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Effect of Water Deficit on Expression of Stress-related Genes in the Cambial Region of Two Contrasting Poplar Clones

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Abstract In this study we investigated the effect of prolonged water shortage in the cambial region of two poplar clones, Dvina (*Populus deltoides*) and I-214 (*Populus x canadensis)* that differ in their response to water deficit. For this purpose we monitored growth parameters in Dvina and I-214 plants under well-watered and water-stress conditions and after rewatering and analyzed the content of malondialdehyde (MDA) and proline and the expression level of genes coding for antioxidant enzymes (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR) and metallothioneins (MT) by quantitative real-time RT-PCR (RT-qPCR). Water deficit resulted in a significant increase of osmotically active solutes in both clones. No significant increase of MDA level was observed in Dvina, whereas a significant enhancement of lipid peroxidation was detected in I-214, in which also a strong fivefold increase of proline was detected. Transcript analysis of stress-related genes indicated a different ability of the two clones to modulate antioxidant genes under stress. In particular, *MT3b* was strongly upregulated by water deficit in Dvina thus suggesting an important role for this gene in the maintenance of cellular redox homeostasis in the clone. On the whole, the two clones exhibited changes at the transcription and physiological levels in the cambial region that suggest the occurrence of different strategies of plant protection from prolonged water deficit.

Keywords Poplar · Drought tolerance · Cambium · Antioxidant genes · Metallothionein

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Introduction

Poplar (*Populus* sp.) is of primary economic importance for the production of wood in temperate regions of the world. Long-lasting periods of drought strongly limit radial growth of plants, decreasing productivity of poplar plantations and wood quality. Although poplars are usually known as one of the most drought-sensitive woody plant groups (Tschaplinski and Tuskan 1994), their drought tolerance varies greatly among species, populations, and clones due to their high genetic diversity (Monclus and others 2006). The adaptive responses to water deficit of different poplar genotypes include morphological, physiological, and biochemical changes, such as changes in growth rate, stomatal conductance, tissue osmotic potential, and antioxidant defense (Kozlowski and Pallardy 2002; Zhang and others 2005). In a previous study, the two poplar clones Dvina (*Populus deltoides*) and I-214 (*Populus* × *canadensis*) displayed different responses to water deficit. Under limited water availability Dvina maintained stem growth longer than I-214 and had lower leaf abscission at the end of the drought period. After irrigation resumed, Dvina showed greater ability to restore stem growth (Giovannelli and others 2007). These two poplar clones, distinct for their response to water deficit, appear a suitable model to improve knowledge of the molecular mechanisms underlying drought tolerance.

Several recent studies have dealt with the identification of genes induced under drought stress in specific poplar clones. They focused primarily on transcriptome and proteome changes in roots and leaves (Brosché and others 2005; Plomion and others 2006; Bogeat-Triboulot and others 2007; Bonhomme and others 2009; Regier and others 2009; Xiao and others 2009), whereas, with a few exceptions, the regulation of genes in the poplar cambial region under water deficit has received little attention (Berta and others 2006, 2009, 2010). The vascular cambium is a secondary meristem that plays a major role in the radial growth of gymnosperm and angiosperm stems and roots. It produces secondary phloem on the outside and secondary xylem, or wood, on the inside. Cambial activity ensures the perennial life of trees through the regular renewal of functional xylem and phloem. Wood characteristics are due to cambium division, cell expansion, and secondary wall production. Drought strongly affects radial increment and xylem anatomy (Corcuera and others 2004; Arend and Fromm 2007). Until now no study has dealt with the effect of genotype on the regulation of genes involved in drought tolerance in the cambial region of poplar.

Osmotically active substances play a crucial role in plant adaptation to water stress, limiting cell dehydration (Kozlowski and Pallardy 2002) and preserving enzyme structure and activity (Samuel and others 2000). Osmotic adjustment involves the synthesis and accumulation of small solutes (osmolytes) such as proline, glycine betaine, sugars, and some inorganic anions (Chaves and others 2003). In poplar leaves and roots osmotically active substances accumulate under water-limited conditions (Lei and others 2006; Regier and others 2009). In addition, drought may induce oxidative stress through the production of reactive oxygen species (ROS) (Foyer and Noctor 2005; Mittler 2006; Miller and others 2008). ROS are highly toxic and can damage many important cellular components such as lipids, proteins, DNA, and RNA (Foyer and others 1994). The main scavengers of oxygen radicals are superoxide dismutase (SOD), catalase (CAT), and the enzymes of the ascorbate-glutathione pathway, such as ascorbate peroxidase (APX) and glutathione reductase (GR) (Foyer and Halliwell 1976; Asada 1999). Recently, a putative role in the defense against oxidative stress has been suggested also for metallothioneins (MTs), a family of low-molecular-weight cysteine-rich proteins (Xue and others 2009; Samardžić and others 2010). The importance of osmotic adjustment and the contribution of antioxidant and MT genes to drought tolerance in the poplar cambial region is still unknown and remains to be clarified.

