

# Drought tolerance of two black poplar (*Populus nigra* L.) clones: contribution of carbohydrates and oxidative stress defence

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## ABSTRACT

Drought is expected to become an increasingly important factor limiting tree growth caused by climate change. Two divergent clones of *Populus nigra* (58-861 and Poli) originating from contrasting environments were subjected to water limitation (WL) to elucidate whether they differ in tolerance to drought, which mechanisms to avoid stress they exhibit and whether drought has an impact on the interactions between roots and shoots. Limiting water availability caused photosynthetic rate and total non-structural carbohydrate (TNC) levels to decrease in 58-861. However, starch-degrading enzyme activity and gene expression were induced in roots, and soluble sugar levels were higher than in well-watered (WW) plants. These data suggest that assimilation and partitioning of carbon to the roots are decreased, resulting in mobilization of stored starch. In contrast, the photosynthetic rate of Poli was reduced only late in the treatment, and carbohydrate levels in WL plants were higher than in WW plants. Superoxide dismutase (SOD) activity and gene expression were higher in Poli than in 58-861, even in WW plants, leading to a higher capacity to defend against oxidative stress.

**Key-words:** gene expression; leaf gas exchange; qRT-PCR; root; starch-degrading enzymes; superoxide dismutase activity.

## INTRODUCTION

Drought is one of the crucial factors limiting plant growth and is expected to become increasingly important in many regions because of the ongoing climate change. Forests contain up to 80% of the global above-ground and 40% of the below-ground carbon in terrestrial ecosystems (Dixon *et al.* 1994). Therefore, the impact of drought on trees might become an important factor influencing the global carbon cycle and thereby further boost global warming because of reduced CO<sub>2</sub> fixation. Trees have evolved

various mechanisms which help them to cope with limited water supply. Responses to drought include: (1) reducing the water deficit by developing root systems able to take up water deep in the soil; (2) minimizing water losses through stomatal closure and producing small leaves; and (3) accumulating osmoprotective substances (Kozlowski & Pallardy 2002).

In recent years, poplar (*Populus* sp.) has emerged as the model tree species. Poplar is fast growing, easy to propagate via stem cuttings and economically important. As the poplar genome has now been completely sequenced and genomic tools are available (Tuskan *et al.* 2006; Jansson & Douglas 2007), poplar is increasingly used to investigate molecular mechanisms of trees and their responses to environmental stress. To complement the findings on the molecular level and to be able to use poplar as an integrated model for tree species, we need to gain detailed knowledge of the biochemical, physiological and morphological properties, and how they change in response to environmental stress.

Several recent studies have dealt with the responses of poplar to drought, but most have been restricted to above-ground parts of the plant. A number of traits that are affected by water shortage are used as indicators for drought. Total biomass, leaf area, shoot height and diameter often are heavily reduced by drought (Monclus *et al.* 2006), but are also affected by other adverse environmental conditions and are therefore not indicators for drought alone. Identification of physiological traits that are specifically affected by drought may help to improve our understanding of adaptive mechanisms and will be particularly useful for breeding strategies which aim to combine high productivity with drought tolerance.

Effects of drought on gas exchange characteristics and leaf water relations of poplar have been frequently evaluated (e.g. Monclus *et al.* 2006; Street *et al.* 2006; Brilli *et al.* 2007). Osmotically active substances, which accumulate in poplar leaves under water-limited conditions, have also been determined (Marron *et al.* 2002; Lei, Yin & Li 2006). Soluble sugars contribute to the osmotic potential, and despite lower assimilation rates, drought-stressed plants have been reported to maintain the total concentration

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of soluble sugars in the leaves (Chaves, Maroco & Pereira 2003). This is accompanied by a drastic decline of leaf starch, which has been explained by altered carbon partitioning (Quick *et al.* 1992; Arndt *et al.* 2001; Li & Li 2005). It is possible that osmotic adjustment is a more important factor in roots than in leaves, as roots are in direct contact with low soil water potential and cannot easily regulate water loss. Roots store carbohydrates mainly in the form of starch, and with lower supply from the shoot, carbon partitioning into sugars and starch is altered (Gaucher *et al.* 2005). However, our knowledge about the effects of drought on the metabolism of stored starch in roots is still marginal and remains to be investigated.

Reactive oxygen species (ROS) are generated by transfer of excess electrons produced during photochemistry in chloroplasts (Edreva 2005) or during respiration in the mitochondria (Navrot *et al.* 2007). ROS production is enhanced in response to several environmental stresses, and causes cellular damage. Effects of drought-induced generation of ROS in poplar and concomitant induction of ROS-detoxifying enzymes in leaves have been investigated (Guerrier *et al.* 2000; Lei *et al.* 2006; Marron *et al.* 2006). However, our understanding of the impact of drought on osmotic adjustment and generation of oxidative stress in roots is still fragmentary. With a few exceptions (Tschaplinski & Tuskan 1994; Bogeat-Triboulot *et al.* 2007; DesRochers, van den Driessche & Thomas 2007), roots have been mostly disregarded in studies concerning drought stress in poplar, but it has been shown that roots also react significantly to water shortage. Morabito & Guerrier (2000) found that roots are the most sensitive organ of poplar to oxidative stress after 12 h drought, but nothing is known about reactions in roots after longer drought events. Therefore, we still need to gain information about responses of poplar roots to drought and the interactions between root and shoot processes.

We analysed two black poplar (*Populus nigra* L.) genotypes with highly divergent phenotypes originating from contrasting environments. The two genotypes were recently crossed for the construction of genetic linkage maps (Gaudet *et al.* 2008). Black poplar is a native European species that pioneers riparian ecosystems. However, some genotypes also grow in arid regions. The differences in drought tolerance of these two clones have not yet been analysed. In this study, we characterized and compared morphological, physiological and molecular traits between the divergent *P. nigra* clones, and analysed the effects of water limitation (WL). We hypothesized that drought would: (1) alter the carbohydrate metabolism and carbon partitioning between starch and soluble sugars in the roots, also influencing related enzyme activities and gene expression; and (2) induce the production of ROS in the roots, leading to higher activity of ROS-degrading enzymes. In order to improve our understanding of the interactions between root and shoot processes, we determined also shoot parameters including growth and leaf gas exchange.

## MATERIALS AND METHODS

### Plant material and experimental design

Two clones of *P. nigra*, previously used as parents for a full-sib progeny for genetic mapping (Gaudet *et al.* 2008) were used for this study. They originate from contrasting environments and have highly divergent phenotypes. The clone 58-861 was collected in Northern Italy at 597 m above sea level close to the Italian Alps and near the Dora Riparia river (45°09'N, 7°01'E), in a region with a mean summer (June–September) rainfall of 315 mm and mean summer temperature of 25.7 °C. Poli grows in Southern Italy close to the Ionian Sea and near the Sinni River at 7 m above sea level (40°09'N, 16°41'E; 116 mm mean summer rainfall and 29.3 °C mean summer temperature).

