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Original article

DNA methylation and histone acetylation: genotypic variations in hybrid poplars, impact of water deficit and relationships with productivity

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Abstract

• Several reports on annual plants have already shown the involvement of epigenetic modifiers such as DNA methylation in their adaptation to abiotic stresses.

• Nevertheless, the genotypic variations of epigenetic modifiers, their possible correlations with morphological traits and the impact of water deficit have not been described for perennial plants.

• Six genotypes of *Populus deltoides* × *P. nigra* were subjected or not to a moderate water deficit treatment. Various morphological traits such as the height of the plants, their biomass and the total leaf area were measured to characterize the productivity in both conditions. Levels of DNA methylation, histone acetylation and the activities and isoform accumulation of the corresponding enzymes were measured at the shoot apex, the site of morphogenesis. Genotypic variation was observed for the morphological traits and the epigenetic variables and correlations were established among them. Genotypic variation for DNA methylation was detected in hybrid poplars. A positive correlation was demonstrated between DNA methylation percentage and productivity under well watered conditions.

• While there was a general decrease of growth for all genotypes in response to a moderate water deficit, genotypic dependant variations of DNA methylation were found suggesting different strategies among hybrids.

Mots-clés :
apex caulinaire /
déficit hydrique /
développement végétatif /
méthylcytosine /
productivité

Résumé – Méthylation de l'ADN et acétylation des histones : variations génotypiques chez des peupliers hybrides, impact d'un déficit hydrique et relations avec la productivité.

• Plusieurs études sur des plantes annuelles ont déjà montré l'implication des modifications épigénétiques telles que la méthylation de l'ADN dans la plasticité de leurs réponses aux contraintes abiotiques.

• Néanmoins, les variations génotypiques de ces modifications épigénétiques, leur possible corrélation avec des variables de croissance et l'impact d'un déficit hydrique n'ont pas été décrits sur une plante pérenne.

• Six génotypes de *Populus deltoïdes* × *P. nigra* ont été soumis ou non à un déficit hydrique modéré et plusieurs variables de croissance ont été mesurées afin de caractériser leur productivité. Les niveaux de méthylation de l'ADN, d'acétylation des histones, les activités enzymatiques et l'accumulation des isoformes correspondantes ont été mesurés sur des apex caulinaires, site de la morphogenèse. Des variations génotypiques ont été observées pour les variables de croissance et épigénétiques. Une corrélation positive a été mise en évidence entre la méthylation de l'ADN et la productivité en condition hydrique favorable.

• Bien qu'il y ait une diminution générale de la croissance de tous les génotypes en réponse à un déficit hydrique modéré, des variations génotype-dépendant de la méthylation de l'ADN ont été trouvées suggérant différentes stratégies entre hybrides.

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1. INTRODUCTION

Poplars are among the fastest growing trees in temperate latitudes but their high productivity is associated with large water requirements (Tschaplinski et al., 1994) and they are one of the most sensitive to water deficit. For this reason, the natural poplar growing area is restricted to river banks and its cultivation to alluvial plains. Nevertheless, significant genotypic variability in the response to water deficit has been reported (Monclus et al., 2006). In order to limit water losses and to optimize water absorption, poplar possesses several mechanisms such as stomatal closure, as well as leaf surface reduction and root surface maintenance via changes of carbon allocation among organs (Braatne et al., 1992; Roden et al., 1990). These mechanisms to adjust to environmental variation define the high developmental plasticity of plants. Studies on annual plants, organs or cell cultures have already demonstrated the role of epigenetic mechanisms in developmental plasticity (Boyko and Kovalchuk, 2007; Chen and Tian, 2007). Indeed, epigenetic modifiers play an important role in genome organization and stability, the control of gene expression and inheritance via chromatin structural reworking (Zilberman et al., 2007).

Chromatin is defined as the association of DNA with histone proteins (H_2A, H_2B, H_3 and H_4) with reversible condensation states (Allis et al., 2007). DNA methylation (Gehring and Henikoff, 2007) or histone acetylation (Chen and Tian, 2007; Loidl, 2004) are epigenetic modifications that lead towards either heterochromatin or euchromatin formation. Indeed, these modifications may ensure the recruitment of multienzymatic complexes responsible for dynamic chromatin changes (Allis et al., 2007). Heterochromatin is associated with a negative transcriptional state, due to the incapacity of transcriptional activators or general transcription machinery to gain entry to the DNA sequence.

DNA methylation corresponds to the addition of a methyl group (-CH_3) from *S*-adenosyl-L-methionine on a cytosine, and is catalyzed by DNA methyltransferases (DNMT; EC 2.1.37) (Allis et al., 2007; Pavlopoulou and Kossida, 2007) at the level of CG, CNG (where N is any base) or non-symmetric CHH genomic sequences (where H is any base except G). Histone acetylation or deacetylation is the addition or the removal of an acetyl group (-CO-CH_3) on various lysine residues of the N-terminal extremity of histones, and is catalyzed by histone acetyltransferases (HAT; EC 2.3.1) and histones deacetylases (HDAC; EC 3.5.1) respectively (Allis et al., 2007; Loidl, 2004).

While a few studies have demonstrated natural variation of DNA methylation in annual plants (Riddle and Richards, 2002; Vaughn et al., 2007; Zhang et al., 2008) or in organ and cell cultures under osmotic or dehydration stresses (Kovarik et al., 1997; Labra et al., 2002; Sabbah et al., 1995), no data are available in perennial species such as poplar concerning genotypic variation of various epigenetic modifiers, or of their possible correlation with growth and the impact of water deficit. Therefore, the aim of this study is to test a possible role of epigenetic mechanisms in the plasticity of hybrid poplars in response to water deficit. Elements of answers will be provided to the following questions: (1) Is there any genotypic variation for epigenetic modifiers such as DNA methylation and histone acetylation in hybrid poplars? (2) If so, could this variability be correlated to the genotypic variability observed in morphological traits? and (3) What is the impact of water deficit on these variables and their eventual correlations?

Six genotypes of *Populus deltoides* × *P. nigra,* which is the best-selling hybrid in Europe and a model of woody perennial plants, were submitted to a moderate water deficit. At the end of the experiment, growth was assessed through morphological traits (stem and root biomasses, plant height, total leaf area and leaf number) and leaf structure (specific leaf area), which were measured for each genotype. In parallel, shoot apices containing meristematic cells and being the site of shoot morphogenesis were collected from each plant. DNA methylation percentages, histones H_3 and H_4 acetylation ratios, and their associated enzymatic activities (DNMT and HDAC) and isoform accumulations were measured. Our results show that genotypic variation of epigenetic modifiers in poplar shoot apex is correlated to productivity. DNA methylation is affected in a genotype dependent way by a moderate water deficit suggesting different strategies among hybrids.

