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PHYSIOLOGICAL AND MULTIGENIC CHARACTERISTICS OF WIDELY USED TREE AND HERBACEOUS PEONIES IN RESPONSE TO FIELD STRESS IN YANQING, BEIJING, CHINA

WENSI TANG[1], LONGJUN CHAO[1], ARSHAD IQBAL[1], WENBIAO ZHANG[2], JING DING[3] AND HUAFANG WANG[1*]

[1]National Engineering Laboratory for Tree Breeding, College of Biological Sciences and Biotechnology, Beijing Forestry University, Beijing 100083, China
[2]Yanqing No.1 Vocational School, Beijing 102100, China
[3]Jiangsu National Beauty and Oil Peony Science and Technology Development Co., Ltd., Jiangsu 213000, China
**Corresponding author's email: hfwang@bjfu.edu.cn*

Abstract

There are nine Chinese indigenous tree peony species including *Paeonia suffruticosa* and *Paeonia rockii*, which have hundreds of traditional ornamental cultivars widely grown in China and other countries we assessed the adaptability of four peonies to this specific site using field trials. Four one-, two-, and three-year old plants of different varieties or cultivars were grown unprotected and their growth, 13 physiological criteria, and the sequence and expression characteristics of three gene homologs in response to the severe field conditions were assessed. The growth of three-year old plants was significantly different from the others. The membership function values of the four peonies in decreasing order were as follows: *P. ostii* var. *lishizhenii* > *P. suffruticosa* 'Wulong Pengsheng' (with *P. ostii* var. *lishizhenii* used as the rootstock). > *P. rockii* 'Bingshan Xuelian' > *P. lactiflora* 'Taohong Yushuang'. Some differences in the nucleotide or amino acid sequences of the dehydration responsive element binding protein 2A (*DREB2A*), *WRKY19*, and xyloglucan endotransglycosylase (*XET*) multigenes were observed between species. The phylogenetic tree based on conserved DREB2A sequences clustered into one branch with all four peony species, branches of *P. ostii* and other varieties for WRKY, and three branches of *P. ostii, P. rockii*, and other identified sub-clusters for XET. Gene expression analysis using quantitative reverse transcription-polymerase chain reaction divided the four peonies into two groups: stress-insensitive (*P. ostii* var. *lishizhenii, P. rockii* 'Bingshan Xuelian') and stress-sensitive (*P. suffruticosa* 'Wulong Pengsheng' and *P. lactiflora* 'Taohong Yushuang'). Notably, both groups adapted to field stress at the site in Yanqing, Beijing, albeit to different degrees. Together, these results indicate the importance of exploring the characteristics of peony adaptability to inform their relative suitability for specific horticultural applications.

Key words: *Paeonia ostii* var. *lishizhenii*, Physiological index, Molecular regulation, Geographic adaptation.

Introduction

China has nine indigenous tree peony species, including *Paeonia suffruticosa* Andrews and *P. rockii* (S.G. Haw & Lauener) T. Hong & J.J. Li ex D.Y. Hong, which have hundreds of traditional ornamental cultivars that are widely grown in China and across the world. *P. suffruticosa*, a popular Chinese traditional flower, has been used for more than 1600 years and cultivated in urban Beijing for over 1000 years (Ghayur *et al*., 2008; Yuan *et al.*, 2011). *P. suffruticosa* cultivars are grown in urban Beijing in protected courtyards and parks during the winter, including Zhongshan Park and the Summer Palace.

When cultivating plants, especially in urban areas, their physiological and molecular biological responses and resistance mechanisms to stress should be studied. This has been previously undertaken for the peony to a certain extent (Yuan *et al*., 2011). The moderate field moisture capacity of *P. suffruticosa* is approximately 70%, indicating a deficit at lower moisture levels (Hou *et al*., 2006). The leaf water content (LFW), chlorophyll a/b content, free proline content, soluble sugar content, relative water content, total malondialdehyde (MDA) content, carotenoid content, and cultivars of *P. rockii* affect plant stress resistance (Peng, 2014). In response to drought stress, the chlorophyll content, net photosynthetic rate, and transpiration rate of 1-year-old seedlings of *P. ostii* var. *lishizhenii* B.A. Shen declined gradually, whereas catalase (CAT) activity increased or decreased in the leaves and roots, respectively (Lu *et al*., 2011). Peony plants exposed to environments that fluctuated between low and high temperatures showed a decrease in the net leaf photosynthetic rate, antioxidant enzyme activities, soluble proteins (including heat shock proteins), and cell membrane permeability, as well as in proline, soluble sugar, and MDA content (Liu *et al.*, 2012; Zhang *et al.*, 2015; Sai *et al*., 2017). Dehydration responsive element binding protein 2A (DREB)/WRKY transcription factors are responsible for independent/dependent ABA signalling in the response to drought, high salt, and cold stress in most plants (Nakashima *et al.*, 2000; Niu *et al*., 2012; Xiu *et al.*, 2016; Zhang, L. *et al.*, 2020). In peonies, the *PsDREB* gene has been cloned from *P. suffruticosa* 'Luo Yanghong' leaves (Liu *et al*., 2015; Iqbal *et al*., 2017). Additionally, the xyloglucan endotransglucosylase (*XET*) target gene product participates in hydrotropic root growth and increases plant drought resistance (Wang, 2009; Cai *et al.*, 2015). These physiological and molecular correlations for peony plants are crucial to the analysis of their resistance to site stress conditions.

Yanqing, a suburban city north-west of Beijing, successfully bid for the 2019 Beijing World Horticultural Exposition and has therefore been the focus of preparations for this international conference. As a well-known traditional flower of China, the tree peony has been chosen as the theme flower to be displayed at the Beijing Exposition, which is significant and symbolically important. However, the year-round temperature of the Yanqing climate is approximately 4°C below that of other urban areas of Beijing, and the minimum temperature

recorded was 33.7°C, which is 12.5°C lower than Beijing urban conditions due to prevailing winter northwest winds. The average annual precipitation is 481.1 mm, 163.1 mm less than Beijing urban levels (Cui *et al*., 2017). As the growth of peonies in Yanqing field sites has not been characterized, it is therefore necessary to study the physiological and molecular biological responses of various peony species to the severe Beijing suburban conditions to confirm their suitability and optimal presentation for the 2019 World Horticulture Exposition.

Among the most widely grown tree peonies, *P. suffruticosa* has > 1000 cultivars (most are double-flowering with few seeds) in Central China and *P. rockii* has > 500 cultivars; both species are used ornamentally. In addition, *P. ostii* var. *lishizhenii is* robust and therefore thrives in most barren sites (Wang, 2009). It is used as a traditional Chinese medicine formulation, to graft stocks for ornamental tree peonies, and is among the top ten ranked woody oil trees in China. In addition, the herbaceous peony *P. lactiflora* Pall., is important as a stock for grafting ornamental tree peonies (Kamenetsky & Dole, 2012; Yuan *et al.*, 2014; Cai *et al.*, 2015).

The aim of the present study was to address the relative performance of these four peony species with regard to growth and environmental stress resistance at the Yanqing site, in preparation for the World Horticulture Exposition. For each species, one to three year old plants were grown on the Yanqing Beijing site and their growth was determined based on four indices: height, number, and length of branches on the ground parts; length of primary roots; and number of secondary roots. Furthermore, 13 physiological indices including leaf water content (LWC), electrolyte leakage (EL), stomatal conductance (SC), chlorophyll content, soluble sugar content, free proline content, soluble protein content, superoxide dismutase (SOD) content, peroxidase (POD) content, CAT, and MDA content were evaluated for drought stress resistance. The membership function method was used to analyse the physiological traits and determine the resistance of the peonies. The stress response genes *DREB2A*, *WRKY19*, and *XET* were sequenced using their conserved domains, and quantitative reverse transcription-polymerase reaction (qRT-PCR) was used to quantitatively determine their expression levels in order to analyse the response characteristics of stress genes. These findings will be useful for determining the relative importance the characteristics of peony adaptability for predicting site-specific performance under stressful conditions in general, and for the Yanqing site in particular.