In early March 2008, 20 woody cuttings of the two clones were planted separately into 10 L plastic pots filled with a mixture of 42% crust humus, 42% wood fibre, 4% clay, 1.25 g Floranid permanent and 1.25 g NP 20:20 with a pH of 5.5–6.2 and grown in the greenhouse under well-watered (WW) conditions. The plants were supplemented with light (400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photon flux density) for 15 h  $\text{d}^{-1}$ , and temperature and humidity were kept constant (20 °C at night and 25 °C during day-time with 40–60% relative humidity). After 6 weeks of growth, plants of similar height were selected for the experiment. The experimental layout was a randomized block design with two factors (clone and watering regime) and five replicates per clone and treatment. At the beginning of the experiment on 21 April 2008, all pots were watered to field capacity. The pots were weighed weekly to determine the amount of water loss, which was completely replenished for the WW pots. From pots assigned to WL, water was withheld for 3 weeks followed by 2 weeks watering with 25% of the amount of water added to the WW plants. Soil water potential ( $\Psi_{\text{soil}}$ ) was measured using tensiometers (TS1 Self Refilling Tensiometer; UMS, Munich, Germany).

### Leaf morphology

For each clone, adaxial and abaxial surfaces of 10 leaves from WW plants were taken near the midvein using a steel borer (8 mm diameter), mounted on aluminium stubs and then immediately frozen in liquid nitrogen. In addition to adaxial and abaxial epidermal tissues of the leaf, freeze-fractures were analysed. In this case, the tissue was mounted vertically on an scanning electron microscopy (SEM) stub and plunge frozen. Low-temperature SEM was performed as described by Frey *et al.* (2000). Epidermal surfaces were analysed using  $\mu\text{Image}$  Micro Image Analysis Software (VWR International AG, Dietikon, Switzerland).

### Plant growth, gas exchange and plant harvesting

Plant height and stem diameter were monitored weekly during the experiment. Leaf gas exchange measurements were recorded weekly between 0900 and 1200 h using an

LI-6400 portable photosynthesis system (Li-Cor Inc., Lincoln, NE, USA) at 25 °C with saturating light (1200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation) and a  $\text{CO}_2$  concentration of 400  $\mu\text{mol mol}^{-1}$  air. Measurements were performed on the same 5 plants per clone and treatment throughout the experiment. Before we started the treatments, the two youngest fully expanded leaves of each plant were labelled and monitored throughout the experiment. Additionally, at each following measurement date, we recorded gas exchange on the youngest fully expanded leaf of 2 plants per clone and treatment. After 5 weeks, 5 WW and 5 WL plants per clone were harvested. Leaves were divided into young and old. Before we started the treatments, we labelled the youngest fully expanded leaf. Leaves present when the experiment started, which were fully expanded and dark green at the time of harvest, were defined as old. The remaining new leaves which were light green, and included those not yet fully expanded, were defined as young. Fine roots were also sampled. All plant material was immediately frozen in liquid nitrogen and stored at  $-80$  °C until analysis.

### Starch and soluble sugars

Starch was extracted from leaves and roots as described by Smith & Zeeman (2006). Approximately 0.2 g of plant material was homogenized in liquid nitrogen and extracted three times in 80% ethanol at 80 °C. Starch amount was determined by measuring the glucose released by treatment with  $\alpha$ -amylase and amyloglucosidase.

The pooled 80% ethanol supernatants obtained by the starch extraction were dried by vacuum centrifugation and resuspended in water. After passing them through DOWEX 50 W and DOWEX 1 (Sigma-Aldrich, Buchs, Switzerland) columns (Harley & Beevers 1963), sugars were analysed using HPAEC-PAD Dionex BioLC system (Dionex, Olten, Switzerland), equipped with an AS50 auto-sampler, a GS50 gradient pump and an ED50 PAD system on a Dionex CarboPac PA-20 column according to the following conditions: eluent A, 100 mM NaOH; eluent B, 150 mM NaOH, 500 mM sodium acetate. The gradient was 0–15 min, 100% A (sugar elution); 15–25 min, 10% A, 90% B (column wash step); 25–30 min step to 100% A (column re-equilibration). The flow rate was 0.5 mL  $\text{min}^{-1}$ . Peaks were identified by co-elution with known glucose, fructose and sucrose standards. Peak areas were determined using Chromeleon software (Dionex, Olten, Switzerland).

### Protein analysis

Frozen fine roots were homogenized with 50 mM potassium phosphate buffer, pH 7.5 containing 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 2-mercaptoethanol, 4% PVP-40 and 0.5% Triton X-100, and cell debris removed by centrifugation at 13 000  $g$  for 20 min at 4 °C. The supernatant was transferred to a fresh, pre-cooled tube, and stored at  $-80$  °C for further analyses. Protein content was determined by the Bradford method (Bradford 1976) using a

commercial protein assay (Bio-Rad Laboratories GmbH, Munich, Germany).

Peroxidase activity was determined according to Lin & Wang (2002) based on oxidation of guaiacol to tetraguaiacol. The reaction mixture consisted of 300  $\mu\text{L}$  100 mM potassium phosphate buffer containing 16 mM guaiacol and 0.03%  $\text{H}_2\text{O}_2$ . Reaction was started by adding 20  $\mu\text{L}$  plant extract, and the absorption increase at 470 nm was monitored in a spectrophotometer (Tecan, Männedorf, Switzerland) for 15 min. A dilution series of peroxidase from horseradish (Fluka, Buchs, Switzerland) was used as standard.

Superoxide dismutase (SOD) was assayed spectrophotometrically based on inhibition of the photochemical reduction of nitroblue tetrazolium (Beauchamp & Fridovich 1971). The reaction mixture was composed of 50 mM phosphate buffer containing 0.1 mM EDTA, 13 mM methionine, 2  $\mu\text{M}$  riboflavin and 75  $\mu\text{M}$  nitroblue tetrazolium according to Lei *et al.* (2006). One unit of SOD is defined as the amount of enzyme that inhibited the rate of nitroblue tetrazolium reduction by 50%.

Native polyacrylamide gels for detection of starch-degrading enzyme activities were used as described previously (Zeeman *et al.* 1998). The gels contained 6% acrylamide and 0.1% amylopectin or  $\beta$ -limit dextrin. After electrophoresis at 4 °C, the gels were incubated overnight at room temperature in 100 mM Tris, pH 7.0, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$  and 5 mM dithiothreitol, and stained with Lugol solution.

### RNA extraction and first-strand cDNA synthesis

Frozen fine roots were ground to a powder in liquid nitrogen. RNA was extracted using the Agilent Plant RNA Isolation Mini Kit according to the manufacturer's instructions (Agilent Technologies AG, Basel, Switzerland). RNA concentration was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and RNA quality was assessed using the Agilent Bioanalyzer (Agilent Technologies AG). First-strand cDNA synthesis was performed with the QuantiTect Reverse Transcription Kit (Qiagen AG, Hombrechtikon, Switzerland) using 200 ng total RNA. The quality of the first-strand cDNA was tested via PCR using primers for actin and elongation factor 1, beta subunit (Table 1). Absence of genomic DNA in the samples was verified by choosing an intron-spanning amplicon for elongation factor 1, beta subunit.