In previous studies on white poplar (Berta and others 2006, 2009, 2010), genes coding for antioxidant enzymes such as SOD, CAT, APX, and GR and for MT type 3 were shown to be differentially regulated by water deficit within the cambial region. In this work we analyzed the effect of a severe water deficit on plant water relations, cellular membrane injury, occurrence of active osmotic adjustment, proline content, and expression patterns of stress defense genes (*Sod*, *Cat*, *Apx*, *Gr*, *MT*) in the cambial region of the two clones Dvina and I-214, with different responses to water deficit, to highlight molecular and metabolic differences that could account for their different sensitivities to stress.

Materials and Methods

Plant Material and Experimental Design

In early June 2009, 64 two-year-old plants of *Populus deltoides* clone Dvina and *Populus x canadensis* Moench clone I-214 were selected for dimensional uniformity (mean height = 144.6 ± 12.3 cm for I-214 and 133.5 ± 10.5 cm for Dvina, mean stem diameter at ¼ of stem height = 17.1 ± 0.6 mm for I-214 and 16 ± 1.1 mm for Dvina, average total leaf area per plant = 77.36 ± 3.8 dm^2 for I-214 and 72.2 ± 3.2 dm^2 for Dvina). Plants were grown in 20-L plastic pots filled with a mixture of peat-sand-perlite (50/40/10 v/v, pH 6.8), fertilized with a commercial slow release fertilizer (18:18:18, N-P-K), and irrigated every second or third day to maintain soil moisture to field capacity.

Each genotype was divided into two homogeneous groups of 32 plants each: at the beginning of the experiment (T0), plants were subjected to different watering regimes for 30 days. In watered plants (Pw), soil moisture was maintained to field capacity (soil water capacity [SWC] = 28–30%), whereas in stressed plants (Pst), watering was suspended for 15 days (Tmax) and then resumed for up to 30 days (Trec). During the experiment, plants were maintained in a nursery in the open air. To avoid soil rehydration by rainfall, the pots of Pst plants were covered with water-repellent plastic covers. During the experiment the mean temperature ranged between 16 and 26°C, with a value of 34°C as the maximum recorded at midday (3 pm) on day 20 (air temperature recorded at LAMMA, Laboratory of monitoring and environmental modeling for sustainable development, Florence, http://www.lamma.rete.toscana.it/eng/index.html). The daily mean of vapor pressure deficit ranged between 0.6 and 2 kPa, whereas the daily global radiation ranged between 13 and 28 MJ/m^2.

Soil–Plant Water Relations and Growth Measurements

Predawn leaf water potential (Ψ_{pd}, MPa) was measured with a pressure chamber (PMS Instruments Co., Corvallis, OR, USA) on two to three fully expanded leaves (leaf

plastochrone index between 5 and 7) collected from randomly selected shoots. At the beginning of the experiment (day 0) and days 15 and 30, four plants from each clone and treatment were used to determine stem water content, calculated following the procedure described in Deslauriers and others (2009). Stem pieces 10 cm long were immediately weighed within 15 min after harvest to determine the fresh mass (g). The fresh volume of samples (cm^3) was assessed by water displacement (Borghetti and others 1991). The dry mass (g) was measured after the samples were maintained at 72°C for 96 h. Relative stem water content (RWCstem) was calculated following Domec and Gartner (2001):

$$\text{RWCstem} = (M_f - M_d)/(V_f - V_s) \times 100$$

where M_f and M_d are the fresh and dry mass of the wood (g), respectively, and V_f and Vs are the volumes of fresh and solid material (cm^3), respectively. V_s was estimated by dividing M_d by 1.53, assuming a density of 1.53 g cm^{-3} for dry cell wall material (Skaar 1988).

Soil water content (SWC) was measured every 2 days for each replicate in the Pw and Pst plants by Hydro-sense probes (Campbell Scientific Inc., Logan, UT, USA) based on time domain reflectometry (TDR). Stem radial growth was determined by point dendrometers as already reported elsewhere (Giovannelli and others 2007). Raw data were recorded every 15 min and hourly averages were calculated. The extraction of maximum daily shrinkage (MDS) and daily stem increment (ΔR) was performed by dividing the stem cycle into three distinct phases (Downes and others 1999; Deslauriers and others 2003) and identifying (1) the expansion phase, total period from the minimum to the following morning maximum; (2) the stem radius increment phase, part of the expansion phase from the time when the stem radius exceeds the morning maximum until the subsequent maximum; and (3) the contraction phase or MDS, period between morning maximum and afternoon minimum.

Cambial Region Collection

The cambial region was extracted following the procedure of Berta and others (2010). Stems are cut at the collar, divided into logs 10–15 cm in length, and immediately immersed in liquid nitrogen and freeze-dried at a constant temperature of −50°C under vacuum (0.15 mbar or less). The cambial region with the differentiating phloem was gently scrapped with a razor blade from the inner side of the bark. From 200 to 500 mg of dried powder was collected from each sample.