2. MATERIALS AND METHODS

2.1. Plant material, growth conditions and water deficit induction

The experiment was conducted on six genotypes of *Populus euramericana* (*Populus deltoides* × *P. nigra*): Carpaccio (female and diploid), I45-51 (male and possible triploid), NL3972 (female and diploid), Triplo (male and triploid), Lambro (male and diploid) and Soligo (male and diploid). Twelve cuttings per genotype were planted in 10L pots containing a mixture of peat and sand (50/50, v/v) amended with magnesian chalk (60 g/100 L) and fertilized with a slow releasing fertilizer (Nutricote T100, 13/13/13/2, N/P/K/Mg + trace elements). Plants were placed in a greenhouse exposed to natural daylight (ranging from 350 to 900 µmol m^{-2} s^{-1}) and where temperature was maintained in the range 15–27 °C with humidity oscillating between 50 and 75%. Plants were watered to field capacity three times a day for 5 weeks, until plant height was between 0.6 and 0.9 m, depending on the genotype. A moderate water deficit was then applied for 17 days to the half of the cuttings, the other half remaining fully watered (controls). While soil volumetric water content (SWC) at field capacity was 32%, it was maintained in the range 7–13% (minimum–maximum) by controlled watering every morning. A correlation between weight of pots (Wt) and SWC measured by time-domain reflectometry was established previously and allowed a control of SWC by weighing the pots.

2.2. Shoot apex harvest and morphological traits measurement

At the end of the experiment, the shoot apex was harvested, frozen in liquid nitrogen and conserved at −80 °C until epigenetic analyses. Then, the height of plants (m) and the length of each individual leaf (m) were measured; the number of leaves was consequently obtained.

The surface area of each individual leaf (m^2) was calculated from abacs previously established on a sample of leaves covering the whole size range (one for each genotype). The total leaf area (m^2) was then calculated for each plant. Roots were washed to remove substrate and then detached from the cutting. Dry weight of leaves, stems and roots (g) were determined after oven-drying for 48 h at 60 °C. Specific leaf area ($cm^2\ g^{-1}$) was calculated as the ratio of total leaf area and leaf number.

2.3. Determination of the genomic DNA methylation percentage

Shoot apices were ground to a fine powder. Genomic DNA was extracted, enzymatically hydrolysed into nucleosides and analysed by High Performance Liquid Chromatography (HPLC) according to Causevic et al. (2005). A new hydrophobic column Gemini^{TM} (150 × 4.6 mm, 5 µm, Phenomenex, Le Pecq, France) was used. Isocratic mobile phase is composed of 0.5% methanol (v/v) and 5 mM acetic acid in water. A flow rate 1.5 mL min^{-1} was used. Identification of cytosine (C) and methylcytosine (mC) were assessed by comigration under the same HPLC conditions with commercial standards (Sigma-Aldrich, Saint-Quentin Fallavier, France). The methylcytosine percentages were calculated using the following formula: %mC = (mC/(C + mC)) × 100, where "C" represents 2′-deoxycytidine content and "mC" 5-methyl-2′-deoxycytidine content.

2.4. Protein extraction, enzymatic activities and immunodetection

Total soluble proteins and chromatin bound histones were extracted separately from shoot apex according to Causevic et al. (2006). The protein concentration was determined using Protein Assay reagent (Bio-Rad, Marnes-la-Coquette, France) and a standard curve established with different solutions of bovine serum albumin (from 0 to 33 µg μL^{-1}).

Similar amounts of protein extracts and a molecular weight marker (Prestained SDS-PAGE Standards, Low Range, Bio-Rad) were separated by SDS-PAGE and electrotransfered on a nitrocellulose membrane (Causevic et al., 2005; 2006). Blotted membranes with soluble proteins were hybridized with different antibodies: carrot anti-DNA methyltransferase 1 (DNMT1; Bernacchia et al., 1998; Causevic et al., 2005) antibodies at 1:300 dilution (v/v) and three maize anti-Histone deacetylase antibodies (HDAC; Pipal et al., 2003; Causevic et al., 2006) raised against the three classes of HDAC (anti-HD1A at 1:2000 dilution (v/v), anti-HD1BI at 1:1500 dilution (v/v) and anti-HD2 at 1:1000 dilution (v/v)). Blotted membranes with histone extracts were hybridized with anti-acetylated lysine at 1:1000 dilution (v/v) or anti-non acetylated H_3 histone at 1:200 dilution (v/v) (Santa Cruz Biotechnology, California, and Cell Signalling Technology, Danvers, USA). Cross-reacting bands were identified using anti-rabbit or anti-goat immunoglobin conjugates labelled with alkaline phosphatase (Sigma-Aldrich) at 1:2000 dilutions (v/v) and stained with BCIP/NBT liquid substrate system (Sigma-Aldrich).

Blots were scanned and the intensity of the immunodetected bands quantified in arbitrary units using imaging software (ImageTool for Windows version 3.00) providing quantitative data. Three independent extractions were analysed by western blot in duplicate for all genotypes, treatments and antibodies. Fixed amounts of a histone H_3 enriched preparation from calf thymus (arginine-rich subgroup f3; Sigma-Aldrich), a maize-HD1A recombinant protein and a carrot-DNMT1 recombinant protein were used as positive controls as already described (Causevic et al., 2005; 2006). These controls served as internal standards for quantification after immunodetection and comparison between membranes. A mean value was calculated for each immunodetected band using the corresponding internal standard and after the subtraction of the aspecific background. Then, mean amounts of the 100 kDa DNMT and 80 kDa HDAC bands were expressed as percentages of the total signal detected for a given protein sample and antibody. This total signal corresponds to the additional mean signal of bands at 100, 70, 50, 30 and 15 kDa for DNMT and 80, 75, 65, 45 and 30 kDa for HDAC. Acetylation ratios of histones H_3 or H_4 were calculated according to the following formula: acetylation ratio of H_3 or H_4 histone (AcH_3 or AcH_4) = amount of acetylated histone H_3 or H_4 / amount of non acetylated histone H_3. Anti-acetylated histone H_3antibodies were also tested to confirm the data as previously published (Causevic et al., 2006).

Using total soluble protein extracts, DNMT and HDAC activities were measured as described by Causevic et al. (2005; 2006) respectively. DNMT activity was measured using non methylated genomic DNA from a *dam*$^-$*dcm*$^-$ *E. coli* strain as substrate, and ^{3}H-SAM as a methyl donor and is expressed in pkatal per µg of protein. HDAC activity was assayed using an artificial substrate, N-(4-methyl-7-coumarinyl)-N-(t-butyloxycarbonyl)-N-acetyllysinamide (MAL) (Sigma-Aldrich). HDAC activity is expressed in percentage of deacetylated MAL min^{-1} per µg protein after HPLC separation and detection.