### Primer design and real-time RT-PCR

Primers for genes involved in carbohydrate metabolism (*BAM3*, *DPE1*, *ADG1*, *BEII*, *ISAI*, *PGM* and *SSII*) and oxidative stress defence (*APX*, *CAT*, *GR* and *SOD*) were designed using the Primer3 software (<http://frodo.wi.mit.edu/primer3>; Rozen & Skaletzki 2000) for the amplification of gene fragments around 100 bp in length and an annealing temperature of 60 °C. Sequences for primer design were downloaded from the *Populus trichocarpa* v1.1 database at the Joint Genome Institute (<http://genome.jgi-psf.org>)

**Table 1.** Sequences of the primers used for qRT-PCR

Gene	Abbr.	Forward primer	Reverse primer	JGI gene model ID
Reference genes				
Actin 2	ACT	ACC CTC CAA TCC AGA CAC TG	TTG CTG ACC GTA TGA GCA AG	estExt_Genewise1_v1.C_1850029
Elongation factor 1, beta subunit	EFI	AAG CCA TGG GAT GAT GAG AC	ACT GGA GCC AAT TTT GAT GC	estExt_fgenes4_pg.C_LG_11178
Ubiquitin	UBQ	CGT GGA GGA ATG CAG ATT TT	GAT CTT GGC CTT CAC GTT GT	estExt_fgenes4_pg.C_188960001
Starch metabolism				
Beta-amylase 3	BAM3*	CCA CAA CAT GCA AAT TGCTC	CAA TGC ATT CTC TCC TGC AA	eugene3.01180078
Disproportionating enzyme	DPE1*	TTT GGA AAC TGG AGG TGG AG	AAC CGC CCA TAT GTT GAG AG	gw1.VII.2145.1
ADP-glucose-pyrophosphorylase	ADG1*	TGA GGA GCA CAA TGT TTT GG	TGC ATC AGT TTC CCT GTG AG	eugene3.00141188
Enzyme 2	BEI1*	GGA AAT CCA GCA ATT CCT CA	TGG CAC ATT TTA TCC AAG CA	fgenes4_pg.C_LG_V0001651
Isoamylase 1	ISAI*	ATG GCA GCT TTT CTC CTG AA	CAC CAA AAT CCC CTC TGC TA	gw1.XVIII.258.1
Phosphoglucomutase	PGM*	TTT TGG AGC TGC AAG TGA TG	TTG GCA GCA ATG ATA GCA AC	estExt_fgenes4_pg.C_LG_XV1054
Starch synthase 2	SSI1*	GTG GAC ATT TGA TGG TGC TG	GGA GTC CCT CCC AAC TTT TC	gw1.XVII.1126.1
Oxidative stress defence				
Ascorbate peroxidase	APX	TCT TGC GAG GAA GTG AAG GT	AAT GGT TGG ACC TCC AGT GA	estExt_fgenes4_pm.C_LG_IV0530
Catalase	CAT	TTG CTT TCT GCC CTG CTA TT	GTG CCT CTG GGT ATC AGC AT	eugene3.00020082
Glutathione reductase	GR	CCG TTC ATT CCT GAC ATT CC	CTC CAC CAA CTA TGG CGA TT	eugene3.00150408
Superoxide dismutase	SOD	GGG TCT CGT CCA ACA CAC TT	AGC CAT GGC GAT AGA TTG AC	estExt_Genewise1_v1.C_LG_XIII1983

Asterisks assign where gene names and sequences are based on *Arabidopsis* orthologs. Primers were designed on JGI gene models as denoted and tested against the poplar genome via *insilico* PCR.

Poptr1\_1/Poptr1\_1.home.html; Tuskan *et al.* 2006). Primer sequences are shown in Table 1. For genes involved in carbohydrate metabolism, the closest homologs to functional *Arabidopsis* genes were identified using the homology search tool at PopulusDB ([http://www.populus.db.umu.se/search\\_homol.php](http://www.populus.db.umu.se/search_homol.php); Sterky *et al.* 2004). Primer specificity was tested against the genome sequence using *insilico* PCR available on <http://www.popgenie.db.umu.se> (Sjödin *et al.* 2009). Primers were evaluated via non-quantitative PCR, and only those primer combinations, which yielded only one clear amplification product of expected length, were used for real-time RT-PCR.

Real-time RT-PCR was performed using the FastStart Universal SYBR Green Master Mix (Roche Diagnostics, Mannheim, Germany) on an ABI 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). We performed three technical replications for each of three biological replicates per clone and treatment combination. Reactions of 15  $\mu$ L total volume contained 5  $\mu$ M of forward and reverse primers, and 1  $\mu$ L of 1:10 or 1:100 diluted cDNA. PCR conditions were 10 min 95  $^{\circ}$ C, 40  $\times$  15 s 95  $^{\circ}$ C, 1 min 60  $^{\circ}$ C, followed by a dissociation curve. PCR efficiencies were determined using the program LinRegPCR (Ramakers *et al.* 2003). Relative gene expression was calculated from the  $C_t$  values with qBase software (Hellemans *et al.* 2007), which allows the inclusion of multiple reference genes for the calculation of relative gene expression. The calculation was conducted according to the manual provided with the software. We used actin2, ubiquitin and elongation factor1, beta subunit as reference genes.

