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RNAi suppression of DNA methylation affects the drought stress response and genome integrity in transgenic poplar

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Abstract :

Trees are long-lived organisms that continuously adapt to their environments, a process in which epigenetic mechanisms are likely to play a key role. Via downregulation of the chromatin remodeler DECREASED IN DNA METHYLATION 1 (DDM1) in poplar (Populus tremula × Populus alba) RNAi lines, we examined how DNA methylation coordinates genomic and physiological responses to moderate water deficit.

We compared the growth and drought response of two RNAi-ddm1 lines to wild-type (WT) trees under well-watered and water deficit/rewatering conditions, and analyzed their methylomes, transcriptomes, mobilomes and phytohormone contents in the shoot apical meristem.

The RNAi-ddm1 lines were more tolerant to drought-induced cavitation but did not differ in height or stem diameter growth. About 5000 differentially methylated regions were consistently detected in both RNAi-ddm1 lines, colocalizing with 910 genes and 89 active transposable elements. Under water deficit conditions, 136 differentially expressed genes were found, including many involved in phytohormone

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pathways; changes in phytohormone concentrations were also detected. Finally, the combination of hypomethylation and drought led to the mobility of two transposable elements.

Our findings suggest major roles for DNA methylation in regulation of genes involved in hormone-related stress responses, and the maintenance of genome integrity through repression of transposable elements.

Keywords : ddm1, DNA methylation, drought, epigenetics, hormone, mobilome, poplar, shoot apical meristem

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Introduction

As long living organisms, trees are subjected to repeated environmental challenges over their lifetime. During the last decades, forest decline has been reported around the world due to heat and drought episodes (Allen *et al.,* 2010; Anderegg *et al.,* 2016). Survival and adaptation of populations will depend on the ability of trees to cope with rapidly changing conditions. Among the potential sources of flexibility in perennials like trees, epigenetics has received growing attention (Bräutigam *et al.,* 2013; Yakovlev *et al.,* 2012, 2016; Plomion *et al.,* 2016; Carneros *et al.,* 2017; Sow *et al.,* 2018a; Amaral *et al.,* 2020). Epigenetics is defined as the study of heritable changes that affect gene expression without changing the DNA sequence (Russo *et al.,* 1996). Considerable efforts have been made to unravel the role of epigenetics in plant developmental processes, stress response and adaptation, but primarily in annuals (Slotkin & Martienssen, 2007; Colomé-Tatché *et al.,* 2012; Cortijo *et al.,* 2014; Kooke *et al.,* 2015; Raju *et al.,* 2018; Schmid *et al.,* 2018) while its role in perennials like trees still needs clarification (Amaral *et al.,* 2020).

DNA methylation is one of the most studied epigenetic marks (Zhang *et al.,* 2018a). It is important in both plants and mammals for many biological processes such as imprinting , and its disruption can lead to developmental abnormalities (Vongs *et al.,* 1993; Zemach *et al.,* 2013; Zhang *et al.,* 2018b). In addition to controlling gene expression, DNA methylation is also involved in the maintenance of genome integrity by silencing Transposable Elements (TEs), preventing them from spreading within the host genome (Ikeda & Nishimura, 2015; Fultz *et al.,* 2015). DNA methylation is required to silence TEs located in the heterochromatin, and a decrease in DNA methylation level can result in their reactivation (Lippman *et al.,* 2004; Mirouze *et al.,* 2009; Mirouze & Paszkowski, 2011). One of the best known examples for the control of TEs by DNA methylation comes from the study of mutants of the maintenance of DNA methylation involving chromatin remodeling complexes such as *Decreased DNA Methylation I (DDM1* a SWI/SNF family member). Its depletion affects the distribution of methylation in all sequence contexts (Vongs *et al.,* 1993; Zhu *et al.,* 2013; Zemach *et al.,* 2013). *DDM1* was first identified in *Arabidopsis* through a forward genetic mutant screen causing a "decrease in DNA methylation" (Vongs *et al.,* 1993). Several studies further characterized *ddm1* mutants in Arabidopsis (Saze & Kakutani, 2007; Yao *et al.,* 2012; Zemach *et al.,* 2013; Cortijo *et al.,* 2014; Ito *et al.,* 2015; Kawanabe *et al.,* 2016), turnip (Fujimoto *et al.,* 2008; Sasaki *et al.,* 2012), maize (Li *et al.,* 2014), and rice (Higo *et al.,* 2012; Tan *et al.,* 2016). Recently DDM1 has been shown

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to mediate the deposition of H2AW, a histone variant important for heterochromatin (Osakabe *et al*., 2021).

In poplar trees, RNAi-*ddm1* lines have been previously obtained by targeting the transcripts of the two orthologous *DDM1* paralogs in *Populus tremula* × *Populus alba* cv. INRA 717-1B4 (Zhu *et al.,* 2013). Under standard greenhouse conditions, the regenerated lines did not show developmental defects although newly formed leaves displayed necrotic spots after a cycle of dormancy (Zhu *et al.,* 2013). These lines, which have been stabilized *in vitro* for several years now, represent valuable tools to clarify the functional role of DNA methylation in perennials such as forest trees. As a model tree with important genomic resources (Tuskan *et al.,* 2006; Jansson & Douglas, 2007), poplar (*Populus* spp.) has been a prime system for the study of the ecophysiological and molecular bases of the drought response (Monclus *et al.,* 2006; Street *et al.,* 2006; Bogeat-Triboulot *et al.,* 2007; Cohen *et al.,* 2010; Hamanishi *et al.,* 2012; Fichot *et al.,* 2015). Differences in global DNA methylation levels among poplar hybrid genotypes have been shown to correlate with biomass production under water deficit (Gourcilleau *et al.,* 2010; Raj *et al.,* 2011; Le Gac *et al.,* 2019). Epigenomic analyses have further shown that water deficit induces targeted changes in DNA methylation patterns at phytohormone-related genes thereby favoring phenotypic plasticity (Lafon-Placette *et al.,* 2018). This has raised the question of a possible link between epigenetics and phytohormone signaling/synthesis in the regulation of plant plasticity, particularly in primary meristems where development is initiated (Maury *et al.,* 2019). In addition, it has been shown that winter-dormant shoot apical meristems (SAMs) of poplars grown under field conditions can keep an epigenetic memory of a summer drought experienced during the growing season through modifications in DNA methylation (Le Gac *et al.,* 2018; Sow *et al.,* 2018b). The role of epigenetic memory in trees, besides poplar, in response to biotic and abiotic stresses or priming is becoming increasingly documented (Yakovlev *et al.,* 2014; Carneros *et al.,* 2017; Gömöry *et al.,* 2017; Yakovlev & Fosdal, 2017; Amaral *et al.,* 2020).