Determination of Malondialdehyde Content

Lipid peroxidation in the cambial region of Pst and Pw plants was determined by measuring the amount of MDA using the thiobarbituric acid (TBA) method described by Zhang and others (2007) with modifications to the original protocol. The powdered cambial region (10 mg) was homogenized in 1 ml of 20% (w/v) trichloroacetic acid (TCA) and 0.5% (w/v) thiobarbituric acid solution. The mixture was incubated in boiling water for 30 min, and the reaction was stopped by placing the reaction tubes in an ice bath. The cooled mixture was centrifuged at 5,000 *g* for 10 min and the absorbance of the supernatant was measured at 532 nm, subtracting the value for nonspecific absorption at 600 nm. The amount of MDA-TBA complex (red pigment) was calculated from the extinction coefficient of 155 mM^{-1} cm^{-1} (Kosugi and Kikugawa 1985).

Determination of Osmotically Active Solutes and Proline Content

The concentration of osmotically active solutes in the cambial region was determined following the procedure proposed by Arend and Fromm (2007). Four milligrams of dried powder from the cambial region was extracted with 0.25 ml of double distilled water. After centrifugation at 20,000 *g* for 3 min, the concentration of osmotically active solutes was measured with a freezing-point osmometer equipped with a 15-µl measuring cell (Osmomat 030, Gonatec, Germany).

Proline content was assessed colorimetrically using the protocol of Bates and others (1973). The lyophilized powdered cambial region (30 mg) was homogenized in 1 ml of 3% (w/v) aqueous sulfosalicylic acid solution and the homogenate was centrifuged at 5,000 *g* for 5 min. The supernatant was reacted with 1 ml acid ninhydrin (2.5 g ninhydrin/100 ml of a solution containing glacial acetic acid, distilled water, and *ortho*-phosphoric acid 85% at a ratio of 6:3:1) and 1 ml of glacial acetic acid in a test tube for 1 h at 100°C, and the reaction was terminated in an ice bath for 5 min. The reaction mixture was extracted with 1 ml toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm. The proline concentration was determined from a standard curve and calculated on a dry weight basis (µmol g^{-1} DW).

RNA Extraction and First-strand cDNA Synthesis

Total RNA was extracted from 100 mg of the powdered cambial region according to the extraction protocol of Kolosova and others (2004), treated with Rnase-Free Dnase Set (Qiagen, Hilden, Germany), and purified using columns from an RNase Clean-up kit (Qiagen). The RNA concentration was spectrophotometrically measured using Gene Quant Pro (Amersham Biosciences, Milan, Italy) and RNA quality was verified by visualization on ethidium

bromide agarose gels. First-strand cDNA synthesis was performed from 5 μg total RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and priming the reaction with oligo(dT)$_{15}$. The quality of the first-strand cDNA was tested via PCR using primers for elongation factor 1 and eukaryotic initiation factor 4B (Table 1). Absence of genomic DNA in the samples was verified by choosing an intron-spanning amplicon for eukaryotic initiation factor 4B (Table 1).

Primer Design and Quantitative Real-time RT-PCR (RT-qPCR)

All primer sequences used in this study are listed in Table 1. Primers for *CuZnSod*, *MnSod*, *Cat1*, and *Gr* were designed using the Primer3 software (http:/frodo.wi.mitedu/primer3) (Rozen and Skaletzki 2000) and downloading gene sequences from the *Populus trichocarpa* v1.1 database (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html) (Tuskan and others 2006). For the amplification of *Cat3*, *Apx*, *MT3a*, and *MT3b* and for the reference genes *EF1*, *EIF4B*, and *GαS* we used previously published primer pairs (Berta and others 2006, 2009, 2010; Brunner and others 2004; Bogeat-Triboulot and others 2007; Regier and others 2009). The identity of amplicons was verified by nonquantitative PCR and by sequencing on an ABI 3130XL instrument (Applied Biosystems, Foster City, CA, USA). Samples were diluted to a final volume of 100 μl, and 1 μl was used as a template for the RT-qPCR in a reaction of 15 μl of total volume. Reactions were carried out using the iQ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) on an iCycler iQ Real Time PCR System (Bio-Rad). One cDNA sample diluted to 1/5, 1/25, 1/125, 1/625, and 1/3125 was employed as a standard to calculate the amplification efficiency for each primer pair. PCR reactions were performed under the following conditions: 2 min at 95°C and 40 cycles of 30 s at 95°C, 20 s at 60°C, and 20 s at 72°C. Melting curve analysis confirmed the specificity of amplification. The relative mRNA levels for each gene were calculated with qBase software (Hellemans and others 2007) with respect to the internal standards *EF1*, *EIF4B*, and *GαS*. The gene expression stability of reference genes was previously evaluated using geNorm software (Vandesompele and others 2002) and qBase software (data not shown).

Statistical Analysis

The hypotheses testing was performed by ANOVA using STATA 9 software (STATA Corp, College Station, TX, USA). A linear regression model and a quadratic model were fitted to the experimental data on time (T0, Tmax, and Trec) to test the hypothesis of no differences between average values within each treatment and clone.