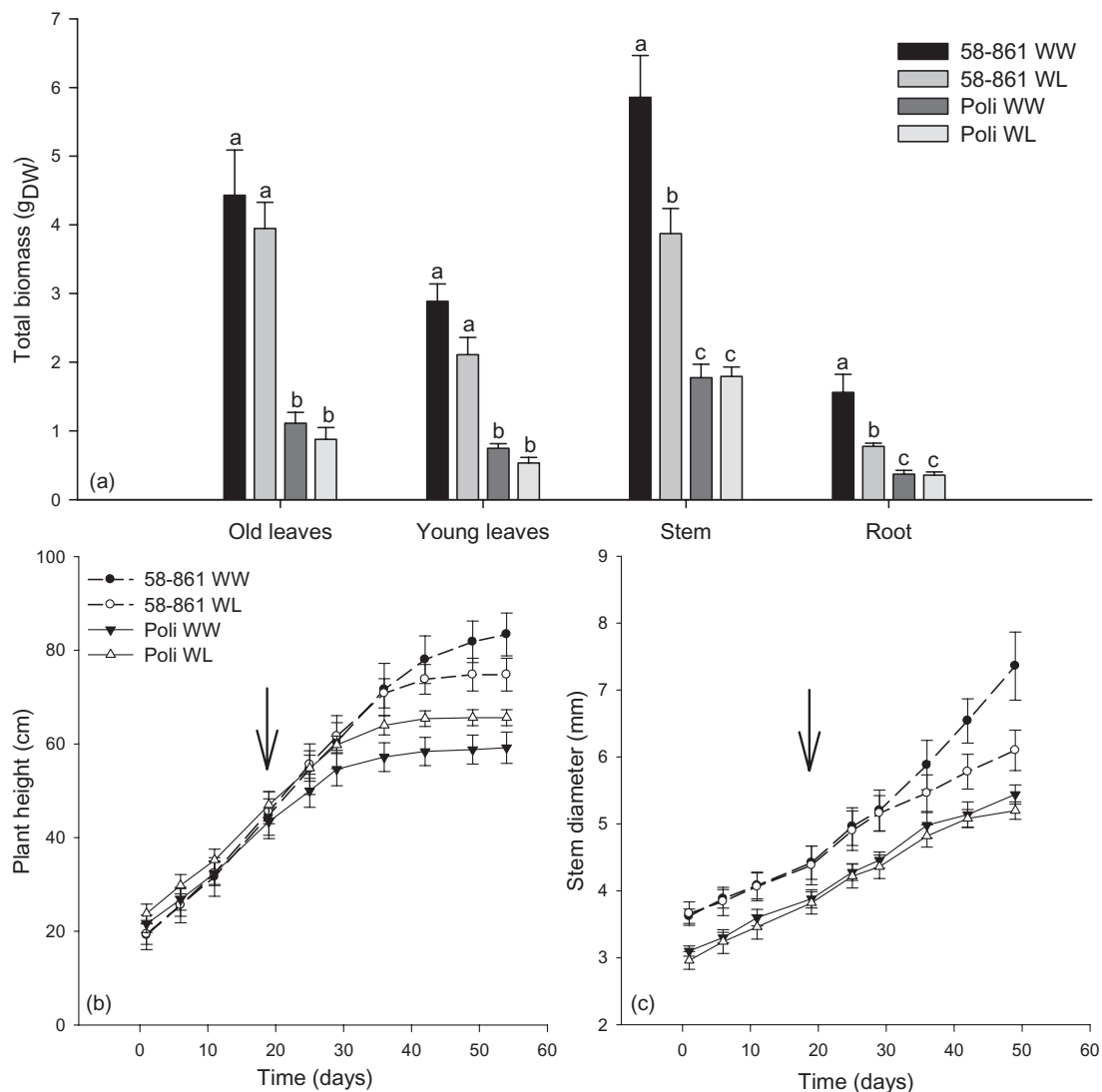
## Statistical analysis

Two-way analysis of variance (ANOVA), with independent factors clone and treatment, was applied to test clone, treatment and interaction effects on biomass, carbohydrate content and enzyme activities. Growth and gas exchange parameters were analysed using GLM repeated measures procedure with two between-subjects factors (clone and treatment) and one within-subject factor (time). Statistical tests were considered significant at  $P < 0.05$ . SPSS 16.0 for Windows statistical software package (SPSS Schweiz AG, Zurich, Switzerland) was used for all analyses.

## RESULTS

### Dry-down cycle

Water loss was determined weekly by weighing the pots. The pot weight greatly exceeded the plant weight such that the increase in weight because of growth of the plants was negligible. After withholding water for 3 weeks,  $\Psi_{\text{soil}}$  was significantly decreased in WL pots of both clones compared to WW pots ( $P < 0.01$ ). In 58-861,  $\Psi_{\text{soil}}$  was lower than in Poli, reaching an extreme of  $-203 \pm 38$  hPa in 58-861, and  $-119 \pm 28$  hPa in Poli ( $P < 0.01$ ), whereas in control pots of both clones  $\Psi_{\text{soil}}$  was maintained close to zero over the whole experiment. After rewatering,  $\Psi_{\text{soil}}$  increased in both



**Figure 1.** (a) Biomass (g<sub>DW</sub>) of old and young leaves, stem and root of *Populus nigra* Poli and 58-861 after 5 weeks of drought treatment. Different letters indicate significant differences within an organ ( $P < 0.05$ ). (b) Plant height and (c) stem diameter. Arrows indicate the start of the experiment. WW, well watered; WL, water limited. Values are mean  $\pm$  standard errors,  $n = 5$ .

clones, leading to similar  $\Psi_{\text{soil}}$  in WL pots of both clones with significant differences to WW pots ( $P < 0.05$ ) until the end of the experiment.

### Effects of WL on plant growth

We found significant differences in total biomass (Fig. 1a;  $P < 0.001$ ), plant height (Fig. 1b;  $P < 0.01$ ) and stem diameter (Fig. 1c;  $P < 0.01$ ) between the clones under WW conditions. The plant height of Poli was about 30% lower than that of 58-861; the difference in stem diameter between the clones was 26%. Under WL conditions, the differences between the clones were reduced to 12% for height and 15% for diameter, although we did not find any significant effects of WL on height ( $P = 0.126$  for Poli and  $P = 0.174$  for 58-861) and diameter ( $P = 0.251$  for Poli and  $P = 0.066$  for 58-861) of both clones. Furthermore, leaf biomass did not

differ significantly between WL and WW plants of both clones. However, stem biomass and root biomass were significantly reduced in WL 58-861 plants, but not in Poli (Fig. 1a). 58-861 showed visible stress symptoms like wilting and leaf abscission.

### Leaf gas exchange and leaf morphology

We found significant effects of the watering regime on net photosynthesis ( $A$ ) and stomatal conductance ( $g_s$ ), while the clone had only significant effects on  $g_s$  and water-use efficiency (WUE; Table 2). Time effects were highly significant ( $P < 0.001$ ), because  $A$  and  $g_s$  had higher values at the beginning than at the end of the experiment in both watering regimes (Table 2, Fig. 2).

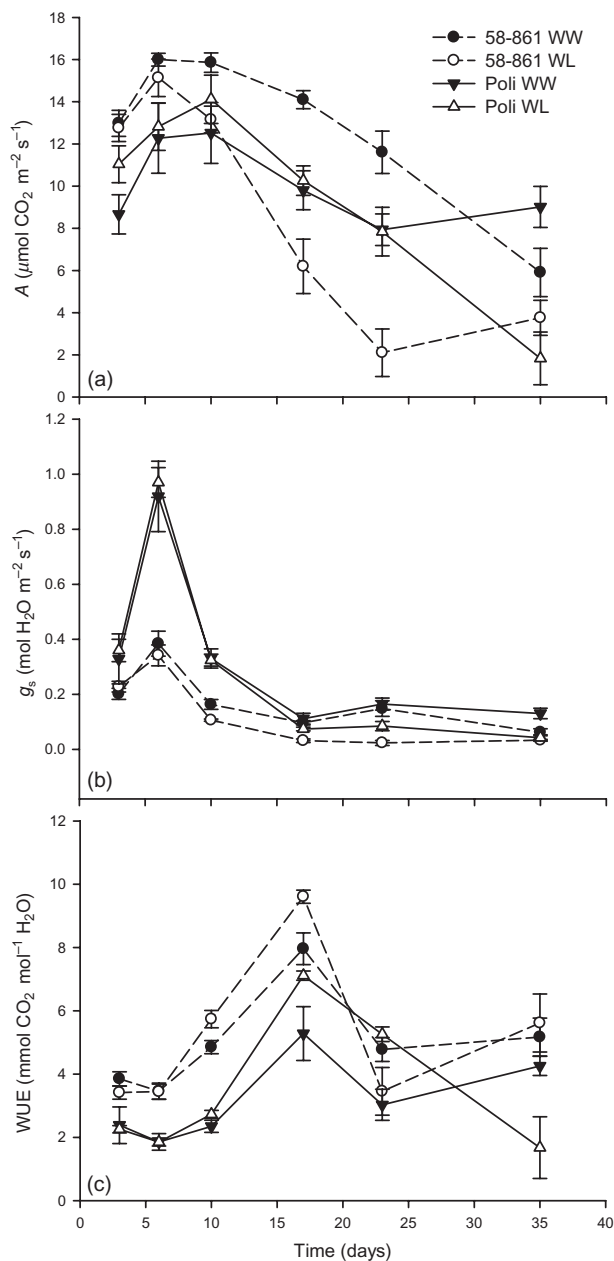
As a result of WL,  $A$  and  $g_s$  of 58-861 were already significantly reduced after 10 d of treatment and decreased

Parameter	T	W	C	T × W	T × C	W × C	T × W × C
A ( $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ )	***	***	ns	***	***	***	***
$g_s$ ( $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ )	***	***	***	***	***	ns	ns
WUE ( $\text{mmol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ )	***	ns	***	*	ns	ns	ns

ns, not significant; \* $P < 0.05$ ; \*\*\* $P < 0.001$ ;  $n = 5$ .

A, photosynthetic rate;  $g_s$ , stomatal conductance; WUE, water-use efficiency; T, time; W, watering regime; C, clone.

