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Dynamic changes in ABA content in water-stressed *Populus nigra*: effects on carbon fixation and soluble carbohydrates

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• **Background and Aims** Hydraulic and chemical signals operate in tandem to regulate systemic plant responses to drought. Transport of abscisic acid (ABA) through the xylem and phloem from the root to shoot has been suggested to serve as the main signal of water deficit. There is evidence that ABA and its ABA-glycosyl-ester (ABA-GE) are also formed in leaves and stems through the chloroplastic 2-C-methylerythritol-5-phosphate (MEP) pathway. This study aimed to evaluate how hormonal and hydraulic signals contribute to optimize stomatal (g_s), mesophyll (g_m) and leaf hydraulic (K_{leaf}) conductance under well-watered and water-stressed conditions in *Populus nigra* (black poplar) plants. In addition, we assessed possible relationships between ABA and soluble carbohydrates within the leaf and stem.
• **Methods** Plants were subjected to three water treatments: well-watered (WW), moderate stress (WS1) and severe stress (WS2). This experimental set-up enabled a time-course analysis of the response to water deficit at the physiological [leaf gas exchange, plant water relations, (K_{leaf})], biochemical (ABA and its metabolite/catabolite quantification in xylem sap, leaves, wood, bark and roots) and molecular (gene expression of ABA biosynthesis) levels.
• **Key Results** Our results showed strong coordination between g_s, g_m and K_{leaf} under water stress, which reduced transpiration and increased intrinsic water use efficiency (WUE_{int}). Analysis of gene expression of 9-*cis*-epoxycarotenoid dioxygenase (*NCED*) and ABA content in different tissues showed a general up-regulation of the biosynthesis of this hormone and its finely-tuned catabolism in response to water stress. Significant linear relationships were found between soluble carbohydrates and ABA contents in both leaves and stems, suggesting a putative function for this hormone in carbohydrate mobilization under severe water stress.
• **Conclusions** This study demonstrates the tight regulation of the photosynthetic machinery by levels of ABA in different plants organs on a daily basis in both well-watered and water stress conditions to optimize WUE_{int} and coordinate whole plant acclimation responses to drought.

Keywords: Abscisic acid (ABA), ABA-GE, bioenergy crop, gene expression, intrinsic water-use efficiency (WUE_{int}), water deficit, leaf gas exchange, leaf hydraulic conductance, 9-*cis*-epoxycarotenoid dioxygenase (*NCED*), *Populus nigra*, soluble carbohydrates.

INTRODUCTION

The ideal biomass crop is not only fast growing but also drought tolerant (McKendry, 2002). These attributes may be incompatible, as the rapid growth of the most productive biomass crop species/varieties is sustained by high rates of water use (Blum, 2005). The plant hormone abscisic acid (ABA) plays a central role in signalling reduced water availability and coordinating plant responses to drought stress (Wilkinson and Davies, 2002). The synthesis of ABA was previously considered to occur largely in the roots prior to transport in the xylem sap to the leaves as a signal of soil drying (Davies and Zhang, 1991). However, recent studies have suggested that the 'whole plant' dynamics of ABA are more complex, with the leaves and stems playing a major role in ABA synthesis and catabolism (Manzi *et al.*, 2015; Mitchell *et al.*, 2016; Marino *et al.*, 2017; Zhang *et al.*, 2018). Analysis of the temporal and spatial dynamics of ABA content in different plant tissues under drought is critical to understanding the function of this hormone in

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coordinating physiological responses to reduced water availability. Furthermore, elucidation of the genetic responses that underpin ABA dynamics and the biochemical/physiological responses elicited by ABA are critical to enhance drought tolerance in fast growing biomass species.

As the availability of water within soil declines, plants decrease the aperture of the stomatal pores to reduce stomatal conductance (g_s), thus minimizing water loss via transpiration. As g_s declines, diffusion of CO_2 into the internal leaf air-space is impaired, resulting in reduced net photosynthesis (A_n) (Flexas *et al.*, 2002; Chaves *et al.*, 2009; Centritto *et al.*, 2011; Lauteri *et al.*, 2014). The stress associated with drought is often the result of an excess of energy that triggers formation of oxidative species as photochemistry declines (Pinheiro and Chaves, 2011). This effect is particularly clear in fast growing biomass species that have high radiation use efficiencies under optimal growth conditions (Silim *et al.*, 2009). Plant responses to drought are regulated by a complex interaction of hydraulic and chemical signals (Comstock, 2002; Rodrigues *et al.*, 2008; Tombesi *et al.*, 2015). Early studies used split-root systems to demonstrate that ABA acts as a chemical signal of soil drying transported from the root to the shoot and acting independently of hydraulic signals in inducing stomatal closure (Zhang *et al.*, 1987; Davies and Zhang, 1991). However, it has been recently shown that g_s values and foliar ABA content in black poplar (*Populus nigra*) grown in a split-root system did not change when half of the root-zone dried (Marino *et al.*, 2017). Similar patterns of g_s and ABA content were observed in leaves of olive trees (*Olea europaea*) subjected to partial root-zone drying (Dbara *et al.*, 2016) in an agronomic extrapolation of the split-root studies. In addition, analysis of *Vitis vinifera* drought responses suggest that initial stomatal closure is induced by hydraulic signals, followed by an increase in foliar levels of ABA that also maintained stomatal closure after leaf water potentials (Ψ_L) had returned to pre-stress levels (Correia *et al.*, 1995; Tombesi *et al.*, 2015). This is consistent with observations in other woody plants (*Acer pseudoplatanus* and *Fagus sylvatica*) that ABA does not act as an immediate root-to-shoot signal of soil drying to induce stomatal closure, but it is preceded by a hydraulic signal (Christmann *et al.*, 2007). Furthermore, changes in the pH of xylem sap have also been proposed to signal soil drying by altering the partitioning of ABA between the leaf apoplast and symplast (Wilkinson and Davies, 1997). The content of free-ABA within a leaf is not only inversely related to g_s, but also modifies stomatal behaviour to environmental stimuli (Haworth *et al.*, 2018).

Mesophyll conductance to CO_2 (g_m) is also related to the content of free-ABA in the leaf. The ABA-induced alterations in g_m are more rapid than those observed in g_s (Sorrentino *et al.*, 2016) and are possibly associated with a reduced activity of aquaporins involved in the transport of CO_2 across the mesophyll (Jang *et al.*, 2004; Flexas *et al.*, 2006; Perez-Martin *et al.*, 2014). ABA has also been shown to decrease leaf hydraulic conductance (K_{leaf}) through reduced biochemical activity of aquaporins involved in the regulation of the permeability of transport tissues to the movement of water (Shatil-Cohen *et al.*, 2011; Pantin *et al.*, 2013).

In addition to the regulation of g_s, g_m and K_{leaf}, ABA could play a role in sugar metabolism, affecting carbohydrate partitioning during drought (Rook *et al.*, 2001). For example, in drought-stressed plants, ABA induces an increase in the activities of β-amylase and vacuolar invertase, leading to higher starch degradation and the release of hexoses in the cytosol (Pelleschi *et al.*, 1999; Kempa *et al.*, 2008).

The levels of ABA involved in all of these processes are regulated by multiple mechanisms: *in situ* ABA biosynthesis by NCED (9-*cis*-epoxycarotenoid dioxygenase) (Pierce and Raschke, 1981; Iuchi *et al.*, 2001; Mitchell *et al.*, 2016; Zhang *et al.*, 2018), root-to-shoot transport (e.g. Zhang *et al.*, 1987), conversion of biologically inactive glucose-conjugated ABA (ABA-GE) (Dietz *et al.*, 2000; Lee *et al.*, 2006; Seiler *et al.*, 2011), and catabolism of free-ABA to phaseic acid (PA) and dihydrophaseic acid (DPA) (Saito *et al.*, 2004; Nambara and Marion-Poll, 2005). Interestingly, *de novo* biosynthesis of ABA may serve as the main mechanism involved in the regulation of the content of this hormone following a reduction in turgor during an air vapour pressure deficit (VPD) transition (Bauerle *et al.*, 2004; Xie *et al.*, 2006; McAdam *et al.*, 2016).

Analysis of the daily dynamic patterns of ABA content in different plant tissues as soil dries may illustrate the complex function of this plant hormone in eliciting and coordinating a wide variety of physiological responses to alleviate the deleterious impact of drought. We subjected black poplar (*Populus nigra*) plants to moderate and severe water stress to: (1) investigate the role of hormonal ABA and hydraulic signals of reduced water availability on plant physiology, in particular on the regulation of g_s and g_m; (2) determine the concomitant diurnal variations in ABA, its breakdown products and regulation of the *de novo* ABA-biosynthetic gene within different plant organs, to understand how these compounds are regulated during water stress progression; and (3) test the hypothesis of a possible relationship between ABA and soluble carbohydrates within the stem.

MATERIAL AND METHODS

Plant material and drought-stress treatment

One-year-old cuttings of *Populus nigra* L. were grown outside in Sesto Fiorentino (Italy, 43°49′N, 11°37′E) in 20-litre pots filled with sandy soil (sand/peat, 60: 40, v/v). The plants were maintained at pot water capacity and fertilized with Hoagland solution once a week to supply mineral nutrients at free access rates, until the onset of the water stress treatment (end of August). The experiment was conducted under minimum/maximum air temperatures of 16.4 ± 2.4/30.8 ± 3.2 °C (mean ± s.d.), midday irradiance of 803 ± 20 W m^{-2} and relative humidity of 27 ± 3.6 %. During the experimental period sunrise occurred between 0625 and 0636 h, and sunset between 2011 and 1954 h. Air temperature, solar irradiance and relative humidity were recorded by the weather station located at the experimental site of the Laboratory of Monitoring and Environmental Modelling for Sustainable Development (http://www.lamma.rete.toscana.it). Forty plants were equally spaced and randomly assigned to three experimental categories: well-watered (WW, 20 plants), moderately water stressed (WS1, ten plants) and severely water stressed (WS2, ten plants). The different water treatments were applied to the plants on the basis

of preliminary leaf gas exchange measurements which allowed us to exclude significant differences in gas exchange parameters among plants ($P > 0.05$, data not shown). Water deficit was then imposed by withholding water (WS1 and WS2 plants), whereas well-watered plants (WW, control) were irrigated to pot capacity each day during the experimental period. Moderate (WS1) and severe (WS2) water-stress conditions were reached when g_s values at 0900 h were respectively ~32 and ~ 6 % of the values observed in WW plants. These declines in g_s were achieved after withholding water for 3–4 and 7–8 d, respectively. To assess their daily trend, physiological measurements and the sampling of plant material for biochemical analyses were conducted on four replicates at three times of the day: 0900, 1300 and 1800 h (solar time).