Table 1 Sequences of the primers used for RT-qPCR

Gene	Abbr.	Forward primer	Reverse primer	JGI gene model ID
CuZn superoxide dismutase	*CuZnSod*	CCTGAGAAGCATAAGGAACAGTG	TCCATGGTCCTTCTTTAATGG	estExt_Genewise1_v1.C_LG_XIII1983
Mn superoxide dismutase	*MnSod*	ACTCCAAGCACACCTGCTCT	CGGCGGAACAAATAGGAGTA	grail3.0095006301
Catalase 1	*Cat1*	TGAAGACGACACCAAAGCTC	AACTGCAAACCACACAGCAG	eugene3.00020082
Catalase 3	*Cat3*	GTCTCAGGCTGATAAATCTTTG	ATTCAGTTTGGTGTCATCTTC	estExt_fgenesh4_pm.C_LG_V0695
Ascorbate peroxidase	*Apx*	AGCTCTCCGAACTGGGATTT	CTCTTCCACCCATCATGAGC	estExt_fgenesh4_pg.C_LG_IX1399
Glutathione reductase	*Gr*	GATGCACCTGAAATCGTGCAG	GGTTTGCCACCAGCAGTAAC	grail3.0008003701
Metallothionein 3°	*MT3a*	TGTCTAGCACCTGCGACAAC	CACATGACGGTTTACGTGCT	eugene3.00110909
Metallothionein 3b	*MT3b*	CTGCACATGCGGTCATTAAG	GTTCACTCCACACCCACAAGT	eugene3.01070053
Elongation factor 1, β-subunit	*EF1*	AAGCCATGGGATGATGAGAC	ACTGGAGCCAATTTTGATGC	estExt_fgenesh4_pg.C_LG_I1178
Eucaryotic translation factor 4B	*EIF4B*	AAAAAGGGGATTTGGGATTG	AACTTCGTCCTCGGTAGCAA	grail3.0001032101
Glucosidase II α-subunit	*GαS*	CTCTCATTGAGCCGGCAAAT	CCCCCCTTCAAGCATAAGG	gw1.VII.1603.1

Results

In the present study, water deficit was imposed for 15 days, followed by a rewatering period of 15 days. The decrease of soil water content (80% water content reduction with respect to the field capacity) significantly affected the leaf and stem water status and growth of Pst plants. However, complete recovery of plant water status and stem growth were reached after 15 days after the resumption of irrigation, as the stress, even if severe, was not sufficient to induce irreversible damage (Table 2). After 15 days (Tmax) of withholding irrigation, leaf Ψ_{pd} of treated plants decreased to values below −1.5 MPa. The leaf Ψ_{pd} decrease was associated with a significant increase in stem water loss (>10% of RWC reduction in Pst with respect to Pw plants in both clones) that gave rise to an increase in maximum daily shrinkage of the stem (Pst plants showed an MDS 10 times higher than control ones) with no significant differences between clones ($P = 0.562$). As a consequence at Tmax, Pst plants showed a significant reduction in daily stem increment (ΔR negative values for both clones). After resumption of the irrigation, SWC of treated plants was maintained at field capacity for 15 days. At Trec, treated plants had similar leaf Ψ_{pd}, RWC, ΔR and MDS, showing a complete recovery of plant water status and growth.

Determination of Malondialdehyde Content

The effect of water deficit on lipid peroxidation of cellular membranes in the cambial region of Dvina and I-214 was estimated by measuring MDA content in stressed and control plants collected at different times. We did not detect a significant increase in levels of lipid peroxidation in plants of Dvina during the time course of the experiment; on the contrary, significant enhancement of MDA was observed in I-214 (Fig. 1) However, the clones exhibited different contents of MDA. In fact, I-214 displayed a MDA content twofold higher than Dvina, independent of treatments (Tables 3, 5).

Determination of Osmotically Active Solutes and Proline Content

In this study, osmotically active solutes and proline content were analyzed in the cambial region of water-stressed (Pst) and control (Pw) plants at Tmax of Dvina and I-214 clones. In parallel with the decrease in predawn leaf water potential and stem water content, the concentration of osmotically active solutes showed a significant increase in Pst plants, without significant differences between clones. Free-proline increased significantly in Pst plants with respect to control (Pw) plants at Tmax, fivefold in Dvina and more than tenfold in I-214 (Tables 3, 5; Fig. 1).