2.5. Statistical analysis

Statistical analyses were carried out using the SPSS statistical software package (SPPS version 11.0.1 PC, Chicago, IL, USA). Genotype and water deficit effects were evaluated by two-way ANOVA (GLM procedure) using the following model: $Y_{ijk} = \mu + G_i + T_j + (G_i \times T_j) + \varepsilon_{ijk}$; where Y_{ijk} are individual values, µ the general mean, G_i the effect of the genotype i, T_j the effect of the treatment j, $G_i \times T_j$ the genotype by treatment interactions and ε_{ijk} the error. Statistical tests were considered significant at * $P \leq 0.05$, ** $P \leq 0.01$ or *** $P \leq 0.001$.

Genotypic variation was described using principal component analysis (PCA) from genotypic means of morphological traits (stem biomass, root biomass, height, total leaf area, leaf number and specific leaf area) measured in well-watered or water-limited conditions. Variables were standardized and orthogonal factors (scores $F1$ and $F2$) were built successively as linear combinations of these variables, maximizing the percentage of the variability explained by these factors. The epigenetic variables were projected as supplementary variables in this $F1 \times F2$ plane. Their coordinates corresponded to their linear correlation coefficients with scores $F1$ and $F2$ axis of the PCA.

3. RESULTS

3.1. Genotypic variation of morphological traits and water deficit effects

Significant genotype and treatment effects were detected for all morphological traits (Fig. 1), except for specific leaf area

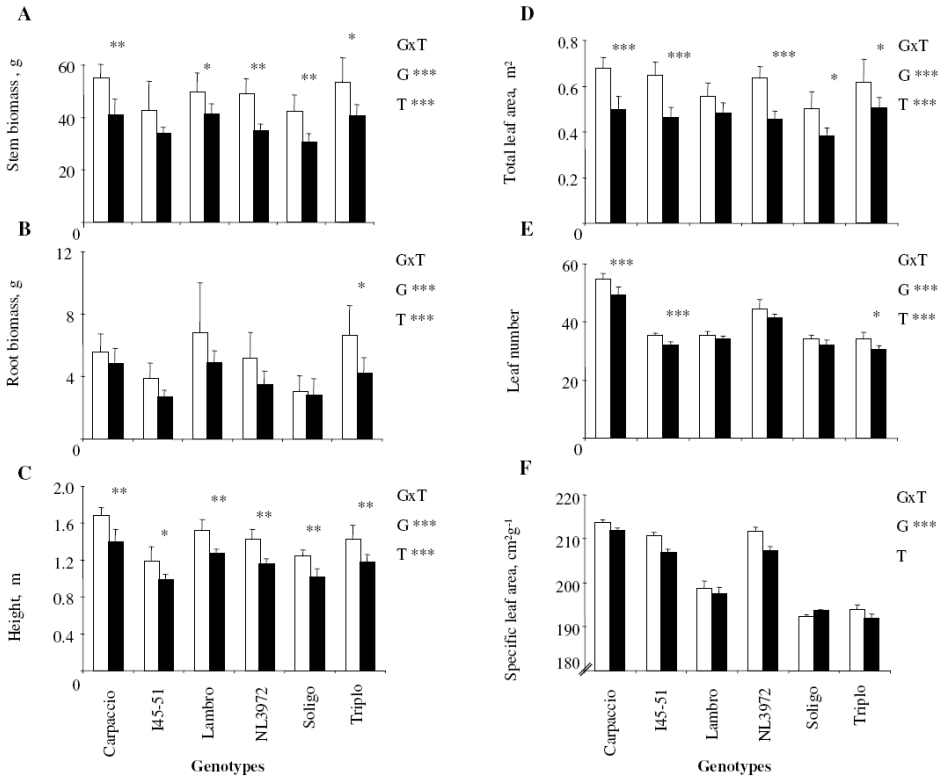

Figure 1. Genotypic variation and water deficit effect on morphological traits related to productivity in six *Populus deltoides* × *P. nigra* hybrids growing in glasshouse in well-watered (white bar) or in water deficit (black bar) conditions. Studied traits encompassed: (A) stem biomass, (B) root biomass, (C) height of plants, (D) total leaf area, (E) leaf number and (F) specific leaf area. For each graph, G indicates the genotype effect, T the treatment effect and (GxT) genotype by treatment effect. Means are accompanied by their standard errors SE (n = 6). Significant differences between well-watered and water deficit conditions are indicated by asterisk: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$.

for which only a genotype effect was observed. No genotype by treatment interaction effect was observed.

Under well-watered conditions, genotypes differed for height, leaf number, total leaf area and specific leaf area (Fig. 1). For most of the traits, Carpaccio and Soligo were the extreme genotypes with height ranging from 1.23 ± 0.06 to 1.68 ± 0.08 m, total leaf area from 0.50 ± 0.07 to 0.67 ± 0.04 m^2, leaf number from 34.2 ± 1.3 to 54.8 ± 1.9 leaves and specific leaf area from 190 ± 5 to 210 ± 5 $cm^2\ g^{-1}$.

The applied water deficit was moderate and leaf predawn water potential of stressed plants did not differ from controls (data not shown). However, height, stem biomass, leaf number and total leaf area decreased significantly in response to water deficit for most of the genotypes. The range of variation depended on the genotype. For example, stem biomass decreased from about 17% for Lambro to 28% for NL3972. Root biomass only decreased significantly for Triplo.

3.2. Genotypic variations of DNA methylation and water deficit effects

A significant genotype by treatment interaction was observed for percentage methylcytosine (%mC) of shoot apices, indicating that the effect of the treatment depended on the genotype (Fig. 2A). In the well-watered samples, %mC ranged from 4.1 ± 1.6% for I45-51 to 11.5 ± 3.4% for Carpaccio. In response to water deficit, %mC decreased significantly for Carpaccio, Lambro and Triplo, while it strongly increased for Soligo and remained unchanged for I45-51 and NL3972 (Fig. 2A).

Neither genotype nor treatment effects were observed for DNA methyltransferase (DNMT) activity except for Carpaccio and Lambro, for which DNMT activity decreased by about 40% after a water deficit treatment (Fig. 2B). Only a tendency is seen between %mC and DNMT activity (r = 0.75 at P = 0.08).

Immunodetection of the isoforms of DNMT class 1 revealed five bands: one over 100 kDa and smaller ones with sizes of 70, 50, 30 and 15 kDa, respectively (Fig. 2C). Distinct patterns of accumulation for these bands were observed between genotypes. These patterns were not profoundly affected by water deficit except for Carpaccio, which showed a strong decrease for all bands. A positive correlation was established between DNMT activity and the amount of the 100 kDa DNMT isoform (expressed as a percentage of total signal) (r = 0.61*; supplementary[1] figure S1A).