Measurements of gas exchange and plant water status

Leaf gas exchange was measured on fully developed and intact leaves using a LI-6400 portable photosynthesis system fitted with a 6400-40 2-cm^2 leaf cuvette (Li-Cor, Lincoln, NE, USA). Measurements were performed at a photosynthetic photon flux density (PPFD) of 1000 µmol m^{-2} s^{-1}at 0900 h, 1800 µmol m^{-2} s^{-1} at 1300 h and 1000 µmol m^{-2} s^{-1} at 1800 h, thus simulating the recorded environmental PPFD. Photosynthesis (A_n), stomatal conductance (g_s) and the intercellular CO_2 concentration (C_i) were calculated using the LI-6400 software; instantaneous water use efficiency (WUE_{ins}) was then calculated as the ratio of net CO_2 assimilation to stomatal conductance (A_n/g_s). Mesophyll conductance to CO_2 (g_m) was calculated using the variable J method (Harley *et al.*, 1992; Loreto *et al.*, 1992) as follows:

$$g_m = \frac{An}{Ci - \frac{\Gamma*[J_F + 8*(An + R_d)]}{J_F - 4*(An + R_d)}}$$

where Γ*, representing the CO_2 compensation point to photorespiration, was measured on leaves of intact plants by determining A_n/C_i curves at four levels of photosynthetically active radiation and constant temperature (Brooks and Farquhar, 1985), while daytime respiration (R_d) was calculated using the Kok method at PPFD steps of 300, 200, 150, 100, 80, 60, 30 and 0 µmol m^{-2} s^{-1} (Kok, 1948). Electron transport rate (J_F) was calculated from chlorophyll fluorescence:

$$J_F = \Phi_{PSII} * PPFD * \alpha * \beta$$

where Φ_{PSII} is the actual photochemical efficiency of photosystem II (Genty *et al.*, 1989), the partitioning factor (β) between photosystems I and II was considered to be 0.5 and leaf absorbance (α) was 0.85.

Leaf hydraulic conductance (K_{leaf}, mmol s^{-1} MPa^{-1} kg^{-1}) was measured in the laboratory (at constant temperature of 25 °C) on detached leaves according to the method described by Brodribb and Holbrook (2003):

$$K_{leaf} = C \times \ln \frac{\left(\frac{\Psi_0}{\Psi_f}\right)}{t}$$

where C is the capacitance, Ψ_o is the leaf water potential prior to partial rehydration, Ψ_f is the leaf water potential after partial rehydration and t is the duration of rehydration. Values of C were calculated from pressure–volume curves (Scholander *et al.*, 1965; Tyree and Hammel, 1972) using the procedure outlined by Johnson *et al.* (2009) and normalized by leaf dry weight (Nardini *et al.*, 2012). Leaf water potential (Ψ_L) was measured on fully developed leaves using a pressure chamber (PMS Instrument, Albany, OR, USA). To quantify the effect of the daily Ψ_L sensed by plants, a cumulative integrated Ψ_L was calculated for each water treatment as reported by Basile *et al.* (2003).

Sample collection for biochemical analyses and pH measurements

Stem xylem sap was collected according to the method described by Secchi and Zwieniecki (2012). Briefly, the stems were initially cut in the air and all leaves were removed. Then, after removing the bark from the first 5-cm basal end of the stem, this was connected to a small vacuum chamber. A vacuum pressure of 0.03 MPa allowed the water to be sucked from the stem during progressive cutting from the top of the base. The exuded xylem sap was collected in an Eppendorf tube sitting in ice and then stored at −80 °C prior to measurement of pH and ABA metabolites. After sap collection, bark (including cambium) and wood tissues were carefully separated using a razor blade, immediately frozen in liquid nitrogen to stop enzymatic activity and then lyophilized. Xylem sap pH was measured with a pH-meter (Mettler-Toledo, Switzerland) equipped with a micro electrode (InLab Micro electrode, Mettler Toledo).

Measurements of free-ABA and its metabolites/catabolites

Abscisic acid (free-ABA), its conjugated form (ABA glucoside ester, ABA-GE), phaseic acid (PA) and dihydrophaseic acid (DPA) were analysed in bark, wood and xylem sap. Sixty milligrams of lyophilized tissue was ground in liquid nitrogen, supplemented with 40 ng of d6-ABA, 40 ng of d5-ABA-GE, 40 ng of d3-PA and 40 ng of d3-DPA (National Research Council of Canada, Ottawa, Canada) and extracted three times with 1 mL of CH_3OH/H_2O (50: 50; v/v, pH 2.5) at 4 °C for 30 min. The extracts were then defatted with 3 × 3 mL of n-hexane and purified through Sep Pak C18-cartridges (Waters, Milford, MA, USA) utilizing ethylacetate as solvent. The eluates were evaporated to dryness, rinsed with 500 µL CH_3OH/H_2O (50: 50 v/v, pH 2.5) and injected (3 µL) into an LC–DAD-MS/MS system, composed of a Shimadzu Nexera HPLC and a Shimadzu LCMS-8030 quadrupole mass spectrometer, functioning in the negative electrospray ionization (ESI) mode (Kyoto, Japan). Mobile phases consisted of H_2O (added with 0.1 % HCOOH, solvent A) and CH_3CN/CH_3OH (1: 1, v/v, added with 0.1 % HCOOH, solvent B). The analysis was performed using a 18-min gradient run (passing from 95 % solvent A to 100 % solvent B) on a Poroshell 120 SB C18 column (2.7 µm, 100 × 3 mm, Agilent Technologies) at a flow rate of 0.3 mL min^{-1}. Quantification was conducted in multiple reaction mode (López-Carbonell *et al.*, 2009). For analysis of xylem sap ABA, samples of 50 µL were used omitting the Sep-Pak C18 purification step. The recovery rates for deuterated

standards are 23 % for d6-ABA and 15 % for d5-ABA-GE, d3-PA and d3-DPA. The ABA delivery flux to the leaves was calculated by multiplying the transpiration rate determined by the Licor device with the concentration of ABA found in xylem sap, as reported by Jackson *et al.* (1995).

Measurements of non-structural carbohydrates

Samples for sugar analysis in leaves were prepared as previously described (Silvente *et al.*, 2012). Briefly, leaves (25 mg of powdered lyophilized tissues) were mixed with 1 mL of CH_3CN/H_2O mixture (1: 1 v/v). After centrifugation at 6700 *g* for 4 min and filtration, the supernatant was dried under N_2 flux. The dry residue was dissolved in 0.7 mL of 400 mM D_2O phosphate buffer (pD = 6.5) containing 1.0 mM of 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSPA). A Bruker AVANCE 600 spectrometer operating at a proton frequency of 600.13 MHz was used to measure the nuclear magnetic resonance (NMR) spectra of the extracts at 300k. The ^{1}H spectra were obtained by co-adding 256 transients with a recycle delay of 7 s, a 45° flip angle pulse of 8.0 µs and 32k data points. A presaturation during the last 2 s of relaxation delay with a long single soft pulse was used to suppress the residual HDO signal. A small line broadening factor (0.4 Hz) was applied before Fourier transformation followed by manual phase and baseline corrections. The spectra were referenced to the signals of TSPA methyl group at δ = 0.00 p.p.m. Bruker TOPSPIN software version 1.3 was utilized for processing of spectra. The integrals of selected signals belonging to sucrose (4.23 p.p.m.), fructose (4.12 p.p.m.), α- and β-glucose (3.42 p.p.m.), and *myo*-inositol (3.29 p.p.m.) were measured and normalized with respect to the TSPA integral (at 0.00 p.p.m.) set to 100. The sugar content (in mg g^{-1} dry weight) was calculated using TSPA as an internal standard taking into account the proportionality between the integrals and molar concentrations.

Sugars were extracted from 30 mg of lyophilized bark and 30 mg of lyophilized wood. Samples were ground with liquid nitrogen and extracted twice with ethanol/water (80: 20). The determination of sucrose, fructose and glucose was performed by means of enzymatic test analysis (Boehringer Mannheim / R-Biopharm; Roche, Darmstadt, Germany) as reported by Steegmans *et al.* (2004). The analysis was full automatized on a ChemWell 2910 Chemistry Analyzer (Awareness Technologies, Palm City, FL, USA).