Transcription Level of Genes Involved in Stress Defense

The expression levels of eight genes coding for proteins (SOD, CAT, APX, GR, and MT) involved in stress response were analyzed in the cambial region of Dvina and I-214 under water deficit conditions and after a period of

Table 2 Predawn leaf water potential (Ψ_{pd}), soil water content (SWC), relative stem water content (RWCstem), daily radial stem variation (ΔR), maximum daily shrinkage (stem MDS) measured at the beginning of the experiment (T_0), 15 days after withholding irrigation (Tmax), and 15 days after resumption of the irrigation (Trec)

	T0	Tmax[a]		Trec	
	Control (Pw)	Control (Pw)	Stress (Pst)	Control (Pw)	Stress (Pst)
I-214					
Leaf Ψ_{pd} (MPa)	−0.28 (0.02)	−0.23 (0.02)	−1.45 (0.70)**	−0.30 (0.04)	−0.25 (0.05)
SWC (%)	31.0	29.0	5.5**	29.0	28.0
Stem RWC (%)	67.2	64.4	57.6**	56.9	56.3
Stem ΔR (μm)	25.3 (1.7)	27.8 (1.1)	−24.6 (2.0)**	20.2 (2.3)	21.7 (3.4)
Stem MDS (μm)	−5.4 (0.4)	−6.2 (1.3)	−48.2 (3.5)**	−5.2 (2.5)	−9.7 (1.2)
DVINA					
Leaf Ψ_{pd} (MPa)	−0.40 (0.08)	−0.18 (0.02)	−1.36 (0.17)**	−0.20 (0.03)	−0.30 (0.03)
SWC (%)	33.7	27.7	5.3**	31.0	29.7
Stem RWC (%)	64.7	67.0	62.2**	57.7	58.6
Stem ΔR (μm)	20.0 (1.3)	19.4 (3.8)	−26.5 (1.4)**	16.3 (2.1)	18.8 (1.4)
Stem MDS (μm)	−5.5 (0.8)	−4.2 (0.9)	−50.0 (4.6)**	−4.1 (0.1)	−5.1 (0.4)

The values are the means (SE) of four replicates

[a] The test of the hypotheses of no differences between average values was performed at Tmax between Pw and Pst (**$P \leq 0.001$)

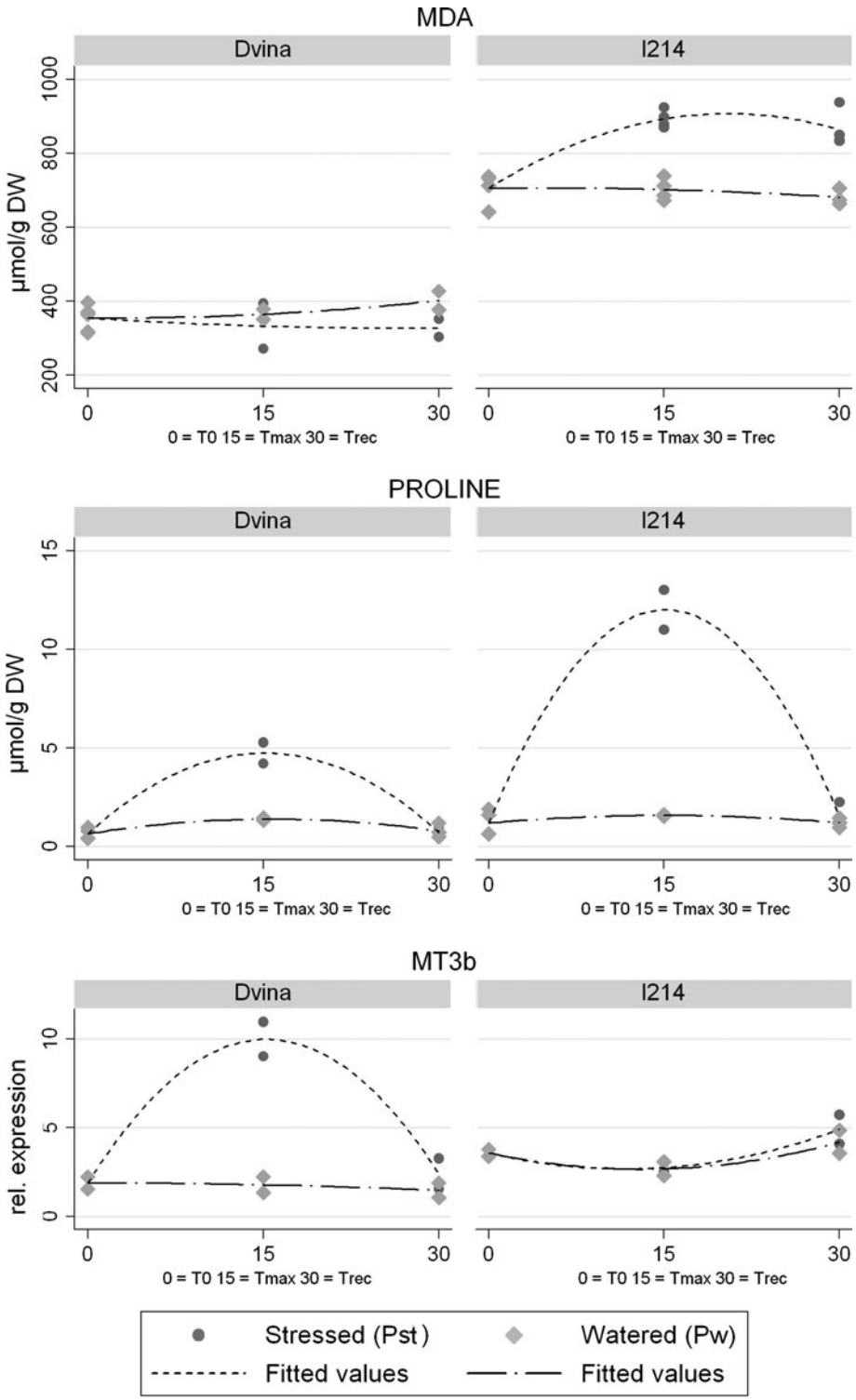

Fig. 1 Responses to treatments of MDA, proline, and *MT3b*. Fitted values refer to quadratic function