Materials and Methods

Plant materials: The peonies used in the experiments were adapted to Beijing urban conditions. *P. suffruticosa* Andr. 'Wulong Pengsheng' grows in the Chinese Central Plains (Ruixian *et al.,* 2017) and the Beijing urban area, and is propagated by grafting scions onto rootstocks of *P. ostii* var. *lishizhenii*. *P. rockii* (S.G. Haw & Lauener) T. Hong et J. J. Li 'Bingshan Xuelian', which grows naturally in Gansu Province and other northwest areas, is hardened against drought and cold, and is suitable for seed propagation. *Paeonia ostii* T. Hong et J. X. Zhang var. *lishizhenii* B. A. Shen grows throughout the country and exhibits strong resistance and flexible adaptability to fluctuating site environmental conditions and can be propagated by sowing seeds. *P. lactiflora* Pall. 'Taohong Yushuang', an herbaceous plant grown in the northern areas of China. All four peonies were studied for three consecutive years.

We grew the four peonies in the same area of the Beijing suburb Yanqing District (115.44°E, 40.16°N), located in the northwest of Beijing, China. The climate of this area is drought-prone with windy and cold winters. The annual average temperature is 8.5°C with extreme minimum and maximum temperatures of 33.7°C and 39°C, respectively (Huo *et al.,* 1988). The recorded annual precipitation is 494 mm. We maintained the plants at the site for 152-175 days. In comparison, the annual average temperature in urban Beijing, where the peonies commonly grow, is 12.8°C, with extreme minimum and maximum values of 21.2°C and 41.6°C, respectively, and the recorded annual precipitation is 644.2 mm. There were approximately 180–200 frost-free days (Huo *et al.,* 1988). The Yanqing climate within the Beijing area is thus severe.

Leaf sampling: For DNA and RNA extraction, nine plants each from the four species varieties/cultivars were randomly selected and the first mature leaf from each apex was sampled. The main veins of the sample leaves were removed and three leaves each from the three varieties/cultivars were combined as one mixed sample, wrapped in aluminium foil, and immediately stored in liquid nitrogen. Each mixed sample was used to produce three replicates.

For the physiological analysis, leaf samples were collected from three random plants from each of the four varieties/cultivars, selected using the same methods described above, and the samples were stored in a cold environment with ice packs.

Soil water, temperature, and salinity determination: The soil water potential of the growing peonies was determined using the PSYPRO water potential system (Wescor, Logan, UT, USA) according to the manufacturer's instructions. The soil moisture content, temperature, and salinity were measured using a W.E.T Sensor Kit (Delta-T Devices Ltd., Cambridge, UK) according to the manufacturer's instructions.

Plant growth index determination: Briefly, 1-, 2-, and 3-year-old plants of the four peony varieties/cultivars were measured in autumn by digging up the plant roots and gently dissolving any adhered soil in water. Ten plants of each variety/cultivar were randomly selected and measured as per the minimum sample requirement. The plant branch numbers and length, and the number and length of primary and lateral roots, were recorded. The length measurement accuracy was ± 1 mm.

Determination of physiological indexes of stress resistance

LWC, cell membrane permeability (EL), and stomatal conductance: LWC was determined by noting the fresh weight (Wf). The leaves were immersed in water to absorb until saturation, excess water was removed using a tissue paper, and the weight (Ws) was then noted. Subsequently, the leaves were dried in the oven at 80°C for about 24 hours in order to ascertain the constant weight (W_d) (Lu *et al.,* 2006). LWC was determined as follows:

$$LWC\ (\%) = (W_f - W_d) / (W_S - W_d) \times 100$$

Leaf EL was determined using the conductivity method (Lu *et al.*, 2009). The leaf sample was washed three times with deionized water, excess water was drained using a tissue paper, and 0.1 g samples were weighed out and immersed in 10 mL deionized water for 24 hours. The conductivity (C_i) of the solution was measured using a conductivity meter (Leici-DDS-J-308A, Shanghai, China). The solution was then placed in a boiling water bath for 20 minutes and the solution conductivity (C_{max}) of the dead tissues was measured. The following formula was used to calculate EL:

$$EL\ (\%) = (C_i / C_{max}) \times 100$$

The stomatal conductance of healthy leaves was determined using a leaf porometer (Decagon Devices, Inc., Pullman, WA, USA) as per the manufacturer's instructions.

Determination of chlorophyll a, b, and ab contents: Fresh leaves were cut and 0.2–0.3 g pieces (W) were measured and decolorized in 10 mL (V) of 80% acetone. The extract solution was poured into a cuvette (1 cm side length) and the OD was measured at wavelengths of 663 nm and 645 nm using a spectrophotometer; 80% acetone was used as the blank. The chlorophyll a, b, and ab contents were calculated according to following formulas (Lu *et al.,* 2009):

$$\text{Chlorophyll a content (mg/g)} = (12.71 \times OD_{663} - 2.59 \times OD_{645})\ V / 1000 \times W$$

$$\text{Chlorophyll b content (mg/g)} = (22.88\ OD_{645} - 4.67\ OD_{663})\ V / 1000 \times W$$

$$\text{Chlorophyll ab content (mg/g)} = (8.04 \times OD_{663} + 20.29 \times OD_{645})\ V/1000 \times W$$

Determination of soluble sugar, proline, and soluble protein content: The soluble sugar content was determined using the anthrone method (Zhang *et al.,* 2015). Briefly, 0.5 g of fresh leaves were weighed, and soaked in 3 mL of 80% ethanol for 30 minutes, followed by centrifugation (10,000 ×*g*) for 15 minutes. The supernatant was extracted and centrifuged again. Then, 0.1 mL of the extract was placed in 3 mL of anthrone reagent (150 mg of anthrone dissolved in 100 mL of 7.74 M 98% H_2SO_4), heated in a boiling water bath at 90°C for 15 minutes, and cooled to room temperature. The OD value at λ620 nm was determined using a spectrophotometer., A.D-glucose standard curve (mg versus OD) was used to determine the soluble sugar content.

The proline content was determined using sulfosalicylic acid (Zhang *et al.*, 2015): 0.5 g of fresh leaves were immersed in 10 mL of 3% aqueous sulfosalicylic acid, homogenized, and filtered. Then, 2 mL of the filtrate was placed in a test tube and mixed with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin. The mixture was heated in a boiling water bath for 40 minutes and cooled to room temperature. The OD value was measured at λ520 nm using a spectrophotometer and the proline content was determined from an L-proline standard curve.

The soluble protein content was determined using the Bradford method (Bradford, 1976): 1.0 g of fresh leaves were ground in a pre-cooled mortar by adding 2 mL of pre-cooled 50 mM phosphate buffer (pH 7.8), and then additional buffer was added to adjust the mixture to a final volume of 10 mL. The mixture was centrifuged at 10,000 ×*g* (4°C) for 15 minutes. The supernatant was used to determine antioxidant enzyme activity and protein content. The Bradford method was used to prepare the standard curve of bovine serum albumin, and the OD value was measured at λ595 nm using a spectrophotometer. The soluble protein content was calculated according to the standard curve.

Free radical oxidation activity and MDA content determination: In total, 0.2 g of fresh leaves with main veins from one or two young leaves were weighed and ground with a mortar and pestle in liquid nitrogen. Leaf powder was then homogenized by adding 3 mL of 100 mM phosphate buffered saline (pH 7.8) and centrifuged at 10,000 × *g* for 20 minutes at 4°C. The supernatant was transferred to new centrifuge tubes for further analysis.