Gene expression analysis

A total of 144 leaf, root, xylem and bark tissues (50–100 mg each) were homogenized using a bead beater for two 30-s runs, flash-frozen in liquid nitrogen and vortexed in 0.8 mL cetyltrimethylammonium bromide (CTAB) buffer (Vincelli and Amsden, 2013). Total RNA was then extracted using the Maxwell 16 LEV simply RNA Tissue Kit (Promega) following the manufacturer's instructions and treated with DNAase to eliminate contaminating genomic DNA. RNA quantity and quality were determined with the Agilent Bioanalyzer. A total of 2 µg of RNA was used for cDNA synthesis (Invitrogen) in a 30-µL system (65 °C, 5 min; 4 °C, 1 min; 40 °C, 2 min; 40 °C, 90 min; 70 °C, 15 min) (Johnson *et al.*, 2012). The resulting cDNA was diluted to 20 ng μL^{-1} and used for quantitative real-time PCR (qPCR) using a Bio-Rad iQ5 real-time PCR detection system and iQ SYBR Green Supermix under the following conditions: 95 °C, 3 min; then 40 cycles of 10 s at 95 °C and 1 min at 60 °C. qPCR was performed in 12.5 µL of reaction mixture, composed of 2 µL of a given cDNA (20 ng μL^{-1}), 6.25 µL 2× iQ SYBR Green Supermix, 0.15 µL each primer (10 µM) and 5.95 µL RNase-free water. BLAST (Altschul *et al.*, 1990) searches for sequence similarities to identify poplar homologues using *A. thaliana NCED3* and 18 sRNA as queries against the *Populus trichocarpa* v3.0 curated in the Phytozome (v9.1) database were performed. Gene-specific primers were then designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) to work under the same PCR conditions. The following primer sequences were used (forward, reverse): *NCED3*: 5′-ATCCGGGGAGAATCTGAAGT-3′, 5′-TGTTCGTGTACTGCCCTCTG-3′; 18S rRNA: 5′-TCAACTTTCGATGGTAGGATAGTG-3′, 5′-CCGTGTCAGGATTGGGTAATTT-3′. A melting curve was produced at the end of every reaction cycle through a temperature ramp from 66 to 95 °C with increments of 0.5 °C s^{-1} and with continuous fluorescence collection to ensure that only single products were formed. PCR products were also separated on a 3 % (w/v) agarose gel to ensure that only a single band was present. Negative controls included the substitution of cDNA with nuclease-free water. All qPCRs were prepared in three biological replicates and technical triplicates/duplicates of each cDNA. The cycle threshold (C_T) values indicating in real-time PCR the cycle number at which fluorescence emission reached a threshold above the baseline emission were determined and the mean C_T values were considered. For data normalization, the data were normalized to the most stable reference gene 18 sRNA (Xu *et al.*, 2011). Data were analysed using iQ5 Optical System Software v2.1 (Bio-Rad). Fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (Pfaffl, 2001).

Statistical analysis

Data were analysed using a repeated-measures ANOVA, with water treatment considered to be the between-subjects effect and time to represent the within-subjects effect (SPSS v.20; IBM, Chicago, IL, USA) for the non-destructive parameters studied (gas exchange). A one-way ANOVA was used to evaluate the effects of water stress on all other parameters. Significant differences among means were estimated at the 5 % ($P < 0.05$) level, using Tukey's test. Linear regression analysis using Sigmaplot (SPSS, Inc., Chicago, IL, USA) was used to assess possible relationships between ABA and g_s/g_m and between ABA and soluble carbohydrates.

RESULTS

Daily trends in gas exchange and water relationships

Water stress induced significant reductions in A_n, g_s, g_m and K_{leaf}, with a strong decrease of all parameters by midday (Fig. 1).

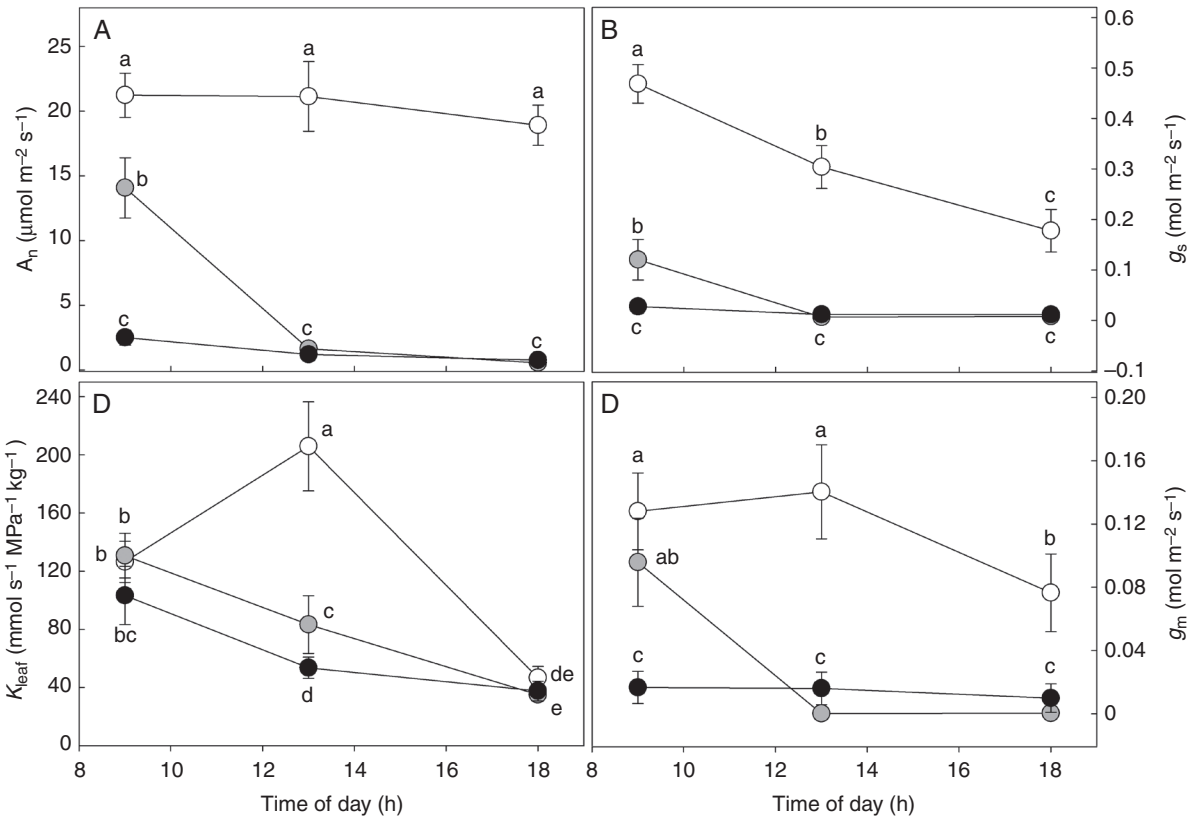

FIG. 1. Daily trend of net photosynthesis (A_n, A), leaf hydraulic conductance (K_{leaf}, B), stomatal conductance (g_s, C) and mesophyll conductance (g_m, D) in *Populus nigra* leaves under well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. Data (means ± s.d., n = 4) were subjected to a repeated-measures ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

In WW plants, A_n consistently remained above 20 µmol m^{-2} s^{-1} during the day, while g_s declined from the morning until 1800 h, g_m remained stable from 00900 to 1300 h before declining by 46.4 % by 1800 h. In WS1 plants, the highest values of A_n, g_s and g_m were recorded at 0900 h, but all parameters dropped by midday, approaching zero in the late afternoon. In WS2 plants, despite g_s and g_m values being close to zero through the day, some photosynthetic activity (A_n = 2.5 µmol m^{-2} s^{-1}) was still recorded at 0900 h. In WW plants, K_{leaf} increased from early morning to midday (+63 %) and declined late in the afternoon. The daily trend of K_{leaf} in WS1 and WS2 plants showed the highest values at 0900 h (157.6 and 83.3 mmol s^{-1} MPa^{-1} kg^{-1}, respectively) prior to significant reductions during the day (Fig. 1D). The leaf to air VPD experienced by the plants was lowest in the morning at 00900h (1.6 ± 0.24 kPa) before rising at midday (3.06 ± 0.62 kPa) until early evening (3.08 ± 0.45 kPa). No significant differences were observed in VPD values among WW, WS1 and WS2 plants. During measurements, the air temperature was 18 ± 3.1 °C at 0900 h, 27 ± 3.3 °C at midday and 21 ± 2.4 °C at 1800 h.

Leaf water potential (Ψ_L) showed a typical trend in all treatments, with the highest values at pre-dawn and towards sunset, and significantly lower values during the day as VPD increased (Fig. 2). As expected, pre-dawn Ψ_L was less negative in WW than in WS1 and WS2 plants. During the middle of the day, Ψ_L also decreased in WW leaves, at levels similar to those of WS1 leaves. There were no significant differences in Ψ_L values of WS1 and WS2 plants measured at pre-dawn and sunset. By contrast, Ψ_L was significantly lower in WS2 than in WS1 plants in the morning and at midday. Significantly lower values of integrated daily Ψ_L were detected in WW plants compared to WS1 and WS2 plants (Fig. 2).

Daily trend of ABA content

Water stress induced an order of magnitude increase in the concentration of ABA in xylem sap (ABA_{sap}) (+97 % in both WS1 and WS2) and roots (ABA_{roots}) (+97 % in WS1 and +94 % in WS2) (Fig. 3A, B). The daily patterns of ABA_{sap}, ABA_{roots} and ABA fluxes varied among irrigation treatments (Fig. 3C–H). In WW plants, ABA_{sap} and ABA_{roots} were an order of magnitude lower than those observed in the WS1 and WS2 treatments (Fig. 3C). ABA increased to a greater extent under moderate (WS1) than severe (WS2) water stress (Fig. 3E, G). In addition, in WW plants, ABA_{sap} was constant throughout the day, while ABA_{roots} rose slightly from 1300 to 1800 h (Fig. 3C). In contrast, in WS1 plants, ABA_{sap} was low in the morning and increased significantly during the day, while ABA_{roots} showed the opposite trend, declining during the day from a very high morning concentration (Fig. 3E). In WS2 plants, ABA_{roots} again declined significantly during the day, while ABA_{sap} did not change, showing values similar to those in WS1 plants (Fig. 3A). Plants grown under all water treatments showed similar

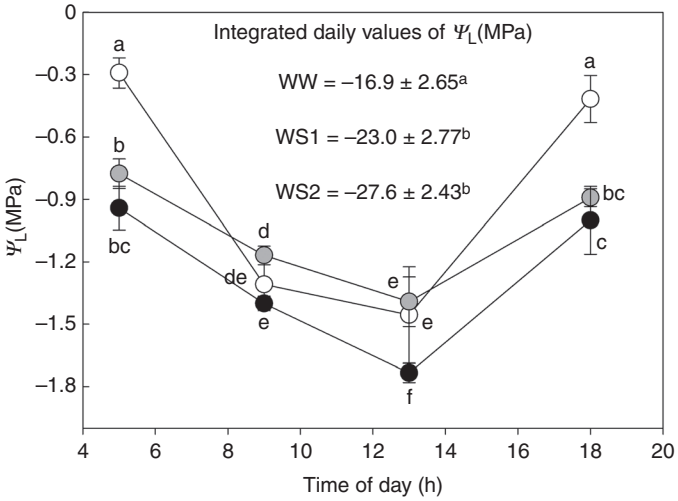

FIG. 2. Daily trend of leaf water potential (Ψ_L) in *Populus nigra* plants subject to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. In the box the integrated daily values of Ψ_L represent the cumulative difference in water potential from 0 MPa integrated over a 24-h period. Data (means ± s.d., n = 4) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

patterns in the flow of ABA delivered to the leaf by xylem sap, with higher values in the morning and subsequent reductions during the afternoon. The overall flux of ABA into the leaves of WS1 plants was ~380 and 200 % greater than in WW and WS2 plants, respectively (Fig. 3D–F).