rewatering (Table 4). The expression of the genes considered in the analysis did not vary significantly in watered plants (Pw) during the time of the experiment (T0, Tmax, Trec), indicating that the transcription rate was not affected by development. Conversely, with the exception of *Cat3*, *CuZnSod*, and *Gr*, differences were observed between the clones in transcript abundance of the genes. Water deficit induced significant changes in the expression level of the genes, and the intensity of variation was genotype-related. In particular, *Cat3* transcript abundance was strongly increased by water shortage in both Dvina and I-214, whereas for *Gr*, transcript levels significantly decreased in

Table 3 Malondialdehyde (MDA), osmotically active solutes, and proline contents in watered (Pw) and stressed (Pst) plants at the beginning of the experiment (T_0), 15 days after withholding irrigation (Tmax), and 15 days after resumption of the irrigation (Trec)

	T0	Tmax		Trec	
	Control (Pw)	Control (Pw)	Stress (Pst)	Control (Pw)	Stress (Pst)
I-214					
MDA (μmol/g DW)	705.96 (31.56)	702.20 (20.43)	864.08 (35.60)	681.38 (15.82)	793.06 (97.65)
Osmotically active solutes (OAS) (mOsm/g DW)	1.96 (0.05)	2.30 (0.11)	2.65 (0.05)	1.67 (0.05)	2.13 (0.08)
Proline (μmol/g DW)	1.20 (0.46)	1.58 (0.04)	12.00 (1.00)	1.20 (0.14)	1.50 (0.37)
Dvina					
MDA (μmol/g DW)	354.79 (23.10)	364.88 (13.74)	332.80 (61.58)	401.80 (25.37)	327.49 (25.00)
Osmotically active solutes (mOsm/g DW)	1.77 (0.12)	2.20 (0.12)	2.44 (0.04)	1.84 (0.09)	1.86 (0.03)
Proline (μmol/g DW)	0.64 (0.20)	1.39 (0.06)	4.74 (0.53)	0.78 (0.20)	0.72 (0.13)

Table 4 Test of the hypotheses of no differences between average values within each treatment condition and clone by means of linear and quadratic models on time (T_0, Tmax, and Trec)

Treatment	Clone	Fitted model	*Cat1*	*Cat3*	*CuZn Sod*	*MnSod*	*MT3a*	*MT3b*	*Gr*	*Apx*	MDA	OAS	Proline
Control (Pw)	I-214	Linear	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		Quadratic	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Dvina	Linear	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		Quadratic	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Stress (Pst)	I-214	Linear	n.s.	n.s.	n.s.	n.s.	n.s	n.s.	n.s.	n.s.	**	n.s	n.s
		Quadratic	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	***
	Dvina	Linear	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
		Quadratic	n.s.	***	**	***	***	***	**	n.s.	n.s.	***	***

n.s. not significant; ** significant at $P \leq 0.001$; *** significant at $P \leq 0.0001$, F test

Dvina and slightly increased in I-214. In contrast, in Dvina, transcript levels for *MnSod*, *CuZnSod*, and *MT3a* were reduced, whereas that for *MT3b* increased in response to withholding water (Table 5; Fig. 1). Rehydration resulted in a return to control levels for all the gene transcripts with the exception of *MnSod*, demonstrating the reversibility of the changes observed.

Discussion

Because of the central role that antioxidant systems play in the defense against stress, we focused our research on the transcriptional analysis of antioxidant enzymes and scavenging proteins, coupled with physiological parameters in the cambial region of two clones with different responses to water deficit. The aim was to highlight tissue crucial for wood formation and particularly sensitive to water deficit in the cambial region, the relationships among molecular and physiological traits, and the response to water deficit of two contrasting genotypes.

The water deficit applied had a strong impact on plant water status, without significant differences between clones at Tmax. In fact, the SWC, RWCstem, and leaf Ψ_{pd} values showed that the water status in the continuum soil–plant–atmosphere did not vary between clones at Tmax, and these data confirmed that they were subjected to the same water stress intensity. Severe water deficit induced a strong water loss from the stem as shown by the high MDS values, and this condition led to some physiological and biochemical changes in the cambial region and differentiating phloem.

MDS represents the more sensitive parameter to define the stem water status in woody plants (Zweifel and others 2000). In a previous analysis under mild water-deficit conditions, Dvina showed a longer growth ability and better leaf and stem water status than I-214 (Giovannelli and others 2007). In this study, to maintain both clones in a comparable growth and water status, a severe, even if reversible, water deficit was imposed; such a condition can account for the comparable response to stress by the clones. Water deficit resulted in a significant enhancement of lipid peroxidation in the cambial zone of I-214 as assessed by the analysis of MDA contents. Damage at the membrane level is observed generally in tissues of plants subjected to water stress, reflecting impairments in the equilibrium between ROS production and scavenging by the antioxidant system.