SOD (EC 1.15.1.1) activity was tested using the nitro tetrazolium (NBT) method (Giannopolitis & Ries, 1977): 20 μL of leaf protein extract was mixed with 0.2 mL of 1.125 mM NBT, 5 mL of 50 mM phosphate buffer, 0.3 mL of 195 mM methionine, 0.1 mL of 60 μM nucleus flavonoids (pH 7.8), and then kept under light (200 μmol $m^{-2}\ s^{-1}$) for 20 minutes, followed by OD measurement at λ560 nm using a spectrophotometer. An SOD activity unit was defined as the amount of enzyme required to inhibit the 50% reduction of NBT photochemical reduction.

POD (EC 1.11.1.7) activity was measured using guaiacol (Giannopolitis & Ries, 1977): 20 μL of the protein extract was mixed with 1 mL of 0.25% (w / v) guaiacol, 3 mL of 100 mM sodium acetate buffer (pH 5.0), and 0.5 mL of 0.75% H_2O_2 for 3 minutes, and the OD value at λ460 nm was recorded using a spectrophotometer. A POD activity unit was defined as the amount of enzyme required for increases of 0.01 in absorbance at λ560 nm as measured using a spectrophotometer.

CAT (EC 1.11.1.6) activity was determined using the H_2O_2 method (Giannopolitis & Ries, 1977): 50 μL of the above protein extract supernatant was reacted with 1.5 mL of 50 mM phosphate buffer (pH 7.0) and 0.25 mL of a 45 mM H_2O_2 mixture for 3 minutes. The dynamic absorbance at 240 nm of H_2O_2 was recorded every 60 seconds for 4 minutes (extinction coefficient 0.0394 $mM^{-1}\ cm^{-1}$)., A.CAT activity unit was defined as the amount of enzyme required to decrease 0.1 of absorbance at λ240 nm per minute.

The MDA content was determined using the thiobarbituric acid (TBA) method: 0.5 g of fresh plant leaves were ground in 2.5 mL of a 0.25% TBA (w / v) and 10% (w / v) trichloroacetic acid mixture. The sample homogenate was boiled at 100°C for 20 minutes, cooled to room temperature, and centrifuged at 10,000 ×*g* for 10 minutes. The OD value was measured at λ532 nm and λ600 nm using a spectrophotometer, and the MDA content was determined using the following formula:

$$MDA\ (mM) = (OD_{532} - OD_{600}) / 155$$

where 155 is the extinction coefficient at 532 nm, units of $mM^{-1}\ cm^{-1}$, to eliminate the interference caused by sucrose.

Conserved sequence characteristics and expression of DREB2A, WRKY19, and XET

Analysis of conserved sequences of DREB2A, WRKY19, and XET: In order to amplify the *DREB2A*, *WRKY19*, and *XET* genes, *FpDREB2A* (AY536056), *TaWRKY19*, *MtXET* (DQ855285), and *PeXET* (EF612703) were used as reference sequences for the Phytozome v.11.0.6 database (http://phytozome.jgi.doe.gov/pz/portal.html) (Goodstein *et al.*, 2012). An online search revealed the homologous conserved sequences of 17 species, based on which primers were designed using Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) as shown in Table 1. The 17 species used for primer design were as follows: *Brassica rapa*, *Arabidopsis thaliana*, *Carica papaya*, *Citrus sinensis*, *Glycine max*, *Gossypium raimondii*, *Medicago truncatula*, *Oryza sativa*, *Populus trichocarpa*, *Prunus persica*, *Ricinus communis*, *Solanum lycopersicum*, *Sorghum bicolor*, *Theobroma cacao*, *Triticum aestivum*, *Vitis vinifera*, and *Zea mays*.

Leaf DNA was extracted from the four peonies using the cetyltrimethylammonium bromide (CTAB)-sodium dodecyl sulfate (SDS) method. Briefly, 200 mg of fresh leaf was weighed and ground in 20% SDS and 20% CTAB extraction lysis buffer. Genomic DNA was then washed with ethanol and dissolved in ddH_2O. Electrophoresis was then used to determine DNA integrity, and an ultra-trace spectrophotometer (SMA3000, Beijing, China) was used to determine DNA purity; the final working concentration was adjusted to 100 ng μL^{-1}.

DREB2A, *WRKY19*, and *XET* conserved sequences were amplified from the genomic DNA of the four peonies by PCR (Veriti 96, Applied Biosystems, Inc., Foster City, CA, USA). The PCR amplification mixture of 25 μL comprised of 200 ng of genomic DNA, 2.5 μL of 10 × $MgSO_4$ (Fermentas, St. Leon-Rot, Germany), 2.5 μL of 2 mM dNTP (Fermentas), 4 μL of 10 μM forward primer, 4 μL of 10 μM reverse primer, and 0.25 μL of 2.5, U Taq DNA polymerase (Fermentas). PCR reaction conditions were as follows: pre-denaturation for 10 minutes at 94°C, denaturation for 45 seconds at 94°C, annealing for 30 seconds (*DREB2A* at 60°C, *WRKY19* at 54°C, *XET* at 58°C), and extension for 45 seconds at 72°C, for 40 cycles, followed by a final extension for 10 minutes at 72°C. The PCR product was electrophoresed on an 0.8% agarose gel and the target product was purified, ligated into the pGEM-T easy vector (Promega, Beijing Branch, China), and transformed into *Escherichia coli* Top10 competent cells (TransGen Biotech, Beijing, China). PCR positive clones were sequenced using the ABI 3730 DNA Sequencer.

For the intron and exon analysis of the conserved sequences, specific primers were designed based on the DNA sequencing results. Total RNA was isolated from plant leaves and the conserved sequences mentioned above were amplified using cDNA as the template, sequenced, and compared with DNA sequences.

The conserved nucleotide/amino acid sequence of the *DREB2A*, *WRKY19*, and *XET* genes of each variety/cultivar were compared with the nucleotide/amino acid sequence of the four peonies. A phylogenetic tree was constructed for the four peonies and 17 other species obtained from the Phytozome database to analyse their evolutionary relationships and functional differences.

Expression analysis of DREB2A, WRKY19, and XET: Absolute expression of the four target genes was determined via the standard curve method (Batista *et al.*, 2014). For the preparation of standard plasmids, total RNA was first isolated from the four peonies. As shown in Table 2, primers were designed, and the sequenced cDNA was used as a template to amplify the target sequence. PCR products were then separated on a 1% agarose gel. The target fragments were purified and ligated to the pGEM-T easy vector (Promega). Next, DH5α competent cells were transformed and a single colony culture was used for plasmid extraction. Plasmid concentration was determined using an ultra-trace spectrophotometer (SMA3000). The cloned fragment was sequenced using the ABI3730 DNA Analyzer. The plasmid copy number was calculated using the following formula:

$$Copies/\mu L = (6.02 \times 10^{23}) \times (MW\ ng/\mu L \times 10^{-9})/(DNA\ length \times 660)$$

where MW ng/μL was the concentration of standard plasmid and the DNA length was the size of the plasmid. A 10-fold serial dilution series of the standard plasmid with nuclease-free water, ranging from 1×10^1 to 1×10^5 copies/L, was used to generate the standard curves.

DREB2A, *WRKY19*, and *XET* expression was then measured using ABI 7000 Real Time Fluorescence qPCR. Briefly, a 20 μL reaction mixture was prepared according to the GoTaq® qPCR Master Mix, comprising 10 μL of GoTaq® qPCR Master Mix (2×), 0.8 μL of the upstream and downstream primers, 0.4 μL of ROX Reference Dye II (50×), 6 μL of nuclease-free water, and 2 μL of template DNA (i.e. the peony DNA). The reaction cycle was set as follows: pre-denaturation for 2 minutes at 95°C, denaturation for 15 seconds at 95°C, annealing extension at 60 seconds at 60°C, for 40 cycles. After the completion of the reaction, the dissolution curve was analysed from 65°C to 95°C at a rate of 0.5°C/s. For each peony sample, three biological and three technical replicates were prepared. Nuclease-free water was used as the negative control for the template. *DREB2A*, *WRKY19*, and *XET* copy numbers of the four peonies were calculated based on the Ct value.