[1] Supplementary material is available on line only at www.afs-journal.org.

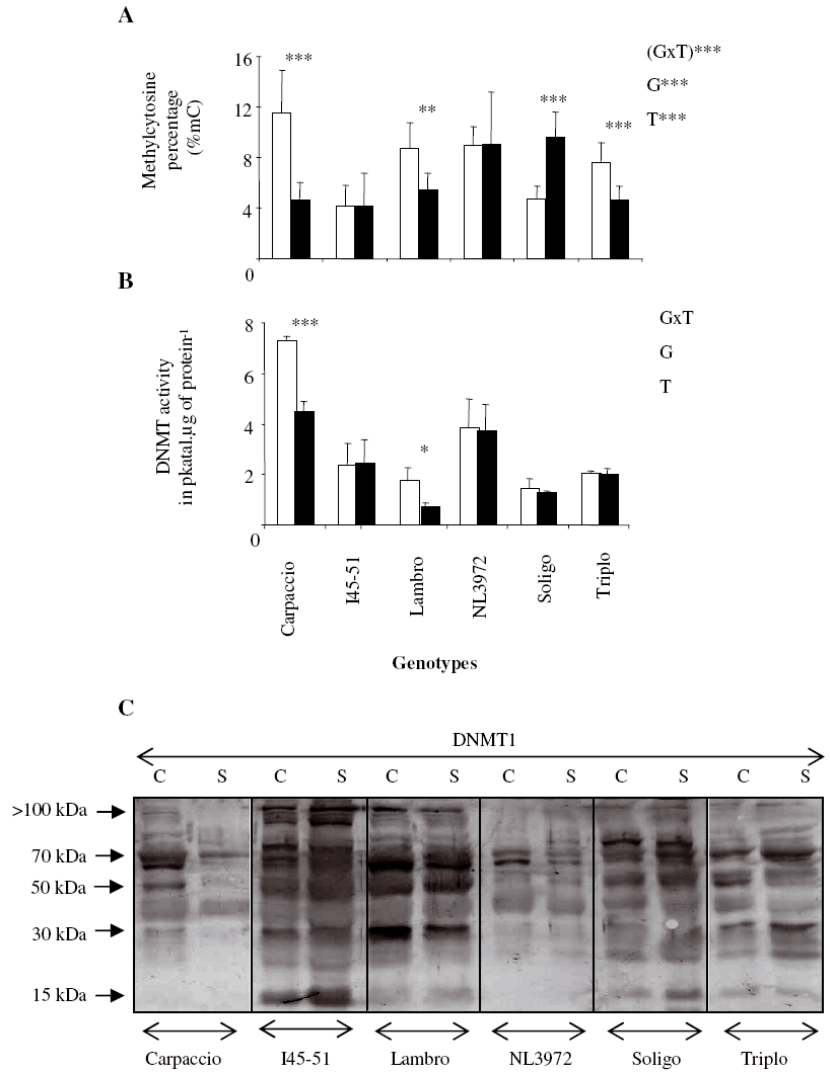

Figure 2. Genotypic variation and water deficit effect on traits related to DNA methylation in six *Populus deltoides* × *P. nigra* hybrids growing in glasshouse in well-watered (white bar) or in water deficit (black bar) conditions. Studied traits encompassed: (A) percentages of methylation of cytosine residues (%mC) and (B) total DNMT activity (see Materials and Methods section for details). For each graph, G indicates the genotype effect, T the treatment effect and (GxT) genotype by treatment effect. Means are accompanied by their standard errors SE (n = 6). Significant differences between well-watered and water deficit conditions are indicated by asterisk: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$ (C) immunodetection in the six genotypes of isoforms of DNMT class I. C (Control) represents plants in well-watered condition and S (Stressed) plants in water deficit condition. Sizes of the different isoforms are indicated in kiloDalton.

3.3. Genotypic variation of histone acetylation and water deficit effects

Using antibodies raised against acetylated forms of histones H_3 and H_4, it was possible to calculate the corresponding acetylation ratios (AcH_3 and AcH_4) by normalising with the amount of non-acetylated histone H_3 (Figs. 3A and 3B; see material and methods for details). Neither genotype nor treatment effects were observed for the ratios of AcH_3 or AcH_4: these were around 0.3, indicating a general hypoacetylation of these histones in poplar.

For Histone deacetylase (HDAC) activity, only a genotype effect was detected. Values ranged from 0.08% MAL deacetylated substrate min^{-1} μg^{-1} of proteins for I45-51 to 11.10% for Carpaccio (Fig. 3C). While no treatment effect was detected by ANOVA for HDAC activity, significant differences were observed for five genotypes in response to water deficit. Thus, an increase in HDAC activity was observed for I45-51 and Triplo while Lambro, NL3972 and Soligo showed a decrease in HDAC activity.

Immunodetection using antibodies raised against each HDAC class described in plants (HD1A, HD1B and HD2, respectively) revealed several bands for each of them ranging from 80 kDa to 30 kDa (Fig. S3D). HD1A antibodies detected isoforms at 80, 75, 65, 45 and 30 kDa, while HD1B antibodies

