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ORIGINAL ARTICLE

Poplars with a *PtDDM1-RNAi* transgene have reduced DNA methylation and show aberrant post-dormancy morphology

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Abstract The *Arabidopsis thaliana DDM1 (Decreased DNA Methylation)* gene is necessary for the maintenance of DNA methylation and heterochromatin assembly. In *Arabidopsis*, *ddm1* mutants exhibit strong but delayed morphological phenotypes. We used RNA interference (RNAi) to suppress transcripts of two orthologous *DDM1* paralogs in *Populus trichocarpa* and examined effects on whole plant phenotypes during perennial growth and seasonal dormancy. The RNAi-*PtDDM1* transgenic poplars showed a wide range of *DDM1* transcript suppression; the most strongly suppressed line had 37.5 % of the expression of the non-transgenic control. Genomic cytosine methylation (mC %) was 11.1 % in the non-transgenic control, compared with 9.1 % for the transgenic event with lowest mC %, a reduction of 18.1 %. An evaluation of greenhouse growth directly after acclimation of in vitro grown plants showed no developmental or growth rate abnormalities associated with the decrease in *PtDDM1* expression. However, after a dormancy cycle and growth outdoors, a mottled leaf phenotype appeared in some of the transgenic insertion events that had strongly reduced *PtDDM1* expression and DNA methylation. The phenotypic consequences of reduced DDM1 activity and DNA methylation appears to increase with cumulative plant propagation and growth.

Keywords *DDM1* · RNA interference · DNA methylation · Leaf mottling · *Populus trichocarpa*

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Introduction

Epigenetic remodeling of chromatin plays a major role in genome regulation during plant development (Feng et al. 2010). DNA methylation and histone modifications are the most commonly assessed epigenetic marks and are associated with formation and maintenance of both euchromatin and heterochromatin. The distribution of these marks defines four main chromatin states by indexing active genes, repressed genes, silent repeat elements, and intergenic regions (Roudier et al. 2011). DNA methylation of promoter regions is generally associated with reduced expression of the corresponding genes (Zhang et al. 2006), whereas DNA methylation within gene bodies has a more complex association with gene expression, varying among species and level or pattern of gene expression (Zhang et al. 2006; Zilberman et al. 2007; Lauria and Rossi 2011; Vining et al. 2012). High levels of cytosine methylation are essential for silencing of transposable elements located in heterochromatin, and reduction of DNA methylation often results in their reactivation (Hirochika et al. 2000; Miura 2001; Singer et al. 2001; Wright and Voytas 2002; Lippman et al. 2004).

DDM1 was first identified in *Arabidopsis* as a mutation that caused a "decrease in DNA methylation", most

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notably in heterochromatin (Vongs et al. 1993; Jeddeloh et al. 1999). Homozygous *ddm1* mutants show a range of morphological abnormalities (Kakutani et al. 1996; Soppe et al. 2000; Saze and Kakutani 2007). The observed phenotypes are thought to be caused by a combination of epigenetic changes at functional genes and insertion of reactivated transposable elements into novel gene-associated locations (Miura et al. 2001; Singer et al. 2001). However, these mutant phenotypes were not immediately apparent but rather appeared several generations after the initial loss of *DDM1* activity (Saze and Kakutani 2007). The remethylation of sequences that were hypomethylated in *ddm1* mutants is extremely slow, even in wild-type *DDM1* backgrounds, and these remethylated loci are associated with high amounts of siRNAs (Teixeira et al. 2009). Unlike *met1* and *cmt3* mutants which show immediate recovery of DNA methylation, the demethylated loci in the *ddm1* mutants remain hypomethylated for at least two generations after crossing to wild type (Kakutani et al. 1999; Mahfouz 2010).

DDM1 is a SWI/SNF family chromatin remodeling factor (Jeddeloh et al. 1999) and has ATP-dependent chromatin remodeling capability both with naked and nucleosomal DNA (Brzeski and Jerzmanowski 2003). The DDM1 protein co-localizes with methyl-CpG binding domain (MBD) proteins, which target methylated cytosines, typically in a CpG context (Zemach et al. 2005). This suggests a possible role in which DDM1 remodels chromatin to provide access for methylated cytosines. However, the precise mechanism by which DDM1 affects DNA methylation, and does so differentially in heterochromatic and specific genic regions, remains unclear (e.g., Saze and Kakutani 2007; Blevins et al. 2009; Melamed-Bessudo and Levy 2012).

Although epigenetic perturbations associated with *ddm1* mutants have been studied in *Arabidopsis (*Vielle-Calzada et al. 1999; Singer et al. 2001; Saze and Kakutani 2007*)* and to a lesser extent in *Brassica* (Fujimoto et al. 2008), their effects in perennial plants and trees are unknown. Gross genomic DNA methylation appears to vary during dedifferentiation and habituation during in vitro propagation of plants, and also during maturation and rejuvenation in forest trees (Fraga et al. 2002; Baurens et al. 2004). This suggests that perturbations to genome methylation might affect a number of basic developmental processes in trees and possibly be a useful tool for biotechnology where alterations to maturation state or redifferentiation are desired. By simultaneous RNAi-mediated suppression of the two *DDM1* homologs of *Populus trichocarpa*, we hoped to alter cytosine methylation levels and study the phenotypic consequences of decreased DNA methylation in poplar. We show here that RNAi-mediated suppression of *PtDDM1* transcript levels caused a significant genome-wide decrease of cytosine DNA methylation and that analogous to *Arabidopsis*, phenotypic consequences were at first invisible, but then became manifest during further propagation and growth.