Table 1. Primers of *DREB2A, WRKY19*, and *XET* conserved domains degenerated for PCR.

Name	Primer sequence	Annealing temperature (°C)	Fragment size (bp)
DREB2A-F	5′-TGAGGTACRTGAGTCACHGA-3′	60	200
DREB2A-R	5′-ACDGCDGAMTGNACRGCST-3′		
WRKY19-F	5′-TCGANANAARTCNGANCTG-3′	54	250
WRKY19-R	5′-GCNGTNCTNAABGCACGN-3′		
XET-F	5′-GCHGAGATNTAYTCSGAATT-3′	58	650
XET-R	5′-GCNGAVCANTAVTAGAGGAAC-3′		

Table 2. Real time-qPCR primers of *DREB2A*, *WRKY19*, and *XET*

Name	Primer sequence	Applicable species	Fragment size (bp)
DREB2A-F1	5′-CCGCCAAAGTCACCTGCAAT -3′	1, 2, 3	160
DREB2A-R1	5′-TTCGACTCCCAAAGAACCGT -3′	1, 2, 3	
WRKY19-F1	5′-AAGGGAATCCTAACCCAAGG-3′	1, 2, 3	165
WRKY19-R1	5′-TCCACATGTTTCCTCACTGG-3′	1, 2, 3	
XET-F1	5′-CGGAACACCTCTGGACTCTG-3′	1, 2, 3	188
XET-R1	5′-CACTGCCGATTTCCACACAT-3′	1, 2, 3	
DREB2A-F	5′ -GTAGTGGTTTGTAATCGCCGC-3′	4	154
DREB2A-R	5′ -CGTCTTTGGCTCGGCACATT-3′	4	
WRKY19-F	5′ -AAGGGAATCCTAACCCAAGG-3′	4	173
WRKY19-R	5′ -TCCACATGTTTCCTCACTGG-3′	4	
XET-F	5′ -TTGGGGTGCCATCTACAGAG-3′	4	199
XET-R	5′ -GGCAAGGGAGATAAGGAGCA-3′	4	

Note: 1: *P. ostii* var. *lishizhenii*; 2: *P. suffruticosa*. 'Wulong Pengsheng'; 3: *P. rockii* 'Bingshan Xuelian'; 4: *P. lactiflora* 'Taohong Yushuang'

Data processing and analysis

Statistical analysis: Data were analysed using SPSS software 12.0 (SPSS, Chicago, IL, USA). The Duncan multiple test was used to determine the significant difference between means ($p<0.05$).

Comprehensive evaluation of peony physiological adaptation to natural site conditions: Data obtained from the 13 physiological parameters of the four peony varieties/cultivars were subjected to a membership function model to integrate the quantitative value, comprehensively order peony values, and to evaluate their adaptation. The 13 physiological parameters included water parameters: LWC, EL, and stomatal conductance; photosynthetic pigment content: chlorophyll a, chlorophyll b, and chlorophyll ab; metabolites: soluble sugar content, proline content, and soluble protein content; and oxidation or anti-oxidation: SOD activity, POD activity, CAT activity, and MDA content.

The higher or lower membership values from the membership function model suggest stronger or weaker adaptation to the sites of the plant growth (---). Negative parameter values (EL, stomatal conductance, and MDA content) were calculated using the anti-membership function values as follows:

$$\text{Membership function value} = (X_i - X_{min}) / (X_{max} - X_{min})$$
$$\text{Anti-membership function value} = 1 - (X_i - X_{min})/(X_{max} - X_{min})$$

where X_i is the measured data of the parameter, and X_{max} and X_{min} are the maximum and minimum values for a particular index in the group data.

Analysis of DREB2A, WRKY19, and XET conserved sequence characteristics and evolutionary relationships: Conserved gene sequences of *DREB2A, WRKY19*, and *XET* of the four varieties/cultivars were analysed using the CD-search tool of the National Center for Biotechnology Information (NCBI) database. MEGA6 was used to analyse differences in the sequences of the varieties/cultivars and a phylogenetic tree was generated based on the neighbour-joining method, computed with a minimum of 1000 Bootstrap replicates (Tamura *et al.*, 2013; Marchler-Bauer *et al.*, 2017).

Results

Growth of peony species in the Yanqing Beijing site: The widely cultivated peony species in China, *P. suffruticosa*, *P. rockii*, *P. ostii*, and *P. lactiflora*, were grown at the same site in Yanqing, Beijing. The site has the driest and windiest in Beijing, with an average temperature at least 4°C lower than that of other urban areas of Beijing. The minimum temperatures were 33.7°C and 20 °C, with minimum precipitation levels of 341 and 424 mm in 2013 and 2014, respectively. The soil temperature was 21.71 ± 1.98°C at the surface layer, with dense root distribution in the summer; the soil water potential and content were 1.21 ± 0.37 Mpa and 20.16 ± 7.22%, respectively, and the salt content was 792.92 ± 9.28 ppm.

The shoot growth of the four peony species is shown in Table 3. There were significant differences in the height of 1-, 2-, and 3-year-old plants. The heights of the three year old plants of the four peony varieties were as follows, in increasing order: 24.45 ± 1.93, 17.32 ± 1.55,

14.85 ± 1.68, and 2.22 ± 0.75 cm for *P. ostii* var. *lishizhenii*, *P. suffruticosa* 'Wulong Pengsheng', *P. rockii* 'Bingshan Xuelian', and *P. lactiflora* 'Taohong Yushuang', respectively. In addition, *P. suffruticosa* 'Wulong Pengsheng' sprouted, with the sprout number being significantly different from those of the other three 1-, 2-, and 3-year-old peonies (p<0.05).

The root growth is shown in Table 4. The primary root length of each species increased from the 1- to 3-year-old plants. The lengths were not significantly different for the 1- and 2-year-old plants of the three peony species, with the exception of *P. suffruticosa* 'Wulong Pengsheng'. Significant differences between all four peony species were noted in 3-year-old plants (p<0.05). The changes in lateral root number of all four peony species showed similar trends (Table 4).

Physiological changes of the four peonies

Changes in LWC criteria: The LWC of *P. ostii* var. *lishizhenii* was 89.80 ± 0.55%, which was significantly higher than the levels in the other three peonies (Fig. 1A). The EL values of the four peonies were 9.46%, 9.86%, 10.33%, and 11.16%, which were not significantly different (Fig. 1B). The SC of *P. suffruticosa* 'Wulong Pengsheng' was 95.33 ± 3.06 $mmol \cdot m^{(-2)-1} \cdot s^{-1}$, which was significantly higher than the other three peonies, (p<0.05, Fig. 1C); the other three peonies did not differ significantly from each other.

Chlorophyll a, b, and ab content in the four peonies: The chlorophyll content of the four peonies is shown in Figure 1D–F. The chlorophyll a, b, and a/b levels of *P. suffruticosa* 'Wulong Pengsheng' were 0.97 ± 0.04, 0.63 ± 0.18, and 1.67 ± 0.11 $mg \cdot g^{-1}$, respectively, which were significantly higher than the other three peonies. *P. ostii* var. *lishizhenii*, *P. rockii* 'Bingshan Xuelian', and *P. lactiflora* 'Taohong Yushuang' did not differ significantly in chlorophyll a or b content (Fig. 1D, E), but showed significantly different chlorophyll a/b content (p<0.05, Fig. 1F).

Soluble sugar, proline, and soluble protein content in the four peonies: The soluble sugar, proline, and soluble protein content of the four investigated peonies are shown in Figure 1G–I. *P. ostii* var. *lishizhenii* had the highest content of these three metabolites with soluble sugar, proline, and soluble protein values of 10.97 ± 0.67 $mg \cdot g^{-1}$ FW, 59.78 ± 5.88 $\mu g \cdot g^{-1}$ FW, and 9.81 ± 0.47 $mg \cdot g^{-1}$ FW, respectively. *P. rockii* 'Bingshan Xuelian' had the lowest soluble sugar content of 2.48 ± 0.16 $mg \cdot g^{-1}$ FW, whereas *P. suffruticosa* 'Wulong Pengsheng' had the lowest soluble protein content of 3.40 ± 0.42 mg g^{-1} FW.