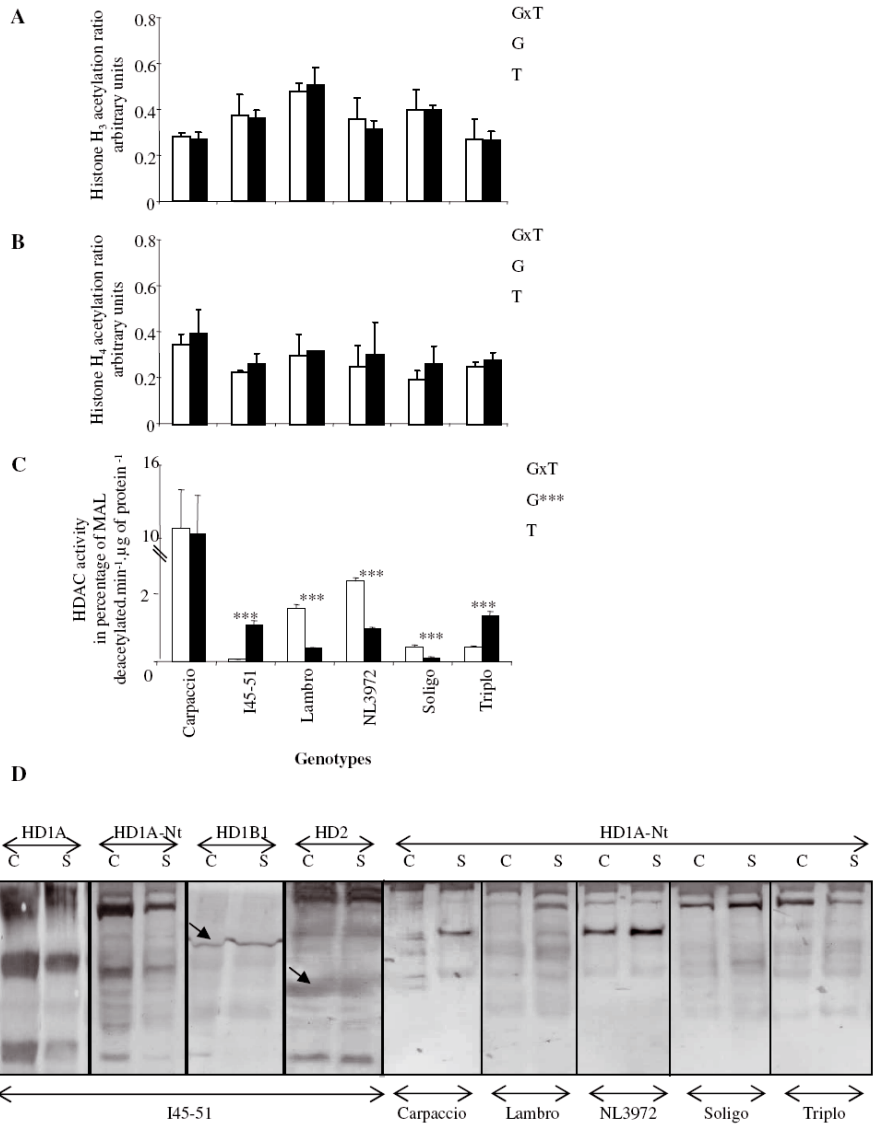

Figure 3. Genotypic variation and water deficit effect on traits related to histone acetylation in six *Populus deltoides* × *P. nigra* hybrids growing in glasshouse in well-watered (white bar) or in water deficit (black bar) conditions. Studied traits encompassed: (A) histone H_3 acetylation ratio (AcH_3). (B) histone H_4 acetylation ratio (AcH_4). (C) HDAC activity. For each graph, G indicates the genotype effect, T the treatment effect and (GxT) genotype by treatment effect. Means are accompanied by their standard errors SE (n = 6). Significant differences between well-watered and water deficit conditions are indicated by asterisk: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. (D) immunodetection of the three classes of HDAC using anti-HD1A antibody, truncated anti-HD1A antibody (HD1A-Nterminal part of the protein), anti-HD1B1 antibody and anti-HD2 antibody. C (Control) represents plants in well-watered condition and S (Stressed) plants in water deficit condition. Sizes of the different isoforms are indicated in kiloDalton. Two arrows indicate specific bands mentioned in the result section.

revealed an additional specific band of about 50 kDa and HD2 one at 40 kDa. Blots with antibodies raised against the N-terminal part of HD1A (HD1A-Nt) presented a different profile, with additional bands of weak intensity, from those with HD1A antibodies. The most significant change between genotypes or treatments was in the relative amounts of the HD1A bands smaller than 80 kDa (Fig. 3D). A positive correlation was established between HDAC activity and the amount of the 80 kDa HDAC isoform expressed in percentage of total signal (r = 0.63*; supplementary[1] figure S1B).

3.4. Correlation between morphological traits and epigenetic modifiers

Principal component analyses (PCA) were performed using genotypic means of the morphological traits measured in well-watered or water deficit conditions to characterize the variability of morphological traits between hybrid poplars (Fig. 4). Under the control conditions, the main plane of PCA explained 88.0% of the variability, with 60.7% for $F1$ alone (Fig. 4A). The $F3$ axis did not differentiate the variables (data

Table I. Linear correlations (Pearson coefficient, *r*) computed between morphological traits, *F*1 and *F*2 axes of PCA (see Fig. 4) and epigenetic variables. Only significant values are indicated by asterisk: * $P \leq 0.05$, ** $P \leq 0.01$ and *** $P \leq 0.001$. "Control" and "Stressed" represent plants in well-watered or in water deficit conditions, respectively.

	%mC		DNMT		AcH_3		AcH_4		HDAC	
	C	S	C	S	C	S	C	S	C	S
SB	0.89*							0.85*		
RB										
Hgh	0.98***						0.83*	0.85*	0.81*	
TLA										
LN			0.98***	0.85*					0.94**	0.85*
SLA										
*F*1 axis	0.92**		0.86*						0.82*	
*F*2 axis					–0.90*	–0.83*				

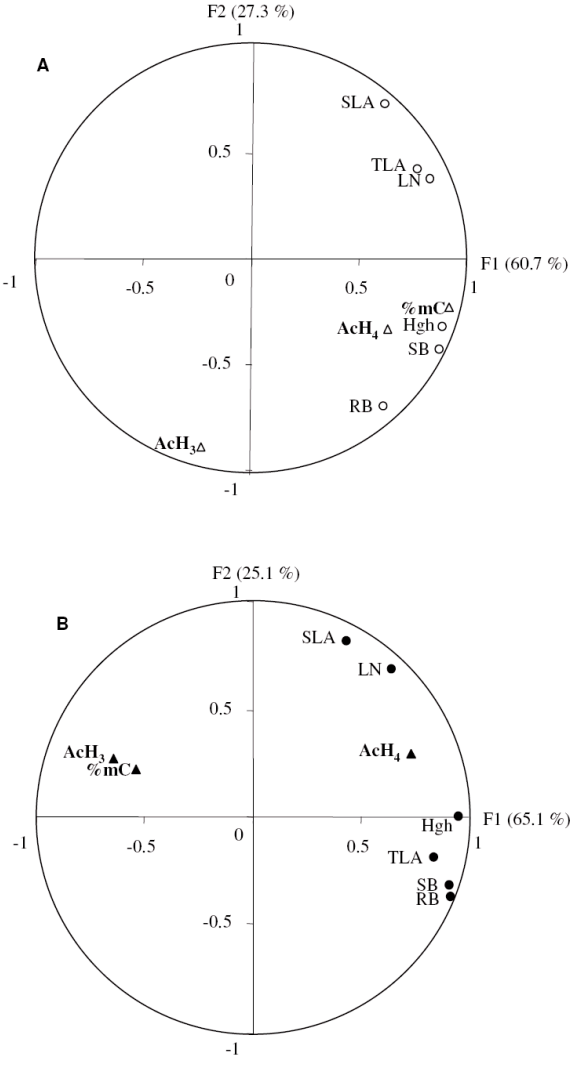

Figure 4. Principal Component Analyses (PCA) built using morphological traits (circles): stem biomass (SB), root biomass (RB), height (Hgh), total leaf area (TLA), leaf number (LN) and specific leaf area (SLA). Epigenetic variables (triangles): percentages of methylation of cytosine residues (%mC), histone H_3 and H_4 acetylation ratios (respectively AcH_3 and AcH_4) have been projected as supplementary variables using their Pearson coefficients with scores of PCA as coordinates. (A) in well-watered condition, (B) in water deficit condition.

not shown). The *F*1 axis was mainly defined by height, leaf number and stem biomass. In this group, most variables scaled positively. The *F*2 axis was mainly defined by specific leaf area. Linear correlations between methylcytosine percentage or histone acetylation ratios and scores of *F*1 and *F*2 axes were computed and used to project them as supplementary variables in these planes. Positive correlations were detected between methylcytosine percentage and stem biomass, height and scores of *F*1 axis from PCA (Tab. I and Fig. 5). AcH_3 was only negatively correlated to scores of *F*2 axis, while AcH_4 scaled positively with height (Tab. I; supplementary[1] figures S2A and S3A).

