Anatomical, physiological and transcriptional responses of two contrasting poplar genotypes to drought and re-watering

Xu Caoa,b, Jingbo Jiaa, Chao Zhananga, Hong Lic, Tongxian Liuc, Xiangning Jiangd, Andrea Polleb, Changhui Penge,f and Zhi-Bin Luoa,e,*

aCollege of Life Sciences and State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, Shaanxi, 712100, China
bBüsgen-Institute, Department of Forest Botany and Tree Physiology, Georg-August University, 37077, Göttingen, Germany
cCollege of Plant Protection, Northwest A&F University, Yangling, Shaanxi, 712100, China
dNational Engineering Laboratory of Tree Breeding, College of Life Sciences and Biotechnology, Beijing Forestry University, Beijing, 100083, China
eKey Laboratory of Environment and Ecology in Western China of Ministry of Education, College of Forestry, Northwest A&F University, Yangling, Shaanxi, 712100, China
fInstitute of Environment Sciences, Department of Biology Science, University of Quebec at Montreal, Montreal, C3H 3P8, Canada

Correspondence
*Corresponding author, e-mail: luozbibil@163.com

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Introduction

Climate changes are predicted to elevate mean temperature by 2–4° C with increases in frequency, length and severity of drought in this century (Seager et al. 2007). Drought is one of the most important abiotic stressors limiting forest productivity and survival of trees (Peng et al. 2011). Accumulating evidence suggests that occurrence of intensified drought is associated with increased forest mortality around the world (Allen et al. 2010). For

Abbreviations – ABA, abscisic acid; APX, ascorbate peroxidase; GPX, guaiacol peroxidase; LPI, leaf plastochron index; MDA, malonaldehyde; NCED3, 9-cis-epoxycarotenoid dioxygenase 3; PCA, principal component analysis; PIP, plasma membrane intrinsic protein; PP2C, protein phosphatase 2C; ROS, reactive oxygen species; SOD, superoxide dismutase; WUEi, intrinsic water use efficiency; δ13C, stable carbon isotope composition; δ15N, stable nitrogen isotope composition.
instance, the widespread die-off of *Populus tremuloides* forest in western United States is mainly ascribed to the intensified drought events that occurred in recent years (Anderegg et al. 2012). Thus, it is of particular importance to better understand the responses of trees to drought in order to select tree species/genotypes with drought tolerance for future climate.

*Populus* plants can produce high biomass and are utilized as woody crops for bioenergy (Luo and Polle 2009). The fast growing poplar species/genotypes require a large amount of water and many species/genotypes are relatively susceptible to drought (Hukin et al. 2005, Monclus et al. 2006, Luo et al. 2009b, Beniwal et al. 2010). Natural occurrences of poplar forests are often found on riparian sites where the water table is easily accessible by poplar roots (Rennenberg et al. 2006, Koyama and Kielland 2011). To meet the increasing biomass demand for biofuels, mitigation of CO₂ emission and pulp and paper industry, however, forest plantations including poplars are often established on marginal lands where water availability can be limited (Amichev et al. 2012). Poplar plantations may be exposed to drought and re-watering cycles. To ensure high biomass yield of poplar plantations, selection of species/genotypes with drought tolerance (the capacity to maintain considerable growth at a given soil moisture level) is of particular interest. In this context, it is crucial to characterize the anatomical, physiological and transcriptional responses of poplar species/genotypes to drought and re-watering conditions.

Soil water deprivation can result in a suite of anatomical and physiological responses in plants. At the anatomical level, drought can cause changes in root morphology, and wood and leaf anatomical properties of woody plants (Beniwal et al. 2010, Plavcová and Hacke 2012). For instance, poplar plants often increase the thickness of leaf lamellae (Tosens et al. 2012) and produce more narrow vessels in stem-wood in acclimation to low water availability in soil (Arend and Fromm 2007). At the physiological level, CO₂ assimilation and plant growth are often inhibited due to stomatal closure under drought (Luo et al. 2009b). Decreasing soil water potential is perceived by plant roots and triggers several signal cascades including the abscisic acid (ABA) signaling pathway (Popko et al. 2010). As a primary signal molecule, ABA concentrations in plants are often elevated in response to soil water deficit (Yin et al. 2009, Coccozza et al. 2010). A 9-cis-epoxycarotenoid dioxygenase 3 (NCED3), a key enzyme for ABA biosynthesis and a protein phosphatase 2C (PP2C) involved in ABA-mediated signaling pathway, play a key role in sensing changes in soil water availability (Soon et al. 2012). Transcriptional regulation of these key genes involved in drought acclimation can occur in plants (Soon et al. 2012). Water entry to cells is controlled by the plasma membrane intrinsic proteins (PIPs) (Secchi et al. 2009). Plants can modulate transcript levels of PIPs to facilitate water uptake under low water availability in soil (Cohen et al. 2013).

