

Physiological and transcriptional regulation in poplar roots and leaves during acclimation to high temperature and drought

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To elucidate the physiological and transcriptional regulatory mechanisms that underlie the responses of poplars to high temperature (HT) and/or drought in woody plants, we exposed *Populus alba* × *Populus tremula* var. *glandulosa* saplings to ambient temperature (AT) or HT under 80 or 40% field capacities (FC), or no watering. HT increased the foliar total carbon (C) concentrations, and foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$. HT triggered heat stress signaling via increasing levels of abscisic acid (ABA) and indole-3-acetic acid (IAA) in poplar roots and leaves. After perception of HT, poplars initiated osmotic adjustment by increasing foliar sucrose and root galactose levels. In agreement with the HT-induced heat stress and the changes in the levels of ABA and carbohydrates, we detected increased transcript levels of *HSP18* and *HSP21*, as well as *NCED3* in the roots and leaves, and the sugar transporter gene *STP14* in the roots. Compared with AT, drought induced greater enhancement of foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in poplars at HT. Similarly, drought caused greater stimulation of the ABA and foliar glucose levels in poplars at HT than at AT. Correspondingly, desiccation led to greater increases in the mRNA levels of *HSP18*, *HSP21*, *NCED3*, *STP14* and *INT1* in poplar roots at HT than at AT. These results suggest that HT has detrimental effects on physiological processes and it induces the transcriptional regulation of key genes involved in heat stress responses, ABA biosynthesis and sugar transport and HT can cause greater changes in drought-induced physiological and transcriptional responses in poplar roots and leaves.

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Abbreviations – ABA, abscisic acid; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; DHA, dehydroascorbate; GA, gibberellin; GR, glutathione reductase; GSSG, oxidized-glutathione; HSPs, heat shock proteins; IAA, indole-3-acetic acid; INT1, Inositol transporter 1; JA, jasmonic acid; LPI, leaf plastochron index; LWP, leaf water potential; NCED3, 9-cis-epoxycarotenoid dioxygenase 3; PCA, principal component analysis; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; SA, salicylic acid; SOD, superoxide dismutase; STP14, Sugar transporter 14; $\delta^{13}\text{C}/\delta^{18}\text{O}$, stable carbon/oxygen isotope composition.

Introduction

Predictions suggest that climate change will increase global average temperatures by 2.4–6.4°C during this century, with increases in the frequency, length and severity of droughts (IPCC 2007). High temperature (HT) can cause heat stress in plants and is often accompanied by drought, which can severely damage plants. Recent research suggests that heat- and drought-induced forest mortality events are widespread around the world (Allen et al. 2010, Anderegg et al. 2012); thus, HT and drought will be major abiotic stresses for most plants including tree species in the near future. Therefore, heat- and drought-tolerant tree species are needed urgently for afforestation. To breed heat- and drought-tolerant woody plants, it is essential for us to obtain a better understanding of the physiological and molecular mechanisms employed by tree species during acclimation to HT and drought.

Temperature is a key environmental factor for tree growth and development (Way and Oren 2010). Particularly, temperature has major effects on CO₂ assimilation by woody plants. Given the balance between the carboxylation and oxygenation of ribulose-1,5-bisphosphate (RuBP), the optimum temperature for net CO₂ assimilation is close to 25°C in most C₃ plants (Yamori and von Caemmerer 2009). In this study, we consider that HT comprises temperatures above the optimum for plant growth. The effects of HT on herbaceous plants including crops have been studied extensively (Bitá and Gerats 2013). HT can cause morphological, physiological and molecular changes in plants. Importantly, HT can accelerate photorespiration, inhibit net CO₂ assimilation rates, differentiate the fractionation of ¹³C and ¹⁸O, induce the synthesis of heat shock proteins (HSPs), trigger the phytohormonal signaling network such as abscisic acid (ABA) signaling, and alter the balance between reactive oxygen species (ROS) and antioxidants compared with AT (Bitá and Gerats 2013). Furthermore, HT can also induce the transcriptional regulation of genes involved in physiological acclimation, such as genes encoding HSPs, ABA biosynthesis and sugar transport (Bhardwaj et al. 2015). Perennial woody plants experience more severe and extreme abiotic stresses including HT during their lives compared with herbaceous plants, which may have evolved more complex stress responses. Currently, little is known about the physiological responses and transcriptional modulation of genes involved in the physiological acclimation of woody plants in response to HT (Rennenberg et al. 2006).

Drought can lead to physiological and molecular changes in tree species, including stomatal closure, reduced photosynthetic rates, altered non-structural

carbohydrate concentrations, ABA induction and differential expression of a number of genes and proteins (Harfouche et al. 2014). Since drought and HT can occur simultaneously under future climatic conditions, it is of necessity to understand the physiological and molecular responses of trees to combined drought and HT. Previous studies showed that the combination of drought and HT modify plant metabolism and gene expression in a distinct manner compared with each separate stress in *Arabidopsis* (Rizhsky et al. 2004, Rasmussen et al. 2013). These reports highlight the importance of studying combined stresses to understand stress-tolerance in trees during acclimation to future climatic conditions. However, little information is currently available about the effects of combined drought and HT on physiological (e.g. photosynthesis, phytohormones, carbohydrates, ROS and antioxidants) and molecular (e.g. the transcriptional regulation of genes involved in physiological acclimation) responses in woody plants. Thus, it is essential to elucidate the physiological and molecular mechanisms of tree species during acclimation to combined drought and HT.

Populus species are fast-growing woody plants that are sensitive to environmental changes (Harfouche et al. 2014). Under natural conditions, poplar plantations may suffer from mild or severe drought because of limited or no precipitation. To simulate mild and severe drought that poplars may experience, it is necessary to treat plants with limited and no watering under controlled experimental conditions. Thus, a short-term greenhouse experiment including limited (mild drought) and no watering (severe drought) treatments was carried out in this study. Here, we exposed *Populus alba* × *P. tremula* var. *glandulosa* saplings to either ambient temperature (AT) or HT in combination with one of three watering regimes (80 or 40% FCs, or no watering). Because HT can often induce stomatal closure leading to lower CO₂ assimilation in plants compared with AT (Bitá and Gerats 2013), it is probable that combined HT and drought can have synergistic effects on physiological and molecular processes in plants. We hypothesized that HT would have detrimental impacts on physiological processes (such as energy metabolism and phytohormonal signaling) and induce the transcriptional regulation of key genes involved in physiological acclimation (i.e. heat stress response, ABA biosynthesis and sugar transport), as well as aggravating the stress effects of drought on poplar roots and leaves. To test this hypothesis, we characterized the physiological responses (total carbon, stable carbon/oxygen isotope compositions, water status, photosynthesis, phytohormones, non-structural carbohydrates, ROS and antioxidants) and the transcriptional regulation of key genes (transcript levels of genes involved in heat stress responses, ABA biosynthesis and sugar transport)

involved in physiological acclimation of poplar roots and leaves exposed to HT and/or drought.