Materials and methods

Identification of *AtDDM1* homologs, construct design and assembly

Two putative *DDM1* orthologs from the *Populus trichocarpa* genome (*PtDDM1-1:* POPTR_0007s12710 and *PtDDM1-2*: POPTR_0019s15030) were identified in Phytozome (Phytozome v7.0 2011) and hypothesized to be *DDM1* homologs after evaluation using NCBI BLAST (Altschul et al. 1990; Fig. 1, Supplemental Table S1). We designed a construct (Fig. 2) to suppress both *DDM1* transcripts in the target female hybrid clone *717-1B4* (*P. tremula* × *P. alba*), which is widely used for poplar transgenic studies (e.g., see Durand et al. 2010; Han et al. 2011). Using cDNA isolated from shoot-tips of *717-1B4*, primers DDM1-F01 and DDM1-R02 (Supplemental Table S2) were used to amplify a 500-bp fragment of both *PtDDM1-1* and *PtDDM1-2* using Platinum Taq HiFi Polymerase (Invitrogen, Carlsbad, CA, USA). The products were cloned into pCR-4 (Topo vector, Invitrogen) and verified by sequencing. We blasted the 500-bp *717-1B4 DDM1* sequence against *P. trichocarpa DDM1* transcripts to design a new primer pair, NDDM-F01 and NDDM-R01 (Supplemental Table S2), which amplified a 190-bp fragment for the RNAi construct, a segment highly conserved between *PtDDM1-1* and *PtDDM1-2* (96 % identity). Using Pfx Polymerase (Invitrogen), the 190-bp PCR product was amplified from *P.trichocarpa* shoot tip cDNA, cloned into the pENTRY vector (Invitrogen), and verified by sequencing. LR recombination was performed between the pCAPD binary vector and the pENTRY using Gateway LR Clonase II enzyme mix (Invitrogen). The orientation of sense and antisense DNA fragments in the final expression vector was confirmed by restriction analysis and sequencing.

Plant material, transformation, regeneration, and screening

The *PtDDM1* RNAi construct was transformed into *A. tumefaciens* strain *AGL1* by electroporation, verified by PCR and then into *717-1B4*, essentially using a previously described method (Filichkin et al. 2006). Non-transgenic plants were used as a control. After 2 months in root induction medium (height of ~10 cm), in vitro propagated candidates of transgenic poplars were harvested for DNA extraction and PCR confirmation of their transgenic status.

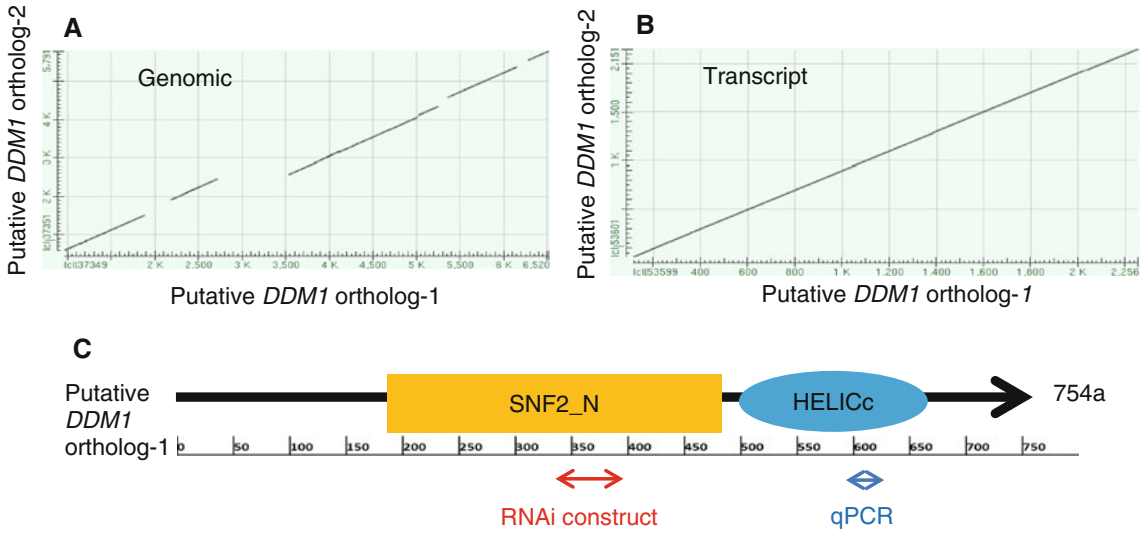

Fig. 1 Alignment and structure of putative *DDM1* orthologs in poplar. **a** Dot matrix alignment of genomic sequences of ortholog-*1* and ortholog-*2*. Numbers on the axes represent positions of the residues. **b** Dot matrix alignment of transcripts of cDNAs of ortholog-*1* and ortholog-*2*. **c** Conserved domains of ortholog-*1* compared with *Arabidopsis DDM1*. The positions of the region targeted by the RNAi construct and used for qPCR are labeled