So far, most of the studies conducted in trees focusing on DNA methylation and gene expression have used a correlative approach. For example, extensive gene-body methylation is found in open chromatin, is linked to structural gene characteristics, and correlates with tissue-specific gene expression or stress (Vining *et al.,* 2012; Bräutigam *et al.,* 2013; Lafon-Placette *et al.,* 2013; Liang *et al.,* 2014; Lafon-Placette *et al.,* 2018). Here, we used a reverse genetic

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approach using RNAi-*ddm1* poplar lines to investigate the functional impact of variations in DNA methylation under water deficit conditions. We combined a fine-scale ecophysiological characterization of water deficit responses and growth dynamics with hormone profiling and integrative epi/genomics in the shoot apical meristem of the hypomethylated RNAi-*ddm1* lines.

Materials and Methods

Plant material, experimental design and control of water deficit

Experiments were conducted on two *PtDDM1* RNAi lines (*ddm1-15* and *ddm1-23*) and a wild type (WT) line of *Populus tremula* × *Populus alba* (clone INRA 717-1B4). These two RNAi-*ddm1* lines were chosen among those previously described by Zhu *et al.* (2013) as they consistently displayed lower levels of *PtDDM1* residual expression (38.0 and 37.5 % for *ddm1-15* and *ddm1-23*, respectively) and a decrease of cytosine methylation levels (decrease of 17.0 and 16.7 %, respectively) compared to WT. These characteristics were confirmed in the present study. The experiment was conducted in a controlled greenhouse on 4-L potted saplings (see Methods S1 for detailed growth conditions). In total, 81 plants were randomly distributed into nine blocks and assigned to either a well-watered control treatment (WW, 1 individual of each line per block) or a water deficit treatment followed by re-watering (WD-RW, 2 individuals of each line per block) (Fig. 1).

Water deficit was initiated at t_0 on three month-old plants and lasted three weeks until t_1. Plants from the WW condition were maintained watered close to field capacity, while plants from the WD-RW condition were watered to a target value of 40% of relative extractable water (REW, see Methods S1 for detailed information on the control of water deficit). At t_1, three blocks were destructively harvested while the six blocks remaining were rewatered and maintained at field capacity before being sampled one week later (t_2). As the focus of this study was on post-stress epigenomic events, the blocks sampled at t_1 were not considered in this study and the measurements reported hereafter were systematically performed on the six remaining blocks (n = 6 for WW, n = 12 for WD-RW) unless another subset is specified.

The intensity of water deficit was evaluated at t_1 by measuring predawn leaf water potential (Ψ_{pd}, MPa) before re-watering. Measurements were performed on a subset of five

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141 randomly selected blocks using a pressure chamber (PMS instruments, Albany, OR, USA; n = 5
142 for WW, n = 5 for WD-RW). Minimum leaf water potential (Ψ_{min}) was estimated for the same
143 plants at midday on the day preceding re-watering.

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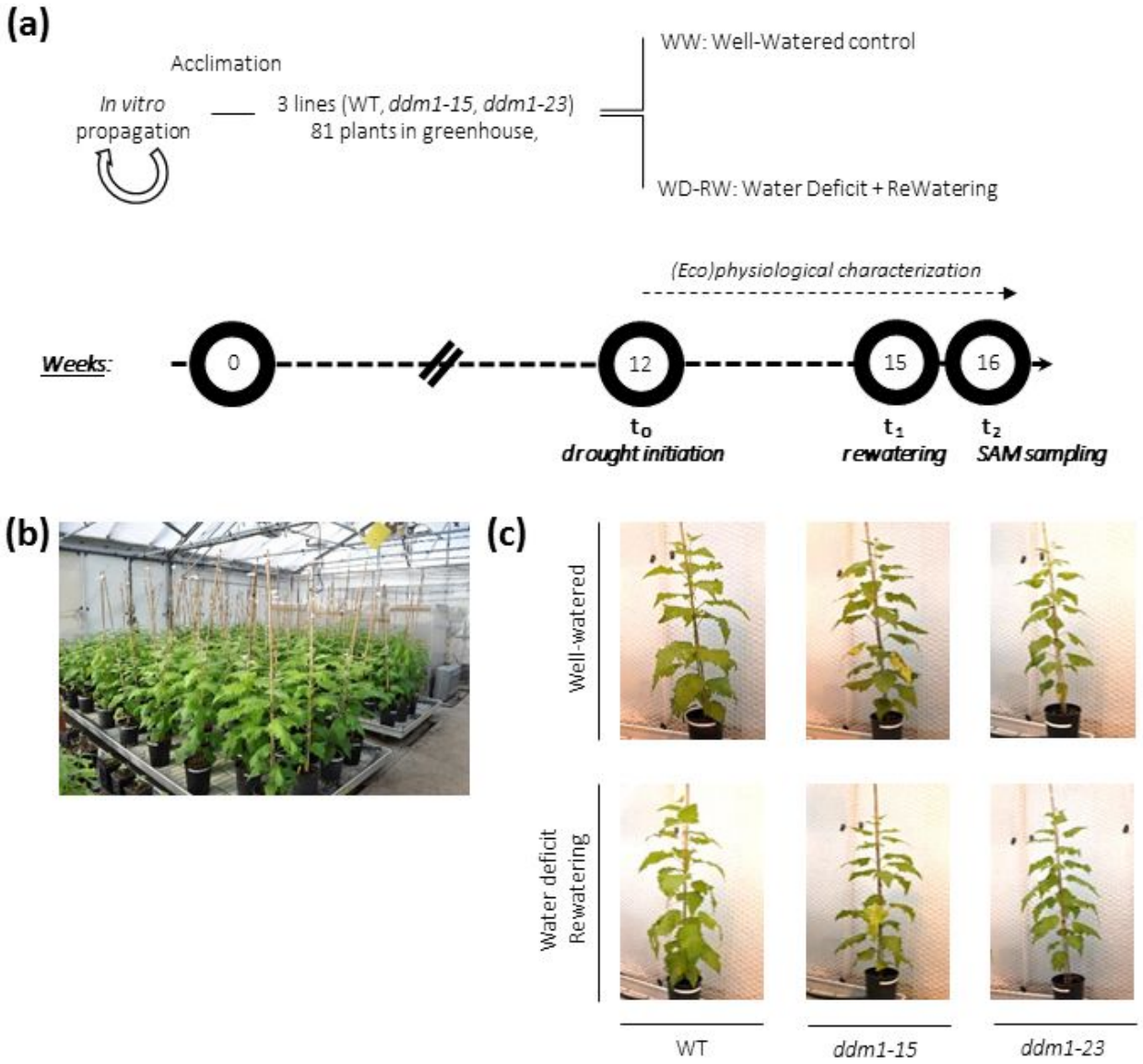

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146 **Fig. 1** General overview of the drought experiment on poplars (*Populus tremula* × *Populus alba*). (a) Timeline of
147 the experiment. Propagated *in vitro* plantlets from the wild type (WT) and the two RNAi-*ddm1* transgenic lines were
148 acclimated in a heated greenhouse, transferred into 4L pots and maintained under control conditions until they were
149 three-months-old. At that time (t_0), water deficit was initiated for the plants of the water deficit / rewatering treatment
150 (WD-RW) while control plants were maintained well-watered (WW). After three weeks of water deficit (t_1), plants
151 of the WD-RW condition were re-watered to field capacity for one week after which the experiment ended (t_2). The
152 ecophysiological characterization of plant material was performed between t_0 and t_2. Sampling of shoot apical
153 meristems (SAMs) for molecular analysis was performed at t_2. (b) Overview of the plants in the greenhouse. (c)
154 Examples of plant phenotypes at t_2 in WW and WD-RW conditions for WT and RNAi-*ddm1* lines.