Table 5 Relative transcript levels for stress-related genes in the cambial region of clones Dvina and I-214 obtained by RT-qPCR

	T0	Tmax		Trec	
	Control (Pw)	Control (Pw)	Stress (Pst)	Control (Pw)	Stress (Pst)
I-214					
Cat1	2.89 (0.42)	2.11 (0.35)	3.08 (0.38)	2.16 (0.47)	1.95 (0.52)
Cat3	4.08 (0.21)	3.98 (0.18)	69.49 (2.39)	2.60 (0.76)	1.89 (0.50)
CuZnSod	4.79 (0.62)	4.21 (1.04)	5.60 (0.43)	4.49 (0.67)	5.75 (0.26)
MnSod	2.26 (0.22)	1.30 (0.30)	2.04 (0.21)	1.93 (0.24)	2.44 (0.15)
Gr	2.98 (0.34)	2.95 (0.11)	3.66 (0.20)	2.72 (0.28)	2.37 (0.26)
Apx	3.89 (0.08)	4.30 (0.28)	5.61 (1.22)	4.45 (0.90)	5.94 (0.5)
MT3a	3.90 (0.25)	4.57 (0.09)	2.81 (0.38)	3.61 (0.09)	4.13 (1.05)
MT3b	3.58 (0.19)	2.67 (0.41)	2.74 (0.25)	4.19 (0.66)	4.90 (0.83)
Dvina					
Cat1	1.60 (0.22)	1.35 (0.14)	2.02 (0.44)	1.61 (0.50)	1.10 (0.10)
Cat3	4.48 (0.78)	2.99 (0.74)	65.83 (1.41)	3.04 (0.97)	5.29 (0.52)
CuZnSod	5.35 (0.56)	4.94 (0.64)	1.26 (0.23)	5.17 (1.70)	5.41 (1.08)
MnSod	3.95 (0.14)	3.61 (0.35)	1.53 (0.36)	3.39 (0.18)	2.33 (0.41)
Gr	2.61 (0.49)	2.50 (0.34)	1.32 (0.32)	3.11 (0.49)	3.33 (0.03)
Apx	1.20 (0.15)	1.21 (0.21)	1.65 (0.51)	2.89 (1.04)	1.41 (0.28)
MT3a	7.78 (0.70)	6.76 (0.52)	1.68 (0.68)	6.13 (0.27)	7.29 (0. 27)
MT3b	1.88 (0.35)	1.78 (0.45)	9.99 (0.98)	1,46 (0.41)	2.39 (0.87)

Values are means (SE) of at least two biological replicates. Expression values are normalized with qBase software against genes coding for *EF1*, *EIF4B*, and *GαS*

However, a low increase in MDA content in response to water deficit was found in leaves of *P. euphratica* and *P. cathayana* that were subjected to prolonged water depletion (Bogeat-Triboulot and others 2007), demonstrating that plant adaptation to drought probably involved the activation of enzymes capable of protecting lipids from peroxidation. The results suggested that defense strategies carried out by Dvina and I-214 to protect cambium cells from ROS or to avoid ROS increase were not equally effective. Interestingly, Dvina plants displayed a lower MDA level than that of I-214, independent of soil water availability. In different plant species, genotypes with a low lipid peroxidation level under a normal irrigation regime showed higher tolerance to the toxic effect of ROS when subjected to water deficit (Malenčić and others 2003; Koca and others 2006). On the basis of the data we obtained, we could hypothesize that Dvina had a membrane system more suitable to face lipid peroxidation induced by ROS, thus contributing to its adaptation to water deficit.

A significant increase of the concentration of osmotically active solutes in the cambial region of stressed plants showed that active osmotic adjustment took place within this zone in response to water loss, without differences between Dvina and I-214. Free proline significantly increased in both clones under water deficit. On the basis of these results we can postulate that the increase in the concentration of osmotically active solutes within the cambial region was due to mainly the increase of free proline content. The active osmotic adjustment is a typical adaptive response of plants to drought (Tschaplinski and Tuskan 1994). Our results are not in agreement with those obtained by Arend and Fromm (2007) in which a decrease of the concentration of active osmolytes in the cambial zone of poplar in response to the decrease in predawn leaf water potential was observed. Moreover, previous studies on leaves of *P. deltoides* indicated that a small osmotic adjustment occurred under water-deficit conditions (Tschaplinski and Tuskan 1994; Gebre and others 1998) and that dehydration rearrangement, such as stomatal control and adjustment of tissue elastic properties, may be the main drought-tolerance mechanism of this poplar species (White and others 1996). Our data for the cambial region suggested a different scenario; in fact, active osmotic adjustments have been found as part of the mechanisms of water-deficit response. This study also showed that I-214 under water deficit accumulated proline much more than did Dvina. In previous works performed on poplar (Ren and others 2007) and other species (Peuke and others 2002; Rampino and others 2006), the proline content was observed to rise strongly in the sensitive genotypes, whereas in the resistant ones the increase was lower or delayed. The difference in proline accumulation observed in this study may reflect the different strategies employed by Dvina and I-214 to achieve adaptation to water deficit. Proline accumulation in the cambial region appears as the most prominent reaction to stress by I-214 clone; nonetheless, the accumulation of this osmolyte proved ineffective in conferring water stress tolerance.