Carbon and nitrogen metabolism is important for plant survival and growth under drought. Soluble sugars and sugar alcohols can be altered in plants in response to drought and re-watering conditions, playing important roles in osmotic homeostasis, signaling and protection against reactive oxygen species (ROS) in plants (Luo et al. 2009b, Regier et al. 2009). Moreover, C starvation, i.e. decreases in carbohydrate concentrations and/or dysfunction of sugar transport leading to less/no carbohydrate supply to meet C demand for maintenance, is proposed to be one of the main mechanisms underlying widespread drought-induced tree mortality (McDowell and Sevanto 2010, McDowell 2011). Additionally, nitrogen uptake by plant roots is coupled with water movement because ammonium or nitrate is dissolved in water, absorbed by roots and translocated to above-ground part of plants. Soil water deficit inevitably affects nitrogen uptake leading to differentiation of nitrogen isotope (Peuke et al. 2006). Additionally, nitrogen metabolism in plants can be affected by drought stress, resulting in further differentiation of nitrogen isotope (Peuke et al. 2006). Plants are frequently found to alter carbon (C) and nitrogen (N) metabolism including _13C and _15N fractionation in acclimation to drought (Luo et al. 2009b, Anderegg et al. 2012, Schifman et al. 2012). While a number of studies have investigated the above-mentioned responses of trees to drought, a better understanding of the anatomical, physiological and transcriptional responses is still needed in fast growing woody plants such as *Populus* to drought and re-watering conditions.

The *Populus* genus consists of 30–40 species which are mainly distributed in the temperate and boreal regions of the north hemisphere (Polle et al. 2013). A large variation in drought tolerance exists among these poplar species/genotypes (Monclus et al. 2006, Silim et al. 2009). In our previous study, it was found that *Populus × euramericana* (Pe) displays higher stable carbon isotope composition (δ¹³C) and intrinsic water use efficiency (WUEi) in comparison with *Populus cathayana* (Pc) under unlimited water conditions (Cao et al. 2012). These findings render us to hypothesize that Pe is better acclimated to water deficiency than Pc. To examine this hypothesis, we compared the responses of both poplar genotypes to drought and re-watering. Saplings of Pc and Pe were exposed to 80, 60 or 40% field capacity for 26 days, and subsequently re-watered to 80% field capacity for 13 days. Measurements of anatomical,
physiological (e.g. soluble sugars and sugar alcohols, δ¹³C) and molecular (e.g. transcript levels of PIPs) parameters known to be important for drought acclimation were conducted. Moreover, multivariate analysis was used to dissect the importance of parameters as contributors to poplars in acclimation to drought and re-watering. Specifically, we address the following questions: (i) Do anatomical, physiological and transcriptional regulation responses of Pc and Pe display different patterns to drought and re-watering conditions? and (ii) By which physiological mechanisms do both poplar genotypes acclimate to drought and re-watering conditions?

**Materials and methods**

**Plant cultivation, drought treatment and harvest**

Cuttings (ca. 15 cm in length, 2 cm in diameter, 1 year old) of *P. cathayana* (Pc) and *P. x eurameriana* (Pe) were rooted. Each rooted cutting was planted in a 10l pot filled with soil (clay : sand, 1:1, v/v). Plants were cultivated in a greenhouse with natural light. After 3 months, uniform saplings were transferred to a climate chamber with a day/night temperature of 25/20 °C. Plants were rooted. Each rooted cutting was planted in a 10l pot filled with soil (clay : sand, 1:1, v/v). Plants were watered to field capacity each day and 50 ml Hoagland nutrient solution was supplied daily to each plant.

For drought treatment, 72 saplings of each genotype were randomly allocated to three groups with 24 plants in each group. Plants in the three groups were exposed to 80% (control), 60% (moderate stress) or 40% (severe stress) field capacity, respectively. To achieve the target field capacity of drought treatment, each pot was weighted every day and the amount of water equal to loss of transpiration and soil evaporation was added. At the beginning of drought treatment, the apex of each plant was marked to distinguish new shoots formed during drought treatment. The drought treatment lasted for 26 days. Subsequently, 12 plants were harvested in each group and the remaining plants were re-watered to 80% field capacity. After 13 days, the remaining plants were also harvested.

During the harvest, the new shoot (stem and leaves) formed during the treatment was separated from each plant. Roots of each plant were carefully cleaned and harvested. Harvested roots or leaves were wrapped with tinfoil and immediately frozen in liquid nitrogen. Frozen samples were ground into fine powder in liquid nitrogen with a mortar and a pestle and stored at −80 °C. Fresh powder (ca. 100 mg) from each tissue per plant was dried at 60 °C to determine the fresh-to-dry mass ratio and water content (water amount in fresh tissue per unit biomass). Equal amounts of fine powder from the same tissue of two plants within each treatment were combined and well mixed to form a sample for further analysis. Subsamples of leaves [leaf plastochron index (LPI) = 5] and stems (ca. 2 cm long above the mark) were also collected for anatomical analysis. For light microscopy, leaf discs (6.0 mm in diameter) were excised from the middle (avoiding to the main vein) of the harvested leaves and preserved in FAE solution (37% formalin : glacial acetic acid : 60% ethanol, 10:5:85, v/v/v). Wood (debarked stem) sections were stored in FAE solution (37% formalin : glacial acetic acid : 70% ethanol, 5:5:90, v/v/v).

Due to space limitation, detailed methods for anatomical analysis, concentrations of ABA, soluble sugars and sugar alcohols, ROS and antioxidants are provided in Appendix S1.