To investigate the dynamics of ABA content in the plants, ABA, ABA-GE, ABA catabolites (PA and DPA) and expression of the *NCED3* gene were determined in different tissues (leaves, wood and bark). Water stress induced a marked boost in leaf ABA content (ABA_{leaf}) (Fig. 4A and Supplementary Data Table S1), reaching values of ~14 nmol g^{-1} DW in both WS1 and WS2 plants between 0900 h and midday. Moreover, a further increase of ABA_{leaf} was observed in the afternoon in WS1 plants (+50 %), while ABA_{leaf} did not change through the day in WS2 plants (Fig. 4A). Water stress also promoted higher metabolism and catabolism of leaf ABA, particularly in WS2 plants where the content of ABA-GE ($ABA\text{-}GE_{leaf}$) and ABA catabolites ($PA+DPA_{leaf}$) were almost four-fold greater than those observed in WW plants (Supplementary Data Table S1). It is noteworthy that ABA-GE in WS2 plants showed a different daily trend in comparison to other plants, with the minimum value observed at midday (Fig. 4B). Finally, in plants exposed to moderate water stress (WS1), the expression of *NCED3* was four times higher than in WW plants throughout the day, and higher than WS2 plants at midday and in the afternoon (Fig. 4D).

The ABA content in bark (ABA_{bark}) increased significantly under water stress, resulting in respectively 26-fold and 13-fold higher levels in WS1 and WS2 plants in comparison to WW plants (Supplementary Data Table S1). Furthermore, the daily trend of ABA_{bark} differed between plants subjected to the three water treatments (Fig. 5A). In contrast to the leaf, ABA-GE content in the bark ($ABA\text{-}GE_{bark}$) was higher in WW plants compared to WS1 (+27 %) and WS2 (+38 %) plants (Table S1). Additionally, the maximum daily values of $ABA\text{-}GE_{bark}$ were observed at midday in WW plants (Fig. 5B). The sum of ABA catabolites in the bark ($PA+DPA_{bark}$) of water-stressed plants was more than double those found with WW treatments (Table S1). No statistically significant daily changes were found in $PA+DPA_{bark}$ in WW and WS2 plants; however, $PA+DPA_{bark}$ decreased significantly at midday and then increased again at 1800 h in WS1 plants (Fig. 5C). An increase in *NCED3* expression similar to that recorded in the leaf was also observed in the bark in response to water stress (Fig. 5D). Expression of *NCED3* at 0900 h in WS2 plants was ten-fold higher than in WW plants. The expression of *NCED3* then declined during the day in WS2 plants. In contrast, the daily expression of *NCED3* was five-fold higher in WS1 plants and remained relatively stable during the day (Fig. 5D).

The content of ABA in wood (ABA_{wood}) increased strongly under water stress (Supplementary Data Table S1), reaching its maximum at 1800 h in WS1 plants (73 nmol g^{-1} DW) and at 1300 h in WS2 plants (53 nmol g^{-1} DW) (Fig. 6A). The trend of ABA-GE content in wood ($ABA\text{-}GE_{wood}$) was similar to that observed in bark. In fact, $ABA\text{-}GE_{wood}$ was more than 50 % higher in WW plants than in WS1 and WS2 plants (Table S1). However, $ABA\text{-}GE_{wood}$ was more than 30-fold lower than $ABA\text{-}GE_{bark}$ (Table S1). The mean content of ABA catabolites in wood ($PA+DPA_{wood}$) did not differ among plants from the three water treatments (Table S1). However, in WW and in WS2 plants, levels of $PA+DPA_{wood}$ were at a minimum at midday, while no significant changes were observed during the day in WS1 plants (Fig. 6C). Similar to the other tissues, water stress increased expression of *NCED3* in wood. The expression of *NCED3* was respectively two- and three-fold higher in WS1 and WS2 plants in comparison to WW plants (Fig. 6D). The expression of *NCED3* underwent different diurnal variations in all water treatments. In particular, in WS1 plants, *NCED3* expression reached its maximum at 1300 h, whereas in WS2 the highest value was observed at 0900 h.

The lowest value of xylem sap pH was observed at predawn in WW plants (Fig. 7). During the day, values of pH in the xylem sap remained relatively unchanged in all treatments, ranging between 6.0 and 6.3, except at 0900 h when WS2 plants showed significantly lower values (pH 5.7).

Relationship between ABA and diffusional limitations to CO_2 transport

Increased ABA concentration in the sap and in the leaf of water-stressed plants was significantly and positively correlated to both g_s (Fig. 8A, B) and g_m (Fig. 8C, D). The highest correlation was found between ABA in xylem sap and g_m (R^2 = 0.84).

Relationship between ABA and soluble carbohydrates in leaves and stem

The content of soluble carbohydrates in the different organs was affected by both water treatment and hour of the day (Supplementary Data Fig. S1). The ABA content in leaves showed a significant positive relationship with glucose ($P \leq 0.05$) and a significant negative relationship with sucrose ($P \leq 0.05$) (Fig. 9A–C). In bark, a significant positive

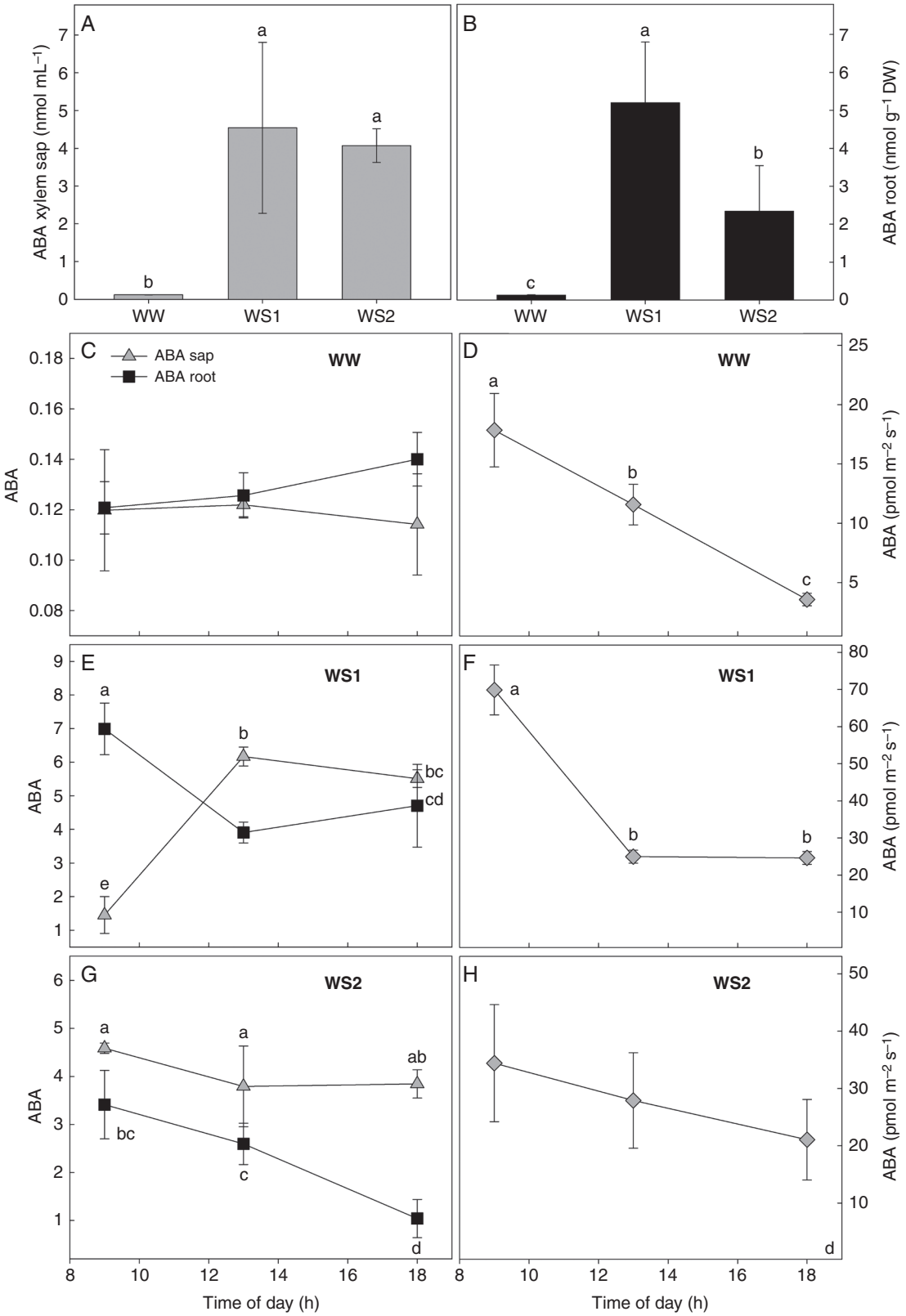

FIG. 3. Daily mean of ABA in xylem sap (nmol mL^{-1}) (A) and roots (nmol g^{-1}DW) (B) and daily trend of ABA concentration in xylem sap (ABA sap, grey triangles) and roots (ABA root, nmol g^{-1}DW, black squares) (C, E, G) and flux of ABA into leaf (D, F, H) in *Populus nigra* plants subject to well-watered (WW), moderate water stress (WS1) and severe water stress (WS2) conditions. Data (means ± s.d., $n = 4$) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