**Table 2.** Significance of time, watering regime, clone and interaction effects on A,  $g_s$  and WUE according to repeated measures multivariate analysis of variance



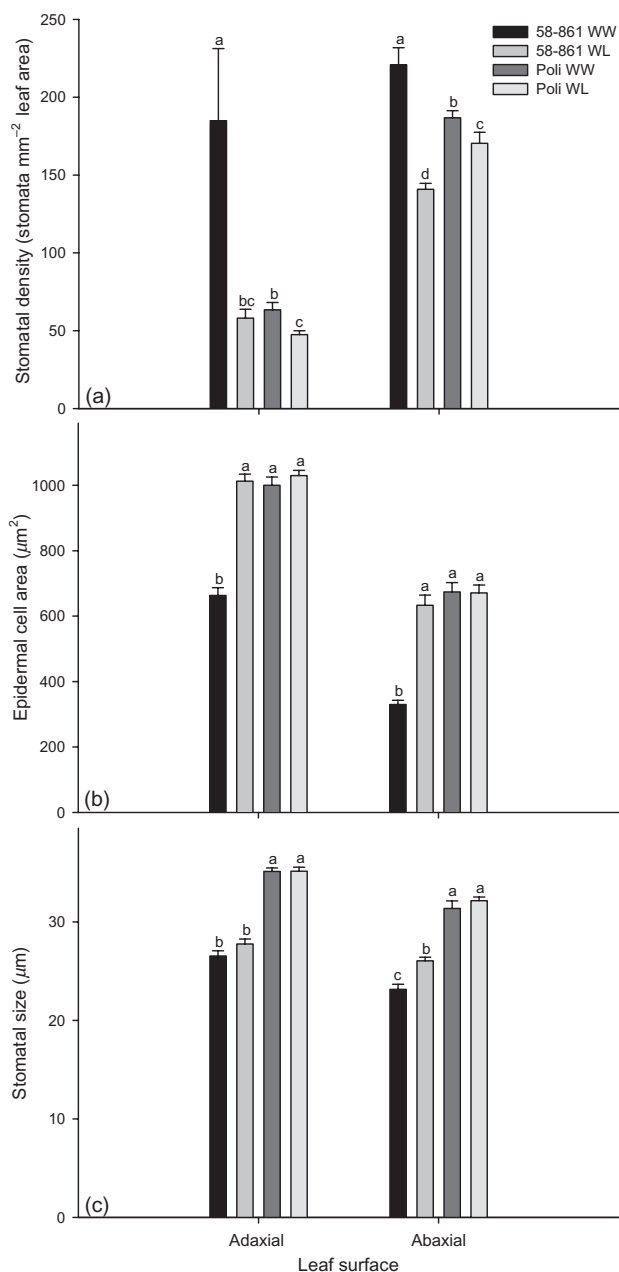
**Figure 2.** (a) Photosynthetic rate (A), (b) stomatal conductance ( $g_s$ ) and (c) water-use efficiency (WUE) of leaves of WW and WL 58-861 and Poli. WW, well watered; WL, water limited. Values are means  $\pm$  standard errors;  $n = 5$ .

further over the whole experiment. The photosynthetic rate of Poli was the same for both WW and WL plants during most of the experimental procedure, and decreased in the WL plants only at the last measurement date (35 d after initiating the treatment), while  $g_s$  was significantly affected after 3 weeks of drought. WUE of WW 58-861 plants was higher than WUE of WW Poli. As we did not find significant differences between leaves of different developmental stages of a plant, we present means of all leaves measured per clone and treatment combination.

Stomatal density, epidermal cell sizes and stomata lengths on the abaxial and adaxial leaf surfaces were analysed (Fig. 3). For WW 58-861, there was no significant difference in stomatal distribution ( $P = 0.39$ ), whereas WW Poli had significantly more stomata on the abaxial than on the adaxial surface of the leaf ( $P < 0.001$ ). Total stomatal density of WW Poli leaves was lower compared to leaves of WW 58-861. Both clones reduced stomatal density on both surfaces of leaves that developed during the WL treatment, whereas the difference was much bigger for 58-861 than for Poli. Stomatal ratio of adaxial : abaxial epidermis of 58-861 decreased from  $0.85 \pm 0.18$  in WW plants to  $0.40 \pm 0.03$  in WL plants. The stomatal ratio of Poli was less affected, namely it was  $0.35 \pm 0.02$  in WW plants and  $0.28 \pm 0.01$  in WL plants. WW 58-861 had significantly smaller epidermal cells than WW Poli, but WL treatment resulted in significantly enlarged epidermal cells of 58-861, reaching values comparable with WW and WL Poli. The stomata of Poli were significantly longer than of 58-861 in both treatments. The leaf thickness of Poli was significantly higher than of 58-861 ( $P < 0.01$ ), while the leaf size of 58-861 greatly exceeded the leaf size of Poli (Fig. 4). WL did not affect the leaf area of either clone (Cocozza, unpublished results).

### Effects of WL on the carbohydrate content in leaves and roots

Total non-structural carbohydrate (TNC, here defined as the sum of starch and the sugars sucrose, glucose and fructose) content of WW plants was higher in all analysed tissues of 58-861 compared to Poli, and in both clones TNC was higher in leaves than in roots (Table 3). Starch was also higher in WW 58-861 than in WW Poli, and in both clones higher in leaves than in roots. The WL treatment had different effects on the two clones. In 58-861, TNC decreased because of WL, whereas in Poli, TNC increased during WL. In WL Poli, starch content was significantly increased in roots and old leaves as compared to WW plants. In contrast,



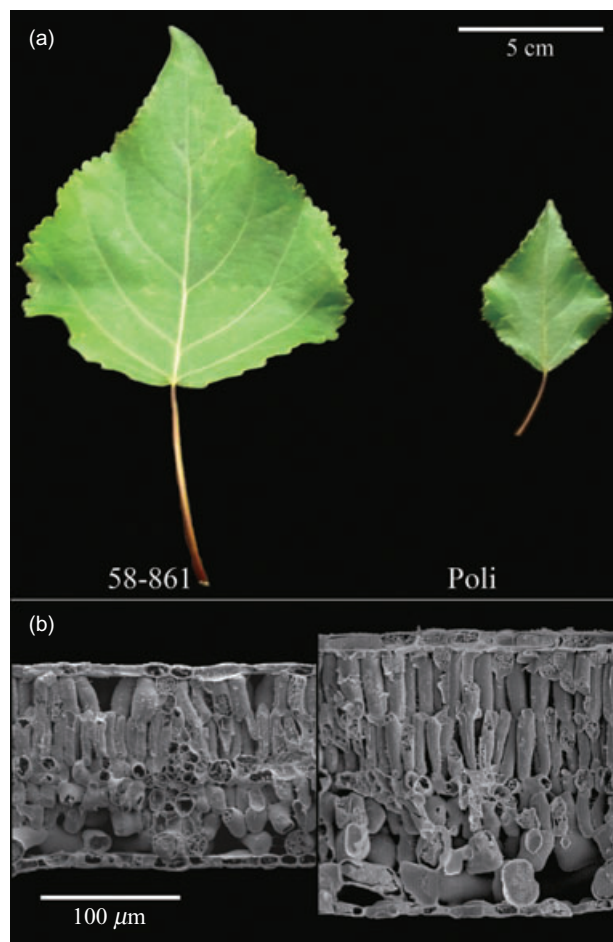
**Figure 3.** (a) Stomatal density, (b) epidermal cell size and (c) stomatal size (length) on the adaxial and abaxial leaf surfaces of well-watered (WW) and water-limited (WL) 58-861 and Poli. All values are mean  $\pm$  standard errors;  $n = 10$ . Means indicated by the same letter for each leaf surface are not significantly different ( $P \geq 0.05$ ).