**Measurements of stem height, gas exchange and photosynthetic pigments**

Stem height of each plant was measured every four days. Gas exchange was determined on three mature leaves formed during the treatment (LPI = 8–10) of each plant before harvest. Net photosynthetic rates (A), stomatal conductance (gs) and transpiration rates (E) were determined with a portable photosynthesis system (LiCor-6400; LiCor, Lincoln, NE) and an attached LED light source (6400–02) as described by He et al. (2011). Concentrations of chlorophyll and carotenoid in leaves were determined spectrophotometrically as suggested by Wellburn (1994). The instantaneous WUEi was calculated as the ratio of A to gs according to the method proposed by Cao et al. (2012).

**Analysis of transcript levels**

The frozen powder of roots or leaves (ca. 200 mg) was used for total RNA extraction. Total RNA was isolated according to the protocol of Chang et al. (1993) and purified with a plant RNA extraction kit (R6827; Omega Bio-Tek, Norcross, GA). Trace genomic DNA in the total RNA extract was digested by DNase I (E1091; Omega Bio-Tek) attached to the RNA extraction kit. The lack of trace genomic DNA in the total RNA was confirmed by a control PCR using total RNA as templates. Aliquots of 1 μg total RNA were used for first strand cDNA synthesis in a total volume of 20 μl, containing 0.5 μg oligo d(T)18-primer and 200 U RevertAid Moloney murine leukemia virus reverse transcriptase (K1621; Fermentas, Burlington, CA) according to the manufacturer’s instruction. The synthesized cDNA was used for real time qPCR as described by Luo et al. (2009a) with minor modification as suggested by Li et al. (2012). Quantitative PCR
was performed in 20 μl reaction system using 10 μl 2× SYBR Green Premix Ex Taq II (DRR081A; Takara, Dalian, China), 0.5 μl cDNA and 0.2 μM primer (Appendix S2) in an iQ5 Real Time system (iQ5, Bio-Rad, Hercules, CA). The 18S rRNA was used as a reference gene. To ensure the primer specificity, PCR products were sequenced and aligned with homologs in other model plants (Appendix S3). The efficiencies of all PCR reactions were between 94 and 110% (Appendix S2). PCR was performed in triplicate together with a dilution series of the reference gene.

Analysis of total C and N, and their stable isotope compositions

Three mature leaves (LPI = 8–10) selected for gas exchange measurements and roots were used for determination of total C and N, 13C and 15N. The fine powder (ca. 50 mg) was dried in an oven at 80°C. The dried powder (ca. 0.8 mg) was analyzed by an elemental analyzer (NA 1110; CE Instruments, Rodano, Italy) and a mass spectrometer (Delta Plus; Finnigan MAT, Bremen, Germany) with an interface (Conflo III; Finnigan MAT) according to the method of Werner et al. (1999). Total C and N concentrations were calculated as described by Cao et al. (2012). Stable N isotope composition was calculated as suggested by Yousfi et al. (2012):

\[ \delta^{15}N = \frac{(R_{sa} - R_{sd})}{R_{sd}} \times 1000 \ [\permil] \]

where \( R_{sa} \) and \( R_{sd} \) are the ratios of \( ^{15}N \) to \( ^{14}N \) of the sample and the standard, respectively. The standard is referred to N\(_2\) in air.

Statistical analysis

Statistical tests were carried out with Statgraphics (STN, St Louis, MO). To examine the effects of genotypes and drought treatment on experimental variables, all variables were analyzed by two-way ANOVAs. Data were tested for normality prior to statistical analysis. Differences between means were considered significant when the P-value of the ANOVA F-test was less than 0.05. Linear correlations were performed using Pearson correlation coefficient. The Cq values obtained after RT-qPCR were normalized and the fold changes of transcripts were calculated using the relative expression software tool REST (Pfaffl et al. 2002). For principal component analysis (PCA), data were standardized and subsequently computed by the command prcomp() in R (http://www.r-project.org/) as described previously (He et al. 2013, Ma et al. 2013). The cluster analysis of gene expression was computed by command heatmap.2() with the package ‘gplots’ in R as described by Luo et al. (2013).

Results

Plant water status, height growth, anatomical and photosynthetic characteristics

Under 80% (control) field capacity conditions, water status was similar in both poplar genotypes, but it reduced more rapidly in Pc than in Pe in response to low water availability (Appendix S4). The water contents, however, increased upon re-watering in roots and leaves of both poplar genotypes (Appendix S4). The stem height increment was similar in both genotypes under 80% field capacity, but it was more sensitive to 40% field capacity (severe drought) in Pc than in Pe (Appendix S4). The vessel frequency was lower in Pe than in Pc under the control conditions, Pe increased vessel frequency in response to water deficiency, but Pc displayed no such response (Appendix S5). The thickness of foliar spongy tissue was higher in Pe than in Pc under the control conditions, Pe increased vessel frequency in response to water deficiency, but Pc displayed no such response (Appendix S5). A in drought-stressed poplars was recovered to the levels of control plants after re-watering in both genotypes (Appendix S5). These results indicate that Pc and Pe display different patterns of anatomical and photosynthetic properties in response to drought and re-watering, which is confirmed by further PCA using plant water content, anatomical and photosynthetic parameters (Fig. 1, Appendix S6).

