classified into five classes: HSP100, HSP90, HSP70, HSP60 and small heat-shock proteins (sHSP) (Al-Whaibi, 2011). Among which, HSP70 was the hottest studied and most widely distributed and most conservative HSP (Wahid *et al*., 2007). It was also confirmed that a positive correlation between its expression level and the heat resistance of plants (Lee and Schoffl, 1996). In this study, *PlHSP70* was isolated from *P. lactiflora* using homologous cloning technology, had high homology with nucleotide and amino acid sequences of other plants, and contained three highly conserved *HSP70* family signatures, which was in accordance with the highly conservative conclusion put forward by predecessors (Wahid *et al*., 2007). Besides *HSP70*, *HSP60* could stabilize advanced structure of proteins, maintain the enzyme activity, help eliminate dangerous free radicals and protect biological macromolecules under high temperature stress (Roy and Raghavan, 1994). Moreover, *HSP90* was mainly involved in signal transduction, cell cycle regulation, protein degradation and transport (Richter and Buchner, 2001; Pratt *et al*., 2001), and also associated with the morphological development and phenotypic variation (Queitsch *et al*., 2002). For example, *ZmHSP*90 expression of maize (*Zea mays*) had obvious response to abiotic stresses including high temperature and drought (Liu *et al*., 2012). In this study, the expression levels of *PlHSP70* and *PlHSP90* in shaded *P. lactiflora* were all higher than those of control, while the expression level of *PlHSP60* was opposite, which might be that shade alleviated the synthesis inhibition of HSP70 and HSP90 at high temperature. In general, under the shade treatment, high protective enzyme activities and expression levels of HSP reduced the thermal damage to leaf internal structure and the photosynthetic pigment degradation, contributing to keep *Pn* high and eventually delay the senescence and death of plants. These results provided an understanding for the biochemical and molecular mechanisms of shade alleviating the thermal damage of high temperature stress to *P. lactiflora*, which might lay a theoretical foundation for *P. lactiflora* cultivated form in summer.

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