The four peonies showed significant differences in soluble sugar content (Fig. 1G) and soluble protein content (Fig. 1I). The proline content in *P. ostii* var. *lishizhenii* was significantly different from that of the other three peonies, whereas no significant differences were found among *P. suffruticosa* 'Wulong Pengsheng', *P. rockii* 'Bingshan Xuelian', and *P. lactiflora* 'Taohong Yushuang' (p<0.05, Fig. 1H).

Antioxidant enzyme activity and MDA content in the four peonies: The enzyme activities of the antioxidants SOD, POD, and CAT of the four investigated peonies are shown in Figure 1J–L. *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' showed the highest SOD activities at 167.38 ± 42.06 and 210.09 ± 34.95 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, respectively, and they were not significantly different (p<0.05, Figure 1J). *P. lactiflora* 'Taohong Yushuang' showed the lowest SOD, but the highest POD and CAT activities at 85.25, 5.43 ± 0.24, and 18.86 ± 0.95 $U \cdot g^{-1}$ $FW \cdot min^{-1}$, respectively. *P. suffruticosa* 'Wulong Pengsheng' exhibited the lowest POD and CAT activities (Fig. 1K, L).

The SOD, POD, and CAT activities of the four peonies were significantly different (p<0.05), as were the SOD activities of *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' (Fig. 1J). *P. suffruticosa* 'Wulong Pengsheng' exhibited the lowest POD and CAT activities of the three tree peonies, with the exception of herbaceous *P. lactiflora* 'Taohong Yushuang', and the values were significantly different (p<0.05, Fig. 1K, L).

The content of the oxidation product, MDA, was highest in *P. lactiflora* 'Taohong Yushuang' at 0.37±0.00 $\mu mol \cdot g^{-1} \cdot FW^{-1}$ and lowest in *P. suffruticosa* 'Wulong Pengsheng' at 0.07±0.01 $\mu mol \cdot g^{-1} \cdot FW^{-1}$, and there were significant differences among the four peonies (p<0.05, Fig. 1M).

Table 3. Plant heights and sprouting number of four peonies widely used in China.

Variety/Cultivar	Plant height (cm)			Number of sprouts		
	1 year	2 years	3 years	1 year	2 years	3 years
Paeonia ostii var. *lishizhenii*	8.62 ± 2.67^{a}	10.30 ± 3.33^{b}	24.45 ± 1.93^{a}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}
Paeonia suffruticosa 'Wulong Pengsheng'	6.24 ± 0.68^{b}	11.84 ± 1.62^{a}	17.32 ± 1.55^{b}	1.60 ± 0.54^{a}	3.60 ± 0.89^{a}	6.00 ± 0.70^{a}
Paeonia rockii 'Bingshan Xuelian'	4.94 ± 0.71^{b}	7.40 ± 0.75^{b}	14.85 ± 1.68^{c}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}
Paeonia lactiflora 'Taohong Yushuang'	2.18 ± 0.45^{c}	2.26 ± 0.78^{c}	2.22 ± 0.75^{d}	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	1.00 ± 0.55^{b}

Note: Data represent the means ± standard deviation (n = 10). Letters indicate significant differences at p<0.05 (Duncan test)

Table 4. Primary root length and lateral root number of the four peonies.

Variety/Cultivar	Primary root length (cm)			Number of lateral roots		
	1 year	2 years	3 years	1 year	2 years	3 years
Paeonia ostii var. *lishizhenii*	16.80 ± 3.38^{ab}	19.70 ± 2.44^{b}	27.00 ± 1.84^{b}	0.80 ± 0.45^{b}	1.20 ± 1.10^{b}	5.80 ± 2.28^{b}
Paeonia suffruticosa 'Wulong Pengsheng'	18.25 ± 3.25^{a}	27.50 ± 7.50^{a}	36.00 ± 1.00^{a}	9.50 ± 0.50^{a}	9.00 ± 1.00^{a}	25.00 ± 5.00^{a}
Paeonia rockii 'Bingshan Xuelian'	14.20 ± 2.22^{b}	16.60 ± 1.88^{b}	24.60 ± 3.94^{b}	0.47 ± 1.06^{b}	2.20 ± 0.84^{b}	3.00 ± 0.71^{c}
Paeonia lactiflora 'Taohong Yushuang'	13.5 ± 2.45^{b}	15.00 ± 1.84^{b}	16.40 ± 3.31^{c}	0.00 ± 0.00^{b}	1.00 ± 1.00^{b}	3.20 ± 0.84^{c}

Note: Data represent the means ± standard deviation (n = 10). Letters indicate significant differences at p<0.05 (Duncan test)