In the water deficit conditions, the main plane of PCA explained 90.2% of the variability, with 65.1% for *F*1 alone (Fig. 4B). The *F*3 axis did not differentiate the variables (data not shown). The *F*1 axis was defined by total leaf area, height, stem biomass and root biomass and most of these variables scaled positively (data not shown); the *F*2 axis was mainly defined by specific leaf area. The negative correlation between scores of *F*2 axis and AcH_3 was conserved (Tab. I; supplementary[1] figure S2B). AcH_4 still scaled positively with height but also with root biomass (Tab. I; supplementary[1] figure S3B and S3C).

4. DISCUSSION

4.1. Morphological traits distinguish hybrid poplars and are decreased by a moderate water deficit

Genotypes differed for morphological traits as already reported in previous studies (Bonhomme et al., 2008; Marron et al., 2005; Monclus et al., 2006). In response to water deficit a general decrease of most of these morphological traits was recorded. The leaf variables adjustment ensures a reduction of water losses but affects the photosynthesis activity and consequently the biomass production (Monclus et al., 2006; Roden et al., 1990). The absence of a genotype specific response may be explained by the fact that the constraint applied was moderate and short. Indeed, previous results show that in response to longer and stronger droughts poplars (and particularly those of this study) are differentially sensitive to water deficit (Marron et al., 2005; Monclus et al., 2006). This highlights that water deficit tolerance is often the result of a temporal integration of

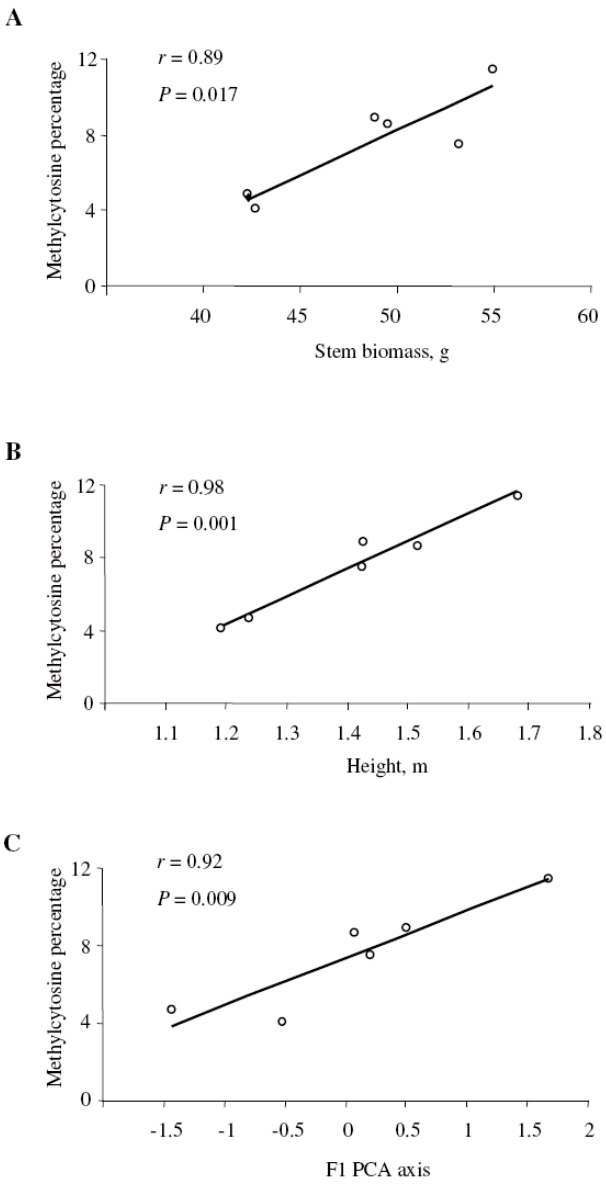

Figure 5. Relationship (linear correlation) between methylation percentages (%mC) and (A) stem biomass, (B) height and (C) *F*1 axis of PCA, measured on the apex of six poplar genotypes in well-watered condition.

fine physiological differences, not easily revealed in short term experiments. Possibly for similar reasons specific leaf area was not affected, either (Marron et al., 2005; Monclus et al., 2006).

The water deficit was moderate and the predawn leaf water potential (Ψ_{pd}) was unaffected (data not shown). Thus, Ψ_{pd} is one of the last parameters to be affected during an increasing water deficit (Bogeat-Triboulot et al., 2007). As the growth of aerial parts is the most sensitive parameter to water deficit, we assessed the impact of the moderate water deficit on morphological traits.

4.2. Differences in DNA methylation levels between genotypes

Because epigenetic patterns participate in the control of gene expression (Zhang et al., 2006; Zilberman et al., 2007) and are transmitted by mitosis, changes that arise in dividing cells in the shoot apex during environmental stress have the possibility of being propagated to daughter cells (Boyko and Kovalchuk, 2007; Chen and Tian, 2007; Gehring and Henikoff, 2007). Thus, epigenetic changes in shoot apex would explain, in part, variation for morphological traits.

Genotypic variation was observed for DNA methylation percentages that ranged from 3.9% for Soligo to 11.1% for Carpaccio, with no relationship with the characteristics of sex or ploidy of the six genotypes. Nevertheless, these percentages are relatively low compared with other plant species where the values ranged from 5% in *Arabidopsis* to more than 37% in *Helianthus* (Causevic et al., 2005). This is usually attributable to differences in repetitive DNA content among species, which is mostly associated with heterochromatin and is the major target of DNA methylation (Gehring and Henikoff, 2007).