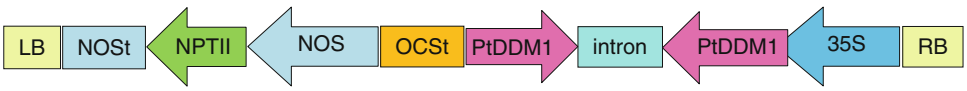

Fig. 2 T-DNA structure and primer sites for the *PtDDM1* RNAi construct within the pCAPD (pART27) binary vector backbone. *LB* left border, *RB* right border

PtDDM1 expression by qPCR

To quantify endogenous *PtDDM1* gene expression, we performed quantitative real-time, reverse transcriptase (RT) PCR (hereafter called "qPCR"). We extracted RNA from shoot tips of 2-month-old, in vitro propagated plants. Three independent extractions were made for each of the transgenic events and the non-transgenic control. RNA was extracted using freshly made extraction buffer (4 M guanidine, 0.2 M sodium acetate [pH 5.0], 25 mM EDTA, 2.5 % polyvinylpyrrolidone [p/v], 1 % [v/v] β-mercaptoethanol) and purified by QIAshredder columns (Qiagen, Hilden, Germany), and a Qiagen RNeasy Mini Kit. RNA quality was checked by electrophoresis for obvious 28S and 18S ribosomal bands and the absence of degradation products, and quantified on an ND-1000 UV–Vis spectrophotometer. From each sample, 10 µg RNA was treated with TURBO DNase I (Ambion, Austin, TX, USA) and again quantified on an ND-1000 UV–Vis spectrophotometer. One µg of DNAse I-treated RNA was used to synthesize cDNA (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen). The reference gene was polyubiquitin (BU879229) in *P. trichocarpa,* as suggested for poplar (Brunner et al. 2004). The primer pairs used to amplify the 107-bp-fragment of endogenous *PtDDM1* transcripts for qPCR were *PtDDM1*_C and *PtDDM1*-NR01 (Supplemental Table 1). The forward primer *PtDDM1*_C has 95 % identity between *PtDDM1-1* and *PtDDM1-2* transcripts, while the reverse primer *PtDDM1*-NR01 has 100 % (Supplemental Table 2). We used three 96-well plates and a randomized block design on each plate. Two technical replicates were performed for each reaction and placed in adjacent wells in plates. qPCR was performed using SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen). The PCR program used was

1. 50 °C for 2 min,
2. 95 °C for 10 min,
3. 95 °C, 15 s,
4. 60 °C, 60 s,
5. 40 cycles of 3 and 4.

Dissociation curves were checked for single peaks for all products. PtDDM1 expression was calculated from

$$\Delta\mathrm{CT} = CT_{\mathrm{target}} - CT_{\mathrm{reference}}$$

$$R_{\mathrm{i}} = \frac{1}{e^{\Delta CT}} \qquad R_{\mathrm{c}} = \frac{1}{e^{\Delta CT_{\mathrm{control}}}} \qquad P_{\mathrm{i}} = \frac{R_{\mathrm{i}}}{R_{\mathrm{c}}}$$

ΔCT is the difference in cycle threshold between the target gene and the reference gene; R_{i} is the normalized starting amount of the target gene transcripts in line *i* or

normalized *PtDDM1* expression for line *i*; R_c is the normalized *PtDDM1* expression for the non-transgenic control; *e* is the mean amplification efficiency. The efficiency was calculated by real-time PCR Miner (Zhao and Fernald 2005). P_i is the percentage of *PtDDM1* expression for line *i* compared with the non-transgenic control. A repeated qPCR study was done on independent cDNA synthesis of the same RNA for selected events.