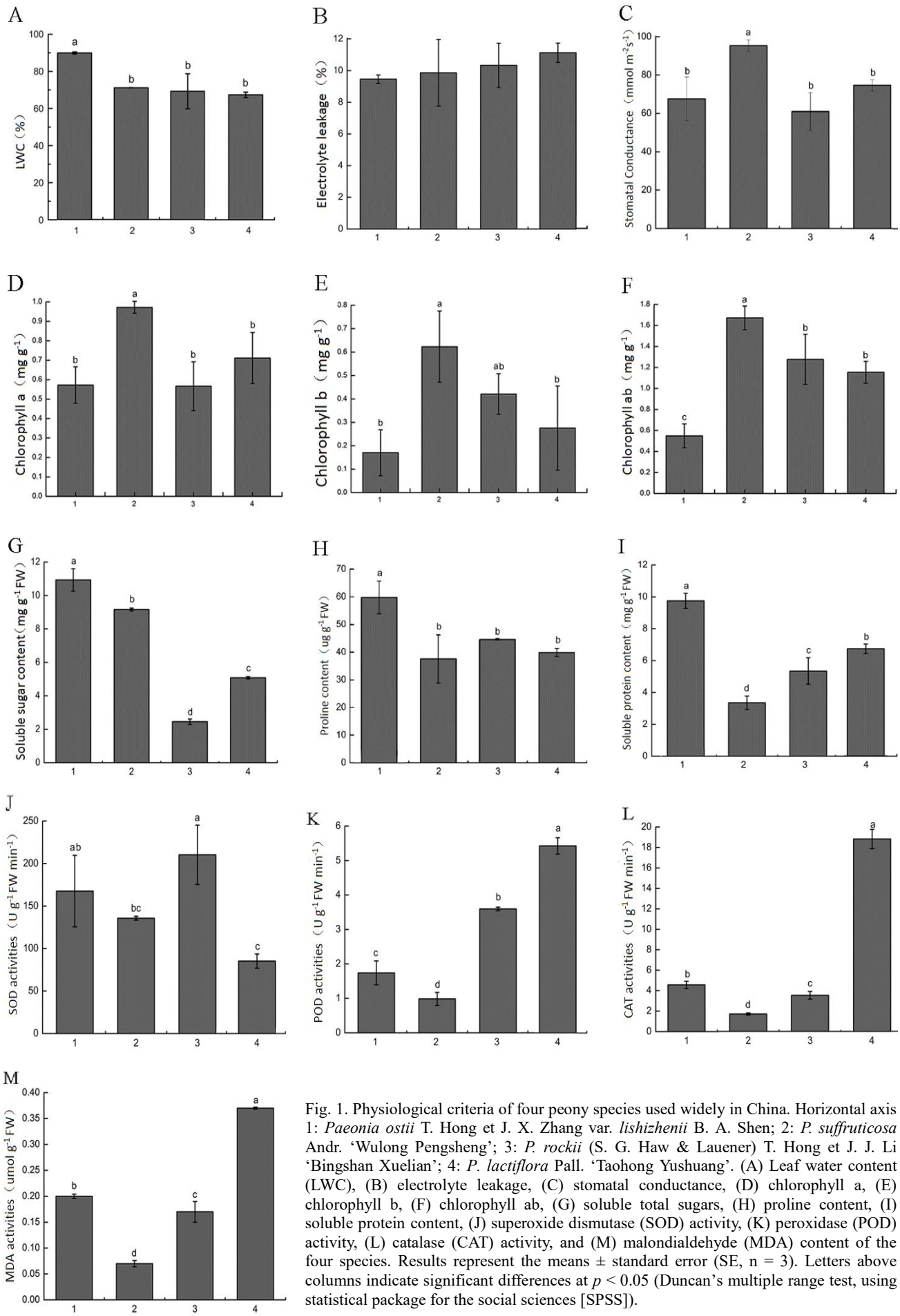

Fig. 1. Physiological criteria of four peony species used widely in China. Horizontal axis 1: *Paeonia ostii* T. Hong et J. X. Zhang var. *lishizhenii* B. A. Shen; 2: *P. suffruticosa* Andr. 'Wulong Pengsheng'; 3: *P. rockii* (S. G. Haw & Lauener) T. Hong et J. J. Li 'Bingshan Xuelian'; 4: *P. lactiflora* Pall. 'Taohong Yushuang'. (A) Leaf water content (LWC), (B) electrolyte leakage, (C) stomatal conductance, (D) chlorophyll a, (E) chlorophyll b, (F) chlorophyll ab, (G) soluble total sugars, (H) proline content, (I) soluble protein content, (J) superoxide dismutase (SOD) activity, (K) peroxidase (POD) activity, (L) catalase (CAT) activity, and (M) malondialdehyde (MDA) content of the four species. Results represent the means ± standard error (SE, n = 3). Letters above columns indicate significant differences at $p < 0.05$ (Duncan's multiple range test, using statistical package for the social sciences [SPSS]).

Table 5. Average membership function (AMF) values of different *Paeonia* species.

No.	LWC	EL	SC	Chl a	Chl b	Chl ab	Sugar	Proline	Protein	SOD	POD	CAT	MDA	AMF
1	1.00	1.00	0.80	0.01	0.00	0.00	1.00	1.00	1.00	0.66	0.17	0.16	0.57	0.57
2	0.19	0.67	0.00	1.00	1.00	1.00	0.77	0.00	0.00	0.40	0.00	0.00	1.00	0.46
3	0.09	0.33	1.00	0.00	0.55	0.65	0.00	0.45	0.42	1.00	0.59	0.12	0.67	0.45
4	0.00	0.00	0.60	0.36	0.23	0.54	0.30	0.14	0.56	0.00	1.00	1.00	0.00	0.36

Note: 1: *Paeonia ostii* var. *lishizhenii*; 2: *Paeonia suffruticosa* 'Wulong Pengsheng'; 3: *Paeonia rockii* 'Bingshan Xuelian'; 4: *Paeonia lactiflora* 'Taohong Yushuang'; LWC, leaf water content; EL, electrolyte leakage; SC, stomatal conductance; Chl, chlorophyll; SOD, superoxide dismutase' POD, peroxidase; CAT, catalase; MDA, malondialdehyde

Comprehensive evaluation of 13 physiological criteria of the four peonies: The membership function of the 13 physiological criteria related to plant stress resistance was established. The subordinate values and their membership function values for each of the four peonies are shown in Table 5. The membership function values of the four peonies in decreasing order were as follows: *P. ostii* var. *lishizhenii* > *P. suffruticosa* 'Wulong Pengsheng' > *P. rockii* 'Bingshan Xuelian' > *P. lactiflora* 'Taohong Yushuang'.

Gene sequences and expression of DREB2A, WRKY19, and XET in the four peonies

DREB2A, WRKY19, and XET sequence characteristics: The *DREB, WRKY,* and *XET* gene families were characterized using the conserved domains "cd00018", "cd03892", and "cd02176", respectively. The domains were contained in the four peony conserved sequences of *DREB2*A (KT890350, KX121327, KX121328, and KX121317), *WRKY19* (KT890351, KX121408, KX121409, KX121399), and *XET* (KT890352, KX121368, KX121369, KX121358).

The *DREB2A* genes of the four peonies exhibited a small number of different nucleotide sites in the conserved sequences (Fig. 2A), whereas their encoded amino acid sequences were identical (Fig. 2B). The introns of the conserved DNA sequence of *WRKY19* in *P. lactiflora* 'Taohong Yushuang' were shorter than those of *P. ostii* var. *lishizhenii*, *P. suffruticosa* 'Wulong Pengsheng', and *P. rockii* 'Bingshan Xuelian' (Fig. 2C); in addition, a few amino acid residues differed between the three tree peonies (Fig. 2D). The *XET* conserved DNA sequences of the four peony species and their encoded amino acid sequences differed (Fig. 2E, F).

Phylogenetic analyses of the four peony *DREB2A*, *WRKY19*, and *XET* conserved sequences were performed using 17 species from the Phytozome v. 11.0.6 database (Fig. 2G–I). The *DREB2A* conserved sequences clustered into a single branch (Fig. 2G). In comparison, the *WRKY*19 conserved sequences clustered into two branches: one containing *P. ostii* var. *lishizhenii*, and the other containing the remaining three peonies along with *Amborella trichopoda* and *Arabidopsis thaliana*. In this second branch, the two tree peonies eventually formed one sub-cluster, and the herbaceous *P. lactiflora* 'Taohong Yushuang' formed another sub-cluster (Fig. 2H). The four peony *XET* conserved sequences were distributed across three branches: *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' formed two branches, while *P. suffruticosa* 'Wulong Pengsheng' and *P. lactiflora* 'Taohong Yushuang' formed another branch, although they were further separated into two branches with a long phylogenetic distance from the database plant species (Fig. 2I).

DREB2A, WRKY19, and XET expression in the four peonies: The *DREB2A, WRKY19,* and *XET* gene expression levels of the four peonies were quantified as shown in Figure 2J–L. The *DREB2A* gene exhibited the highest expression in *P. suffruticosa* 'Wulong Pengsheng' and the lowest in *P. rockii* 'Bingshan Xuelian' at 67.25 ± 5.9 × 10^{14} and 1.62 ± 0.35 × 10^{14} copies/μL, respectively. The level of *P. ostii* var. *lishizhenii* was 13.03 ± 0.81 × 10^{14} copies/μL, which was higher than that of *P. rockii* 'Bingshan Xuelian' at 1.62 ± 0.35 × 10^{14} copies/μL (p<0.05). Furthermore, the herbaceous *P. lactiflora* 'Taohong Yushuang' exhibited expression levels of 33.00 ± 7.92 × 10^{14} copies/μL, ranking as the second highest (p<0.05, Fig. 2J).

The *WRKY19* gene expression of the four peonies was highest in *P. lactiflora* 'Taohong Yushuang' (38.16 ± 4.97 ×10^{14} copies/μL). *P. suffruticosa* 'Wulong Pengsheng' exhibited an expression level of 12.62 ± 1.62 ×10^{14} copies/μL, which was the highest among the three tree peonies. *P. ostii* var. *lishizhenii* exhibited an expression level of 4.35 ± 0.52 × 10^{14} copies/μL, which was higher than that of *P. rockii* 'Bingshan Xuelian' (2.07 ± 0.19 × 10^{14} copies/μL, p<0.05, Fig. 2K).

The *XET* gene was expressed at the highest level of any of the four peonies in *P. lactiflora* 'Taohong Yushuang' at 10.27 ± 0.56 × 10^{14} copies/μL. The three tree peonies exhibited gene expression levels in decreasing order are as follows: 3.29 ± 0.29, 3.01 ± 0.31, and 2.24 ± 0.11 ×10^{14} copies/μL for *P. suffruticosa* 'Wulong Pengsheng', *P. rockii* 'Bingshan Xuelian', and *P. ostii* var. *lishizhenii*, respectively (p<0.05, Fig. 2L).