![Fig. 1. PCA plots of plant water contents, anatomical and photosynthetic characteristics of Populus cathayana (Pc, triangle) and Populus × euramericana (Pe, circle) exposed to 80% (light cyan), 60% (green) and 40% (olive) field capacity for 26 days (A) and re-watered to 80% field capacity for 13 days (B), respectively. PCA was conducted using plant water content, anatomical and photosynthetic parameters presented in Appendix S6.](http://www.r-project.org/)
Fig. 2. Fold changes of transcript levels of PIPs in roots (A, B) and leaves (C, D) of Populus cathayana (Pc) and Populus × euramericana (Pe) exposed to 80, 60 and 40% field capacity for 26 days (A, C) and re-watered to 80% field capacity (denoted as R80%, R60% and R40%) for 13 days (B, D), respectively. Fold changes of transcript levels of PIPs in roots or leaves of Pc exposed to 80% field capacity were defined to be 1, and subsequently fold changes of transcript levels of PIPs were calculated under other conditions.

The PCA results demonstrate that during drought treatment PC1 tended to separate the effect of water availability, and PC2 tended to uncouple the variation of genotypes (Fig. 1A). PC1 and PC2 accounted for 35 and 17% of the variation, respectively (Fig. 1A). A, water contents of roots and leaves and thickness of foliar spongy tissue were essential contributors to PC1, whereas leaf thickness, stem height increment and vessel frequency were important factors to PC2 (Appendix S6). During re-watering phase, PC1 and PC2 accounted for 27 and 22% of the variation, respectively (Fig. 1B). Root biomass and foliar water content were important contributors to PC1, and thickness of palisade and fiber length were essential factors to PC2 under the re-watering conditions (Appendix S6). These data suggest that Pc and Pe differentially respond to drought and re-watering, which is probably associated with differences of Pc and Pe in physiological and transcriptional regulation in acclimation to drought and re-watering conditions.

Transcript levels of PIPs, NCED3 and PP2C and ABA concentrations

Under drought and re-watering conditions, transcriptional regulation of PIPs may play a role in water acquisition in plants (Secchi et al. 2007a, 2007b). The transcript abundance of key PIPs was analyzed in roots and leaves of Pc and Pe under drought and re-watering conditions. On the basis of our preliminary experiments and previous studies (Secchi et al. 2009, Cohen et al. 2013), PIP1;2, PIP1;3, PIP2;2, PIP2;3, PIP2;4 and PIP2;5 were selected for assessment of mRNA levels in both poplar genotypes (Fig. 2). The cluster analysis clearly separated the effects of genotypes and water deficit based on the responsiveness of transcript levels of PIPs in roots (Fig. 2A, B) or leaves (Fig. 2C, D) of both poplar genotypes under drought (Fig. 2A, C) or re-watering (Fig. 2B, D) conditions. During the drought phase, the transcript levels of PIP2;4 and PIP1;2 in Pc roots were lower than those of Pe roots under any given field capacity, and in both genotypes these genes were induced in response to decreasing water availability (Fig. 2A). Under 80% field capacity, PIP2;5 transcript abundance in Pc roots was higher than that of Pe, and the transcript levels were elevated in both genotypes under lower water availability (Fig. 2A). PIP1;3 and PIP2;2 transcript levels were similar in both genotypes exposed to 80% field capacity, and both genes were downregulated under 60% field capacity but slightly upregulated in response to 40% field capacity (Fig. 2A). The transcript abundance of PIP2;3 was similar in roots of Pc and Pe under control conditions, and it was lower in Pc but higher in Pe in response to moderate stress, and increased in both genotypes under severe stress (Fig. 2A). During the re-watering phase, transcriptional regulation of key PIPs...
also occurred in roots of Pc and Pe (Fig. 2B). The mRNA levels of PIP2;3 and PIP2;5 were lower in Pc roots than those of Pe roots under R80% conditions, and increased in both genotypes in response to R60% and/or R40% conditions (Fig. 2B). The mRNA levels of PIP1;2, PIP2;2, PIP1;3 and PIP2;4 were higher in Pc roots than those in Pe roots under R80% conditions, and decreased in both genotypes under R60% and/or R40% conditions in comparison with those under R80% condition (Fig. 2B).

In leaves, the transcriptional regulation of PIPs was different from that in roots of Pc and Pe under drought conditions (Fig. 2C). The transcript levels of all analyzed PIPs except PIP1;3 were lower in Pc than those in Pe under control conditions, and most genes in Pc were over-expressed in response to decreasing water availability, but transcript levels of genes in Pe were induced under moderate stress and suppressed under severe stress (Fig. 2C). During the re-watering phase, Pc leaves had lower transcript levels of PIP1;2, PIP2;4 and PIP2;5, and higher mRNA levels of PIP2;3, PIP2;2 and PIP1;3 than Pe leaves under R80% condition (Fig. 2D). Most PIPs were downexpressed in leaves of both genotypes under R40% compared to R80% condition (Fig. 2D).