Physiological and phenotypic characterization

Assessment of growth and leaf phenotypes

Stem height was measured every two days using a telescopic ruler, while stem diameter was measured every four days using a digital caliper. We also repeatedly measured the number of leaves showing necrotic spots (mottled leaves, see Zhu *et al.,* 2013) during the whole duration of the experiment and counted the number of leaves showing a 'folded' morphology. These measurements were performed on all plants (n = 6 for WW, n = 12 for WD-RW).

Leaf traits

Leaf gas exchange were assessed every day during the experiment (n = 5 per line for WW and WD-RW). Bulk leaf carbon isotope composition (δ13C) was measured at t_2 from a mature leaf on all plants (n = 6 for WW, n = 12 for WD-RW) while stomatal density was assessed on a subset of three blocks (n = 3 for WW and WD-RW). See Methods S1 for detailed procedures for stomatal conductance, carbon isotope composition and stomatal counts.

Xylem vulnerability to drought-induced cavitation

Xylem vulnerability to drought-induced cavitation was assessed at t_2 on the stems of all plants undergoing well-watered condition only (n = 6) (INRAE Phenobois Platform, Clermont-Ferrand, France). We used the Cavitron technique which is well suited for poplars (Cochard *et al.,* 2005, Fichot *et al.,* 2015). The xylem tension causing 50% loss of hydraulic conductance (P50, MPa) was used to compare vulnerability. See Methods S1.

Phytohormone quantification

Phytohormone assays for abscisic acid (ABA), free auxin, salicylic acid (SA), jasmonic acid (JA) and cytokinins were performed on the individual SAMs collected at t_1 (n = 3 for each line and condition) using LC-MS according to a published procedure (OVCM platform, IJPB, INRAE Versailles, France; Li-Marchetti *et al*., 2015; Trapet *et al*., 2016). See Methods S1.

DNA extraction and determination of global DNA methylation levels by HPLC

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Genomic DNA was extracted from individual SAM (n = 3 for each line for each water condition at t_2) with a CTAB protocol (Doyle & Doyle, 1987), and stored at -80°C. Quantity and quality were approximated using a NanoDrop spectrometer (NanoDrop Instrument, France). For the HPLC determination of global DNA methylation, we followed Zhu *et al.* (2013).

Methylome analyses using Whole Genome Bisulfite Sequencing (WGBS)

An equimolar pool of 2 µg DNA at approximately 100 ng/µl was made for each line in each treatment from four individual SAMs (n = 4; at t_2). Whole-genome bisulfite sequencing was performed at the CNRGH (Evry, France) in accordance with the published procedure Daviaud *et al*. (2018) adapted from (http://www.nugen.com/products/ovation-ultralow-methyl-seq-library-systems) See Methods S1. The sequencing was performed in paired-end mode (2×150bp) on an Illumina HiSeq4000 platform. Raw data were stored as FASTQ files with a minimal theoretical coverage of 30X.

The bioinformatic pipeline for methylome analysis was adapted from the ENCODE pipeline (https://www.encodeproject.org/data-standards/wgbs) and installed on the Galaxy instance, accessible of IHPE (http://galaxy.univ-perp.fr/, Perpignan, France) using the reference genome *Populus tremula* × *Populus alba* (http://aspendb.uga.edu/index.php/databases/spta-717-genome). See Methods S1.

Transcriptomic analyses

We performed RNAseq on samples obtained in WD-RW conditions to compare one RNAi-*ddm*1 line (*ddm1-23*) to the WT. The line *ddm1-23* was chosen as the most representative of the two lines as it showed a lower decrease in methylation compared to *ddm1-15*, and most of its DMRs were common to *ddm1-15* (see Results section). Total RNA was extracted from SAMs from three biological replicates per line (n = 3; at t_2) using a modified protocol of Chang *et al.* (1993). See Methods S1.

Mobilome-seq and copy number variation of TEs

We sequenced extrachromosomal circular DNAs (eccDNAs) in order to identify active TEs, as described in Lanciano *et al.* (2017). We used approximately 6 µg of genomic DNA (the same pools used for WGBS) obtained for each line in each condition from individual SAMs (n = 4; at

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208 t_2) to perform mobilome-seq libraries. eccDNAs were isolated and amplified and libraries were
209 prepared and sequenced following Lanciano *et al.* (2017). Bioinformatic analyses were carried
210 out on the *Populus tremula* × *Populus alba* genome (SPta717 v1.1) using the pipelines described
211 in Lanciano *et al.* (2017). DNA copy number variation of TEs was assessed for all studied lines
212 in both conditions by quantitative PCR (qPCR) in triplicates. See Methods S1.

213 **Statistical analyses**

214 Statistical analyses were performed using the R statistical software under R Studio integrated
215 development environment (R Development Core Team, 2015, RStudio: Integrated Development
216 for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/). Means are expressed with their
217 standard errors (SE). When measurements were available for more than one individual replicate
218 per block under the WD-RW condition, pseudoreplicates were averaged to yield a single value
219 per block. Differences between lines and conditions for phenotypic traits were evaluated by
220 analysis of variance (ANOVA) on individual values adjusted for block effects. Tukey's post-hoc
221 test was used to identify differences between groups when ANOVAs indicated significant effects.
222 Statistical tests and *P* values are indicated according to Wasserstein & Lazar (2016).

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Results

Phenotypic and physiological differences among lines under well-watered conditions

We performed a drought experiment on WT and RNAi-*ddm1* poplar lines (Fig. 1). Plants from the control condition remained close to field capacity during the whole experiment. Relative extractable water (REW) never dropped below 70% (Fig. 2a) and predawn leaf water potential (Ψ_{pd}) values remained above -0.5 MPa (Fig. 2b). There was no significant difference in either REW or Ψ_{pd} among lines (Fig. 2a,b). The WT and RNAi-*ddm1* lines all showed linear growth during the experiment (Fig. S1a) and there was no significant difference for height growth rates (1.27 ± 0.03 cm.day^{-1}, $P = 0.797$) and diameter growth rates (0.09 ± 0.02 mm.day^{-1}, $P = 0.091$) (Fig. 3a,b). Differences among lines were however visible for total leaf area, RNAi-*ddm1* lines exhibiting on average 28% lower values compared to the WT ($P = 0.021$, Fig. S2a) which was mainly explained by smaller leaves in the middle canopy (Fig. S2b).

Significant differences were observed for xylem vulnerability to cavitation ($P < 0.001$, Fig. 3c). The WT was about 10% more vulnerable ($P_{50} = -2.16 \pm 0.05$ MPa) compared to the RNA-*ddm1* mean; *ddm1-23* was the most resistant ($P_{50} = -2.45 \pm 0.04$ MPa) while *ddm1-15* was intermediate ($P_{50} = -2.28 \pm 0.04$ MPa). In contrast, there were no significant differences between lines for leaf traits (Fig. S3) and xylem structural or biochemical traits (Table S1).