Discussion

Three tree peonies and one herbaceous peony were cultivated during field trails in Yanqing, Beijing, the scheduled site for the World Horticultural Exposition in 2019. Conditions at this site were severe, with the lowest annual precipitation and winter temperatures in the Beijing area. The peonies grew at the test site showed differences in growth and physiological responses of the 13 physiological criteria measured, and in the conserved gene sequences and expression levels of *DREB, WRKY,* and *XET,* in response to the harsh, stressful environment. *P. ostii* var. *lishizhenii* was shown to be the most resistant of the four investigated peony varieties grown in Yanqing, Beijing.

Fig. 2. Dehydration responsive element binding protein 2A (*DREB2A*), *WRKY19,* and xyloglucan endotransglycosylase (*XET*) nucleic acid and amino acid sequence alignment (A–F), conserved gene sequence phylogenetic tree (G–I), and absolute quantitative expression levels (J–L) of the four peonies (1: *Paeonia ostii* T. Hong et J. X. Zhang var. *lishizhenii* B. A. Shen; 2: *P. suffruticosa* Andr. 'Wulong Pengsheng'; 3: *P. rockii* (S. G. Haw & Lauener) T. Hong et J. J. Li 'Bingshan Xuelian'; 4: *P. lactiflora* Pall. 'Taohong Yushuang').

Growth adaptation to the new site with severe climate conditions in Yanqing, Beijing: Some of the three year old peony plants bloomed. The height of the *P. ostii* var. *lishizhenii* plants was significantly more than those of the other varieties tested, whereas their plant sprout numbers were similar to those of *P. rockii* 'Bingshan Xuelian' (Table 3). These findings are consistent with those reported previously (Xiu *et al.,* 2017). Shoot sprout numbers were negatively related to plant drought resistance (Ramya *et al*., 2016), which suggested that the three year old *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' plants exhibited stronger environmental adaptability than *P. suffruticosa* 'Wulong Pengsheng' plants.

The three year old *P. ostii* var. *lishizhenii* plants showed no difference in primary root length to *P. rockii* 'Bingshan Xuelian' plants but were significantly lower than those of *P. suffruticosa* 'Wulong Pengsheng' (Table 4). The lateral root number of *P. ostii* var. *lishizhenii* plants was significantly higher than those of *P. rockii* 'Bingshan Xuelian' and *P. lactiflora* 'Taohong Yushuang' but lower than that of *P. suffruticosa* 'Wulong Pengsheng' (Table 4). Large root systems with elongations or expansions reflect enhanced soil water uptake and increase the drought avoidance or water deficit tolerance of the plant (Yamaguchi & Sharp, 2010). Although the root system of *P. suffruticosa* 'Wulong Pengsheng' showed longer primary roots and increased numbers of lateral roots, but these roots consisted of those grafted from the buds of *P. suffruticosa* 'Wulong Pengsheng' on to the root stocks of *P. ostii* var. *lishizhenii*, and the grafted plants were a year older than the seedlings. Therefore, the enhanced root system and environmental adaptability appeared to be mainly provided by the seedling roots of *P. ostii* var. *lishizhenii*, a finding that is consistent with what has been previously observed with *P. suffruticosa* 'Roufurong' (Dong *et al*., 2013). Therefore, we consider that the *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' showed the strongest resistance to the harsh climatic conditions of the Yanqing, Beijing site.

Physiological adaptation to the Yanqing, Beijing site: The Yanqing, Beijing site exhibits the least annual precipitation and the coldest minimum temperature, as well as the strongest northwest winds, during the winter in Beijing. Consequently, the results of the 13 physiological criteria evaluated for the four peonies showed that, with the exception of the EL values (Fig. 1B), the other 12 criteria differed significantly among the four peonies in response to the field environmental conditions.

The 3-year-old seedlings of *P. ostii* var. *lishizhenii* showed high LWC, soluble sugar, protein, and proline content (Fig. 1A and G–I), indicating that the bio-reactions of sugar accumulation and conversion to proline and protein were highly effective at the leaf water conditions experienced at the site. Low EL (Fig. 1B) is related to cell membrane permeability and indicates a strong cellular water-retaining property (Doody *et al*., 2013). This characteristic is vital for maintaining physiological and biochemical processes, as has been observed in *Arabidopsis* under water deficit conditions (Yoo *et al*., 2010). This could be used to identify plants among the tested peonies that exhibit strong resistance and wide adaptability. High soluble sugar, protein, and proline content reduce the cellular water potential of plants (DaCosta & Huang, 2006). Furthermore, this effect enhances the water absorption by the roots and the retention capacity of the leaves to aid drought resistance, as has been observed in maize plants (De Sousa *et al.*, 2016). The SOD and CAT antioxidant activity of the tree peonies were almost proportional to their MDA content (Fig. 1J–M), although the POD activity level was in between that of *P. rockii* 'Bingshan Xuelian' and *P. suffruticosa* 'Wulong Pengsheng'. This suggested that *P. ostii* var. *lishizhenii* was highly capable of maintaining cell membrane structure during the antioxidation process. As a result of this, *P. ostii* var. *lishizhenii* plants ranked first in the membership function of the 13 physiological criteria.

The leaves of *P. suffruticosa* 'Wulong Pengsheng' exhibited high chlorophyll levels (Fig. 1D–F) and soluble sugar content, indicating that its photosynthesis level was high. The SC is directly proportional to the transpiration rate and inversely related to the stomatal resistance. This was highest among the four peonies (Fig. 1C), indicating that *P. suffruticosa* 'Wulong Pengsheng' exhibited rapid transpiration with low stomatal resistance, and appeared to utilize water efficiently. This observation might explain why this plant exhibited weak drought resistance. Moreover, its SOD enzyme activity was high, and its POD and CAT activities (Fig. 1J–L), were the lowest of all the plants, as were as its MDA content (Fig. 1M). These observations have been associated with minimal cellular damage due to lipid peroxidation and antioxidant levels in litchi and perennial flowers (Ye *et al.*, 2008; Ma *et al.*, 2015). Although *P. suffruticosa* 'Wulong Pengsheng' ranked second in the membership functions of the 13 physiological criteria among the four peonies (Table 5), the plant scions were grafted on the rootstocks of *P. ostii* var. *lishizhenii* and, therefore, the rootstocks contributed to the long primary roots and higher number of lateral roots. Therefore, the stress resistance of the *P. suffruticosa* 'Wulong Pengsheng' itself was actually the weakest of the three tree peonies tested.

P. rockii 'Bingshan Xuelian' exhibited a lower LWC than *P. ostii* var. *lishizhenii*. Compared with the other two peonies (Fig. 1A), *P. rockii* 'Bingshan Xuelian' exhibited higher EL (Fig. 1B), indicating a higher cell membrane permeability or lower water retaining capacity. This implies that the drought resistance of *P. rockii* was inferior to that of *P. ostii* var. *lishizhenii*. Although its soluble sugar content was the lowest, its SC was also low (Fig. 1C), which contributed to its efficient water use. Coupled with the higher proline and protein content (Fig. 1G-I), this result indicated active bioconversion and effective osmotic adjustment and homeostasis in the cells. The SOD activity of the *P. rockii* cultivar leaves was markedly high, whereas the POD of the peonies tested was even higher (Fig. 1J, K). This observation suggested that the plant was resistant to the effects of high MDA (Fig. 1M), which reduced oxidative damage to the cell membranes or prevented cell death (Finkel & Holbrook, 2000; Shi *et al.*, 2012). This cultivar thus exhibited characteristics that matched the membership function value of stress resistance plants, as shown in Table 5.