Under drought and re-watering conditions, transcriptional regulation of genes involved in signaling pathway is important for plants to perceive the changes of water status in soil. On the basis of our preliminary assays and previous studies (Tan et al. 2003, Soon et al. 2012), NCED3 and PP2C were selected to analyze their transcript changes in roots and leaves of Pc and Pe under control, severe drought and re-watering conditions (Appendix S7). In roots of Pc and Pe, the transcript levels of NCED3 and PP2C were induced under severe drought compared to control condition, but repressed under R40% vs R80% condition. In leaves of both genotypes, the transcript levels of NCED3 were unaltered under severe drought compared to control conditions, but suppressed under R40% vs R80% condition. In leaves, the PP2C mRNA levels were stimulated under severe drought compared to control condition.

ABA is a key signaling molecule and plays a role in signaling pathway in plants in response to soil water deficit (Arend et al. 2009). ABA consists of cis- (c-ABA) and trans-ABA (t-ABA), c-ABA is a naturally occurring active form and t-ABA is inactive and inter-convertible with c-ABA in plants (Taiz and Zeiger 2010). Concentrations of c- and t-ABA were determined in roots and leaves of both genotypes under control, severe drought, R80 and R40% conditions (Appendix S7). The c-ABA concentrations were similar in roots of Pc and Pe under control condition and induced ca. 10-folds in Pc roots and 15-folds in Pe roots under severe drought condition. The c-ABA concentration was higher in Pc roots than that in Pe roots under R80% condition. In leaves, Pc had higher concentrations of c-ABA than Pe under either control or severe drought condition. The severe drought induced c-ABA concentrations by ca. fivetimes in leaves of both genotypes compared to those under control condition. Pc leaves had higher c-ABA concentration than Pe leaves under R80% condition, and foliar c-ABA concentration was lower in Pc but higher in Pe under R40% vs R80% conditions.

**Soluble sugars and sugar alcohols, total C and δ13C and total N and δ15N**

Plants acclimate to low water availability by partial closure of stomata, leading to reduced CO2 gain and changed C metabolism such as soluble sugars and sugar alcohols, total C and δ13C. Under soil drought conditions, decreased water uptake can result in lower N absorption and assimilation in plants. Thus, metabolism of C and N can be altered in plants in acclimation to drought and re-watering conditions. Soluble sugars and sugar alcohols, total C and δ13C and total N and δ15N (stable nitrogen isotope composition) were analyzed in roots and leaves of Pc and Pe (Figs. 3 and 4, Appendix S8). Glucose, fructose, sucrose, galactose, inositol and mannitol were detected in roots or leaves, and sorbitol and trehalose were only found in roots of both genotypes (Appendix S8). Although concentrations of some carbohydrates decreased in roots of both genotypes in response to decreasing water availability, no clear patterns for changes in concentrations of most sugars and sugar alcohols were observed in roots and leaves of both genotypes under drought or re-watering conditions (Appendix S8).

Total C concentrations were lower in roots of Pc than those of Pe under drought treatment, but the opposite was true under re-watering conditions (Fig. 3A, B). Limiting water availability resulted in decreased total C concentrations in roots of both genotypes (Fig. 3A). The R40% or R60% treatments caused increases in total C concentrations in Pe root compared to R80% treatment (Fig. 3B). Total C concentrations in leaves were slightly higher in Pc than in Pe under re-watering conditions, and total C concentrations increased in leaves of both genotypes in response to lower water availability (Fig. 3C, D). The δ13C was higher in roots or leaves of Pe than that of Pc under drought or re-watering conditions (Fig. 3E–H). The δ13C was higher in leaves under drought compared to control conditions, and in roots or leaves of both genotypes under R40% or R60% compared to R80% conditions (Fig. 3F–H).

Total N concentrations were higher in roots or leaves of Pc than those of Pe under drought or...
re-watering conditions (Fig. 4A–D). Total N concentrations decreased in leaves of both genotypes under severe drought conditions compared to those under control conditions (Fig. 4C). The δ¹⁵N was lower in roots of Pc than that of Pe under drought conditions, but the opposite was true under re-watering conditions (Fig. 4E–F). The δ¹⁵N increased in roots of Pc under drought compared to control, and under R40% or R60% in comparison with R80% conditions (Fig. 4E–F). Foliar δ¹⁵N was higher in Pc than in Pe under drought or re-watering conditions (Fig. 4G–H). Foliar δ¹³C was induced in both genotypes under severe drought or R40% conditions compared to those under control conditions (Fig. 4G–H).

The δ¹³C is linked with WUEᵢ. Thus, WUEᵢ and correlation analysis were determined in Pc and Pe (Fig. 5). WUEᵢ in both genotypes increased in response to lower water availability (Fig. 5A). WUEᵢ was lower in Pc than in Pe under re-watering conditions (Fig. 5B). Positive linear correlations were found between WUEᵢ and δ¹³C in both genotypes (Fig. 5C, D). Interestingly, similar correlations between WUEᵢ and δ¹⁵N were also detected in both poplar genotypes (Fig. 5E, F). These data indicate that WUEᵢ is tightly coupled with fractionation of ¹³C or ¹⁵N, and metabolism of C or N is associated with water loss in Pc and Pe under the current experimental conditions.