Total cytosine methylation by HPLC

We measured percent cytosine methylation (mC %) using HPLC. For plants growing in an outdoor covered nursery, we used young but fully expanded leaves from five selected events and the non-transgenic control. The selected events included two of the most highly suppressed events based on qPCR, two intermediate suppression events, and one event with no detectable change compared with the control. In contrast, because of limited material available in vitro, we used both leaves and stems from three selected transgenic poplars and one non-transgenic control that were being micropropagated in sterile Magenta boxes. We used a CTAB method to extract genomic DNA (Porebski et al. 1997). The quality of DNA was checked by gel electrophoresis and quantified using an ND-1000 UV–Vis spectrophotometer. About 500 ng/μL of DNA in 1 mL was treated with 1 μL of RNase A (100 mg/mL) and 1 μL Ribonuclease T1 (0.5 mg/mL), extracted with chloroform:octanol (24:1), precipitated with 95 % ethanol and 3 M Sodium Acetate, washed twice with 70 % ethanol, centrifuged, and air-dried. The sample was resuspended with 170 μL 1 × Turbo DNase I buffer (Ambion) overnight and incubated with Turbo DNaseI for 12 h at 37 °C. At the end of the incubation period, 20 μL of 100 mM Tris–HCl (pH 10.2) and 10 μL snake venom phosphodiesterase I (10 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) were added to the mixture and incubated for 8 h at 37 °C. For the separation of nucleosides, we used the following buffers: Buffer A, 8 mM TBA-OH (tributylammonium hydroxide), 0.01 M KH_2PO_4; Buffer B, 2 mM TBA-OH, 0.10 M KH_2PO_4. The HPLC elution program was (1) 0–35 min, mobile phase change from 10 % buffer B and 90 % buffer A to 25 % buffer B and 75 % buffer A; (2) 36–40 min, from 25 % buffer B and 75 % buffer A back to 10 % buffer B and 90 % buffer A. The column used was an AlltimaTM C18 5u (Alltech, Lexington, KY), nucleosides were detected at 280 nm, and nucleoside standards were processed together with the hydrolysate. Standard curves were based on nucleoside standards (HPLC grade, Sigma-Aldrich) at 0.5, 1.0, 1.5, and 2.5 nM. The amount of cytosine and methyl-cytosine was calculated as $\%mC = (mC/(mC + C)) \times 100$ based on estimates of peak area, where "C" represents 2′-deoxycytidine content and "mC" 5-methyl-2′-deoxycytidine content (Gentil and Maury 2007).

To verify our HPLC results, leaf material taken from plants growing in vitro and in the covered outdoor growth area described above was collected and immediately lyophilized before shipping to the Maury laboratory in Orleans, France. There, genomic DNA was purified from poplar samples using a published method (Causevic et al. 2005; Gourcilleau et al. 2010) employing RNase A digestion, phenol/chloroform extraction, and ethanol precipitation. Hydrolysis of purified DNA into nucleosides was performed using successively DNase I (700 U, Roche Diagnostics, Meylan, France), phosphodiesterase I (0.05 U, SerLabo Technologies, Entraigues sur la Sorgue, France), and alkaline phosphatase type III (0.5 U, Sigma-Aldrich, Saint-Quentin Fallavier, France). The global percentage of methylation was determined by HPLC (Gentil and Maury 2007) using a Gemini^{TM} column (150 × 4.6 mm, 5 μm, Phenomenex, Le Pecq, France) with an isocratic mobile phase composed of 0.5 % methanol (v/v) and 5 mM acetic acid in water according to the methods of Gourcilleau et al. (2010). Controls for this procedure included co-migration with commercial standards (Sigma-Aldrich), confirmation by enzyme restriction analysis (Causevic et al. 2005), and tests for RNA contamination using HPLC detection of ribonucleosides. The methylcytosine percentages were calculated as above. Three independent analyses with three replicates each were performed for each measurement.

Greenhouse study

We moved 2-month-old in vitro propagated transgenic poplars from the in vitro sterile environment to gradually become acclimated to an open environment starting in early January 2010. By the end of January 2010, we moved the plants to the greenhouse with a mean temperature of ~25 °C during the day and ~21 °C at night. Each transgenic line had at least five ramets, and the non-transgenic control had 20 ramets. They were randomly arranged on two benches in the greenhouse, and the plants were watered every day and fertilized every week using liquid fertilizer (400–500 ppm of N:P:K, 20:20:20).

Height (cm, from top leaf to the border of container) and diameter (cm, at ground level) were measured every week for the first 6 weeks once the plants were moved into the greenhouse. We calculated volume index (diameter^2 × height, cm^3) for analysis of overall productivity. We also visually compared transgenic trees and controls carefully regarding leaf color, leaf shape, and leaf pubescence but were not able to find any obvious differences (thus no quantitative data was taken for these traits). Because we observed a high rate of sylleptic branching in the transgenic population compared with other studies conducted under

similar conditions using the same poplar genotypes, we measured sylleptic branching frequency and length of the longest sylleptic branch of each tree. Sylleptic branches are those which grow out from the main stem during the same season that the main stem is also elongating: Cline and Dong-IL (2002). We also observed variation among the color of the leaves; thus chlorophyll content of fully expanded leaves was measured by a chlorophyllometer (Konica Minolta brand SPAD-502, Spectrum Technologies, Inc). At the end of the greenhouse study (October 2010), we moved the plants to a walk-in growth chamber, where the plants went through a 6-month dormancy induction period at a constant temperature of 4 °C under continuous darkness. In March 2011, the plants were moved out of doors to a covered growth area. They were water every other day and fertilized with a slow release fertilizer according to supplier recommendations (Osmocote, The Scotts Company, Marysvill, OH, USA). Following winter dormancy and regrowth, plants were scored for the presence of mottled leaves according to the following criteria:

1. "+", only 1–5 leaves were mottled, and mottled area was <1/3 of the whole leaf;
2. "++", 5–10 leaves were mottled, and the mottled area on average was more than 1/3 of the whole leaf and <2/3 of the whole leaf;
3. "+++", over 10 leaves were mottled, and the mottled area on average was more than 2/3 of the whole leaf.