P. lactiflora 'Taohong Yushuang' is a perennial herbaceous plant. Its LWC was lower than that of *P. ostii* var. *lishizhenii* and similar to that of the tree peonies *P. suffruticosa* 'Wulong Pengsheng' and *P. rockii* 'Bingshan Xuelian', but its EL was highest and SC was high. Furthermore, its soluble sugar content was lower and its protein content was higher. This suggested that the plant was water efficient, with high cell membrane permeability and transpiration rates. The herbaceous peony exhibited marked POD and CAT enzyme activities in order to reduce hydrogen peroxide (H_2O_2) levels, leading to the detoxification of H_2O_2 in proportion to the content of MDA (Fig. 1J–M). MDA reflects the lipid peroxidation of the cell membrane and the degree of cell distress injury (Stewart & Bewley, 1980). The data showed the *P. lactiflora* 'Taohong Yushuang' to be drought sensitive, similarly to bermudagrass (*Cynodon dactylon* (L.) Pers.) and tended to exhibit enhanced enzymatic activities by oxygen-scavenging enzymes. This decreased the reactive oxygen species levels and reduced cell injury levels (Xu *et al.*, 2007; Ouyang *et al.*, 2010). The plant was ranked fourth in the membership function values of the 13 physiological criteria, and it flourished under conditions with sufficient water supply.

Multiple homolog expression of DREB2A, WRKY19, and XET genes in peonies in coordinated response to field trial conditions in Yanqing, Beijing: The transcriptional factors DREB and WRKY mediate drought signalling pathways that are either independent or dependent on ABA, respectively (Aguado *et al*., 2014). *AtDREB2*A (AB007790.1) and *OsDREB2A* (JQ341059.1) were verified as key genes for improving drought resistance in herbaceous crops, pattern plants, and some trees (Liu *et al*., 1998; Raj *et al.*, 2015). *FpDREB2A* (AY536056.1) had a similar effect on the taproot architecture of forest trees (Xiu *et al.*, 2016). *TaWRKY19* (EU665430) enhanced transgenic plant drought-resistance mediated by the ABA-dependent signalling pathway (Niu *et al.*, 2012). XET loosened the cell wall by transiently cleaving and re-ligating xyloglucan-cellulose cross links (Kaewthai *et al.*, 2013). *MtXET* and *PeXET* genes have been shown to promote the root growth of transgenic *Medicago truncatula* and tobacco, which improved plant drought resistance (Iqbal *et al.*, 2017). In the current study, the homologous genes *DREB, WRKY,* and *XET* were analysed to determine their regulatory functions in the four peonies and were confirmed to coordinately regulate stress resistance. This study thus revealed some of the mechanisms underlying the molecular drought resistance of peonies.

The *DREB2A* sequences of the four peonies tested differed in a few nucleotides, but no differences were observed in their encoded amino acid sequences (Figs. 2A, B). This observation suggested that these *DREB2A* sequences were highly conserved in all four peonies. The phylogenetic tree analysis showed that the four peonies clustered into a single branch (Fig. 2G) with high homology. Moreover, the four peonies shared low *DREB2A* amino acid sequence homology with other species. This suggested that the *DREB2A* sequences of the four investigated peonies have the characteristics of the *DREB2A* family, whereas their species characteristics and functions were closer to other species. The gene expression levels of *DREB2A* in *P. suffruticosa* 'Wulong Pengsheng' were the highest in the qRT-PCR analysis, followed by *P. lactiflora* 'Taohong Yushuang' (Fig. 2J). Therefore, it was inferred that *DREB2A* expression was induced by drought stress (Liu *et al*., 1998, Xiu *et al*., 2016). *P. ostii* var. *lishizhenii*. and *P. rockii* 'Bingshan Xuelian' were not more sensitive to a water deficit; as they exhibited more tolerance to drought than the other two peonies.

The *WRKY19* sequences showed that introns of the three investigated tree peonies differed from that of the herbaceous peony *P. lactiflora* 'Taohong Yushuang'. The amino acid residues encoded by the *WRKY* gene sequences differed in a few amino acids between the tree and herbaceous peonies. The *WRKY19* phylogenetic tree analysis indicated that *P. ostii* var. *lishizhenii* formed a branch by itself and showed low homology with the other three cultivars. This revealed, that *P. ostii* var. *lishizhenii* differed somewhat from the other three peonies with regard to stress resistance and adaptability. The qRT-PCR analysis of *WRKY19* expression in *P. lactiflora* 'Taohong Yushuang' showed it was the highest, followed by *P. suffruticosa* 'Wulong Pengsheng' (Fig. 2K), indicating their sensitivity. *P. ostii* and *P. rockii* exhibited the strongest stress resistance at the field trial site.

The *XET* gene and its encoded amino acid sequences differed from those of the four tested peonies (Figs. 2E, F). The combined phylogenetic analysis revealed that *P. ostii* var. *lishizhenii*, *P. rockii* 'Bingshan Xuelian', and *P. suffruticosa* 'Wulong Pengsheng', and *P. lactiflora* 'Taohong Yushuang' were grouped into three branches (Fig. 2I). The differences in the gene sequences of these varieties/cultivars can be identified using gene functions such as *GPAT* gene sequences and cold resistance, techniques that have been used for rice cultivars (Wu & Liu, 2010). The qRT-PCR analysis revealed that *XET* expression levels were highest in *P. lactiflora* 'Taohong Yushuang', followed by *P. suffruticosa* 'Wulong Pengsheng' (Fig. 2L), consistent with the results of *WRKY* and almost comparable to those of *DREB2A*. This suggested that coordinated multiple gene expression occurred in response to the field trial site conditions. As a consequence, *P. ostii* var. *lishizhenii*. and *P. rockii* 'Bingshan Xuelian', which have insensitive gene expression profiles, demonstrated stronger resistance to environmental stresses than the other peonies trialled.

Conclusions

The four peonies investigated, consisting of three tree peonies and one herbaceous peony, grow widely in China. Therefore, we investigated their adaptability to severe climatic conditions using a field trail at the Yanqing, Beijing site. The site was characterized by a soil water potential of −1.21 ± 0.37 MPa, root soil temperature of 21.71 ± 1.98°C in summer, and a salt content of 792.92 ± 9.28 ppm. The three year old plants were highly adapted to the site as follows, in decreasing order: *P. ostii* var. *lishizhenii*, *P. suffruticosa* 'Wulong Pengsheng', *P. rockii* 'Bingshan Xuelian', and *P. lactiflora* 'Taohong Yushuang'.

The 13 physiological criteria tested in the four peonies and their membership function values showed that *P. ostii* var. *lishizhenii* had the highest water retention, lowest cell membrane permeability, and highest soluble sugar, proline, and protein content. *P. rockii* 'Bingshan Xuelian' showed markedly low SC and high SOD activity. *P. suffruticosa* 'Wulong Pengsheng' and *P. lactiflora* 'Taohong Yushuang' were characterized by high SC values, higher cell permeability, and balanced MDA contents with POD CAT and SOD enzyme activities than the other varieties.

We also detected three homologous genes, *DREB2A*, *WRKY19*, and *XET,* in the four peonies tested based on conserved domain sequences. Although the three gene sequences were different in the four peonies, their phylogenetic trees identified all four peonies as belonging to one DREB2A branch. In contrast, WRKY formed two branches with *P. ostii* var. *lishizhenii* forming one branch and the other three peonies forming the second; *XET* formed three branches, with *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' forming two branches, and *P. suffruticosa* 'Wulong Pengsheng' and *P. lactiflora* 'Taohong Yushuang' together forming the third branch. The qRT-PCR analysis of the three gene expression levels in the four tested peonies revealed that they could primarily be divided into two groups, depending on whether they were sensitive or insensitive to environmental stresses at the field trial site. *P. ostii* var. *lishizhenii* and *P. rockii* 'Bingshan Xuelian' were insensitive and exhibited stronger stress resistance, whereas *P. suffruticosa* 'Wulong Pengsheng' and *P. lactiflora* 'Taohong Yushuang' were sensitive and exhibited lower stress resistance at the Yingqing, Beijing site.

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