Materials and methods

Plant cultivation and treatments

Cuttings (ca. 15 cm in length, 2 cm in diameter, 1-year-old stem) were rooted from a hybrid poplar (*P. alba* × *P. tremula* var. *glandulosa*). Subsequently, each cutting was planted in a separate plastic pot (10 l) filled with soil (clay:sand, 1:1, v/v). Plants were cultivated in a glasshouse (day/night temperature, 25/18°C; light/dark, 16/8 h; light intensity, 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at plant height; relative humidity, 50–60%) for 3 months. Next, 108 plants with similar heights and growth performance were selected and randomly assigned to six climate chambers, with 18 plants in each. Before the stress treatments, plants were grown for 2 weeks with daily irrigation to FC and supplied with 50 ml Hoagland nutrient solution every 2 days in the climate chambers (day/night temperature, 25/20°C; light/dark, 16/8 h; light intensity, 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at plant height; relative humidity, 60%). In the temperature treatments, three climate chambers were assigned to HT and the other three were set as AT. The day/night temperatures of the chambers treated with HT and AT were set as $30 \pm 0.5/25 \pm 0.5^\circ\text{C}$ and $25 \pm 0.5/20 \pm 0.4^\circ\text{C}$, respectively. For the drought treatments, 18 plants (ca. 70 cm in height) in each chamber were divided into three random groups (six plants in each group) and the plants in each group were treated with one of three watering regimes [80% (the control) or 40% (mild drought) FCs, and no watering (severe drought, denoted as 0% FC)]. In a preliminary experiment, the times required for the soil to initially reach 40% FC at HT and at AT were 1 and 2 days, respectively, indicating that the water consumption of plant soil at HT is faster than that at AT. To achieve the target FCs (80 and 40%) in the drought treatments, the pot was weighed and the amount of water added was equal to the loss due to transpiration and soil evaporation, as described previously (Chen et al. 1997). Owing to the greater water consumption of plants at HT, more frequent addition of water to the pot was carried out to achieve 80 and 40% FCs at HT. The temperature and drought treatments were initiated on the same day.

Determination of gas exchange and predawn leaf water potential

During the temperature and drought treatments, three plants from each drought condition in each chamber were selected for daily monitoring of stomatal conductance (g_s). Three mature leaves (leaf plastochron

index, $\text{LPI}=7-9$) were analyzed from each selected plant using a portable photosynthesis system (LI-6400; LiCor, Lincoln, NE) with an attached LED light source (6400-02), as described previously (He et al. 2011). Before harvesting, the CO_2 assimilation rate (A), transpiration rate (E), g_s and the ratio of the intercellular relative to the ambient CO_2 concentrations (C_i/C_a) were measured. The measurements were performed from 8:00 to 11:00 h with a light intensity of 1000 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$. The air flow through the sample chamber was set at 500 $\mu\text{mol s}^{-1}$ and the CO_2 concentration in the sample chamber was 400 $\mu\text{mol mol}^{-1}$. The adaptation time of the leaf in the sample chamber was 5 min and the measurement lasted 5 min for each leaf.

Before harvesting, the respiration rates (R_s) of the leaves used for the gas exchange analysis were measured after the plants were adapted to darkness for 30 min using the same chamber and instrument. The accuracy of this method has been validated in previous studies (Loreto et al. 2001, Centritto et al. 2011). The leaves ($\text{LPI}=8$) used for gas exchange measurements were tested to determine the predawn leaf water potential (LWP). LWP was measured after daily irrigation at predawn on the harvest day using a Scholander pressure chamber (model 600; PMS, Albany, OR), as described previously (Hinckley et al. 1978).

Harvesting and determination of the relative water content (RWC)

The temperature and drought treatments were continued until g_s reached zero for the plants exposed to AT and no watering. The temperature and drought treatments lasted 8 days, after which all the plants were harvested. The fine roots of each plant were cleaned carefully and harvested. The harvested roots or leaves were then wrapped with tinfoil and frozen immediately in liquid nitrogen. Frozen samples were ground into a fine powder in liquid nitrogen with a mortar and a pestle, before storing at -80°C . Aliquots of powdered (ca. 80 mg) fine root or leaf samples were dried at 60°C for 72 h to determine the ratios of fresh relative to dry biomass and the relative water contents (RWCs), which were calculated as follows: $\text{RWC}\% = (\text{FW} - \text{DW})/\text{FW} \times 100$. Equal amounts of fine powder from the same tissues of three plants that received each treatment in each chamber were combined and mixed thoroughly before use in the biochemical and molecular analyses.

Analysis of total carbon and stable carbon/oxygen isotope compositions

The dried fine powdered (ca. 0.8 mg) root and leaf samples were used to determine the total carbon (C) and

stable C isotope composition ($\delta^{13}\text{C}$). The dried powders were analyzed using an elemental analyzer (NA 2500; CE Instruments, Rodano, Italy) and a mass spectrometer (Delta Plus; Finnigan MAT, Bremen, Germany) with an interface (Conflo III; Finnigan MAT), according to a published method (Werner et al. 1999) with minor modifications (Cao et al. 2014). The total C concentrations were calculated according to a published method (Luo et al. 2013). The stable C isotope composition was calculated as follows:

$$\delta^{13}\text{C} = (R_{\text{sa}} - R_{\text{sd}}) / R_{\text{sd}} \times 1000 [\text{‰}]$$

where R_{sa} and R_{sd} are the ratios of ^{13}C relative to ^{12}C for the sample and the standard, respectively. The standard for C was referred to CO_2 in air.

In addition, ca. 1 mg of dry fine root or leaf sample was analyzed to determine $\delta^{18}\text{O}$ using an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT) following pyrolysis in a high temperature furnace (Thermoquest TC/EA, Finnigan MAT), according to a published method (Cernusak et al. 2009). The $\delta^{18}\text{O}$ values were calculated according to the formula given above and the standard was referred to Vienna Standard Mean Ocean Water.

Analysis of phytohormone concentrations

The concentrations of ABA, indole-3-acetic acid (IAA), gibberellin (GA_3), salicylic acid (SA) and jasmonic acid (JA) were determined by high performance liquid chromatography (HPLC) (LC-20AT, Shimadzu, Kinoh Do, Japan)–electrospray tandem mass spectrometry (API 2000TM, ip), according to a published method (Shi et al. 2015). In brief, phytohormones in roots or leaves were extracted in 4 ml of precooled 80% methanol (containing 200 mg l^{-1} butylated hydroxytoluene and 500 mg l^{-1} citric acid monohydrate) three times, and the supernatants were collected and combined. The supernatants were dried under N_2 and the residues were dissolved in 0.8 ml of 80% methanol. The extract (ca. 2 μl) was separated with a C18 column (4.6 \times 150 mm, 5 μM ; WondasilTM, Shimadzu, Kinoh Do, Japan) and further analyzed using the HPLC-MS system. The standard curves were established for phytohormones using ABA ((\pm)-ABA, A1049), IAA (I2886), GA_3 (G7645), SA (S7401) and JA (J2500) standards purchased from Sigma (St Louis, MO).

Determination of soluble sugars, sugar alcohols and starch

Soluble sugars and sugar alcohols were analyzed using a GC-MS system (Thermo Electron Corporation,

Austin, TX), as described previously (Luo et al. 2009, He et al. 2013a). Briefly, fine powdered (ca. 80 mg) fresh samples were extracted in an extraction solution (methanol:chloroform:water, 12:5:3, v/v/v). Next, the extracted compounds were acetylation derivatized, separated in a DB-17 capillary column (30 mm \times 0.25 mm \times 0.25 μm ; J&W Scientific, Folsom, California, CA) attached to a Finnigan Trace GC ultra, and quantified with a Finnigan Trace GC ultra-Trace DSQ GC-MS system (Thermo Electron Corporation). Ribitol was used as the internal standard in the analysis, and mannitol, galactose, sorbitol, fructose, myoinositol, glucose, sucrose and trehalose were used as standards to identify and quantify the concentrations of sugars and sugar alcohols.

The starch concentrations in fine roots and leaves were analyzed using the anthrone method, as described previously (He et al. 2013b). Absorption was determined spectrophotometrically at 620 nm. A standard curve was established using serial diluted solutions of glucose and the starch concentrations were expressed as glucose equivalents.

Determination of $\text{O}_2^{\bullet-}$ and H_2O_2

$\text{O}_2^{\bullet-}$ and H_2O_2 are important ROS in plants, particularly under stress conditions. The concentrations of the superoxide anion ($\text{O}_2^{\bullet-}$) and H_2O_2 in the fine roots and leaves were determined spectrophotometrically at 530 and 410 nm, respectively, according to a published method (He et al. 2013b).

Determination of non-enzymatic antioxidants and activities of antioxidative enzymes

In plants, the ascorbate–glutathione cycle plays a pivotal role in scavenging overproduced ROS. Thus, ascorbate (ASC), dehydroascorbate (DHA), reduced- (GSH) and oxidized-glutathione (GSSG), and the relevant enzymes involved in this cycle were analyzed in poplars. ASC and DHA were determined based on a published protocol (Stamm and Kumar 2010) with minor modifications (He et al. 2015). GSH and GSSG were determined according to a previously reported method (Stamm and Kumar 2010).