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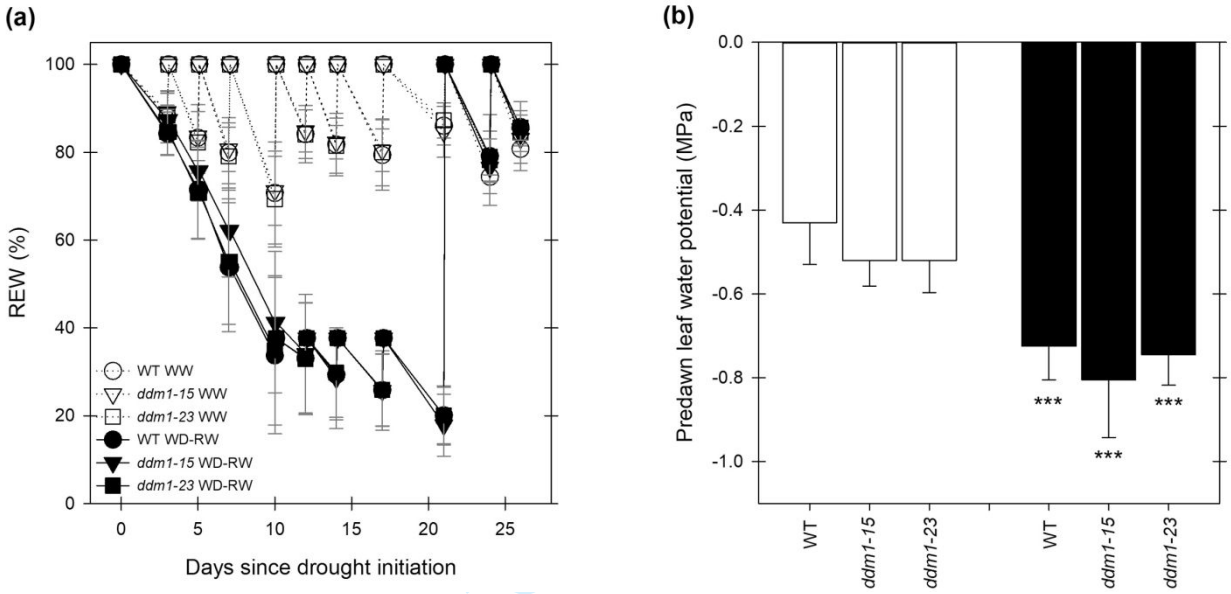

Fig. 2 Measurements on soil water content and leaf water potential during the experiment. (a) Time course of soil relative extractable water (REW) during the experiment for the wild type (WT) and the two RNAi-*ddm1* (*ddm1-15*, *ddm1-23*) transgenic poplar lines in control (Well-Watered, WW) and stress (moderate Water Deficit followed by ReWatering, WD-RW) conditions Water deficit in the WD-RW condition started on day 0 (t_0) and lasted for three weeks after which plants were rewatered (t_1) for one additional week (t_2). Values are genotypic means ± SE (n = 6 for WW, n = 12 for WD-RW). (b) Predawn leaf water potential measured at the drought peak (i.e. before rewatering on day 21) for the WT and the two RNAi-*ddm1* (*ddm1-15*, *ddm1-23*) poplar lines. Open bars for control conditions and black bars for stress conditions (moderate water deficit followed by rewatering). Values are genotypic means ± SE (n = 5). Asterisks indicate significant differences between conditions for each line (level of significance: *** P < 0.001).

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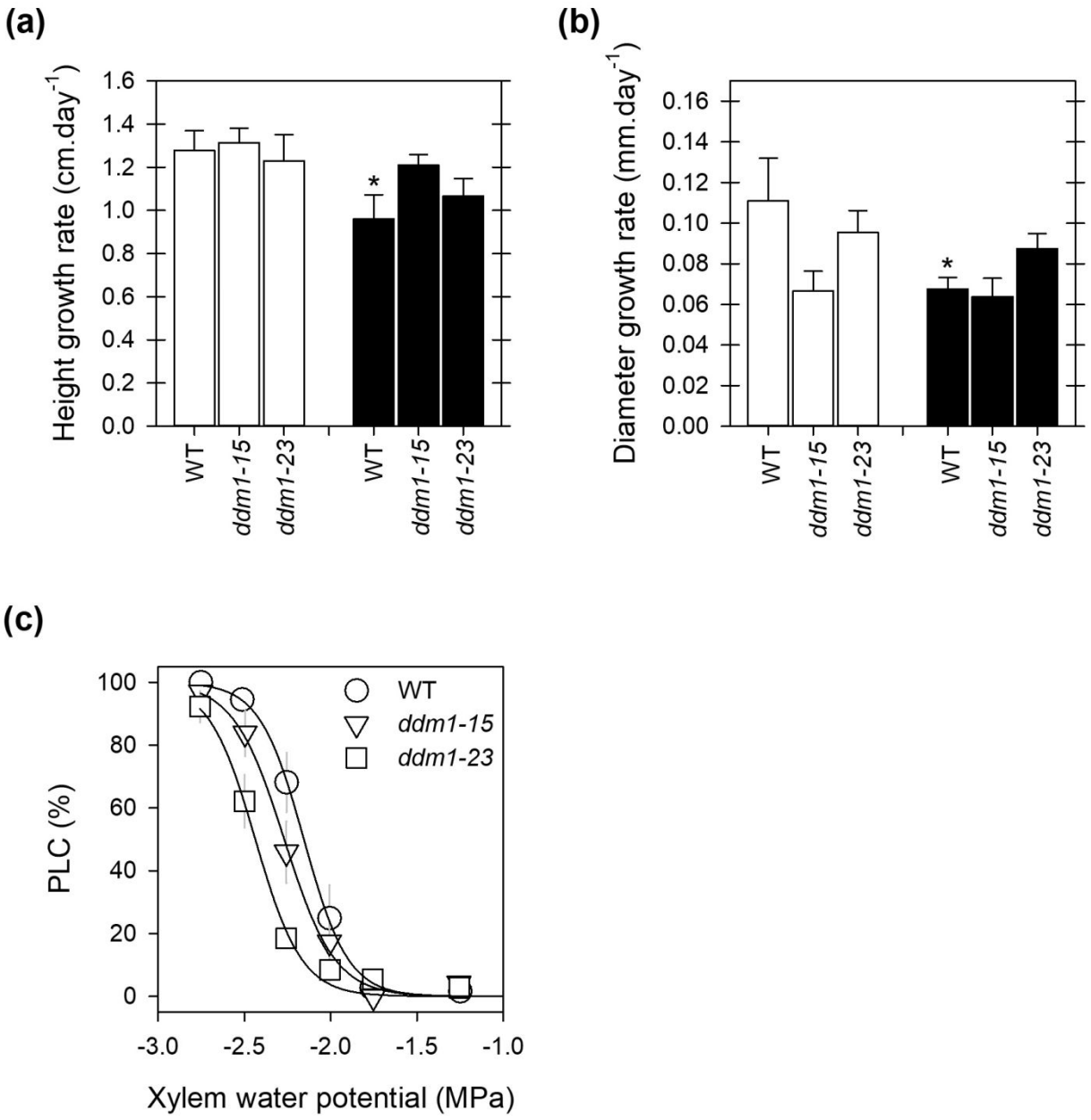