Variations of DNA methylation have already been reported on *Arabidopsis* ecotypes for selected loci (Riddle and Richards, 2002; Vaughn et al., 2007). A similar degree of methylation polymorphism was observed, and gene expression was not generally affected by differences in DNA methylation. In contrast, in two *Arabidopsis* accessions and their reciprocal *F*1 hybrids Zhang et al. (2008) found a significant negative correlation between the degree of methylation variation within immediate upstream/downstream coding regions and the degree of expression variation. Furthermore, methylation polymorphism within genic regions showed a weak positive correlation with expression variation. These data suggest a possible relationship between natural CG methylation variation and gene expression variation that should be tested in poplars. Indeed, data on the *Arabidopsis* methylome have revealed that transposons and other repeats are heavily methylated, more than one-third of all genes contain methylation whereas fewer than 5% of expressed genes were shown to have methylated promoters (Zhang et al., 2006). Interestingly, genes methylated in transcribed regions are highly expressed and constitutively active, whereas promoter-methylated genes show a greater degree of tissue specific expression.

In addition to genetic variations among genotypes, the variations of global DNA methylation percentages observed in this study between poplar genotypes could have two main origins: (i) the heterogeneity of the plant material. Indeed, shoot apex of these genotypes could differ for their ratio of differentiated/undifferentiated cells. Two results seem to render this possibility less likely (but do not exclude it): the direct observation of shoot apex organization revealed no differences between genotypes (data not shown) and the histone H_3 and H_4 acetylation ratios were not significantly different between genotypes; (ii) the activity of DNA methyltransferases (Pavlopoulou and Kossida, 2007). Several DNMT isoforms were assessed in hybrid poplars in accordance with databases (Tuskan et al., 2006) and a process of limited proteolysis at the C-terminal part (Bernacchia et al., 1998;

Causevic et al., 2005). The relative amount of a 100 kDa DNMT class 1 isoform was positively correlated with the total DNMT enzymatic activity, in agreement with a previous report (Causevic et al., 2005). Nevertheless, no significant correlation was found between the total DNMT activity and the global methylation percentage. This suggests the involvement of distinct mitotic index between poplar genotypes, cell replication without methylation maintenance and/or variable DNA glycosylase-lyase activities.

4.3. Genotype specific changes in DNA methylation levels in response to water deficit

The water deficit also affected the levels of global DNA methylation percentages, with a significant interaction between genotype and treatment. Effects of environmental variations such as temperature, diseases, light, salinity, ABA and water stress on epigenetic parameters have already been shown in plants (Boyko and Kovalchuk, 2007; Chen and Tian, 2007). The few studies on water stress have analysed overall levels of DNA hypermethylation during osmotic stress in tobacco and potato cell cultures (Kovarik et al., 1997; Sabbah et al., 1995) or during dehydratation in pea root tip cells (Labra et al., 2002). The two fold variation in DNA methylation levels in our system are in agreement with the previous reports. While poplar genotypes showed reduced growth in water deficit conditions, a significant genotype effect was observed for DNA methylation variations. This suggests that DNA methylation could participate to the fine-tuning of the reported control of gene expression in poplar during water stress (Plomion et al., 2006; Bogeat-Triboulot et al., 2007) including genotype-specific differences (Bonhomme et al., 2009). A complementary study should be done on the same plant material to confirm this hypothesis.

No correlation between DNA methylation and morphological traits was observed in response to a moderate water deficit certainly in relation to the genotype specific variations of DNA methylation. Nevertheless, morphological traits have already been shown to be genotype specific under intense water deficit (Monclus et al., 2006). It is then possible that epigenetic variations are more sensitive or precocious. Furthermore, genotype specific variations of molecular markers such as DNA methylation, mRNA and proteins reflect different strategies even if the morphological consequences are similar. Indeed, different kinetics and amplitude of the response between genotypes reflect their variable phenotypic plasticity (Monclus et al., 2006).

4.4. Histones H_3 and H_4 acetylation change neither between hybrids nor in response to moderate water deficit

Using polyclonal antibodies raised against acetylated lysine, we showed that acetylation ratios of histones H_3 and H_4 were consistent with previously-reported values (Causevic et al., 2006). No significant difference could be observed between genotypes or in response to a moderate water deficit. This result apparently contrasts with the dynamic and reversible changes of histone H_3 acetylation and methylation reported during submergence of rice plants (Tsuji et al., 2006). Nevertheless, a detailed kinetic study of the variation of histone acetylation ratios on various lysine positions during water deficit as well as a gene specific analysis are needed to reach firm conclusions. Several HDAC isoforms were assessed in hybrid poplars in accordance with poplar databases (Tuskan et al., 2006) and a limited proteolysis of the C-terminal part previously reported in other plants (Causevic et al., 2006; Loidl, 2004; Pipal et al., 2003). The relative amount of a 80 kDa HDAC isoform was positively correlated to the total HDAC enzymatic activity. This is in agreement with previous reports showing that immunoprecipitation of the corresponding isoform is followed by a strong decrease of HDAC activity in the residual supernatant (Causevic, 2005; Causevic et al., 2006). This isoform and HDAC activity were affected by water deficit in poplar in accordance with a report in *Arabidopsis* about the role of this enzyme in the abscisic acid response (Sridha and Wu, 2006).

4.5. Epigenetic traits scaled with morphological traits

Linear correlations were established between DNA methylation percentages, histones H_3 or H_4 acetylation ratios and morphological traits. Significant linear correlations were observed between epigenetic variables and the scores of $F1$ or $F2$ axes of PCAs representing the variability of morphological traits. Furthermore, some traits were highly correlated such as %mC with height or stem biomass. The occurrence of correlations do not mean a possible causal link between these variables and the biological significance, if any, remains to be established. Nevertheless, considering the role of DNA methylation in the fine-tuning control of gene expression, we hypothesise that epigenetic traits may be determinants of poplar productivity. The identification of the genomic sequences that show differential methylation should now be envisaged using recently published approaches (Gentil and Maury, 2007; Zhang et al., 2006). This will permit the discovery of biomarkers that could represent valuable tools for poplar breeders.

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REFERENCES

Allis C.D., Jenuwein T., Reinberg D., and Caparros M.-L., 2007. Epigenetics, Cold Spring Harbor Laboratory Press, New York, 502 p.

Bernacchia G., Primo A., Giorgetti L., Pitto L., and Cella R., 1998. Carrot DNA methyltransferase is encoded by two classes of genes with differing patterns of expression. Plant J. 13: 317–329.

Bogeat-Triboulot M.-B., Brosche M., Renaut J., Jouve L., Le Thiec D., Fayyaz P., Vinocur B., Witters E., Laukens K., Teichmann T., Altman A., Hausman J.-F., Polle A., Kangasjärvi J., and Dreyer E., 2007. Gradual soil water depletion results in reversible changes of gene expression, protein profiles, ecophysiology, and growth performance in *Populus euphratica*, a poplar growing in arid regions. Plant Physiol. 143: 876–892.

Bonhomme L., Barbaroux C., Monclus R., Morabito D., Berthelot A., Villar M., Dreyer E., and Brignolas F., 2008. Genetic variation in productivity, leaf traits and carbon isotope discrimination in hybrid poplars cultivated on contrasting sites. Ann. For. Sci. 65: 503.