WL 58-861 plants contained less starch than the WW plants. Starch amount was highest in the roots of WL Poli, while WL 58-861 plants still had a higher starch amount in the leaves than in roots. Overall, both clones showed reduced soluble sugar concentrations when grown under WL. Because of WL, sucrose, glucose and fructose significantly decreased in old leaves of both clones, while in young leaves, the three analysed sugars were only reduced in

58-861. In the roots, WL Poli had a lower amount of soluble sugars than the WW plants ( $P < 0.01$ ), while in the clone 58-861 the root sugar concentration tended to increase compared to WW plants ( $P = 0.068$ ).

### Changes in starch-degrading enzyme activities in roots induced by WL

Native polyacrylamide gel electrophoresis (PAGE) was conducted using gels containing amylopectin or  $\beta$ -limit dextrin in order to evaluate differences in glucan-hydrolysing enzymes. We found that amylolytic activity was higher in roots of WL than of WW 58-861 plants with a strong induction of band A3 on gels containing amylopectin (Fig. 5a). This correlates with the depletion of starch in roots of WL 58-861 compared to WW plants. In roots of Poli, amylopectin gels revealed a slightly higher activity of starch-degrading enzymes in WW plants. On gels containing  $\beta$ -limit dextrin, band A3 was not visible (Fig. 5b) suggesting that it is a  $\beta$ -amylase.



**Figure 4.** (a) Leaves of 58-861 (left) and Poli (right). (b) Representative leaf cross-sections of 58-861 (left) and Poli (right).

**Table 3.** Starch, soluble sugar (sucrose, glucose and fructose) and total non-structural carbohydrate (TNC, here defined as the sum of starch and the sugars glucose, fructose and sucrose) content in young and old leaves, and roots of *Populus nigra* clones Poli and 58-861 after 5 weeks of drought treatment

Parameter	Clone				ANOVA		
	58-861		Poli		Watering regime (W)	Clone (C)	W × C
	WW	WL	WW	WL			
<b>Young leaves (mg g<sub>DW</sub><sup>-1</sup>)</b>							
Starch	84.3 ± 16.4 a	77.6 ± 38.0 ab	16.3 ± 2.0 bc	28.1 ± 2.8 ab	ns	*	ns
Sucrose	32.1 ± 3.0 a	20.4 ± 1.2 b	18.0 ± 2.2 b	11.9 ± 1.8 bc	**	**	ns
Glucose	2.7 ± 0.1 a	1.8 ± 0.3 a	2.2 ± 0.2 a	1.7 ± 0.2 ab	*	ns	ns
Fructose	1.5 ± 0.1 a	0.9 ± 0.0 b	1.0 ± 0.1 b	0.8 ± 0.1 b	**	**	*
TNC	120.5 ± 13.1a	100.6 ± 38.3 a	37.5 ± 4.4 ab	42.5 ± 3.2 ab	ns	**	ns
<b>Old leaves (mg g<sub>DW</sub><sup>-1</sup>)</b>							
Starch	134.5 ± 10.1 a	78.5 ± 22.1 a	18.7 ± 3.3 c	34.7 ± 2.7 ab	ns	***	*
Sucrose	33.2 ± 2.5 a	19.6 ± 2.6 b	16.5 ± 0.9 b	10.0 ± 0.1 c	**	***	ns
Glucose	1.6 ± 0.1 ab	1.0 ± 0.2 b	2.5 ± 0.2 a	1.1 ± 0.0 b	***	**	*
Fructose	0.9 ± 0.1 b	0.5 ± 0.1 c	1.2 ± 0.1 a	0.5 ± 0.0 c	***	*	ns
TNC	170.1 ± 10.5 a	99.4 ± 19.8 b	38.9 ± 2.8 bc	46.3 ± 2.8 b	*	***	**
<b>Root (mg g<sub>DW</sub><sup>-1</sup>)</b>							
Starch	46.9 ± 6.1 a	23.9 ± 1.0 b	13.3 ± 2.4 c	41.6 ± 2.2 a	ns	ns	***
Sucrose	4.7 ± 1.4 a	8.1 ± 1.0 a	5.6 ± 0.4 a	2.7 ± 0.3 b	ns	*	**
Glucose	0.9 ± 0.3 a	1.3 ± 0.3 a	0.5 ± 0.1 a	0.2 ± 0.0 b	ns	*	ns
Fructose	0.4 ± 0.1 a	0.9 ± 0.2 a	0.4 ± 0.1 a	0.1 ± 0.0 b	ns	*	*
TNC	52.9 ± 7.6 a	34.1 ± 0.5 ac	19.9 ± 2.8 c	44.6 ± 2.4 ab	ns	*	**

ns, not significant; \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ;  $n = 5$ .

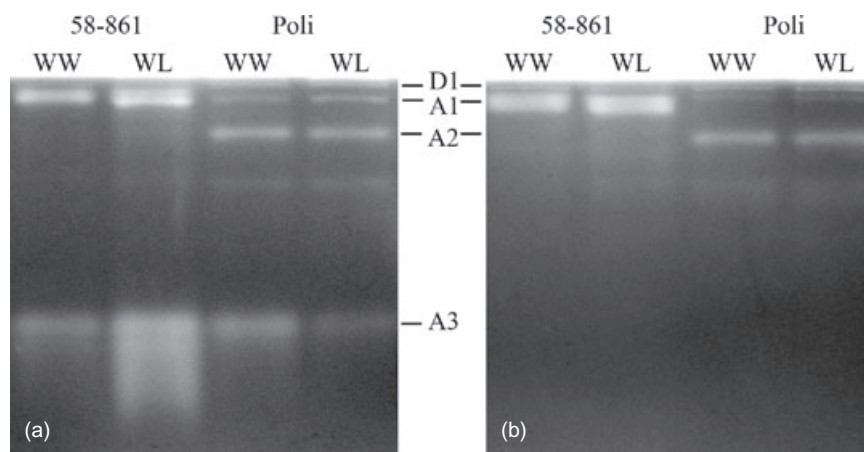
Mean values ± standard errors followed by the same letter within a line are not significantly different. Data were analysed with two-way ANOVA.

ANOVA, analysis of variance; WW, well watered; WL, water limited.