Fig. 3 Physiological traits measured during the drought experiment. (a) Height and (b) diameter growth rates during the three weeks of water deficit from t_0 to t_1 for the wild type (WT) and the two RNAi-*ddm1* (*ddm1-15*, *ddm1-23*) poplar lines. Open bars for Well-Watered conditions (WW) and black bars for stress conditions (moderate Water Deficit followed by ReWatering, WD-RW). Values are means ± SE (n = 6 per line for WW, n = 12 per line for WD-RW). The effects of water deficit were evaluated for each line using a t-test and are only indicated if significant. Levels of significance are * $0.01 < P < 0.05$. Statistical tests did not reveal significant differences between lines when considering conditions separately, but the WT was the only line to be significantly affected by water deficit. (c) Average xylem vulnerability curves for the WT and the two RNAi-*ddm1* (*ddm1-15*, *ddm1-23*) poplar lines. PLC: percent loss of hydraulic conductance. Measurements were performed at t_2 on well-watered plants only. Values are means ± SE (n = 6 per line).

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The proportion of leaves showing necrotic spots reached 40% at the end of the experiment for *ddm1-23* and more than 60% for *ddm1-15,* while it remained close to zero for the WT (Fig. 4). Symptom occurrence was not linear but tended to increase at a specific physiological stage (Fig. 4), mainly on mature leaves and not in the top third upper part of the plant. The RNAi-*ddm1* lines (mostly *ddm1-23*) also exhibited leaves that tended to fold around the midvein (Fig. 4).

In summary, under well-watered conditions, RNAi-*ddm1* lines showed growth and leaf physiology similar to WT. However, RNAi-*ddm1* lines displayed necrotic spots on mature leaves and higher xylem resistance to cavitation (Table 1).

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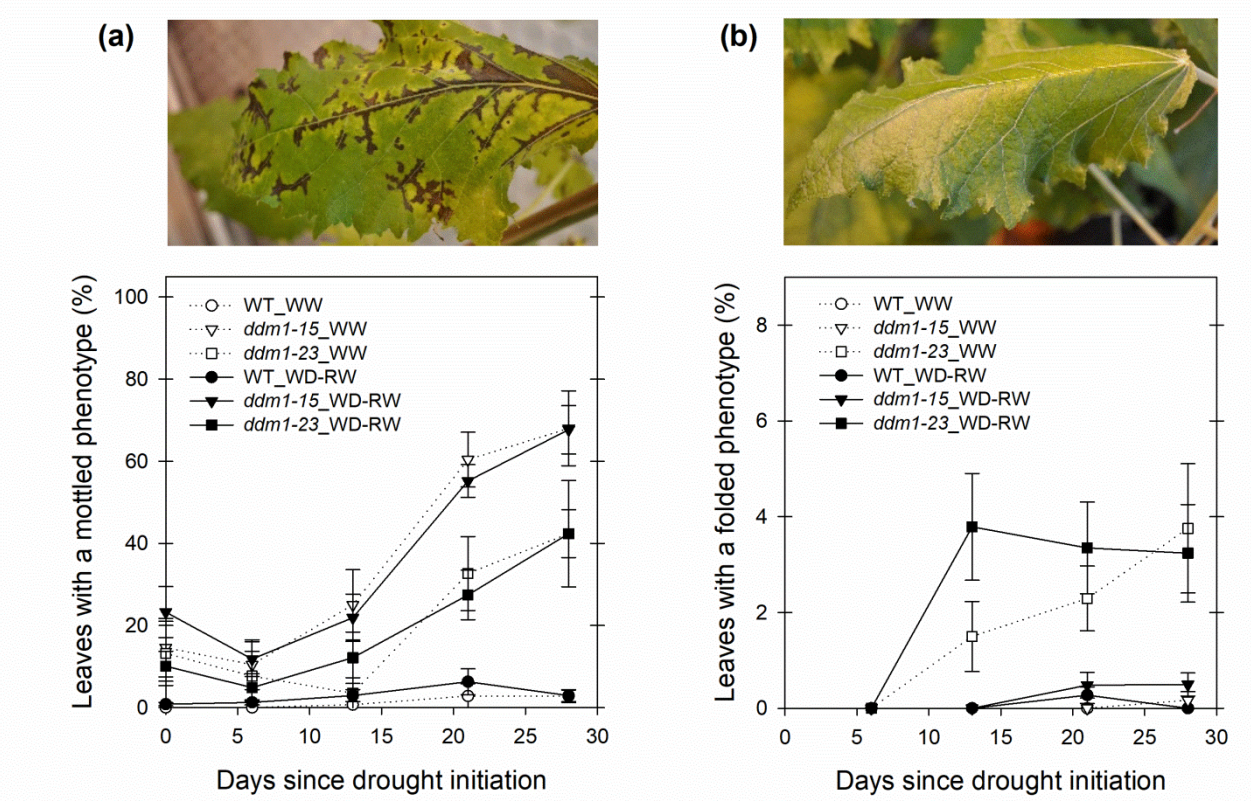

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274 **Fig. 4** Leaves phenotypes during the drought experiment. Plants were phenotyped for the occurrence of necrotic
275 spots (mottled phenotype) (a) and folded phenotype (b). For each panel the upper part shows a picture of a typical
276 phenotype, the graph shows a time course of leaf phenotypic alterations for the wild type (WT) and the two RNAi-
277 *ddm1* (*ddm1-15*, *ddm1-23*) poplar lines under control (Well-Watered, WW) and stress (moderate Water Deficit
278 followed by ReWatering, WD-RW) conditions . Values are line means ± SE (n = 6 for WW, n = 12 for WD-RW). At
279 the end of the experiment (t_2), all lines differed significantly from each other for the proportion of necrotic spots
280 (mottled phenotype) but water deficit had no significant effect; only *ddm1-23* showed a significantly higher
281 proportion of folded leaves, but water deficit still had no significant effect.