Soluble proteins were extracted from the fresh samples and used to quantify the activities of antioxidative enzymes as reported previously (He et al. 2011). The enzyme activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.8.1.7) were determined spectrophotometrically at 560, 240, 290 and 340

nm, respectively, as described previously (He et al. 2013b).

Analysis of the transcript levels of key genes involved in physiological acclimation

Because poplar plants were exposed to HT which is related to heat stress and the concentrations of ABA and non-structural carbohydrates were determined, the transcript levels of genes involved in heat stress responses, ABA biosynthesis and sugar transport were also analyzed in the roots and leaves of poplars. These included four genes implicated in heat stress responses, i.e. *HSP18* (*HSP 18.2*), *HSP21*, *CRK1* (*CDPK-related kinase 1*) and *BOB1* (*HSP20-like chaperones superfamily protein BOBBER1*), two genes involved in the ABA biosynthesis pathway, i.e. *ZEP* (*Zeaxanthin epoxidase*) and *NCED3* (*9-cis-epoxycarotenoid dioxygenase 3*), and six genes involved in the transport of sugars and sugar alcohols, i.e. *SUC2* (*Sucrose transporter 2*), *STP1* (*Sugar transporter 1*), *STP14* (*Sugar transporter 14*), *PMT1* (*Polyol/monosaccharide transporter 1*), *INT1* (*Inositol transporter 1*) and *TMT1* (*Tonoplast monosaccharide transporter 1*). The mRNA levels of these genes were analyzed using quantitative RT-PCR, as described previously (Ma et al. 2014). Briefly, total RNA was extracted from the fine powdered fresh samples (ca. 100 mg) and purified with a plant RNA extraction kit (R6827, Omega Bio-Tek, GA). To remove DNA contamination, the purified total RNA was digested with deoxyribonuclease from the TURBO DNA-free kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The success of the DNA-free treatment was evaluated by a control real-time PCR. After DNA-free treatment, the RNA (ca. 1 µg) was used to synthesize the first strand cDNA with a PrimeScript RT reagent kit (RR047A, Takara, Dalian, China), according to the manufacturer's instructions. The synthesized cDNAs were used for real-time qPCR, as described previously (Li et al. 2012). Quantitative RT-PCR was performed using 10 µl 2× SYBR Green Premix Ex Taq II (DRR820A, Takara, Dalian, China), 2 µl cDNAs and 0.2 µl of 20 mM primers in a 20 µl reaction volume with an IQ5 real-time system (Bio-Rad, Hercules, CA). The primers were designed specifically for each gene (Table S1, Supporting Information). To ensure the primer specificity, the PCR products were sequenced and aligned with homologues from model plants, i.e. *Populus trichocarpa* and *Arabidopsis thaliana* (Fig. S1, Supporting Information). We used 18S rRNA as the reference gene. The efficiencies of all the PCR reactions were between 92 and 109% (Table S1). PCR was performed in triplicate together with a dilution series of the reference gene.

Statistical analysis

Statistical tests were performed using STATGRAPHICS (STN, St Louis, MO). The data were tested to confirm their normality before statistical analyses. For the experimental variables, two-way ANOVAS were applied with temperature and drought as the two main factors. Differences between means were considered significant when $P < 0.05$ according to the ANOVA F-test. If interactions were significant, posteriori comparisons of means were performed using Tukey's method. The fold changes in the transcripts of genes were calculated using the relative expression software tool REST (Pfaffl et al. 2002). The gene expression heatmap was generated using the heatmap.2 () command in the package 'gplots' in R (<http://www.r-project.org/>), as described previously (Luo et al. 2013). Before principal component analysis (PCA), the data were standardized and subsequently computed using the command prcomp() in R, as described previously (He et al. 2013a).

Results

Total C, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in relation to photosynthesis

The total C concentration is sensitive to changes in environmental factors because abiotic stresses can often induce alterations in CO_2 assimilation in plants. The $\delta^{13}\text{C}$ measured in dry matter can indicate the long-term transpiration efficiency of plants, thereby serving as an indicator of water use efficiency (WUE) (Farquhar and Richards 1984), while the $\delta^{18}\text{O}$ in plant tissues can reflect the isotope composition of soil water, evaporative enrichment in transpiring leaves, and isotopic exchange between oxygen atoms in organic molecules and the local water in cells (Barbour 2007). The $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ levels in plants are sensitive to changes in temperature and/or water availability. Therefore, we determined the total C, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ levels in poplar roots and leaves exposed to HT and/or drought (Fig. 1). Although the total C concentrations and $\delta^{13}\text{C}$ in roots were unaffected by HT, the root $\delta^{18}\text{O}$ level was slightly lower at HT compared with AT (Fig. 1A–C). In contrast to the marginal effects of HT on roots, HT yielded higher total C, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ levels in the leaves compared with AT (Fig. 1D–F). Severe drought decreased the total C and $\delta^{18}\text{O}$ levels in roots, and the total C concentrations in leaves, as well as increasing the foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ levels, irrespective of the temperature (Fig. 1A–F). The $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ levels in plants are associated with plant water status and photosynthetic processes, so these results indicate that water status and photosynthesis in poplar can be affected by HT and/or drought. Thus, we assessed the plant water status and photosynthetic characteristics in

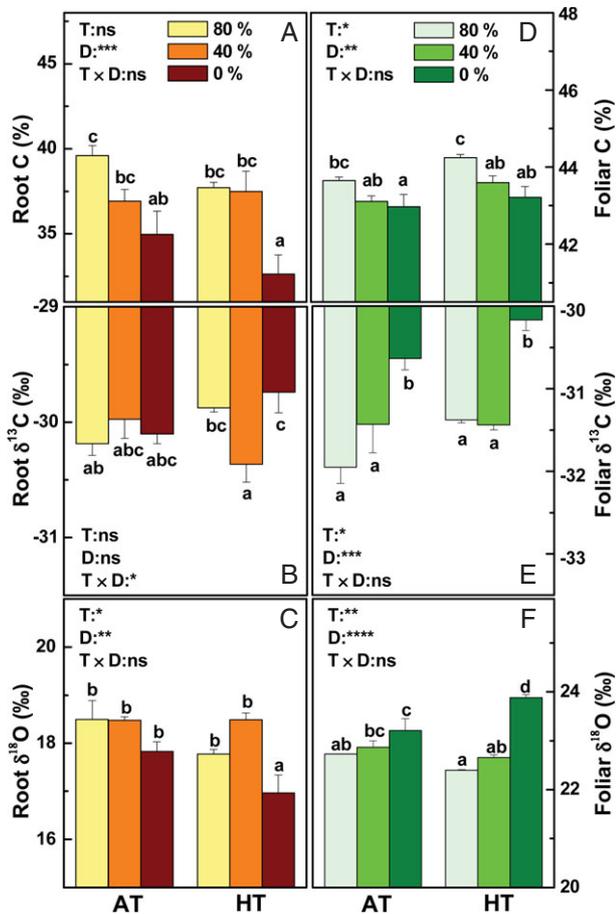


Fig. 1. Total C, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in roots (A–C) and leaves (D–F) of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. Bars indicate means \pm SE ($n=6$). Different letters on the bars indicate significant differences. *P*-values obtained from ANOVAs based on temperature (T), drought (D) and their interactions (T \times D) are also indicated. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns: not significant.

poplars treated with HT and/or drought (Tables S2 and S3). LWP was lower at HT compared with AT (Table S2). Mild and severe drought caused a more rapid reduction in the LWP at HT than AT (Table S2). The lower water status in poplars exposed to HT inhibited the photosynthetic rate (A), transpiration rate (E) and g_s (Fig. S2, Table S3). Moreover, mild and severe drought led to greater reductions in A at HT compared with that at AT (Table S3).