ROS and antioxidants

The balance of ROS and antioxidants are often shifted in plants under drought and re-watering conditions. Thus, O₂•⁻, H₂O₂, malonaldehyde (MDA), free proline and antioxidant enzymes including superoxide dismutase (SOD), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) were determined in roots and leaves of Pc and Pe (Appendix S9). Concentrations of O₂•⁻ were lower in roots of both genotypes exposed to limiting water availability than those under control condition, and were also lower in Pc roots under R40% or R60% compared to R80% conditions, but were recovered in Pe roots under re-watering conditions. Concentrations of foliar O₂•⁻ were lower in Pc than in Pe under drought conditions, and decreased in Pc under moderate or severe drought compared to control conditions. Concentrations of root H₂O₂ were lower in Pc than in Pe during drought conditions, and decreased in both genotypes under drought compared to control conditions. In contrast, concentrations of foliar H₂O₂ were higher in Pc than in Pe during drought and re-watering conditions. Concentrations of MDA in roots or leaves were lower in Pc than in Pe. The free proline concentrations were lower in Pc roots than in Pe roots during re-watering phase, but higher in leaves of Pc than of Pe under drought. Activities of antioxidant enzymes
(SOD, GPX and APX) also responded to water availability in roots and leaves of Pc and Pe.

**PCA of physiological responses**

To find out whether Pc and Pe displayed different physiological response patterns to drought or re-watering, PCA was performed using data of root- and leaf-related parameters (Fig. 6, Appendix S10). Under drought conditions, PC1 and PC2 accounted for 35 and 22% of the variation, respectively, in roots (Fig. 6A). PC1 tended to separate the drought effects, and PC2 uncoupled the variation of genotypes (Fig. 6A). Root galactose, fructose and PIP2;2 were key contributors to PC1, whereas root total N, MDA and δ13C were important factors to PC2 (Appendix S10). Under re-watering conditions, PC1 and PC2 contributed to 28 and 22% of the variation in roots, respectively (Fig. 6B). PC1 tended to separate the re-watering effects and PC2 for genotype effects, respectively (Fig. 6B). Root sucrose, SOD activities and δ15N were essential factors to PC1, whereas root MDA, total N and O2•− were important contributors to PC2 (Appendix S10). In leaves, PC1 and PC2 contributed to 29 and 26% of the variations, respectively, under drought conditions (Fig. 6C). PC1 separated the drought effects, whereas PC2 uncoupled the variation of genotypes (Fig. 6C). WUEi, foliar δ13C and sucrose in leaves were key factors to PC1, whereas foliar PIP1;2 and PIP2;2 and fructose were important contributors to PC2 (Appendix S10). Under re-watering conditions, PC1 contributed to 30% of the variation and PC2 19% of the variation in leaves (Fig. 6D). WUEi, foliar glucose and fructose were critical factors to PC1, but foliar total C, PIP1;2 and PIP2;4 were key contributors to PC2 (Appendix S10). These PCA results indicate that Pc and Pe have distinct responsiveness to drought or re-watering and that Pe displays a stronger physiological responsiveness to limiting water availability than Pc.

**Discussion**

**Do anatomical, physiological and transcriptional regulation responses of Pc and Pe display different patterns to drought and re-watering conditions?**

Although Pe displays higher δ13C and WUEi than Pc under unlimited water conditions (Cao et al. 2012), indicating that Pe is probably better acclimated to drought than Pc, it remains unknown whether Pc and Pe differ in responses of anatomical, physiological and transcriptional regulation to drought and/or re-watering conditions. Using data based on plant water content,
Fig. 5. WUE$_i$ (μmol CO$_2$ mol$^{-1}$ H$_2$O, A, B) and correlations between WUE$_i$ and δ$^{13}$C (C, D) or δ$^{15}$N (E, F) in *Populus cathayana* (Pc) and *Populus × euramericana* (Pe) exposed to 80, 60 and 40% field capacity for 26 days (A, C, E) and re-watered to 80% field capacity for 13 days (B, D, F), respectively. The bar indicates mean ± SE (n = 6). Different letters on the bars indicate significant difference. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; ns, not significant.

Fig. 6. PCA plots of the first two principal components in roots (A, B) and leaves (C, D) of *Populus cathayana* (Pc, triangle) and *Populus × euramericana* (Pe, circle) exposed to 80% (light cyan), 60% (green) and 40% (olive) field capacity for 26 days (A, C) and re-watered to 80% field capacity for 13 days (B, D), respectively. PCA was conducted based on data (both values were averaged in the same treatment) of parameters presented in Appendix S10.

Pc and Pe in physiological and transcriptional regulation in acclimation to drought and re-watering conditions.

The PIPs play an important role in water transport across plasma membrane in poplar plants (Secchi et al. 2009, Cohen et al. 2013). The transcriptional regulation of PIPs in plants can facilitate water uptake under drought, which may contribute to the differences of poplar genotypes in response to drought and re-watering conditions (Bae et al. 2011). The transcriptional modulation of PIPs in roots and/or leaves of Pe displayed a stronger responsiveness to drought and re-watering conditions in comparison with that of Pc (Fig. 2), suggesting that Pe possesses a stronger ability to fine-tune water absorption via transcriptional regulation of PIPs.