Statistical analysis

Data obtained from qPCR, in vitro, greenhouse, and HPLC analyses were analyzed by one-way ANOVA, linear regression, and/or a two-sample *t* test using the R statistical package (v2.12, R Foundation for Statistical Computing, Vienna, Austria). For gene expression, we used one-way ANOVA to test the null hypothesis of homogeneity of *PtDDM1* expression among transgenic events and the control line *717-1B4*. The assumptions of ANOVA, specifically a normal distribution and homogeneity of variances, were checked before each analysis by visual inspection, and necessary transformations of data were performed to better meet the model assumptions. Holm corrections were made when there were multiple comparisons examined. One-way ANOVA was used to test the hypothesis that DNA methylation did not vary among events. Expecting a decrease in DNA methylation and gene expression in our transgenic plants based on RNAi and the known function of *DDM1* in *Arabidopsis*, we employed one-tailed, two-sample t-tests to compare each transgenic event with the *717-1B4* non-transgenic control, and for analysis of the correlation between *PtDDM1* expression and DNA methylation. A Fisher-Freeman-Halton exact test was used to test the association between mottled leaf phenotype and the mC % because of the small sample size.

Results

Identification of *PtDDM1* genes and suppression in transgenic poplars

Two putative *DDM1* orthologs in *Populus trichocarpa* genomes were identified by BLAST. Alignment of genomic sequences of the two putative poplar *DDM1*orthologs showed 86 % identity in their overlapping region (Supplemental Table S1A*)*. The genomic sequence of putative ortholog-1 was ~ 800 bp longer than that of putative ortholog-2 (Fig. 1a), but cDNA of their transcripts was both about 2.2 kb (Fig. 1b). In their overlapping region, the cDNAs showed 87 % nucleotide identity (Supplemental Table 1B) and 89 % putative amino acid identity (Supplemental Table 1C*)*. The two putative *DDM1* orthologs from poplar were also highly conserved relative to *Arabidopsis DDM1(*≥71 % identity and ≥91 % coverage at the transcript level and ≥73 % and ≥78 % at protein level; Supplemental Table 1). The two major domains of DDM1 from poplar orthologs were especially highly conserved compared with *Arabidopsis DDM1*: ≥77 % (SNF2_N) and ≥89 % (HELICc) identity, with ≥100 % coverage (Supplemental Table 1D, E; Fig. 1c). We, therefore, conclude that the two putative orthologs in poplar are very likely to be evolutionary homologs of *DDM1* and were thus named *PtDDM1-1* and *PtDDM1-2,* respectively. This is similar to the situation in *Oliva sativa* and *Brassica rapa*, where two homologs of *DDM1* gene were identified (Tuskan et al. 2006; Johzuka-Hisatomi et al. 2008; Fujimoto et al. 2008).

Table 1 Transformation efficiency of RNAi *PtDDM1* construct

Type of explant	Explants co-cultivated	Explants that produced shoots	Explants that produced roots	Transgenic lines confirmed by PCR	Transformation efficiency (%)
Leaves	558	308	120	14	2.5
Stems	497	126	50	8	1.6

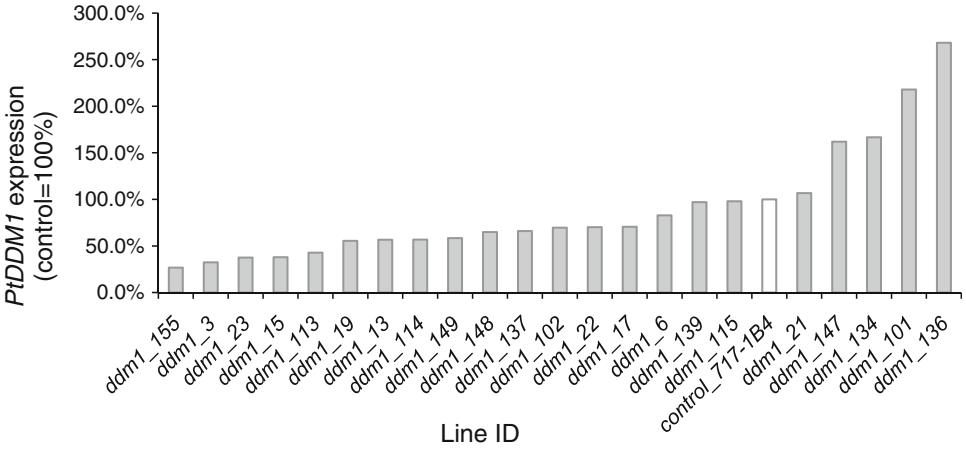

Fig. 3 Endogenous *PtDDM1* expression in transgenic events (*gray bars*) vs. non-transgenic controls (*white bar*) measured by qPCR. RNA was extracted from combined in vitro leaf and stem tissues