The stable carbon/oxygen isotope compositions and photosynthetic processes are often coupled; thus, we assessed the linear correlations between these parameters (Fig. 2). There was a significant negative linear correlation between the intrinsic water use efficiency ($\text{WUE}_i = A/g_s$) and foliar $\delta^{13}\text{C}$ (Fig. 2A). By contrast, there was a significant positive linear correlation between foliar $\delta^{13}\text{C}$ and C_i/C_a (Fig. 2B). There was a negative

linear correlation between foliar $\delta^{18}\text{O}$ and g_s (Fig. 2C). Furthermore, we detected a significantly positive linear correlation between foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in poplars (Fig. 2D).

Phytohormones

Plant hormones play key roles in sensing abiotic stresses via changes in their levels. Thus, we determined the concentrations of ABA, IAA, GA_3 , SA and JA in poplar roots and leaves exposed to HT and/or drought (Fig. 3). In roots, HT increased the concentration of ABA under 40% FC, and of IAA under 80 and 40% FCs, whereas it decreased the levels of GA_3 and JA (Fig. 3A–E). Mild and severe drought elevated the levels of ABA and SA but decreased the concentrations of IAA, GA_3 and JA in roots, irrespective of temperature (Fig. 3A–E). In leaves, HT increased the concentrations of IAA under 80 and 40% FCs, and of GA_3 under 80 and 0% FCs, whereas it decreased the levels of SA and JA under 80% FC (Fig. 3F–J). Mild drought induced higher concentrations of foliar ABA at HT compared with AT (Fig. 3F). Mild drought decreased the IAA level at AT but increased the IAA concentration at HT in leaves (Fig. 3G). Mild and severe drought decreased the concentrations of foliar GA_3 , SA and JA, irrespective of temperature (Fig. 3H–J).

Carbohydrates

After sensing abiotic stresses, plants must trigger physiological acclimation, which includes changes in the carbohydrate concentrations to facilitate osmotic adjustment. Therefore, we determined the levels of soluble sugars and sugar alcohols in poplars (Fig. 4, Table S4). In roots, HT decreased the sucrose concentration under 40% FC, but it was increased under 0% FC (Fig. 4A). HT led to lower glucose concentrations in the roots compared with AT (Fig. 4B). Moreover, HT elevated the galactose concentration but decreased the inositol level in roots (Table S4). Mild and severe drought increased the sucrose concentrations in roots at both AT and HT (Fig. 4A). Mild drought yielded enhanced concentrations of galactose and inositol in roots at both temperatures (Table S4).

In leaves, HT induced the concentration of sucrose (Fig. 4C). Mild drought resulted in lower concentration of foliar sucrose compared with the control (80% FC) (Fig. 4C). Severe desiccation led to greater increases in the glucose and galactose concentrations in leaves at HT compared with AT (Fig. 4D, Table S4).

ROS and antioxidants

Abiotic stresses often disrupt the balance between ROS and antioxidants in plants. Thus, we analyzed

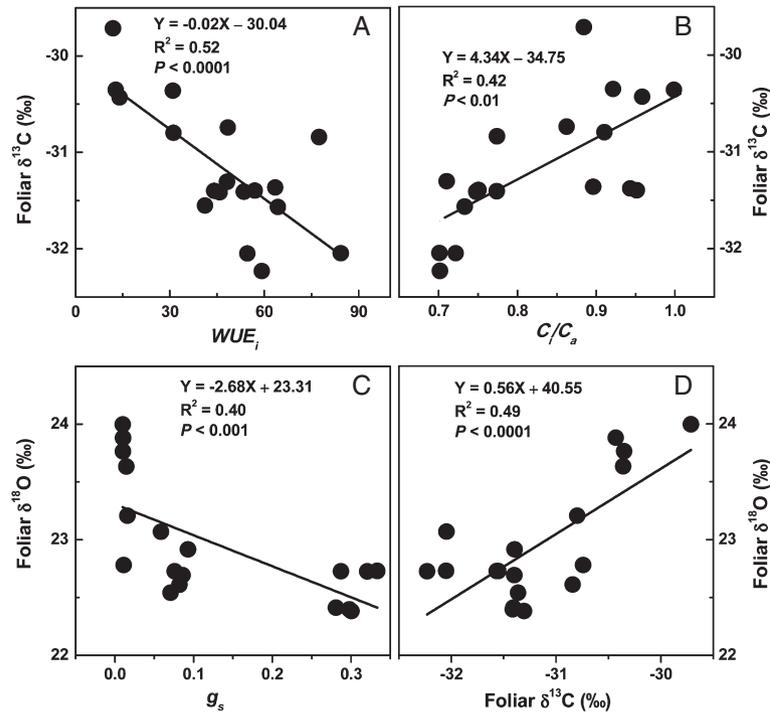


Fig. 2. Correlations between intrinsic water use efficiency ($WUE_i = A/g_s$) and foliar $\delta^{13}C$ (A), foliar $\delta^{13}C$ and C_i/C_a (B), foliar $\delta^{18}O$ and g_s (C) and foliar $\delta^{18}O$ and foliar $\delta^{13}C$ (D) in poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. The data points in each chart are from the measurements in the leaves of poplars treated with the combined temperature and drought.

the concentrations of $O_2^{\bullet-}$ and H_2O_2 in poplar roots and leaves (Fig. 5). In roots, HT decreased the $O_2^{\bullet-}$ concentration but elevated the H_2O_2 level under both 80 and 40% FCs (Fig. 5A, B). Mild and severe drought induced the concentrations of $O_2^{\bullet-}$ and H_2O_2 in roots at AT and HT (Fig. 5A, B). In leaves, HT had no effects on the $O_2^{\bullet-}$ and H_2O_2 levels (Fig. 5C, D). Mild drought induced the concentrations of foliar $O_2^{\bullet-}$ and H_2O_2 , irrespective of temperature (Fig. 5C, D).

To scavenge the ROS that is overproduced in plants exposed to stress, plants can activate the antioxidant system, which includes the overproduction of non-enzymatic antioxidants and changes in the activities of antioxidative enzymes. The ascorbate-glutathione cycle plays a key role in providing antioxidants. Therefore, we assessed non-enzymatic antioxidants such as ASC, DHA, GSH and GSSG, as well as the activities of SOD, CAT, APX and GR in poplar roots and leaves (Fig. 6 and Figs. S3, S4). In roots, HT decreased the ratio of ASC relative to DHA, which was due mainly to HT-suppressing ASC but inducing DHA (Fig. 6A and Fig. S3). Mild drought elevated the ratio of ASC relative to DHA in roots at AT and at HT, which was due to greater reductions in the concentrations of DHA than ASC in roots under drought conditions (Fig. 6A and

Fig. S3). HT had no effects on the ratio of GSH relative to GSSG in roots (Fig. 6B). Mild and severe drought reduced ratio of GSH relative to GSSG in roots at AT and at HT, which was mainly due to decreased GSH levels and elevated GSSG concentrations in roots under desiccation (Fig. 6B and Fig. S3). In leaves, HT induced the ratio of ASC relative to DHA, irrespective of water availability (Fig. 6C). Similarly, severe drought increased the ratio of ASC relative to DHA in leaves, which was mainly due to an elevated concentration of ASC (Fig. 6C and Fig. S3).

Antioxidative enzymes also contributed to the shifted homeostasis of ROS and antioxidants in poplars exposed to HT and/or drought (Fig. S4). For instance, HT increased the activity of CAT in roots under severe drought and of foliar GR in the control (80% FC). Mild and severe drought led to higher activities of root CAT and foliar SOD at HT than at AT.