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283 **Table 1** Main characteristics of RNAi-*ddm1 vs.* wild type (WT) lines. CK: Cytokinin; DEG:
284 Differentially Expressed Gene; DMG: Differentially Methylated Gene; DMR: Differentially
285 Methylated Region; GO: Gene Ontology; SA: Salicylic Acid; SAM: Shoot Apical Meristem; TE:
286 Transposable Element; WD-RW: Water Deficit-ReWatering; WW: Well-Watered;

Study scale and traits	Key results
Growth and drought tolerance	• RNAi-*ddm1* lines height and diameter growth are not significantly affected by moderate water deficit RNAi-*ddm1* lines are intrinsically more resistant to drought-induced cavitation
Leaf phenotypes	• RNAi-*ddm1* lines show necrotic spots independently of water availability
Phytohormones in SAM	• RNAi-*ddm1* lines have higher SA concentrations in all tested conditions • RNAi-*ddm1* lines have lower zeatine riboside and lower zeatine-O-glucoside riboside (CKs) concentrations, but only under WD-RW
Methylome in SAM	• RNAi-*ddm1* lines have hypomethylated SAMs • RNAi-*ddm1* lines display 5,374 (WW) / 5,172 (WD-RW) common DMRs compared to the WT; 1,736 were common to both conditions • RNAi-*ddm1* DMRs are context-dependent: CHG>CG>CHH • RNAi-*ddm1* DMRs colocalize with 879 (WW) / 910 (WD-RW) genes (DMGs) and 23 (WW) / 89 TEs (WD-RW) • DMGs show GO enrichment in development, regulation of biological process and response to abiotic stress including hormones
Transcriptome in SAM	• 136 genes are differentially expressed in RNAi-*ddm1-23 versus* WT in WD-RW conditions with GO enrichment in defense response, response to hormones and regulation of RNA metabolism • 53 differentially expressed genes correlate with the presence of RNAi-*ddm1* DMRs (at +/- 10 kbp) of with a correlation between methylation and expression (Spearman's *rho* = -0.32 at *P* = 0.0004)

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Mobilome in SAM and qPCR	• 414 active TEs were detected in total (all lines and conditions) with 21% that colocalize with RNAi-*ddm1* DMRs • 92% of active TEs in DMRs are hypomethylated (CG and CHG) in RNAi-*ddm1* lines but show CHH hypermethylation under WD-RW conditions • 2 highly active TEs (*Gypsy* retrotransposons) show DNA copy number variation specifically in stressed (WD-RW) RNAi-*ddm1* lines

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288 **Differences in drought response**

289 Soil water content of drought stressed plants started to be significantly lower than the control four
290 days after the initiation of water deficit. Values of REW then fluctuated between 20 and 40 %
291 until t_1 (Fig. 2a); re-watering at t_1 increased REW back to control values (Fig. 2a). Predawn leaf
292 water potential at t_1 was significantly lower than in well-watered plants ($P < 0.001$ for each line)
293 and reached approximately -0.8 MPa with no significant difference among lines (Fig. 2b). Height
294 and diameter growth rates during the three-week water deficit were significantly reduced by 25%
295 and 39% in the WT trees (Fig. 3, $P = 0.037$ and $P = 0.026$). In contrast, height and diameter
296 growth rates of RNAi-*ddm1* lines were not significantly affected by water deficit (8 and 13%
297 reduction in height growth rates with $P = 0.204$ and $P = 0.244$ for RNAi-*ddm1-15* and RNAi-
298 *ddm1-23*, respectively; 4 and 8% reduction in diameter growth rates with $P = 0.828$ and $P =$
299 0.516 for *ddm1-15* and *ddm1-23*, respectively). Rewatering brought growth back to the levels of
300 the controls (Fig. S1).

301 In response to water deficit, stomatal conductance started to decrease approximately 10
302 days after drought initiation, *i.e.* once REW had dropped below 40% (Fig. 2a, Fig. S3). The WT
303 and RNAi-*ddm1* lines showed relatively comparable dynamics and reached almost an 80%
304 decrease relative to controls (Fig. S3). Net CO_2 assimilation rates were less impacted in
305 agreement with the moderate intensity of water deficit (Fig. S3). The WT and RNAi-*ddm1* lines
306 all showed comparable leaf traits as in well-watered conditions, although the WT did show a
307 significant effect of water deficit for leaf $\delta^{13}C$ and stomatal density (Fig. S3). Xylem traits were
308 only seldom affected by water deficit and were not statistically different among the WT and

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RNAi-*ddm1* lines (Table S1). Water deficit also had no significant effect on the occurrence of leaf symptoms (Fig. 4).

In summary, RNAi-*ddm1* lines exhibited a drought response mostly similar to the WT in terms of leaf physiology and xylem structure/biochemical composition. However, height and diameter growth were not significantly decreased by the moderate water deficit, in contrast to the WT, suggesting enhanced stress tolerance (Table 1).

Phytohormone concentrations in shoot apices

There were no significant differences among lines in phytohormone concentrations under well-watered conditions. Salicylic acid (SA) contents, however, were on average almost 2.5 times higher in RNAi-*ddm1* lines (Fig. 5). Under water deficit-rewatering conditions, significant differences among lines were observed for SA (Fig. 5); SA was still almost two times higher in RNAi-*ddm1* lines, while zeatine riboside and zeatine-O-glucoside riboside levels were on average one third lower in RNAi-*ddm1* lines (Fig. 5). Although the levels for ABA were on average 20% lower for the RNAi-*ddm1* lines compared to WT, the results did not reach statistical significance (P = 0.267). Free auxin levels were similar among all lines (Fig. 5). The WT exhibited the lowest values among all lines for SA and the SA level did not change significantly in function of the stress conditions (Fig. 5). Concentrations in zeatine riboside and zeatine-O-glucoside riboside were higher in the WT because of a treatment-induced increase (Fig. 5). Concentrations in isopentenyladenosine showed a more complex pattern with RNAi-*ddm1-15* and RNAi-*ddm1-23* exhibiting the lowest and the highest values, respectively (Fig. 5).

In summary, RNAi-*ddm1* lines differed from the WT mostly under water deficit for SA (higher) and cytokinins (lower) concentrations (Table 1; Fig. 5) suggesting a physiological change in the hormonal balance.