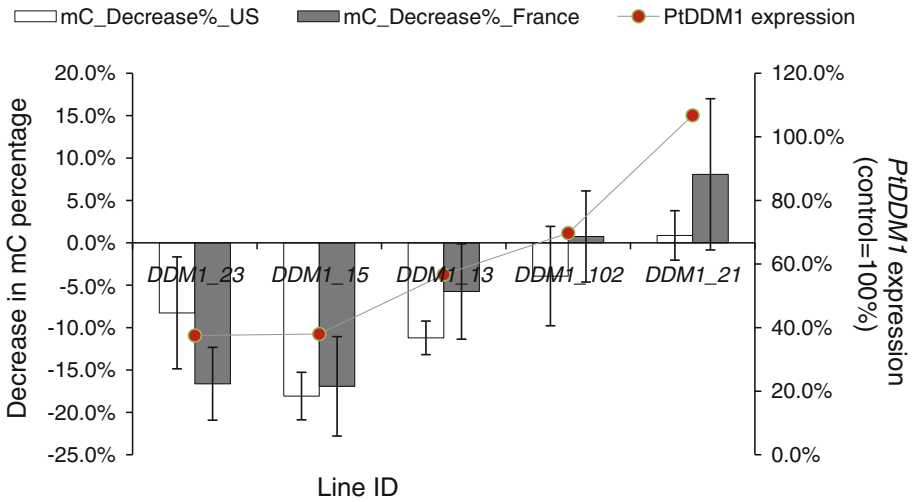

Fig. 4 Percent decrease in mC % in *PtDDM1* transgenic poplars compared to non-transgenic control *717-1B4*

The *PtDDM1* RNAi construct was designed to suppress both *DDM1* transcripts in *717-1B4* (Fig. 2). The absence or presence of intact constructs (Table 1) was tested in the genome of control and non-transgenic poplars, respectively. Only events that had both the sense and antisense copies of the 190-bp *PtDDM1* sequence were further studied. There were 22 confirmed transgenic events. Compared with the number of starting explants, the transformation efficiency was 2.5 % for leaf explants and 1.6 % for stem explants.

In order to select strongly *PtDDM1*-suppressed transgenic events, we performed qPCR to quantify endogenous *PtDDM1* gene expression. RNAi suppression efficiency varied widely among the transgenic events, with the strongest reduction of 62.5 % compared with that of the wild type (Fig. 3). The high *PtDDM1* expression events included the control and events *21, 147, 134, 101, and 136*. These events, except the control and event *21*, had a large amount of variance among replicates. The lowest *PtDDM1* expression events included event *155*, *3, 23,* and *15,* and these events had low variance among replicates. A repeated analysis done on independent cDNA syntheses of the same RNA for selected events confirmed the relative expression levels among the events, except for event *155* (data not shown). One-way ANOVA analysis suggested a possible but inconclusive effect of event on *PtDDM1* expression for the *23* genotypes [$F(21,43) = 1.73$, $P = 0.06$]. However, two sample, one-tailed *t* tests suggest that *PtDDM1* expression of event *15* and *23* were significantly different from control *717-1B4* ($P < 0.05$ after Holm correction). Based both on the mean and variances within each event, six genotypes were selected to represent low- (event *15* and *23*, with 38.0 and 37.5 % *PtDDM1* expression), intermediate- (event *13* and *102*, with 56.7 and 69.7 %), and high-expression groups (*717-1B4* control and event *21*, with 100 and 106.8 %) for further molecular analysis.

Genomic cytosine methylation in *PtDDM1* suppressed poplars

Percent cytosine methylation (mC %) was measured by HPLC (Fig. 4). The low and intermediate *PtDDM1*

expression lines showed only a moderate decrease in mC % that ranged from −0.9 to 18.1 % based on young but fully expanded leaves (Fig. 4, Fig. S1A). Lines *15*, which had among the lowest *PtDDM1* expression, showed the greatest decrease (18.1 %), but a one-tailed Student's *t* tests vs. controls (with Holm correction) showed a *P value* of 0.08. Nevertheless, there was a statistically significant linear (Pearson, *r*) correlation between *PtDDM1* expression and mC % ($r = 0.84$, $P < 0.05$, one-tailed test).

Analysis of mC % was also performed in parallel in France with young leaves giving similar results to that in the USA (Fig. 4, Fig. S1B). One-way ANOVA analysis between lines showed that events were now a significant source of variance [$F(5, 15) = 3.11$, $P = 0.04$]. Thus, both of the low-expression lines, *15* and *23*, exhibited large and significant (one-sided $P < 0.05$ after Holm correction) decreases of mC % compared with the control line (17.0 and 16.7 %, respectively). Pearson correlations between *PtDDM1* expression and mC % were also confirmed ($r = 0.91$ at $P < 0.001$).

Leaves from plants regenerating in vitro were also analyzed for their mC %; their methylation levels were similar to those found in adult plants grown out-of-doors (Fig. S2). An analysis of leaf size in relation to methylation showed that it, however, can have a large effect on methylation levels (Fig. S3). Deviations from means from both USA and France estimates were correlated (Pearson one-sided test, $P = 0.02$), and the grand event averages over analyses from both countries showed a highly significant correlation with *PtDDM1* expression (Fig. S4: Pearson one-tailed test, with $P = 0.003$).