PCA of physiological responses

PCA can provide a clear pattern of multiple parameters in response to experimental treatments by the reduction in data dimension. To gain the response pattern of poplars to HT and/or drought, we conducted PCA based

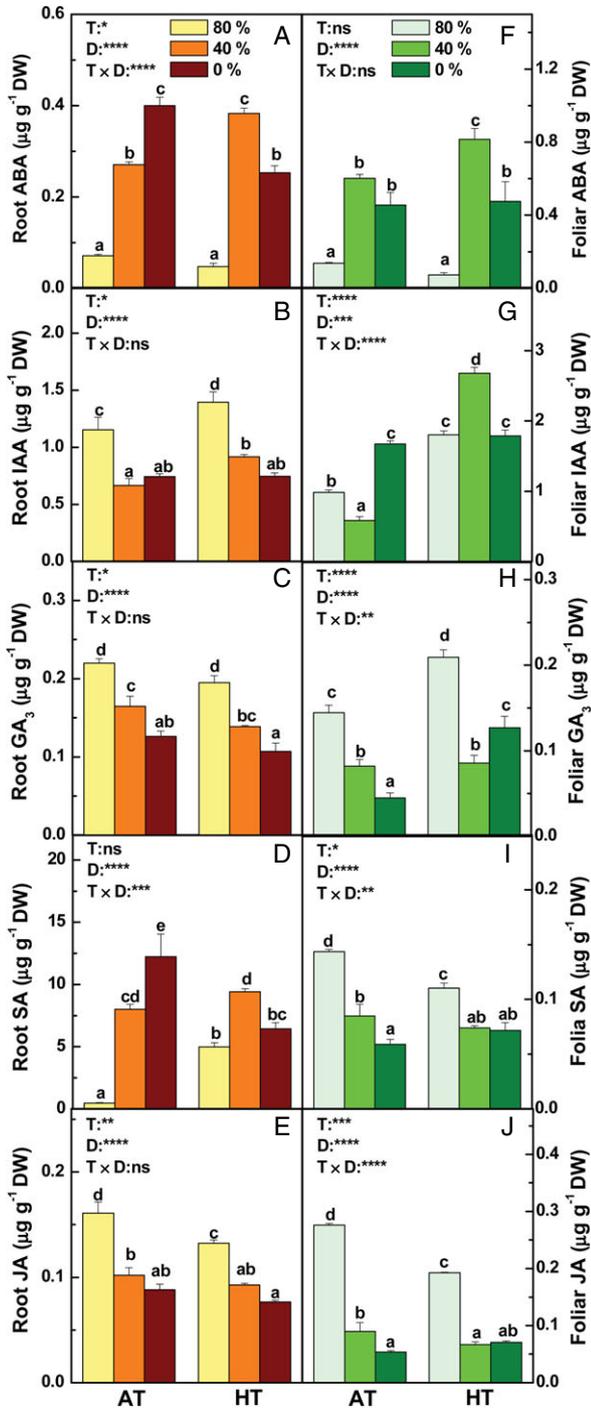


Fig. 3. ABA, IAA, GA₃, SA and JA concentrations in the roots (A–E) and leaves (F–J) of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. Bars indicate means ± se (n=6). Different letters on the bars indicate significant differences. *P*-values obtained from the ANOVAs based on temperature (T), drought (D) and their interactions (T×D) are also indicated. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns: not significant.

on the RWC, photosynthetic parameters, respiration rate, total C concentration, δ¹³C, δ¹⁸O, soluble sugars, sugar alcohols and starch, phytohormones, ROS and antioxidants (Fig. 7, Table S5). PC1 clearly separated the effects of drought treatments and PC2 accounted for the variation in temperature effects. PC1 and PC2 accounted for 37 and 18% of the total variation, respectively. The root ABA and SA, and foliar ABA concentrations were key contributors to PC1, whereas the foliar CAT activity, root ASC/DHA and foliar sucrose concentrations were important factors for PC2. The results of the PCA analysis indicated that drought had more pronounced effects on physiological processes in poplars compared with HT exposure, which were attributable mainly to the root ABA and SA concentrations, foliar CAT activity and root ASC/DHA.

Changes in the transcript levels of key genes involved in physiological acclimation

In addition to physiological acclimation to HT and/or drought, the transcriptional regulation of key genes plays a pivotal role in the physiological responses of poplars. Thus, the effects of HT and/or drought on the transcript levels of genes involved in heat stress responses, ABA biosynthesis and sugar transport were assessed in poplar roots and leaves (Fig. 8). In roots, HT induced the transcript levels of *HSP18* and *HSP21* (Fig. 8A). Mild and severe drought also led to increased transcript levels of *BOB1*, *HSP18* and *HSP21* in roots, irrespective of temperature (Fig. 8A). HT elevated the transcript levels of *ZEP* and *NCED3* in roots under three watering regimes, but HT reduced the mRNA of *NCED3* under 80% FC (Fig. 8A). Similarly, mild and severe drought brought about higher mRNA levels of *ZEP* and *NCED3* in roots at HT compared with AT (Fig. 8A), which is in agreement with the drought-induced ABA levels in roots. In roots, HT elevated the transcript levels of *SUC2*, *STP14*, *PMT1* and *INT1* under 40 and 0% FCs (Fig. 8A). Mild and severe desiccation caused greater increases in the mRNA levels of *SUC2*, *STP14*, *PMT1* and *INT1* in roots at HT compared with AT (Fig. 8A), which is consistent with the drought-induced changes in the levels of glucose, galactose and inositol in roots.

In leaves, HT induced the mRNA levels of *HSP18* and *HSP21* under 40 and 0% FCs (Fig. 8B), which is consistent with elevated air temperature causing heat stress in poplar leaves. Moreover, mild and severe drought caused greater increases in the transcript levels of *HSP18* and *HSP21* at HT compared with AT (Fig. 8B). HT repressed the mRNA levels of *ZEP* and *NCED3* in leaves under 80 and 0% FCs (Fig. 8B). By contrast, mild water deficit caused greater increases in the mRNA levels of *ZEP* and

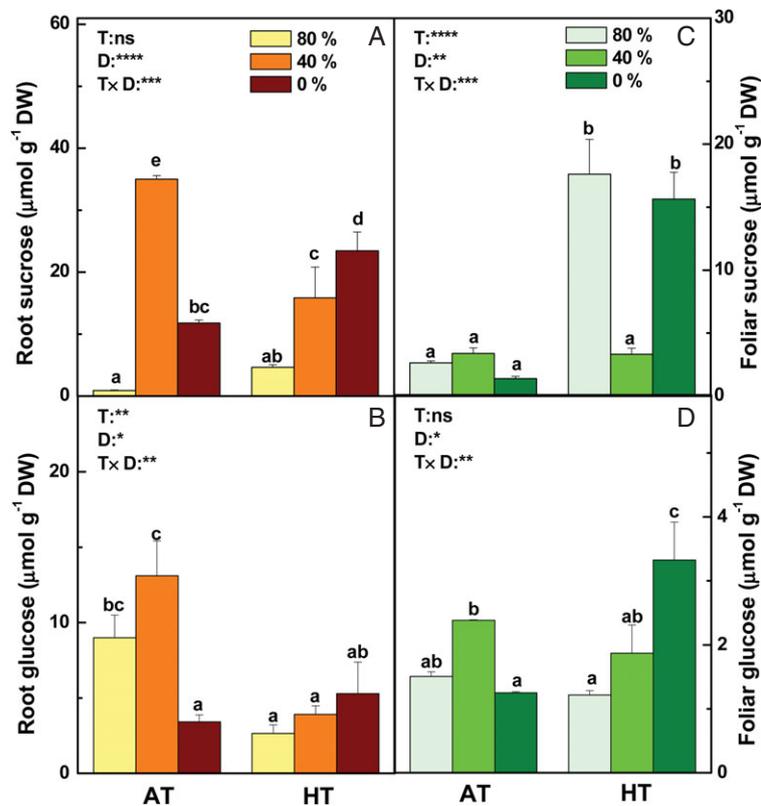


Fig. 4. Concentrations of sucrose and glucose in roots (A, B) and leaves (C, D) of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. Bars indicate means \pm SE (n = 6). Different letters on the bars indicate significant differences. *P*-values obtained from the ANOVAs based on temperature (T), drought (D) and their interactions (T \times D) are also indicated. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns: not significant.