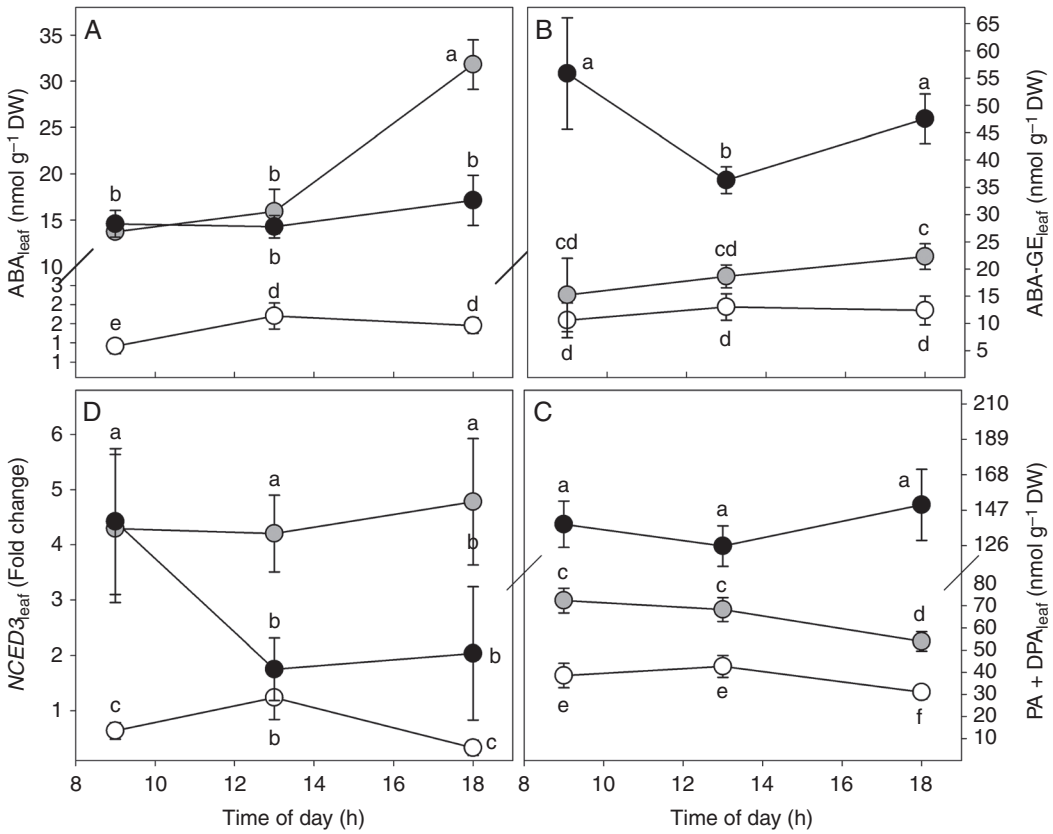

FIG. 4. Daily trend of ABA (ABA_{leaf}, A), ABA-GE ($ABA\text{-}GE_{leaf}$, B), ABA catabolites ($PA+DPA_{leaf}$, C) and *NCED3* expression ($NCED3_{leaf}$, D) in leaves of *Populus nigra* plants subjected to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. Data (means ± s.d., $n = 4$) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

relationship was only found between ABA and fructose ($P \leq 0.05$) (Fig. 9D–F). Stronger linear relationships were found in woody tissues between ABA_{wood} with fructose ($P = 0.003$), sucrose ($P = 0.02$) and glucose ($P = 0.01$) (Fig. 9G–I).

DISCUSSION

In vascular plants, tight coordination between hydraulic and photosynthetic systems allows optimization of water transport and CO_2 assimilation in the leaf (Brodribb, 2009; McDowell, 2011). In particular, g_s, g_m and K_{leaf} all contribute to regulate maximum rates of photosynthesis and water balance under drought (Flexas *et al.*, 2008, 2013*a*; Gago *et al.*, 2016). Indeed, K_{leaf} represents the efficiency of water flow through the leaf mesophyll towards the sites of evaporation, and thus influences stomatal aperture, as it accounts for as much as 80 % of the whole plant hydraulic resistance (Nardini, 2001). Concurrently, g_m represents the CO_2 transfer conductance from the intercellular airspaces of the leaf into the chloroplast, thus strongly influencing biochemical assimilation rates (Centritto *et al.*, 2003; Flexas *et al.*, 2013*a*).

ABA and hydraulic coordination of the physiological response to water deficit

In the present study, photosynthesis was constant during the day in WW plants, despite a decline in g_s values through the day (Fig. 1). The observed reduction in g_s was probably associated with the pronounced increase in VPD (above 1.4 kPa) (Bauerle *et al.*, 2004). Moreover, ABA_{leaf} increased at midday (Fig. 4A), suggesting participation of a biochemical factor in reducing g_s (Fig. 8). Even if the precise temporal pattern of this signalling cascade is not clear, the strict regulation of g_s in this poplar genotype allowed maintenance of high intrinsic water-use efficiency (WUE_{int}) without an excessive Ψ_L drop during the day to minimize the risk of hydraulic failure (Fig. 2 and Supplementary Data Table S2). We suggest that the high photosynthetic rates of WW plants throughout the day derived from the synchronous optimization of g_m and K_{leaf} at midday (Fig. 1C, D). Indeed, previous studies have shown that g_m and K_{leaf} respond positively to both light (Lo Gullo *et al.*, 2005; Scoffoni *et al.*, 2008; Sellin *et al.*, 2008; Loreto *et al.*, 2009; Ben Baaziz *et al.*, 2012; Xiong *et al.*, 2018) and temperature (Sellin and Kupper, 2007; Flexas *et al.*, 2013*b*; Scafaro *et al.*, 2011). Specifically, the increase in temperature (+8 °C) from 0900h to 1300h may have increased the permeability of membranes in the outside-xylem pathway (Sack *et al.*, 2004), also enhancing g_m by reducing the resistance to diffusion of HCO_3^- through a solution. Plasma membrane intrinsic proteins (PIPs) are *trans*-membrane water channels that facilitate water transport across membranes and determine the permeability of membranes and cell tissues to water, and thus strongly influence K_{leaf} (Uehlein *et al.*, 2003; Cochard *et al.*, 2007; Voicu *et al.*, 2008; Heckwolf *et al.*, 2011; Maurel and Prado, 2017). Several PIP isoforms

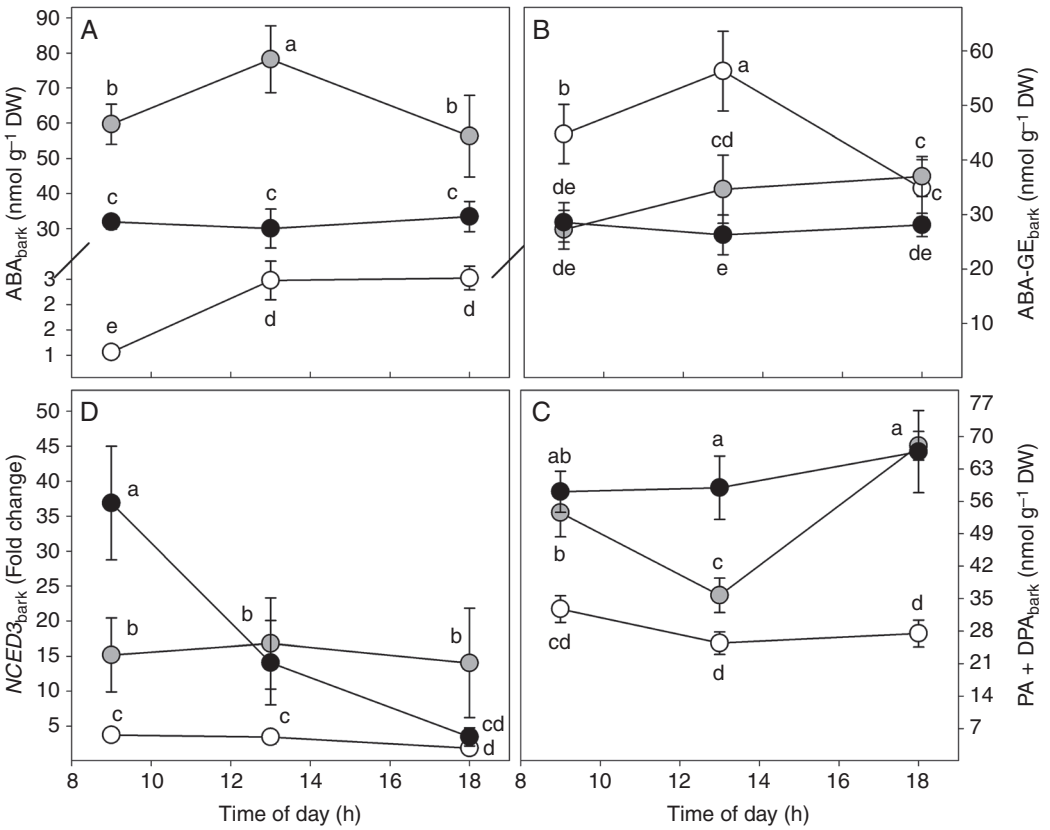

FIG. 5. Daily trend of ABA (ABA$_{bark}$, A), ABA-GE (ABA-GE$_{bark}$, B), ABA catabolites (PA+DPA$_{bark}$, C) and *NCED3* expression (*NCED3*$_{bark}$, D) in the bark of *Populus nigra* plants subject to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. Data (means ± s.d., n = 4) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

have been detected in poplar, which are modulated differently on a daily basis (Lopez *et al.*, 2013). The diurnal expression or activation patterns of PIPs could have been coordinated to maximize K_{leaf} and g_m under WW and drought conditions (Perez Martin *et al.*, 2014; Sade *et al.*, 2015). The strict interconnection of hydraulic and chemical signals may also be due to the effects of ABA on g_m and K_{leaf} through the inactivation of bundle sheath acquaporins during the day (Shatil-Cohen *et al.*, 2011; Grondin *et al.*, 2015; Mizokami *et al.*, 2015; Sorrentino *et al.*, 2016; Qiu *et al.*, 2017). We surmise that the observed daily K_{leaf} and g_m adjustments in the leaves of moderately water stressed plants (WS1) allowed the optimization of carbon gain in the morning before the subsequent rise in temperature and VPD (Flexas *et al.*, 2013*b*; Meitern *et al.*, 2017).