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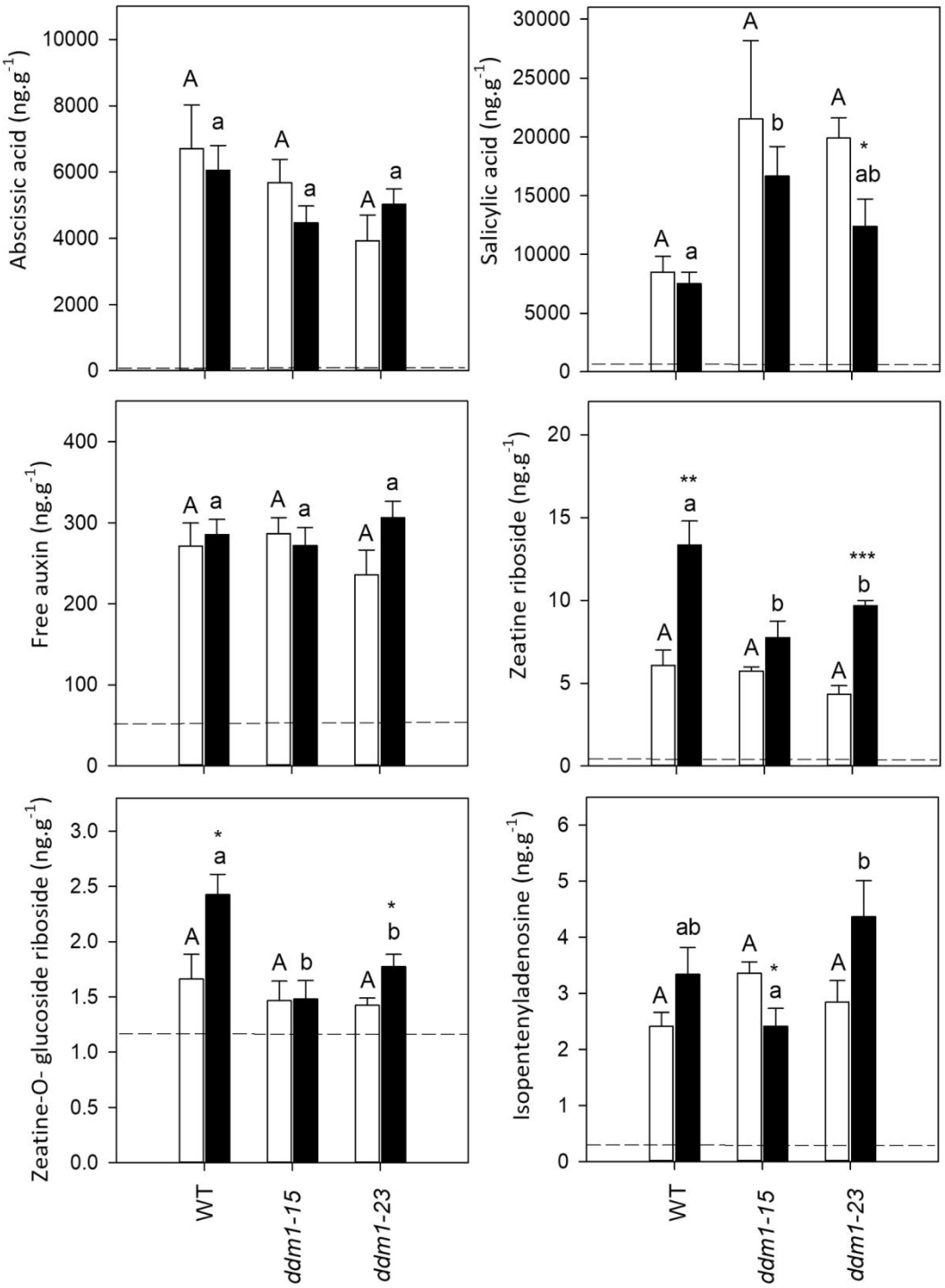

332

333 **Fig. 5** Phytohormone contents in the shoot apex meristems. Measures were performed at rewatering (t_1) for the wild
334 type (WT) and the two RNAi-*ddm1* (*ddm1-15*, *ddm1-23*) poplar lines for 6 phytohormones as indicated. Open bars
335 show the control condition (WW) and black bars the stress conditions (moderate Water Deficit followed by
336 ReWatering, WD-RW). Values are genotypic means ± SE (n = 3). The effects of water deficit were evaluated within

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each line using a t-test and are only indicated when significant. Levels of significance are * $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$. Different letters indicate significant differences between lines within each condition (uppercase for WW, lowercase for WD-RW) following a Tukey's post hoc test. The dashed lines indicate the detection threshold for each phytohormone.

Methylome analysis and identification of constitutive and stress specific RNAi-*ddm1* Differentially Methylated Regions (DMRs) and Differentially Methylated Genes (DMGs) in SAM

Global DNA methylation using the HPLC method in the SAM ranged from 17.5 to 21.3% depending on lines and conditions (Fig. S-4a) confirming a reduction of whole genome methylation up to 17.8% in agreement with Zhu *et al.* (2013). There was no significant line×condition interaction. Values were significantly lower in RNAi-*ddm1* lines compared to the WT under water deficit/rewatering (WD-RW) only, although there was no significant general effect of water deficit (Fig. S4a). WGBS analysis was performed on SAM (Fig. S5) and cytosine methylation percentages for the three contexts ranged from 18.6 to 19.6% in CpG, 4.4 to 6.0% in CHG and 1.6 to 2.0% in CHH contexts, with RNAi-*ddm1-15* systematically displaying the lowest values as with a 25.0% decrease for the CHG context (Table S2; Fig. S5d).

We considered different types of Differentially Methylated Regions (DMRs) between lines and conditions (Figs. 6, S4b, S6a and Tables 1,S3). In order to focus on DMRs specifically related to *PtDDM1* down-regulation, common DMRs to both *ddm1-15* and *ddm1-23* lines *vs*. WT were identified and used for further analysis (Table 1, Fig. 6). In total, 5,374 common DMRs were identified under well-watered conditions (hereafter named "constitutive RNAi-*ddm1*" DMRs; Table 1, Figs. 6a, S6a). Under water deficit/rewatering condition, 5,172 DMRs were similarly identified, among which 1,736 were common to RNAi-*ddm1* constitutive DMRs, while 3,436 were specific to the WD-RW condition (hereafter named "stress-specific RNAi-*ddm1*" DMRs) (Table 1, Figs. 6a, S6a). Altogether, most of the DMRs were hypomethylated and context-dependent with higher values found in CHG, CG and CHH context, respectively (Fig. 6a). However, in the CpG context, these DMRs presented both significant hypo and hypermethylated patterns (Fig. 6a). In addition, 2,592 DMRs that were common to the WT and RNAi-*ddm1* lines were identified in response to water deficit / rewatering (Table 1, Fig. 6b).

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However, as this type of DMRs has already been reported for the WT (Lafon-Placette *et al.,* 2018), we further focused our attention on constitutive and stress-specific RNAi-*ddm1* DMRs (Tables 1,S3 and Figs. 6,S7).