In line with our results, the dry climate adapted clone of *Populus nigra* increases water conservation in roots by repressed transcript levels of PIP1-2, whereas the clone from the moist climate appears to enhance the water permeability by overexpression of PIP1-2 under drought (Cocozza et al. 2010). *Populus simonii × balsamifera* with high stomatal sensitivity and *Populus balsamifera* with less sensitivity to drought, transcript abundances of PIP1;2, PIP1;3 and PIP2;2 were increased in leaves of *P. simonii × balsamifera*, but unaltered of *P. balsamifera* in response to drought. In contrast, the mRNA level of foliar PIP2;5 was stimulated in *P. balsamifera*, but
showed no changes in *P. simonii × balsamifera* under drought (Almeida-Rodriguez et al. 2010). These results suggest that different poplar genotypes can differentially modulate gene expression of *PIPs* to acclimate to changes in soil water availability.

Metabolites of C and N in plants can act not only as basic blocks for growth but also as osmolytes or signal molecules under abiotic stress (Nunes-Nesi et al. 2010). Carbohydrates are important C metabolites, which can contribute to differences of plants in drought tolerance (Keunen et al. 2013). Although Pc and Pe had no differences in concentrations of total soluble sugars and sugar alcohols, higher starch concentrations were detected in roots or leaves of Pe compared to those of Pc (Appendix S8), suggesting that more monosaccharides have been converted to starch in roots or leaves of Pe. Consistently, higher total C concentrations were found in roots of Pe than of Pc under drought conditions (Fig. 3). These data reflect the association between total C concentrations and starch accumulation in roots under drought conditions. Both divergent and similar response patterns of metabolism of C and N have been documented in various tree species/genotypes in acclimation to drought and re-watering conditions (Regier et al. 2009, Warren et al. 2011, 2012). Higher δ¹³C in roots or leaves of Pe under drought or re-watering conditions in comparison with those of Pc (Fig. 3) indicates that ¹³C is more rapidly enriched in Pe under these conditions, which is probably associated with more active C metabolism processes in Pe than those in Pc. The δ¹³C in plants is regarded as an indicator for WUE (Seibt et al. 2008). The positive linear relationships between δ¹³C and WUEi (Fig. 5) validate the use of δ¹³C as an estimate of WUEi, which is consistent with results obtained from other woody plants under various environmental conditions (Fichot et al. 2009, Roussel et al. 2009). Under re-watering conditions, higher WUEi in Pe is consistent with higher δ¹³C compared to those of Pc. It is known that ¹⁵N is depleted in plant dry mass during N metabolism compared to that in the soil (Tcherkez and Hodges 2008, Falka-Raymond et al. 2012, Gauthier et al. 2013). Higher δ¹⁵N in roots and leaves of Pe (Fig. 4) indicates less depletion of ¹⁵N in Pe than in Pc under drought. The positive linear correlations between δ¹⁵N and WUEi (Fig. 5) indicate that ¹⁵N enrichment is tightly associated with water consumption in these poplar genotypes, probably due to the coupling transport process of N and water in the transpiration stream. Negative correlations between δ¹⁵N and transpiration rate were observed in salt-stressed wheat and low N-treated poplar plants (Luo et al. 2013, Yousfi et al. 2013). As WUEi is the ratio of A to E (transpiration rate), these observations are consistent on correlations between δ¹⁵N and WUEi or E.

Overall, higher plant water contents, the stronger responsiveness of transcriptional regulation of *PIPs*, higher starch accumulation, δ¹³C, δ¹⁵N, WUEi and lower ROS accumulation and scavenging in Pe in comparison with those in Pc can contribute to the different physiological response patterns of both genotypes in acclimation to drought and re-watering conditions.

**By which physiological mechanisms do both genotypes acclimate to drought and re-watering conditions?**

Changes in anatomical characteristics including vessel dimension and leaf microstructure can result in acclimation of plants to drought and re-watering conditions (Beniwal et al. 2011). Increased vessel frequency, decreased vessel lumen area due to narrower vessel lumen diameter, lower predicted hydraulic conductivity and thickening leaf lamellae in Pc and Pe under drought conditions suggest that both poplar genotypes reduce water transport from roots to shoots in response to low water availability. In acclimation to lower water availability and higher risk of embolism under soil drought conditions, woody plants including *Populus* species/genotypes often produce wood with narrower vessel lumen and higher vessel frequency which can maintain water transport and reduce risk of embolism (Beniwal et al. 2010, Pinto et al. 2012). In line with modified anatomical changes in Pc and Pe, decreased photosynthetic activities and height growth of both poplar genotypes under drought and growth recovery after re-watering indicate that poplars can rapidly adjust growth according to soil water status, which is critical for survival under decreasing soil water availability.

The transcriptional regulation of *PIPs* and activation of ABA signaling pathway are crucial for plants to acclimate to drought and re-watering conditions. Upregulated mRNA levels of most analyzed *PIPs* in roots and/or leaves of Pc and Pe in response to drought and decreased transcript levels of most *PIPs* under re-watering conditions (Fig. 2) suggest that these PIPs in poplar genotypes can play key roles in facilitating water movement across plasma membrane in roots and in water re-distribution in leaf cells under changing soil water availability conditions. Previous studies have detected upregulation, downexpression or no changes of transcript levels of *PIP1;2, PIP1;3, PIP2;2, PIP2;3, PIP2;4 and PIP2;5* in roots and/or leaves of various poplar genotypes in response to drought and/or re-watering (Almeida-Rodriguez et al. 2010, Bae et al. 2011, Cohen...
et al. 2013). These results suggest that poplar genotypes can modulate transcript levels of PIPs to acclimate to changes in water availability in soil.