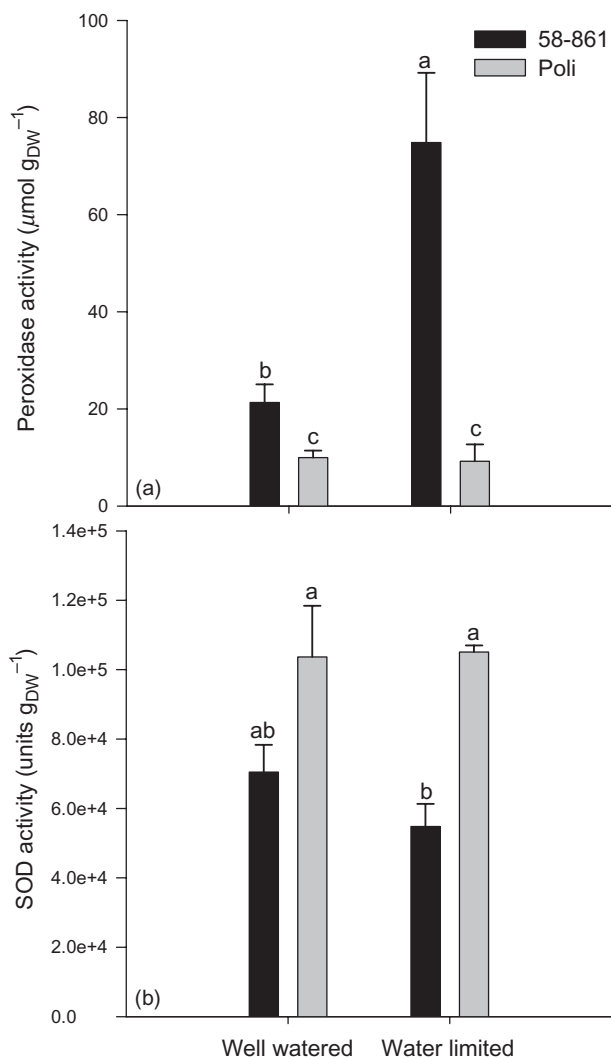
### Changes in ROS-detoxifying enzyme activities in roots induced by WL

Total peroxidase activity was significantly induced in roots of WL 58-861 plants compared to WW 58-861 ( $P < 0.05$ ; Fig. 6a), whereas in Poli, the WL treatment did not have an

effect on peroxidase activity. Peroxidase activity in both treatments was significantly lower in roots of Poli than in roots of 58-861. WL did not have an effect on the activity of SOD in roots of either clones, but overall, SOD activity was significantly higher in roots of Poli than in roots of 58-861 ( $P < 0.01$ ; Fig. 6b).



**Figure 5.** Protein extracts from roots of Poli and 58-861 were separated by native polyacrylamide gel electrophoresis (PAGE) on gels containing amylopectin (a) or  $\beta$ -limit dextrin (b). After incubation, starch-degrading enzyme activities were visualized using iodine staining. Representative samples of three replicates are shown on the gels. D1, putative debranching enzyme activity; A1–A3, putative amylase activities. WW, well watered; WL, water limited.



**Figure 6.** (a) Peroxidase activity in roots of WW and WL Poli and 58-861. (b) Superoxide dismutase (SOD) activity in roots of WW and WL Poli and 58-861. WW, well watered; WL, water limited. All values are mean  $\pm$  standard errors;  $n = 3$ . Means indicated by the same letter are not significantly different ( $P \geq 0.05$ ).

### Effects of WL on expression of genes involved in starch metabolism and oxidative stress defence

WL up-regulated the expression of genes involved in starch synthesis (*ADG1*, *ISAI*, *PGM*, *BEII* and *SSII*) in roots of both clones (Table 4). Expression of two genes encoding proteins involved in starch degradation (*BAM3* and *DPEI*) did not show a consistent pattern. Transcripts of *BAM3* were induced nearly twofold in roots of WL 58-861 plants, while expression of *BAM3* was not changed in roots of Poli because of WL. *DPEI* was repressed in WL 58-861, but up-regulated in Poli.

In accordance with total peroxidase activity, we showed that expression of ascorbate peroxidase was about 2.5 times higher in roots of 58-861 than in roots of Poli, but in contrast

to enzyme activity, *APX* gene expression was not induced by drought. Analysis of *SOD* gene expression supported the findings of the SOD assay, which revealed higher SOD activity in roots of Poli than of 58-861. Relative expression of *SOD* was 9.2- and 4.5-fold higher in roots of Poli than of 58-861 in the WW and the WL treatment, respectively (Table 4).

We analysed a larger set of genes. Here, we present data only for those genes, which yielded reliable results.

## DISCUSSION

### WL differentially affects growth, gas exchange and leaf morphological characteristics of 58-861 and Poli

Drought tolerance is often linked with lower productivity (Tschaplinski *et al.* 1998; Zhang, Wu & Li 2005; Lambs *et al.* 2006; Monclus *et al.* 2006). The first indicator of potential drought tolerance in Poli is the decreased plant height, stem diameter and total biomass of Poli compared with 58-861. Growth of 58-861 was inhibited by WL, while Poli was not affected. Radial growth of WL 58-861 tended to decrease earlier than height growth, which confirmed previous findings that secondary growth is more sensitive to water shortage than height growth (Bogeat-Triboulot *et al.* 2007). Stem and root biomass of WL 58-861 were significantly reduced. Contrasting to previous studies (Lei *et al.* 2006; Bogeat-Triboulot *et al.* 2007), root/shoot ratio of WL 58-861 tended to decrease compared to WW plants. However, it has been shown that carbon and biomass partitioning can be affected by pot size (McConnaughay, Nicotra & Bazzaz 1996), and although the pots used in our study should have had sufficient size, we should not attach too much importance on biomass partitioning when using pots. It is well known that reduced photosynthetic rates lead to reduction of plant growth (McCree 1986). Photosynthetic rate of 58-861 was affected much more strongly by drought than that of Poli, which might be a reason for reduced growth of WL 58-861. This was likely caused by higher water consumption of 58-861 compared to Poli, and therefore leading to faster soil desiccation between watering. Leaf gas exchange was rapidly affected in 58-861, presumably resulting from stomatal closure – one of the first responses of leaves to WL. Assimilate accumulation in leaves has been shown to reduce photosynthetic rate in many plant species, which might explain the decrease of photosynthesis in leaves of WL Poli at the end of the experiment (Goldschmidt & Huber 1992; Iglesias *et al.* 2002; Proietti 2003). We also found that stomatal density of WW Poli was lower than that of 58-861, which is known to be another mechanism for avoiding water loss. Reduced stomatal density of WL 58-861 might have been achieved by enlarging epidermal cell size in response to drought, as has been observed by Yu *et al.* (2008) in *Arabidopsis*. The stomata of Poli were significantly longer than those of 58-861. For tomatoes, it also has been shown that drought-tolerant cultivars have less but larger stomata than drought-susceptible cultivars

Gene	58-861		Poli	
	WW	WL	WW	WL
<b>Starch metabolism</b>				
<i>BAM3</i>	1.0 ± 0.1 b	1.9 ± 0.2 a	1.4 ± 0.2 ab	1.5 ± 0.5 ab
<i>DPE1</i>	2.7 ± 0.4 a	1.7 ± 0.2 b	1.0 ± 0.1 c	1.6 ± 0.2 b
<i>ADG1</i>	1.9 ± 0.1 a	2.3 ± 0.4 a	1.0 ± 0.4 b	2.1 ± 0.2 a
<i>BE11</i>	1.0 ± 0.1 b	1.8 ± 0.3 a	1.0 ± 0.1 b	1.5 ± 0.2 a
<i>ISAI</i>	1.0 ± 0.3 c	1.8 ± 0.4 a	1.3 ± 0.4 abc	1.4 ± 0.1 ab
<i>PGM</i>	1.5 ± 0.2 b	2.0 ± 0.4 a	1.0 ± 0.2 c	1.7 ± 0.2 ab
<i>SSII</i>	1.2 ± 0.2 ab	1.2 ± 0.6 ab	1.0 ± 0.2 b	1.7 ± 0.2 a
<b>Oxidative stress defence</b>				
<i>APX</i>	2.5 ± 0.4 a	2.7 ± 0.2 a	1.0 ± 0.1 c	1.3 ± 0.2 b
<i>CAT</i>	1.2 ± 0.1a	1.5 ± 0.1 a	1.4 ± 0.2 a	1.0 ± 0.1 b
<i>GR</i>	1.3 ± 0.1 ab	1.1 ± 0.1 b	1.0 ± 0.1 b	1.4 ± 0.2 a
<i>SOD</i>	1.0 ± 0.2 c	1.1 ± 0.2 c	9.2 ± 1.2 a	4.5 ± 1.2 b

Mean values ± standard errors, normalized to the lowest value for each gene are given for three replicates. Values followed by the same letter for each gene are not significantly different ( $P \geq 0.05$ ).