Analysis of the integral of diurnal Ψ_L values indicates that under water deficit poplar showed typical isohydric behaviour, with plants subject to the WS1 and WS2 treatments maintaining a near constant minimum daily Ψ_L by reducing g_s in both WS1 and WS2 conditions (Fig. 2) (Attia *et al.*, 2015). Nonetheless, the watering regimes induced different degrees of water stress, as evidenced by significant differences in predawn Ψ_L between water-stressed and well-watered plants and also in midday Ψ_L between WS1 and WS2 plants. Furthermore, irrespective of the severity of water stress, g_m and K_{leaf} decreased strongly in poplar as soil water availability declined, consistent with previous results observed in a wide range of species (Brodribb and Holbrook, 2003; Diaz-Espejo *et al.*, 2007; Misson *et al.*, 2010; Galle *et al.*, 2011; Scoffoni *et al.*, 2012; Wang *et al.*, 2018). It is noteworthy that at 0900 h WS1 plants showed A_n values of ~15 µmol m^{-2} s^{-1} (Fig. 1) and lower C_i values than WW plants and WS2 plants, which resulted in a higher WUE$_{int}$ (Supplementary Data Table S2). Conversely, in WS2 plants g_s and g_m were strongly depressed (Figs 1C, D and 8), imposing a higher limitation to photosynthesis during the whole day (Centritto *et al.*, 2009).

ABA dynamics in different plant tissues under water stress

Alterations in delivery, synthesis and catabolism mechanisms regulated the content of foliar ABA among different water treatments. In WW leaves, ABA$_{sap}$ contributed to total ABA$_{leaf}$ content mostly in the morning, before a subsequent reduction in transpiration flow that decreased the amount of ABA$_{sap}$ delivered to leaves. Therefore, the increase in ABA$_{leaf}$ observed at midday was probably associated with higher *in situ* ABA biosynthesis (Bauerle *et al.*, 2004; Xie *et al.*, 2006; Marino *et al.*, 2017), probably directly made from the dedicated chloroplastic isoprenoid (MEP) pathway, as first argued by Barta and Loreto (2006). Reduced Ψ_L at 1300 h and higher VPD may have triggered ABA biosynthesis in WW leaves, thus decreasing g_s to prevent excessive dehydration. This could indicate that, under

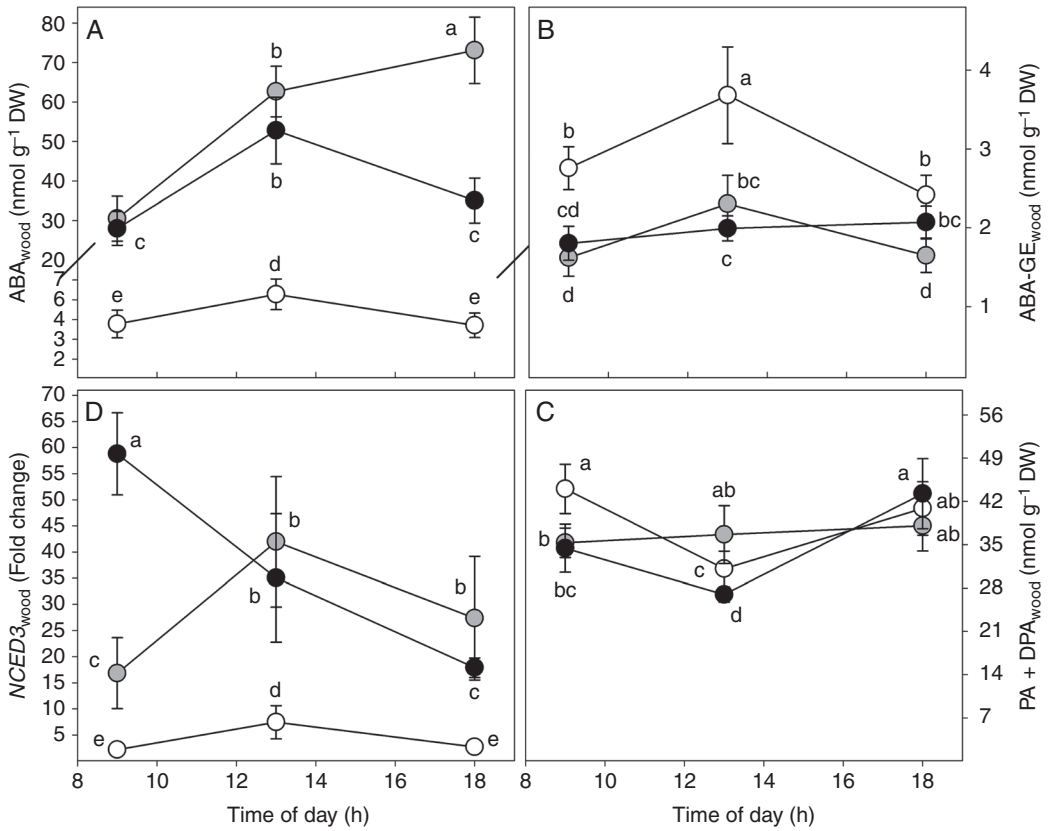

FIG. 6. Daily trend of ABA (ABA$_{wood}$, A), ABA-GE (ABA-GE$_{wood}$, B), ABA catabolites (PA+DPA$_{wood}$, C) and *NCED3* expression (*NCED3*$_{wood}$, D) in the wood of *Populus nigra* plants subjected to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. Data (means ± s.d., $n = 4$) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

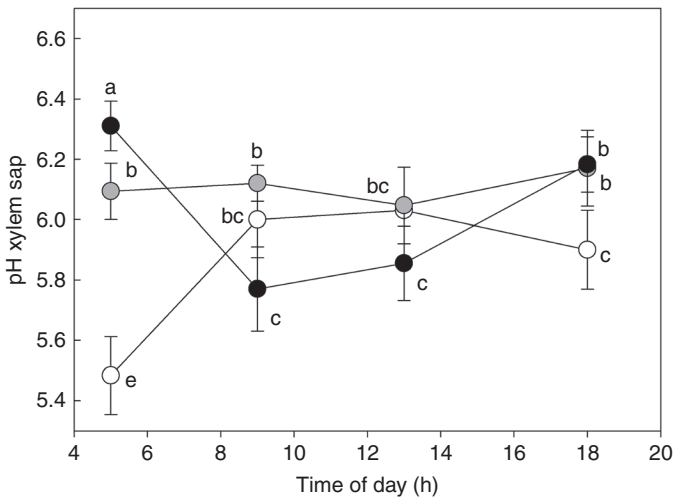

FIG. 7. Daily trend of xylem sap pH in *Populus nigra* plants subject to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. Data (means ± s.d., $n = 4$) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

WW conditions, even a 'low' ABA$_{leaf}$ pool (~0.80 nmol g^{-1} DW) leads to a strong g_s decrease (−36 %) (Figs 1B and 4A). This reduction, which was paralleled by an increase in K_{leaf} and a reduction in C_i, could optimize WUE$_{int}$ (Yang *et al.*, 2016) and reduce the risk of xylem embolism formation on a daily basis in WW plants (Bond and Kavanagh, 1999; Nardini and Salleo, 2000; Davies *et al.*, 2002) (Fig. 1 and Supplementary Data Table S2).