NCED3 in leaves at HT compared with AT (Fig. 8B), which is line with drought-induced higher ABA levels at HT compared with AT. HT decreased the mRNA levels of *SUC2* and *STP1* in leaves (Fig. 8B). Mild and severe drought also repressed the transcript levels of *SUC2* and *STP1*, but elevated the mRNA levels of *PMT1* and *INT1* in leaves at AT and at HT (Fig. 8B), which is consistent with the reduced sucrose concentrations and increased inositol levels under drought conditions.

Discussion

HT had detrimental effects on energy metabolism and phytohormonal signaling, and triggered the transcriptional regulation of key genes involved in heat stress response, ABA biosynthesis and sugar transport

Energy metabolism is tightly linked with carbon and water metabolism in plants. The total C, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ concentrations are important indicators of changes in the metabolism of C and H_2O under abiotic stresses in plants. In the current study, the increases in the

total foliar C concentrations in HT-exposed poplars are probably related to the enhancement of protection from HT in poplar leaves based on C-enriched molecules such as lignin and phenolics (Luo et al. 2008). The positive relationship between foliar $\delta^{13}\text{C}$ and C_i/C_a in poplar leaves confirms that foliar $\delta^{13}\text{C}$ depends on the relative ratio (C_i/C_a) of the partial pressures of CO_2 at the carboxylation sites and in ambient air (Farquhar and Richards 1984). The foliar $\delta^{13}\text{C}$ level is often used as a proxy for the leaf-level intrinsic water use efficiency under natural conditions (Cao et al. 2012). In this study, however, there was a negative linear correlation between foliar $\delta^{13}\text{C}$ and WUE_i in poplars, which was probably because the HT and/or severe drought stresses in leaves during gas exchange measurements disrupted the positive linear correlation between foliar $\delta^{13}\text{C}$ and WUE_i which is often found in plants in normal growth conditions (Seibt et al. 2008, Roussel et al. 2009). The ^{18}O can be enriched in leaves during transpiration and via the exchange of oxygen atoms between water originating from the soil and carbonyl groups (Barbour 2007). The increases in the foliar $\delta^{18}\text{O}$ in HT-exposed

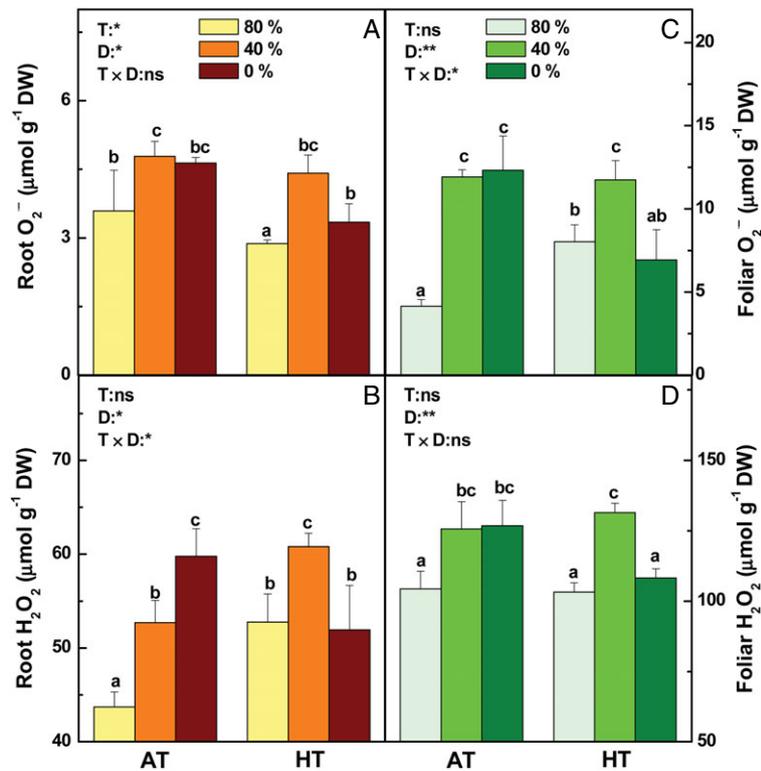


Fig. 5. Concentrations of $O_2^{\bullet-}$ and H_2O_2 in the roots (A, B) and leaves (C, D) of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. Bars indicate means \pm SE (n = 6). Different letters on the bars indicate significant differences. P-values obtained from the ANOVAs based on temperature (T), drought (D) and their interactions (T x D) are also indicated. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; ns: not significant.

poplars indicate the accelerated enrichment of ^{18}O in poplar leaves at HT, which is probably associated with the decreased g_s at HT. Similarly, Barbour et al. (2000) reported a negative linear correlation between the foliar $\delta^{18}O$ and g_s , and a positive correlation between the foliar $\delta^{18}O$ and $\delta^{13}C$ in wheat seedlings. These results demonstrate that HT led to decreases in g_s , which further caused enrichment of ^{13}C and ^{18}O in poplar leaves.

Phytohormones play key roles in perceiving elevated temperatures and mediating plant physiological acclimation to HT (Bitá and Gerats 2013). In this study, the increased levels of ABA correspond well to the elevated transcript levels of *ZEP* and *NCED3* involved in ABA biosynthesis in roots and leaves of poplars at HT, which is consistent with the findings in herbaceous plants (Talanova et al. 2003, Larkindale and Huang 2004, Finch-Savage et al. 2007, Asensi-Fabado et al. 2013). The accumulation of ABA in poplar roots and leaves at HT can initiate stress signaling and lead to physiological acclimation. Particularly, we found that foliar ABA induction at HT triggered stomatal closure, thereby inhibiting CO_2 assimilation and altering the levels of soluble sugars and sugar alcohols in poplars. Changes in

levels of other phytohormones such as IAA, GAs and SA have also been reported in herbaceous plants in other studies (Asensi-Fabado et al. 2013) and in poplars of this study, but the signaling network of these hormones remains less explored in HTs-exposed plants. Overall, HT-triggered ABA increases in poplar leaves resulting in stomatal closure, subsequently inhibiting CO_2 assimilation and changes in carbohydrate levels suggest that ABA plays a central role in mediating the physiological acclimation of plants to HTs.

Although reduced A and foliar starch levels were found in poplars exposed to HT, HT-induced levels of sucrose and glucose in poplar leaves were detected, which is probably due to the hydrolysis of starch, further contributing to osmotic adjustment in poplar leaves at HT. These results are in line with findings of previous studies where HT was found to reduce starch storage in the xylem of *Picea mariana* (Deslauriers et al. 2014) and to stimulate the concentrations of some carbohydrates in the needles of *Picea abies* (Riikonen et al. 2012). STP1 is essential for the uptake of external hexoses in roots (Sherson et al. 2000) and STP14 is a galactose-specific transporter in *Arabidopsis* (Poschet