Phenotype of *PtDDM1* suppressed poplars

PtDDM1 poplars did not show significant difference from non-transgenic control during in vitro propagation (data not shown). The greenhouse phenotypes of *PtDDM1*-suppressed poplars showed differences among events in diameter [$F(21,106) = 2.57$, $P = 0.0008$], height [$F(21,106) = 2.88$, $P = 0.0002$], and volume index [$F(21,106) = 3.11$, $P < 0.0001$]. However, we found no evidence of differences among events in the number of sylleptic branches produced per tree [$F(21,106) = 1.45$, $P = 0.11$] or in chlorophyll content [$F(21, 106) = 0.93$, $P = 0.55$]. Two sample *t* tests also showed no evidence of overall differences between transgenic poplars and the non-transgenic control in height ($P = 0.16$), diameter ($P = 0.32$), number of sylleptic branches ($P = 0.13$), or chlorophyll content ($P = 0.62$). *PtDDM1* expression was also not statistically associated with mean event diameter ($P = 0.13$), height ($P = 0.58$), volume index ($P = 0.27$), or sylleptic branching ($P = 0.92$). Pearson correlations (two-tailed) between average deviation from sample means for mC % and volume index showed a *P value* of 0.13, indicating a lack of significant association. During the greenhouse study, transgenic poplars were also visually inspected for abnormal development or stem/leaf morphology, but we found no obvious differences (data not shown).

Plants were put in a cold chamber to induce dormancy for 5 months and subsequently moved out of doors for 3 months resuming growth in spring 2011. The newly expanded leaves were observed to have a severe mottled leaf phenotype in all the ramets of event *15* (Table 2; Fig. 5c). Four out of five ramets of event *23* and one out of five ramets of event *102* also showed similar but significantly less severe symptoms (Table 2; Fig. 5d). For event *13*, *21,* and the controls, no mutant phenotypes were observed (Table 2; Fig. 5e). A Fisher-Freeman-Halton exact test of the *PtDDM1* expression among events in relation to the presence of mottling of ramets showed a one-sided *P* value of 0.03.

Discussion

Genomic DNA methylation has a major role in development in animals; however, its effects in plants are poorly known, based primarily on a single, annual model species. We, therefore, sought to expand knowledge of its role in plants by RNAi suppression in poplar-a phylogenetically distinct, perennial angiosperm. Our results show that RNAi was effective at suppressing gene expression and cellular cytosine methylation in poplar; however, phenotypic consequences were at first invisible, occurring only after months of additional growth.

PtDDM1 RNAi suppression efficiency

RNAi *DDM1* suppression efficiency varied widely among transgenic events, similar to observations in *Brassica* (Fujimoto et al. 2008). RNAi was used to down-regulate both *DDM1* genes in *Brassica rapa* and they observed strong and variable suppression, with a maximum of 88 %

Table 2 Distribution of mottled leaves among *PtDDM1*transgenic events

PtDDM1 expression	Event ID	Mottling severity	No. ramets mottled	Total ramets
Low	Event 15	+++	5	5
	Event 23	+	4	5
Intermediate	Event 13	–	0	5
	Event 102	+	1	5
High	717-1B4	–	0	20
	Event 21	–	0	5

Fig. 5 Growth of RNAi *PtDDM1* transgenic poplars. **a** May 2010, 3 months after moving to the greenhouse. **b** June 2011, 3 months after moving the plants outdoors post-dormancy (note fiberglass roof covering). Examples of mottled leaf phenotypes observed after growth ourdoors following dormancy. **c** Mutant leaves of event *15*, which showed a severe mottled leaf phenotype in all of its ramets. **d** Mutant leaves of event *23*. Event *23* and *102* showed a moderate mottled phenotype. **e** Wild type leaves of control *717-1B4*

based on qPCR. This is similar to the strongest suppression observed in our study of 62 % for event *15*. The similarity in maximum suppression between *Brassica* and poplar occurred in spite of some significant differences in RNAi construct design. In our construct, a 190-bp sequence homologous to both *PtDDM1-1* and *PtDDM1-2* was used for the inverted repeats; in the *Brassica* study a 300-bp sequence was used for generation of an inverted repeat (Fujimoto et al. 2008). Suppression efficiency and frequency tend to increase with the length of the inverted repeats (Bleys et al. 2006). In addition, the 300-bp sequence used in *Brassica* was 100 % identical between the two co-expressed *DDM1* homologs (*BrDDM1a* and *BrDDM1b*). However, the 190-bp sequence we targeted in *PtDDM1-1* and *PtDDM1-2* had only 96 % identity between transcripts of the two gene models. Both genes are highly correlated in their expression patterns over physiological gradients (Fig. S5A) and developmental states (Fig. S5B). Although the rate of *Brassica* transformation was not reported (Fujimoto et al. 2008), our transformation rate was 2 %, considerably lower than we typically observe for this poplar clone in our laboratory (usually 10–30 %, unpubl. data). It is possible that the more highly suppressed transgenic events might have had difficulty regenerating in both taxa, and thus events with only modest levels of *DDM1* suppression were recovered.