(Kulkarni & Deshpande 2006). Moreover, Poli had a very low stomatal density on the adaxial leaf surface, which is in accordance with the fact that plants growing in hot and dry climates mostly have stomata only on the abaxial leaf surface to reduce transpiration. Poli had significantly smaller and thicker leaves than 58-861, a common adaptation of plants originating from xeric environments compared with the same species growing in mesic environments (Kubiske & Abrams 1992).

#### Effects of WL on root carbohydrate metabolism: carbohydrate levels increase in Poli and decrease in 58-861 during drought

Carbohydrate metabolism of drought-stressed tree roots has not been described previously. Our analyses at the gene, protein and metabolite level show that there are changes in carbon assimilation in leaves and carbohydrate levels in roots in response to drought. We observed large differences between the clones. Concentrations of soluble sugars increased in roots of WL 58-861 compared to WW plants, despite the fact that TNC had decreased. In the leaves, sugar concentrations in WL plants were significantly lower than in WW plants. These results contrast with previous studies, which found an accumulation of soluble sugars, and suggested that they serve a role in osmotic adjustment in response to drought (Tschaplinski & Tuskan 1994; Marron *et al.* 2002). In this case, we suggest that in 58-861, drought-induced inhibition of carbon assimilation causes the large decrease of TNC (including starch) concentrations, and that osmotic adjustment through sugar accumulation either could not occur or could not be sustained for the duration of the experiment. Gebre *et al.* (1998) suggested that instead of osmotic adjustment, the poplar clones used in their study might use other avoidance mechanisms like leaf abscission and stomatal control, which was also the case for 58-861. However, the elevated sugar levels in roots of WL 58-861 suggest that osmotic adjustment has occurred. It may

**Table 4.** Relative expression of genes involved in starch metabolism, oxidative stress defence and water transport in roots of well-watered (WW) and water-limited (WL) 58-861 and Poli plants obtained by quantitative RT-PCR

be that this is particularly important in roots as they are directly confronted with low soil water potential. For eucalyptus trees, it also has been shown that soluble sugars only accumulated in roots, but not in leaves of drought-stressed plants (Shvaleva *et al.* 2006). We found that starch content in WL 58-861 roots was strongly decreased as compared to WW plants. As photosynthesis was inhibited, we conclude that carbohydrate allocation to the roots was reduced, and starch reserves were degraded to maintain root respiration, as well as the concentration of soluble sugars contributing to osmotic potential. Starch-degrading enzyme activity in roots of WL 58-861 was strongly enhanced, and on gels containing  $\beta$ -limit dextrin we were able to show that the induced enzyme was a  $\beta$ -amylase. Furthermore, gene expression of *BAM3* was induced in roots of WL 58-861 plants. In contrast to other  $\beta$ -amylases with unidentified functions, *BAM3* is an isoform of  $\beta$ -amylase, which is active in starch breakdown in potato and *Arabidopsis* (Scheidig *et al.* 2002; Kaplan & Guy 2005; Fulton *et al.* 2008). Because we analysed the closest homolog to *AtBAM3* in poplar, we suggest that this gene is important for starch degradation also in poplar, and therefore expression of *BAM3* in roots of WL 58-861 plants was induced leading to higher starch break-down rates. Poli, however, did not seem to be stressed by water shortage at all. TNC and starch concentrations were higher in WL compared to WW plants in all tissues. In the roots, amyolytic activity was slightly higher in WW than in WL plants, which is in agreement with the lower starch concentration in WW roots. Possibly, Poli is adapted to drought to such an extent that it is not suited to soil water contents reaching field capacity, as applied in our study. Water saturation can reduce oxygen availability, during which roots can switch from respiration to alcoholic fermentation, which leads to a considerably higher demand for carbohydrates (Kreuzwieser, Papadopoulou & Rennenberg 2004). This might explain the significantly lower starch concentrations in WW relative to WL Poli. Interestingly, expression of most genes involved in starch synthesis was

up-regulated in roots of WL plants of both clones, whereas *ADG1*, which is thought to control the flux of carbon into starch biosynthesis (Lin *et al.* 1988; Neuhaus & Stitt 1990; Tiessen *et al.* 2002), was much more strongly induced in Poli than in 58-861. This might explain why starch accumulated only in WL Poli and not in 58-861.

### Poli has a higher potential to defend against drought-induced oxidative stress than 58-861

Total SOD activity was significantly higher in Poli than in 58-861, but we did not find any effects of WL on SOD activity in roots of either clone. Consistent with the activity measurements, qRT-PCR revealed that *SOD* gene expression was higher in roots of Poli than of 58-861, but the expression of the  $\text{Cu}^{2+}/\text{Zn}^{2+}$  *SOD* we studied was twice as high in roots of WW as of WL Poli. Thus, although total SOD activity was not influenced by drought, shifts between different isoforms could have occurred. SODs catalyse the first step in the detoxification of ROS (Alscher, Erturk & Heath 2002). Therefore, the capacity of Poli to degrade ROS is higher than that of 58-861, and might be sufficient to cope with higher superoxide generation under water shortage. Alternatively, SOD might have been induced by drought only transiently, as it has been proposed for eucalyptus (Shvaleva *et al.* 2006), and therefore not observed in our experiments. Peroxidase activity was generally higher in roots of 58-861 than in roots of Poli. Furthermore, peroxidase activity was strongly enhanced in WL 58-861, but not in WL Poli. This suggests that either generation of ROS was negligible and insufficient to induce peroxidase activity in Poli, or a phase of induction might have occurred only earlier during the experiment. In agreement with enzyme activity, also *APX* gene expression was higher in roots of 58-861, but was not further induced by drought. Therefore, we conclude that ROS production was high enough to induce peroxidase activity in roots of 58-861, but either we did not find the gene responsible for enhanced peroxidase activity, or regulation might be post-transcriptional and hence not detectable by qRT-PCR. Until now, in poplar, only one study investigated drought-induced oxidative stress in roots (Morabito & Guerrier 2000). They found that poplar roots are the most sensitive organ to oxidative stress, but drought was applied only for 12 h. We complemented their findings on anti-oxidant status of poplar roots with information about the reactions of roots after a longer drought period.

In conclusion, we were able to demonstrate that the contrasting clones used in our study reacted very differently to water deprivation. Poli exhibited several traits indicating drought tolerance, while 58-861 was severely stressed by water deficit, showing visible symptoms like wilting and leaf abscission. While carbon fixation of 58-861 was constrained, Poli may even have benefited from lower soil water content. Although photosynthesis of WL Poli was not increased, TNC accumulated in all analysed tissues. Poli had a higher capacity to detoxify ROS than 58-861, regardless of the water availability. This could be interpreted to mean that in

Poli mechanisms to cope with ROS (drought induced or otherwise) are constitutively up-regulated.

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