The up-regulation of NCED3 is likely to be the major cause of higher levels of ABA$_{leaf}$ found under water stress conditions (Fig. 4). It is notable that at 0900 h, the highest daily values of K_{leaf}, g_s and g_m were also recorded (Fig. 1) in WS1 plants, when conditions were most suited to photosynthetic activity as Ψ_L was relatively high. However, ABA$_{leaf}$ at 0900 h was identical to values at midday, while g_s declined, and this is consistent with previous suggestions that stomata are less sensitive to ABA in the morning (Correia *et al.*, 1995). We also observed the maximum level of ABA in the roots of water-stressed plants at 0900 h. Previous studies have shown that ABA stimulates root hydraulic conductivity (L_p) (Aroca *et al.*, 2006), thus enabling water influx from the soil and promoting stomatal opening early in the morning (Hose *et al.*, 2000; Thompson *et al.*, 2007; Parent *et al.*, 2009; Tardieu *et al.*, 2015). At midday, the decrease in g_s, g_m and K_{leaf} might depend mostly on the maintenance of high ABA levels, as reported in isohydric grapevine (Coupel-Ledru *et al.*, 2017), but the significant reduction in Ψ_L in WS2 could have also contributed to this regulation. In addition, WS2 leaves utilized ABA-GE to maintain constantly high values of ABA$_{leaf}$ throughout the day (Fig. 4B) (Hansen and Dörffling, 1999; Lee

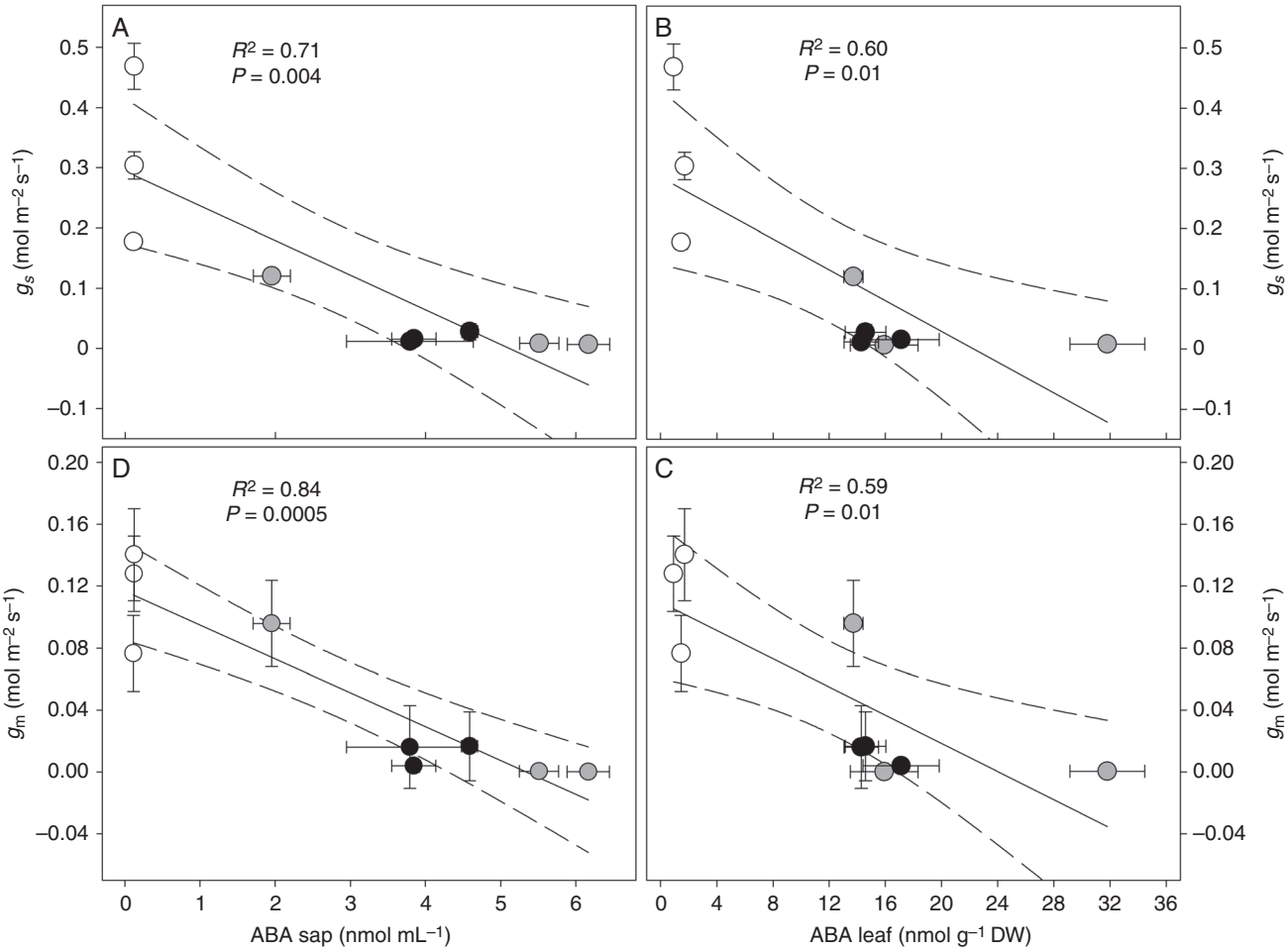

FIG. 8. Relationships between ABA content in the xylem sap (A, B) and leaf (C, D) with stomatal conductance (g_s) and mesophyll conductance (g_m) in *Populus nigra* plants under well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions measured in the morning (0900 h), midday (1300 h) and afternoon (1800 h) sampling points. P and R^2 values indicate the results of linear regression. The central black line indicates the line of best fit. The dotted lines either side of the best-fit line indicate 95 % confidence intervals of the mean. Data are the means ± s.d. (n = 4).

et al., 2006; Xu *et al.*, 2011), whereas WS1 plants showed a peak of ABA_{leaf} at 1800 h, probably due to lower catabolism (Fig. 4C) and/or reduced ABA export to the bark.

Levels of ABA found in the bark could indicate the potential flow of ABA and ABA-GE loaded into the phloem from leaves (Mwange *et al.*, 2003). In fact, we observed a midday increase of ABA and higher levels of ABA-GE within the bark without changes in *NCED3* expression, making the leaves the most likely source of ABA and ABA-GE under WW conditions. Furthermore, ABA-GE decreased in both bark and wood under water stress, while the increase in the content of this metabolite in leaf under severe water stress could be due to reduced rates of phloem loading (Dinat and Lemoine, 2010; Sevanto, 2018) or to an increased rate of ABA glycosylation (Xu *et al.*, 2011). Conversely, ABA appears to be synthesized locally in the wood of WW plants, in which the midday drop of water potential may be related to the peak of ABA_{wood} at 1300 h (Pantin *et al.*, 2013; Fig. 6A). Water stress boosted ABA biosynthesis in both the bark and the wood (Endo *et al.*, 2008; Galvez-Valdivieso *et al.*, 2009; Shatil-Cohen *et al.*, 2011). In these tissues, the catabolism of ABA probably played a major role in the regulation of ABA levels as observed in both WS1 and WS2 plants (Figs 5D and 6D). In particular, it is possible that water stress-induced ABA production in vascular parenchyma (Endo *et al.*, 2008) resulted in the secretion of ABA to the xylem vessels, thus contributing to the strong increase of ABA_{sap}. This ABA secretion could be the result of the release of the uncharged form of the hormone from vascular parechyma cells to xylem vessels (Lacombe and Achard, 2016; Kuromori *et al.*, 2018) induced by the reduction in sap pH by 0.3 units (Fig. 7). The decline in sap pH recorded in our study is similar to results observed in poplar by Secchi and Zwieniecki (2016) and in other deciduous species by Thomas and Eamus (2002). The reduced pH in the xylem sap of poplar contrasts with observations of the opposite trend in other species (Wilkinson and Davies, 1997), suggesting that pH signals of water stress are species-specific, with the charged form of the ABA molecule being more prevalent in herbaceous and evergreen species (Bahrun *et al.*, 2002; Thomas and Eamus, 2002), whilst in woody deciduous species the uncharged form is more commonly utilized as a signal of reduced water availability (Boursiac *et al.*, 2013). Nonetheless, xylem sap pH is related to numerous factors other than ABA concentration, such as the degree of respiration (Salomón *et al.*, 2016), flow rate and the content of minerals/compounds (Peuke,