Transcript analysis of genes involved in the stress response was performed to investigate whether the genotypes exhibited different expression in response to water deficit. The choice of genes was driven by the results previously obtained by a microarray analysis of cambial transcriptome of white poplar in response to water deficit (Berta and others 2010). In general, we observed a large influence of genotype on the level of gene expression independent of treatments. Transcript levels of *Cat1* and *Apx* were higher in I-214 than in Dvina, whereas the expression of *MnSod* and *MT3a* was lower. At Tmax, a fivefold upregulation of *MT3b* in stressed plants of Dvina compared with irrigated plants was observed. In contrast, the expression level of this gene did not change in I-214. *MT3a* transcription, on the other hand, decreased in Pst plants of both clones, but only the decrease in Dvina was significant. MTs belong to a small multigene family consisting of different genes. In poplar they are both constitutively expressed and respond differentially to environmental stimuli (Kohler and others 2004); our data are in agreement with that finding. Besides their role in heavy-metal detoxification, they probably contribute to cell homeostasis in response to water deficit in poplar roots and leaves (Brosché and others 2005; Plomion and others 2006; Bogeat-Triboulot and others 2007). Recently, the increase of MT type 3 transcripts was observed also in the cambial region of *P. alba* under water deficit (Berta and others 2009, 2010). Although the exact role of MT-encoding genes in the defense against water deficit is still not clarified, their involvement in redox homeostasis of plant cells has been recently suggested (Xue and others 2009; Samardžić and others 2010). The upregulation of *MT3b* induced by the stress in Dvina, and not observed in I-214, suggests that this gene plays a key role in the protection of this clone from water deficit. *Cat3* was the only gene strongly upregulated by water deficit in both genotypes, confirming its important role in ROS control and the strong tissue specificity of this isoform, as previously observed in a Northern analysis of catalase genes in different poplar tissues (Caparrini 2004).

The other genes involved in stress response did not change their expression level under drought in I-214, whereas in Dvina they appeared downregulated (*CuZnSod*, *MnSod*, and *MT3a*) or not regulated (*Cat1*, *Apx*). Finally, the analysis of Pw and Pst plants at Trec revealed that the transcriptional changes observed under stress were fully reversible.

Stress tolerance is generally related to control of oxidant levels in tissues through the coordinate expression of antioxidant genes and/or a higher activity of their enzymes (Cheeseman 2007). Our data are in agreement with those obtained by other authors on sensitive and tolerant poplar genotypes subjected to long-lasting drought periods, showing that adaptation to water deficit involved the regulation of few and specific isoforms of stress defense genes (Berta and others 2006; Plomion and others 2006; Bogeat-Triboulot and others 2007; Xiao and others 2009; Berta and others 2010).

The data obtained showed strong differences between the clones in transcription rates of genes related to ROS detoxification, highlighting the genetic control of molecular plasticity in the drought response. Differences among genotypes with respect to a number of proteins involved in protection against oxidative damage were underlined in a study performed on the leaf proteome of *Populus* × *euramericana* genotypes (Bonhomme and others 2009). However, so far, the transcriptome and proteome analyses in poplar focused mainly on leaf and root, whereas no similar attention was paid to cell biology of wood formation in stress conditions. The transcription studies coupled with biochemical and physiological data at the cambial region level in response to water stress presented here represent an element of novelty in particular when performed on poplar genotypes, contrasting their tolerance of water deficit. The data obtained in this study suggest that the two clones faced oxidative stress in the cambial region and exploited different metabolic tools and were not equally effective in response to stress.

Conclusions

The responses to water deficit within the cambial region of Dvina and I-214 were investigated through the analysis of physiological and biochemical parameters and the transcription analysis of a set of antioxidant and MT genes. The results revealed that the clones under severe water deficit carried out different strategies to protect cambial region cells from oxidative stress. I-214 accumulated more free proline than Dvina, suggesting a key role for this amino acid in the drought response of this genotype. On the other hand, the transcript analysis of antioxidant and MT genes showed a different ability of the two clones to modulate antioxidant genes under stress. In particular, *MT3b* was strongly upregulated by water deficit in Dvina thus suggesting an important role in the maintenance of cellular redox homeostasis of the clone. In contrast, I-214 appeared to respond to water stress mainly by means of proline accumulation and osmotic adjustment, but this action has limited effectiveness as shown by the enhancement of lipid peroxidation. On the whole, genetic background appears to be a major factor in response to drought, highlighting the genetic control of molecular plasticity in these responses.

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