Bonhomme L., Monclus R., Vincent D., Carpin S., Lomenech A.-M., Plomion C., Brignolas F., and Morabito D., 2009. Leaf proteome analysis of eight *Populus* × *euramericana* genotypes: genetic variation in drought response and in water-use efficiency involves photosynthesis-related proteins. Proteomics 9: 4121–4142.

Boyko A. and Kovalchuk I., 2007. Epigenetic control of plant stress response. Environ. Mol. Mutagen. 49: 61–72.

Braatne J.H., Hinckley T.M., and Stettler R.F., 1992. Influence of soil water on the physiological and morphological components of plant water balance in *Populus trichocarpa, Populus deltoides* and their F1 hybrids. Tree Physiol. 11: 325–339.

Causevic A., 2005. Étude comparative de quelques paramètres du contrôle épigénétique en relation avec le développement de lignées de betterave sucrière. Thèse de doctorat. Faculté des Sciences, Université Orléans, France, 170 p.

Causevic A., Delaunay A., Ounnar S., Righezza M., Delmotte F.M., Brignolas F., Hagège D., and Maury S., 2005. DNA methylating and demethylating treatments modify phenotype and cell wall differentiation state in sugarbeet cell lines. Plant Physiol. Biochem. 43: 681–691.

Causevic A., Gentil M.-V., Delaunay A., El Soud W.A., Garcia Z., Pannetier C., Brignolas F., Hagège D., and Maury S., 2006. Relationship between DNA methylation and histone acetylation levels, cell redox and cell differentiation states in sugarbeet. Planta 224: 812–827.

Chen Z.J. and Tian L., 2007. Roles of dynamic and reversible histone acetylation in plant development and polyploidy. Biochim. Biophys. Acta 1769: 295–307.

Gehring M. and Henikoff S., 2007. DNA methylation dynamics in plant genomes. Biochim. Biophys. Acta 1769: 276–286.

Gentil M.-V. and Maury S., 2007. Characterization of epigenetic biomarkers using new molecular approaches. In: Varshney R. and Tuberosa R. (Eds.), Genomics-Assisted Crop Improvement: Genomics Approaches and Platforms, Springer, Dordrecht, pp. 351–377.

Kovarik A., Koukalovà B., Bezdìk M., and Opatrny Z., 1997. Hypermethylation of tobacco heterochromatic loci in response to osmotic stress. Theor. Appl. Genet. 95: 301–306.

Labra M., Ghiani A., Citterio S., Sgorbati S., Sala F., Vannini C., Ruffini-Castiglione M., and Bracale M., 2002. Analysis of cytosine methylation pattern in response to water deficit in pea root tips. Plant Biol. (Stuttg.) 4: 694–699.

Loidl P., 2004. A plant dialect of the histone language. Trends Plant Sci. 9: 84–90.

Marron N., Villar M., Dreyer E., Delay D., Boudouresque E., Petit J.M., Delmotte F.M., Guehl J., and Brignolas F., 2005. Diversity of leaf traits related to productivity in 31 *Populus deltoides* × *Populus nigra* clones. Tree Physiol. 25: 425–435.

Monclus R., Dreyer E., Villar M., Delmotte F.M., Delay D., Petit J.M., Barbaroux C., Le Thiec D., Bréchet C., and Brignolas F., 2006. Impact of drought on productivity and water use efficiency in 29 genotypes of *Populus deltoides* × *Populus nigra*. New Phytol. 169: 765–777.

Pavlopoulou A. and Kossida S., 2007. Plant cytosine-5 DNA methyltransferases: structure, function, and molecular evolution. Genomics 90: 530–541.

Pipal A., Goralik-Schramel M., Lusser A., Lanzanova C., Sarg B., Loidl A., Lindner H., Rossi V., and Loidl P., 2003. Regulation and processing of maize histone deacetylase Hda1 by limited proteolysis. Plant Cell. 15: 1904–1917.

Plomion C., Lalanne C., Claverol S., Meddour H., Kohler A., Bogeat-Triboulot M.-B., Barre A., Le Provost G., Dumazet H., Jacob D., Bastien C., Dreyer E., de Daruvar A., Guehl J.-M., Schmitter J.-M., Martin F., and Bonneu M., 2006. Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. Proteomics 6: 6509–6527.

Riddle N.C. and Richards E.J., 2002. The control of natural variation in cytosine methylation in *Arabidopsis*. Genetics 162: 355–363.

Roden J., van Volkenburgh E., and Hinckley T.M., 1990. Cellular basis for limitation of poplar leaf growth by water deficit. Tree Physiol. 6: 211–219.

Sabbah S., Raise M., and Tal M., 1995. Methylation of DNA in NaCl-adapted cells of potato. Plant Cell Rep. 14: 467–470.

Sridha S. and Wu K., 2006. Identification of AtHD2C as a novel regulator of abscisic acid responses in *Arabidopsis*. Plant J. 46: 124–133.

Tschaplinski T.J., Tuskan G.A., and Gunderson C.A., 1994. Water-stress tolerance of black and eastern cottonwood clone and four hybrid progeny. I. Growth, water relations and gas exchange. Can. J. For. Res. 24: 364–371.

Tsuji H., Saika H., Tsutsumi N., Hirai A., and Nakazono M., 2006. Dynamic and reversible changes in histone H3-Lys4 methylation and H3 acetylation occurring at submergence-inducible genes in rice. Plant Cell Physiol. 47: 995–1003.

Tuskan G.A. et al., 2006. The Genome of Black Cottonwood, *Populus trichocarpa* (Torr. & Gray). Science 313: 1596–1604.

Vaughn M.W., Tanurdzic M., Lippman Z., Jiang H., Carrasquillo R., Rabinowicz P.D., Dedhia N., McCombie W.R., Agier N., Bulski A., Colot V., Doerge R.W., and Martienssen R.A., 2007. Epigenetic natural variation in *Arabidopsis thaliana*. PLoS Biology 5: e174.

Zhang X., Yazaki J., Sundaresan A., Cokus S., Chan S.W.-L., Chen H., Henderson I.R., Shinn P., Pellegrini M., Jacobsen S.E., and Ecker J.R., 2006. Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. Cell 126: 1189–1201.

Zhang X., Shiu S., Cal A., and Borevitz J.O., 2008. Global analysis of genetic, epigenetic and transcriptional polymorphisms in *Arabidopsis thaliana* using whole genome tiling arrays. PLoS Genetics 4: e1000032. Doi:10.1371/journal.pgen.1000032.

Zilberman D., Gehring M., Tran R.K., Ballinger T., and Henikoff S., 2007. Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. Nature Genetics 39: 61–69.