Activation of ABA signaling pathway is also critical for poplar plants to perceive changes in soil water availability (Popko et al. 2010). The ABA concentrations are often increased in poplars in response to decreasing soil water availability and recovered to normal levels upon re-watering (Arend et al. 2009, Cocozza et al. 2010). Along with changes in ABA concentrations,
transcriptional regulation of key genes involved in ABA signaling pathway such as NCED and PP2C in plants also occurs in response to drought and/or re-watering conditions (Tan et al. 2003, Soon et al. 2012). Consistently, induction of c-ABA in roots and leaves of Pc and Pe under drought and recovery to normal levels upon re-watering has been observed, but no such changes of t-ABA occur (Appendix S7), suggesting that c-ABA is the isoform which functions in ABA signaling pathway in poplars. In concert with alterations in c-ABA, c-ABA is the isoform which functions in ABA signaling changes of t-ABA occur (Appendix S7), suggesting that c-ABA, NCED3 and PP2C are essential players involved in ABA signaling pathway in poplars to acclimate to changes in soil water availability.

After sensing reduced soil water availability, plants have to alter metabolism of C and N, and homeostasis of ROS production and scavenging. Although drought in a short term can induce soluble sugars and sugar alcohol as osmolytes in plants to facilitate water uptake, long-term water deprivation can lead to reduction of carbohydrates because of decreased CO2 assimilation in plants (Regier et al. 2009, Warren et al. 2011). In this study, drought continued for ca. 4 weeks, which results in decreased concentrations of soluble sugars and sugar alcohols, starch and total C and N in roots and/or leaves of Pc and Pe due to declined photosynthesis under drought. Under drought conditions, plants are in a dilemma because on the one hand they have to minimize water loss by stomatal closure (reduced CO2 assimilation), on the other hand they need photosynthates to support several key processes for survival (Sala et al. 2010). To compromise the shifted balance between water loss and CO2 gain, plants have to increase WUE in acclimation to drought. Consistently, Pc and Pe increased δ13C and WUEi in response to drought, indicating that higher WUE is important for poplar genotypes to survive under drought conditions. Additionally, altered homeostasis of ROS production and scavenging can occur in plants in acclimation to drought and re-watering conditions (Keunen et al. 2013). Although ROS induction is often detected in plants exposed to drought, decreases in ROS are also found in plants after drought exposure (Keunen et al. 2013). The decreases in concentrations of O2•− and H2O2 in roots and/or leaves correspond well to lower activities of root SOD and foliar APX of Pc and Pe under drought conditions, suggesting that a new homeostasis of ROS production and scavenging has been probably reached in these poplars in acclimation to drought.

Taken together, Pc and Pe can acclimate to decreasing soil water availability by changes in anatomical properties, declined growth, transcriptional regulation of PIPs, activation of ABA signaling pathway, decreased total soluble sugars and starch, increased δ13C, δ15N and WUEi and altered homeostasis of ROS production and scavenging, whereas these physiological changes can be recovered under re-watering conditions.

As summarized in Fig. 7, Pc and Pe exhibited distinct anatomical, physiological and transcriptional response patterns in acclimation to drought and re-watering conditions, which is mainly ascribed to higher plant water contents, a stronger responsiveness of transcriptional regulation of PIPs, higher starch accumulation, δ13C, δ15N and WUEi and lower ROS accumulation and scavenging in Pe than those in Pc. Both poplar genotypes demonstrated altered anatomical properties, declined height growth, differentially transcriptional regulation of PIPs, activation of ABA signaling pathway, decreased total soluble sugars and starch, increased δ13C, δ15N and WUEi and lower ROS accumulation and scavenging in acclimation to decreasing soil water availability. However, these anatomical, physiological and transcriptional changes can be recovered under re-watering conditions. These data indicate that Pe is better acclimated to drought stress than Pc and that anatomical, physiological and transcriptional acclimation to decreasing water availability and re-watering is essential for poplars to survive and grow under projected dry climate scenarios in the future.

**Author contributions**

Z. B. L. conceived and designed the experiments. X. C., J. B. J., C. Z. and H. L. performed the experiments. X. C., J. B. J., C. Z., H. L., T. X. L., X. N. J., A. P., C. H. P. and Z. B. L. analyzed the data. X. C. and Z. B. L. wrote the paper.

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Supporting Information
Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplementary materials and methods.

Appendix S2. Primers used for RTqPCR.

Appendix S3. Alignments of PIPs, NCED3 and PP2C.

Appendix S4. The water contents and stem height increments.

Appendix S5. Anatomical and photosynthetic characteristics.

Appendix S6. PCA of anatomical and photosynthetic properties.

Appendix S7. NCED3 and PP2C transcripts, and ABA concentrations.

Appendix S8. Concentrations of carbohydrates.

Appendix S9. ROS and antioxidants.

Appendix S10. PCA of physiological parameters.