PtDDM1 suppression is correlated with reduced DNA methylation

We observed a maximum loss of approximately 18 % in genomic DNA methylation in leaves of transgenic plants compared with controls. This change was observed in samples from distinct developmental stages and in both greenhouse and in vitro grown plants. These reductions were much less than the 70 % loss observed in homozygous loss of function *ddm1* mutants in *Arabidopsis* (Kakutani et al. 1999). However, the demethylation effect in *Arabidopsis ddm1* mutants was seen only after several self-crosses and thus occurred gradually (Saze and

Kakutani 2007). We thus might expect to see substantial further decreases in mC % after sexual reproduction or additional cycles of dormancy and propagation. As discussed above, it is also possible that further reduction of DNA methylation in transgenic tissues might have impaired the ability to regenerate shoots or roots, biasing our transgenic population toward higher levels of DNA methylation that might be possible in alternative regeneration systems (e.g., *in planta* transformation as is common in *Arabidopsis*).

DDM1 is required for maintenance of DNA methylation in *Arabidopsis* (Vongs et al. 1993; Jeddeloh et al. 1998). However, a quantitative relationship between *DDM1* expression and total cellular DNA methylation has not been reported previously. In our study, we produced transgenic poplars with a range of *PtDDM1* expression through RNAi and selected six representative events to measure total cell mC % by HPLC. Our results showed a positive correlation between *PtDDM1* expression and DNA methylation. The outlier (event *23*) had low *PtDDM1* expression, but high DNA methylation; however, this event was not an outlier when the analysis was repeated in France.

Despite a strong reduction in *PtDDM1* expression (62 %), there was only modest decrease of DNA methylation (less than 20 %). This suggests that *PtDDM1* transcription is not linearly related to DNA methylation. However, further loss of DNA methylation may occur with further growth or reproduction; substantial reductions required a number of generations in *Arabidopsis* (Saze and Kakutani 2007).

PtDDM1 suppression elicits post-dormancy onset of morphological disturbance

The mottled phenotypes observed in the greenhouse were significantly associated with reductions in cytosine methylation. To our knowledge, such phenotypes have not been previously described in plants that have reduced DNA methylation. The mottled phenotype was similar to what was observed as "lesion mimics" (Wu et al. 2008). This phenotype may have resulted from demethylation and hyperactivation of disease resistance genes, which can be a trigger for programmed cell death (PCD) (Lorrain et al. 2003). In a genome-scale methylation density analysis of poplar a cluster of leucine-rich repeat (LRR) disease resistance genes were hypermethylated in vegetative tissues and hypomethylated in male flowers (Vining et al. 2012). If normally high methylation levels in leaves were disturbed by reduced *PtDDM1* gene expression, this may have led to ectopic expression of LRR genes and this could have triggered PCD.

Another possibility is demethylation-induced reactivation of a retrotransposon and their associated retroviruses. A striking feature of the mottled leaf phenotype is the vein-associated lesions with sharp boundaries, which are similar to what is seen with many virus infections. In *Arabidopsis, ddm1* mutants were found to activate plant retrotransposons that retained viral envelope-like protein coding regions (Vicient et al. 2001). Perhaps virus-like elements were activated by demethylation-induced retrotransposon expression and retain the potential to infect cells (Wright and Voytas 1998; Vicient et al. 2001; Wright and Voytas 2002). The mottled leaf phenotype appeared after winter dormancy period and in an environment where trees were root-bound and had limited water and nutrients; thus epigenetic or transcriptional modification during dormancy and stress might also be triggering factors. Stressful environments, such as cold temperature, high temperature, and drought, are widely known to reactivate transposable elements and induce epigenetic change (Guo et al. 2003; Wu et al. 2003; Arnholdt-Schmitt 2004; Chinnusamy and Zhu 2009; Tittel-Elmer et al. 2010).

Although we found that the growth in diameter, height, and volume index did vary significantly among gene insertion events in the greenhouse, these were not statistically associated with *PtDDM1* expression or cytosine methylation. Apart from mottling, we did not observe any unusual morphological phenotypes in the transgenic population. Although the *Arabidopsis* studies did not include randomized a quantitative assessment such as that we performed, the results generally agree with our observations, as *ddm1* mutants produced no obvious mutant phenotypes for several generations (Kakutani et al. 1996; Miura et al. 2001; Singer et al. 2001; Saze and Kakutani 2007). The significant variation that we observed among events could have been the result of transgene insertion or somaclonal effects. However, such variation, for example, when transgenic trees contain only reporter or selectable marker genes, are rare in the Strauss laboratory's two decades years of experience with transgenic poplars (unpublished data). Perhaps the epigenetic perturbations of transformation interacted with *PtDDM1* effects in a non-linear manner, causing high event-associated variance but the absence of a simple expression/growth association.

Conclusion

Our study shows that transgenic methods are powerful tools for producing epigenomic modification in trees and that these modifications can have developmental consequences. However, our study only gives a first glance at how modification of the epigenome can affect tree development and whether such modifications could be of value in tree breeding and biotechnology. It is likely that longer term experiments, analysis of the effects of additional

epigenetic control genes, and study of a much wider range of environmental and developmental gradients would reveal many additional phenotypes. Bräutigam et al. (2013) recently reviewed epigenetic processes in relation to forest tree biology and genetic improvement, and suggested that epigenetic modifications could be important means for helping trees adapt to rapid climate shifts. If true, our study presents only a first step in what should be an aggressive and global effort to understand, and begin to use, epigenetic modifications in forest conservation and management.

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