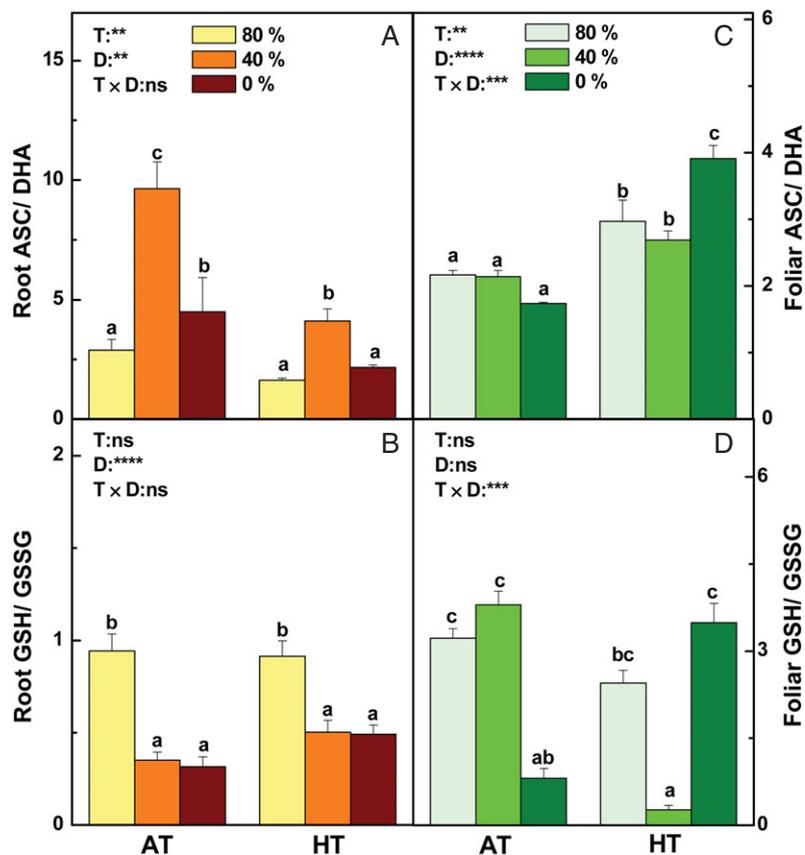


Fig. 6. Ratios of ASC relative to DHA, and of GSH relative to oxidized GSH (GSSG) in the roots (A and B) and leaves (C and D) of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. Bars indicate means \pm SE (n = 6). Different letters on the bars indicate significant differences. *P*-values obtained from the ANOVAS based on temperature (T), drought (D) and their interactions (T \times D) are also indicated. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001; ns: not significant.

et al. 2010). In agreement with the decreases in glucose concentrations in poplar roots at HT, the HT-induced reductions in the mRNA levels of *STP1* were found in poplar roots. In the same line, the HT-induced mRNA levels of *STP14* are consistent with the increased galactose concentrations in poplar roots at HT. These results demonstrate that HT inhibited photosynthesis, altered carbohydrate metabolism and triggered differential expression of mRNA levels of transporter genes involved in translocation of sugars and sugar alcohols in poplars.

In agreement with the HT-induced heat stress, we detected changes in the transcript levels of several key genes involved in heat stress responses in poplars at HT. To prevent HT-induced protein denaturation, plants can produce HSPs, which are molecular chaperones related to thermotolerance (Perez et al. 2009). BOB1, HSP18 and HSP21 are key HSPs in herbaceous plants during the response to elevated temperatures (Sarkar et al. 2009). Thus, the increased transcript levels of *HSP18* and *HSP21* in the roots and leaves of poplars at HT suggest

that these HSPs can help to prevent protein denaturation in poplars in response to HT.

Taken together, our results suggest that HT had detrimental effects on energy metabolism and phytohormonal signaling, and triggered the transcriptional regulation of key genes involved in heat stress response, ABA biosynthesis and sugar transport in poplar roots and leaves during the acclimation.

HT exacerbated the effects of mild and/or severe drought stress in poplar roots and leaves

HT is often accompanied by drought stress, and the combination of these stresses can have synergistic effects on physiological and molecular processes in plants (Vile et al. 2012, Hu et al. 2013). In this study, severe drought caused greater reductions in total C in roots and enhanced the foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in poplars at HT compared with AT, demonstrating that HT aggravated the effects of severe drought on the metabolism of C and water. In agreements, severe drought caused

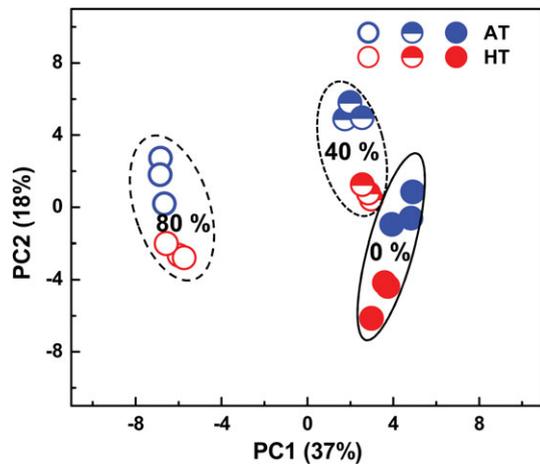


Fig. 7. PCA plot of the physiological parameters determined in the roots and leaves of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. The PCA loadings are presented in Table S5.

greater reductions in the LWP, the RWCs of the roots and leaves, A and R in poplars at HT compared with AT. The synergistic negative effects of HT and drought on the water status and A have also been demonstrated in other woody plants (Duan et al. 2013, Deslauriers et al. 2014). Furthermore, HT can affect the drought-induced changes in phytohormone levels in plants. For instance, the greater increases in the ABA levels in the roots and leaves under 40% FC at HT compared with those at AT suggest that HT can exacerbate the effects of mild drought on ABA induction in poplars. Accordingly, higher mRNA levels of *ZEP* and *NCED3* were detected in the mild drought-exposed roots of poplars at HT compared with AT, which agreed with the greater stimulation of mild-drought-induced ABA levels at HT compared with AT. Similarly, HT caused greater accumulation of severe drought-induced foliar glucose in poplars, thereby making a greater contribution to osmotic adjustment in foliar cells. Moreover, the greater induction of the mRNA levels of genes encoding sugar transporters, such as *SUC2*, *STP14* and *INT1*, was found in the mild and severe drought-treated roots of poplars at HT compared with AT. Recently, it was reported that drought increased the mRNA levels of genes encoding hexose and inositol transporters in seedlings of *Pinus pinaster* and *Pinus pinea* (Perdiguero et al. 2013).

In addition, HT can modify the drought-induced changes in the balance between ROS and antioxidants. A water deficit in the soil can increase the ROS concentrations in poplars (Cao et al. 2014). Thus, the higher level of H_2O_2 in poplar roots exposed to 40% FC at HT compared with that at AT demonstrated that HT could further enhance the mild drought-induced ROS levels in

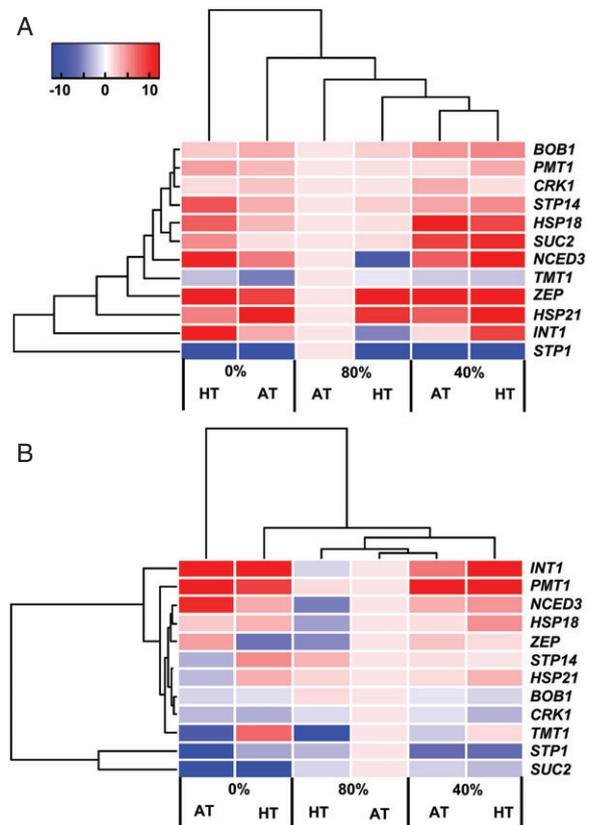


Fig. 8. Fold changes in the transcript levels of genes in the roots (A) and leaves (B) of poplars treated with either AT or HT in combination with one of three watering regimes [80 or 40% FCs, and no watering (denoted as 0% FC)]. The transcript levels of all genes in the roots or leaves treated with AT and 80% FC were defined as 1, and the relative fold changes in the transcript levels of genes were then calculated under other conditions (a negative inverse transform of the fold change was conducted when the gene was downregulated.).

poplars. Non-enzymatic antioxidants such as ASC and GSH play crucial roles in scavenging overproduced ROS in plants. The ASC and GSH concentrations decrease, whereas their oxidative forms (DHA and GSSG) increase during ROS scavenging in plants exposed to various stresses (Haberer et al. 2008). In our study, lower ratios of ASC relative to DHA were detected in the roots of mild and severe drought-treated poplars at HT compared with AT, which was probably attributable to the enhanced conversion of ASC to DHA during accelerated ROS scavenging under combined HT and drought.