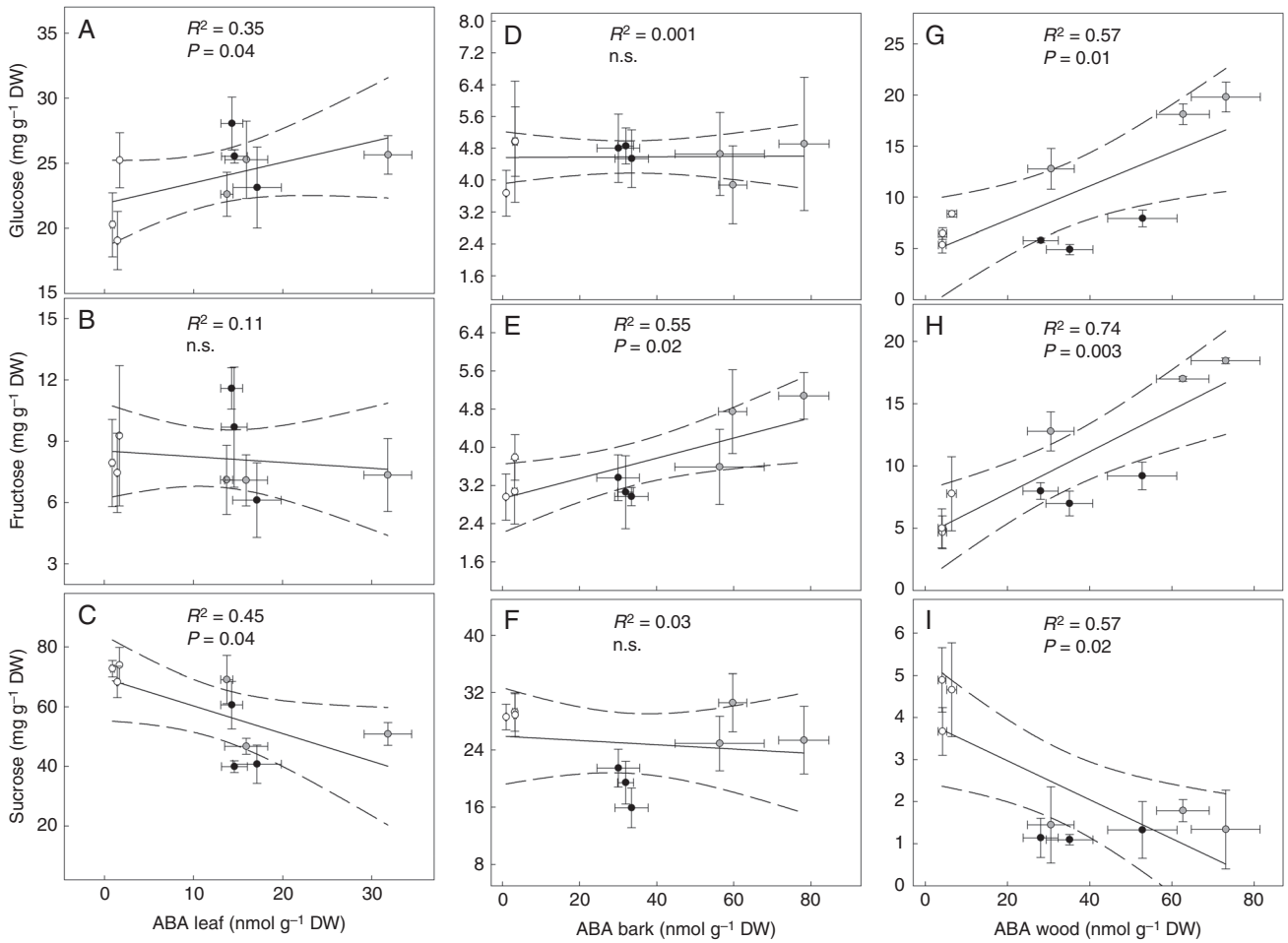

FIG. 9. Relationship between ABA content and soluble carbohydrates in the leaf (A–C), bark (D–F) and wood (G–I) of *Populus nigra* plants subjected to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions measured in the morning (0900 h), midday (1300 h) and afternoon (1800 h) sampling points. P and R^2 values indicate the results of linear regression. The central black line indicates the line of best fit. The dotted lines either side of the best-fit line indicate 95 % confidence intervals of the mean. Data are the means ± s.d. (n = 4).

2016), possibly accounting for the wide variation observed in pre-dawn xylem sap pH values in the present study.

Our results are consistent with the model proposed by Pantin *et al.* (2013), as the observed increase in ABA_{wood} and ABA_{sap} from 0900 to 1300 h in WS1 plants coincided with a decrease in K_{leaf}. According to this model, under drought conditions, ABA chemical signals could be converted into a hydraulic signal in the leaf by down-regulation of the activity of bundle-sheath aquaporins, resulting in the decreased permeability of vascular bundle-sheath cells (Shatil-Cohen *et al.*, 2011; Negin and Moshelion, 2016).

Exploring the role of ABA in carbohydrate metabolism

It is noteworthy that similar ABA levels in both leaf and wood correspond to different water status in plants characterized by different g_s values. To investigate different roles of ABA, possible correlations between ABA and soluble carbohydrates were analysed. In addition to the activation of a multitude of genes involved in primary carbohydrate metabolism (Zeller *et al.*, 2009; Choudhury and Lahiri, 2011; Yoshida *et al.*, 2015), the accumulation of ABA in leaves under drought increases the activity of β-amylase and vacuolar invertase (Pelleschi *et al.*, 1999; Kempa *et al.*, 2008; Thalmann *et al.*, 2016), thus leading to an increase in hexoses. Our results support the hypothesis that ABA may be responsible for the alteration of soluble carbohydrate metabolism (Fig. 9) (Finkelstein and Gibson, 2002). It is possible that during the night ABA induced remobilization of starch reserves from the chloroplast (Robertson *et al.*, 2009) and maintained the size of leaf sugar pools during water stress (Smith and Stitt, 2007). The maintenance of high levels of sugars in the leaf may result in the down-regulation of photosynthetic genes during water deficit (Van Oosten *et al.*, 1994). This relationship between ABA and sugars emerged not only in the leaf, as previously found in transgenic tobacco leaves exposed to water stress (Tattini *et al.*, 2014), but also in the stem, particularly in the wood (Fig. 9C). We suggest a possible role for this hormone in mobilizing non-structural carbohydrates in the wood to preserve xylem hydraulic integrity under water stress (Secchi *et al.*, 2013). It is important to mention that a putative connection between ABA, PIPs and hexoses has been recently put forward (Kelly *et al.*, 2017; Wang *et al.*, 2017), suggesting that a cross-talk between the glucose and ABA

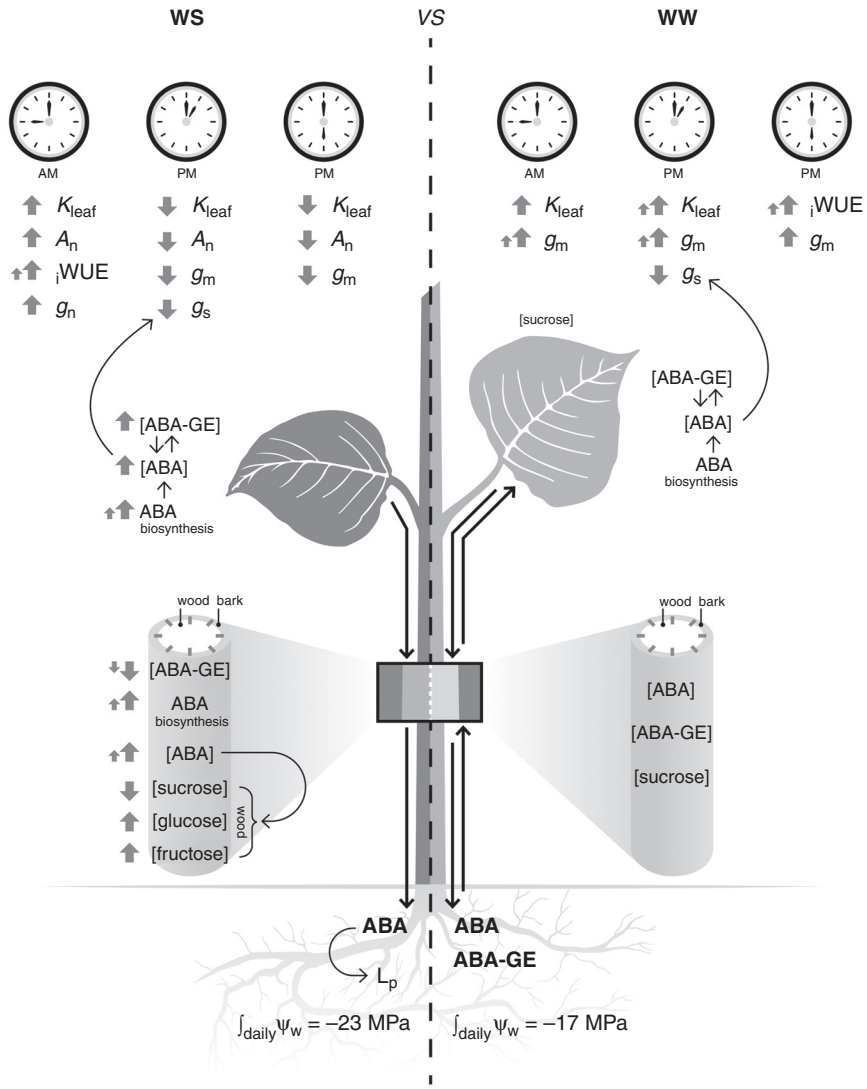

FIG. 10. Schematic overview of the main experimental results on *Populus nigra* plants under moderate water stress (WS) and well-watered (WW) conditions: (1) in the leaf there is a clear daily trend where ABA regulates physiological parameters [stomatal conductance (g_s), mesophyll conductance (g_m) and leaf hydraulic conductance (K_{leaf})] to optimize water transport and CO_2 assimilation; (2) in the stem ABA content may have a role in the regulation of water transport and carbohydrate metabolism; (3) ABA accumulates in the roots due to transport from the leaves where this hormone is synthesized. The increase in ABA content in the roots under water-stress conditions may enhance root hydraulic conductivity (L_p).

signalling pathways may regulate the level of sucrose (Wang *et al.*, 2017), and in turn the level of hexoses may affect the expression of PIP genes and reduce leaf hydraulic conductance (Kelly *et al.*, 2017). As a consequence, a fine tuning of carbohydrate metabolism by ABA to modulate K_{leaf} under drought is likely to occur.

In conclusion, the results of this study expand our understanding of the impact of water stress on the daily trends of g_s, g_m and K_{leaf} and the close coordination of these parameters in the optimization of water transport and CO_2 assimilation in water-stressed plants. In addition, the finding that WW plants restrict g_s during the day while keeping photosynthesis constant provides evidence that unstressed plants can operate in a more water-efficient mode by increasing WUE_{int} without curbing CO_2 fixation (Fig. 10). This study supports previous results showing a role of both hydraulic and hormonal signals in the regulation of g_s under water stress (Wilkinson and Davies, 2002; Christmann *et al.*, 2007). Moreover, these two signals are strictly coupled and have a different influence on g_s and g_m depending on the time of the day and the intensity of the stress. In particular, we suggest that in well-watered plants a slight increase in leaf ABA content is enough to decrease g_s, but reducing g_m in water-stressed plants may require higher leaf levels of ABA, probably associated with reductions in water potential. The hypothesis of a key role for ABA in directly or indirectly regulating stomatal closure in isohydric plants is also supported, as both ABA biosynthesis/catabolism and ABA-GE metabolism are fine-tuned to adjust the content of free-ABA in leaves.

Finally, ABA content and diurnal pattern in different plant organs were also modified in response to water stress. Our study corroborates the current hypothesis that ABA synthesis takes

place in several plant organs such as leaves, roots and vascular tissues, and ABA can move to target cells through both the xylem and the phloem, allowing a two-way transportation between roots and shoots (Fig. 10). To the best of knowledge, our data provide the first report of a possible relationship between ABA and soluble carbohydrates in the leaves and stem, suggesting further potential roles of this hormone in carbohydrate metabolism.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: Daily trend of soluble carbohydrates in the leaf (A–C), wood (D, E) and bark (F, G) in *Populus nigra* plants subject to well-watered (WW, open circles), moderate water stress (WS1, grey circles) and severe water stress (WS2, black circles) conditions. Data (means ± s.d., $n = 4$) were subjected to one-way ANOVA; different letters indicate significant differences between groups at a significance level of $P < 0.05$ using Tukey's test.

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