At the molecular level, HT also affected the drought-induced regulation of the mRNA levels of key genes involved in heat stress response in poplars. HT caused greater increases in mild drought-induced transcript levels of three genes encoding *BOB1*, *HSP18* and *HSP21*, in poplar roots compared with the control (80% FC) condition at AT. In line with our results, the

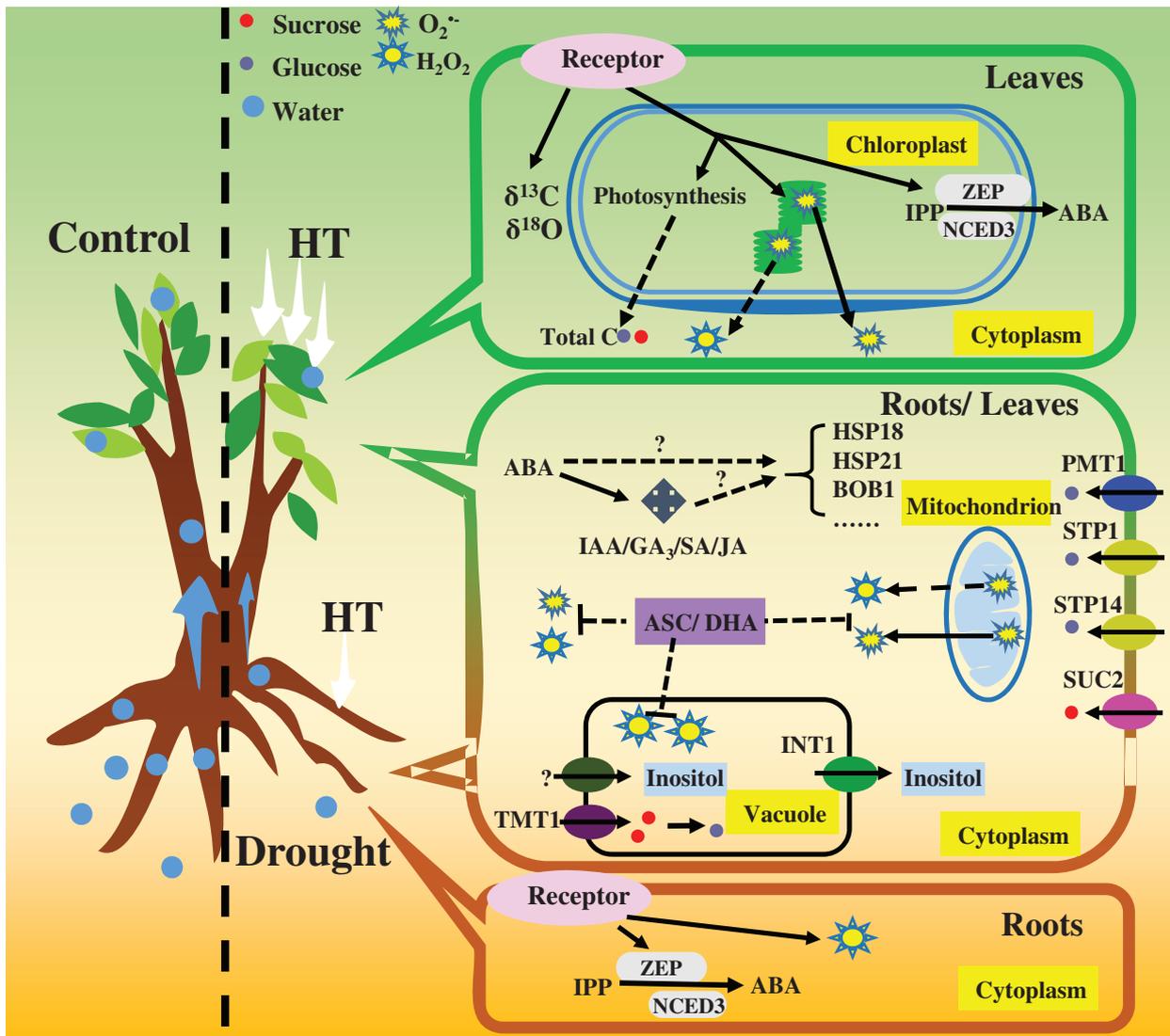


Fig. 9. Schematic model at the cellular level of the physiological and transcriptional responses of poplar to HT and/or drought.

upregulated transcription of genes encoding HSPs has been reported in plants subjected to multiple stresses compared with a single stress (Sewelam et al. 2014).

Overall, our results suggest that HT can exacerbate drought-induced reductions in water contents, total C in roots, A and R, and increases in the foliar $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, ABA and H_2O_2 and transcript levels of key genes involved in heat stress response, ABA biosynthesis and sugar transport in poplars.

As summarized in Fig. 9, HT increased the total C, $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ concentrations in poplar leaves. The positive linear correlation between the foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ showed that the variations in foliar $\delta^{13}\text{C}$ were due mainly to fluctuations in g_s with changes in the temperature and water availability. HT triggered heat stress signaling by

increasing the levels of ABA and IAA in the roots and leaves, GA_3 in the leaves and SA in the roots, as well as decreasing the concentrations of GA_3 in the roots, SA in the leaves and JA in the roots and leaves. After perceiving HT, the poplars initiated osmotic adjustment by increasing the concentrations of foliar sucrose and root galactose. To scavenge HT-induced ROS, the poplars reduced the ASC levels, thereby leading to decreases in the ratio of ASC relative to DHA in the roots. In agreement with the HT-induced heat stress and changes in the levels of ABA and carbohydrates, increased transcript levels of *HSP18*, *HSP21* and *NCED3* in the roots and leaves, *STP14* in the roots and *INT1* in the roots and leaves, but decreased mRNA levels of *STP1* and *INT1* in the roots, were detected of poplars at HT. Severe drought

induced greater reductions in total C in the roots and stimulation of the foliar $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in poplars at HT compared with AT. Similarly, HT caused greater accumulations of severe drought-induced foliar glucose in poplars. Correspondingly, greater increases in the mRNA levels of genes encoding HSPs (BOB1, HSP18 and HSP21), ABA biosynthetic enzymes (ZEP and NCED3) and sugar transporters (STP14 and INT1), were found in mild drought-treated poplar roots at HT compared with AT. These results suggest that HT has detrimental effects on physiological processes and it induces the transcriptional regulation of key genes involved in heat stress response, ABA biosynthesis, and sugar transport, and HT can exacerbate mild and/or severe drought-induced physiological changes and the transcriptional regulation of key genes involved in the physiological acclimation of poplar roots and leaves. It is anticipated that HT and drought can impose synergistic stress effects on woody plants under future climatic scenarios.

Author contributions

Z.-B. L. conceived and designed the experiments. J. J., S. L., X. C., H. L. and W. S. performed the experiments. J. J., S. L., X. C., H. L., W. S., A. P., T.-X. L., C.-H. P. and Z.-B. L. analyzed the data. J. J., S. L. 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:

Table S1. Primers used for qRT-PCR.

Table S2. Predawn LWP and RWC in roots and leaves.

Table S3. Photosynthesis and respiration.

Table S4. Concentrations of sugars, sugar alcohols, and starch.

Table S5. The loadings of PCA.

Figure S1. Sequence alignments of genes.

Figure S2. Stomatal conductance during treatments.

Figure S3. Concentrations of ASC, DHA, GSH and GSSG.

Figure S4. Activities of SOD, CAT, APX and GR.