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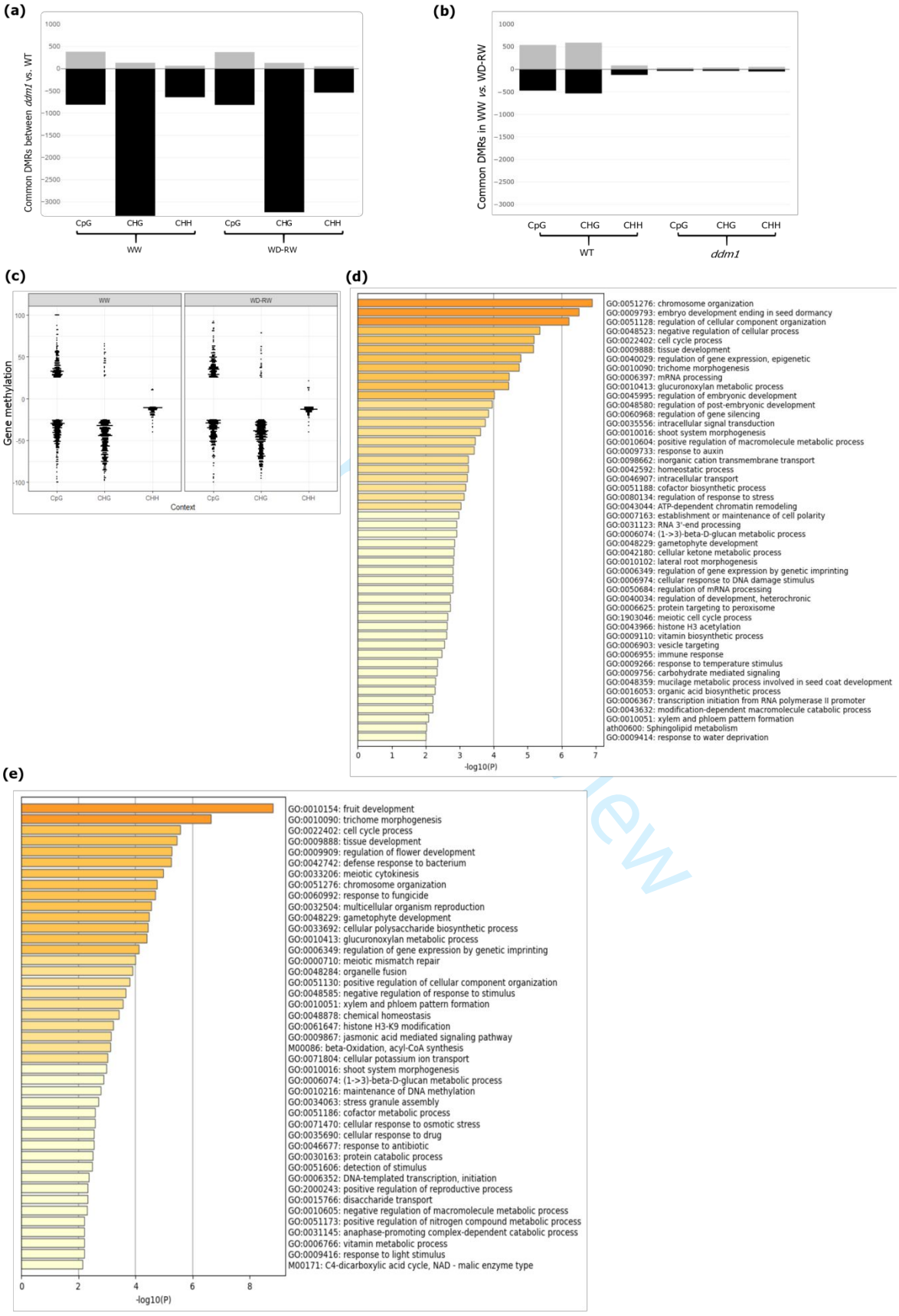

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Fig. 6 Variations in DNA methylation among RNAi-*ddm1* and WT lines in shoot apical meristems one week after rewatering (t_2). (a) Common DMRs between the two RNAi-*ddm1* lines (*ddm1-15* and *ddm1-23*) *vs*. the WT in all contexts (CG, CHG and CHH) in Well-Watered (WW) and stress (moderate Water Deficit followed by ReWatering, WD-RW) conditions. Black bars depict hypomethylated DMRs and grey bars show hypermethylated DMRs. (b) Identification of DMRs in WD-RW conditions compared to WW conditions for WT and RNAi-*ddm1* lines. Black bars depict hypomethylated DMRs and grey bars show hypermethylated DMRs. (c) Genic DNA methylation variation of the common DMRs in the RNAi-*ddm1* lines *vs*. the WT line in WW and WD-RW conditions for each methylation context. Only DMRs with at least a 25 % difference were kept except for CHH where a threshold of 10% was applied due to the low proportion of DMRs in that context. (d) Heatmap view of enriched clusters on constitutive (i.e., not stress induced) RNAi-*ddm1* Differentially Methylated Genes (DMGs) (in WW) (n=879 annotations of homologous Arabidopsis genes). Each bar is a single cluster representative of TAIR10 corresponding to sPta717 v1.1 annotations. (e) Heatmap view of enriched clusters on stress-specific RNAi-*ddm1* DMGs (in WD-RW). (n=910 annotations of homologous Arabidopsis genes). Enriched clusters GO were generated using Metascape (Zhou *et al*., 2019; see Methods S1).

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Regardless of the treatment, constitutive and stress specific, RNAi-*ddm1* DMRs were found in intergenic regions (73 and 71%, for WW and WD-RW conditions, respectively), genes (19 and 21%), promoters (+/-2kb from the TSS; 7 and 8%) and TEs (1%, 47 TEs in WW and 48 TEs in WD-RW) (Fig. S7a). Under control conditions, 879 genes were strictly included within the RNAi-*ddm1* constitutive DMRs (hereafter called DMGs for "Differentially Methylated Genes"), while 910 DMGs were found in stress RNAi-*ddm1* DMRs (Tables 1,S3). About one-third of DMGs (390 genes) were common between conditions and represented constitutive and stable RNAi-*ddm1*-DMGs, while 520 new DMGs were observed (stress specific RNAi-*ddm1* DMGs) (Tables 1,S3). These numbers increased considerably (up to more than 13,000 out of 41,335 genes) when enlarging the windows for DMR identification from 2 kb to 25 kb (Fig. S7b). Under both conditions, a similar number of hypo and hypermethylated DMGs was found in the CG context, while in CHG and CHH contexts, DMGs were mostly hypomethylated (Fig. 6c). In the CHH context, DMGs were slightly methylated (25 to 50% difference) compared to the CG and CHG (Fig. 6c). Gene Ontology annotation of DMGs (879 in WW or 910 in WD-RW) revealed significant enrichment in similar biological functions such as development (including shoot system morphogenesis), regulation of gene expression (epigenetics, ATP-dependent chromatin remodeling, histone modifications), response to hormones, immune response and abiotic stress (Fig. 6d,e). Under WD-RW condition, GO terms for the lost DMGs (489, Fig. S7c) were related to development (meristem development), hormones (regulation of hormone levels, response to auxin) and gene silencing. GO terms for stable DMGs (390, Fig. S7d) were related to development, jasmonic acid, epigenetics, immune response and metabolic process, while the new DMGs (520, Fig. S7e) were associated with development, and defense/immune/abiotic responses suggesting that stable and new DMRs target preferentially stress related genes.

In summary, the analysis of the SAM methylome highlighted that *DDM1* knock-down mostly led to hypomethylated CHG DMRs in RNAi-*ddm1* lines irrespective of the treatment. However, the constitutive *ddm1*-DMRs were largely (about two-thirds, Table 1) affected in water deficit conditions (WD-RW), suggesting that alteration of DDM1 machinery in SAM has the potential to affect development and stress responsive genes including hormone pathways under stress conditions (Table 1 and Fig. S6b).

Transcriptome analysis in SAM

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To analyze the impact of a water deficit and rewatering cycle on SAM, we performed a RNAseq experiment between RNAi-*ddm1* (*ddm1-23*) and WT SAMs under WD-RW condition. The identification of differentially expressed genes (DEGs) revealed limited but clear differences (Fig. 7). Out of 32,048 analyzed genes, only 136 genes (Fig. 7, Table S3) were significantly differentially expressed (76 up-regulated and 60 down-regulated genes in RNAi-*ddm1* as compared to the WT, $P < 0.05$). Gene Ontology annotation revealed significant enrichment in functions such as defense response, immune and wounding responses, response to hormones and signaling, leaf senescence and programmed cell death (Fig. 7a).

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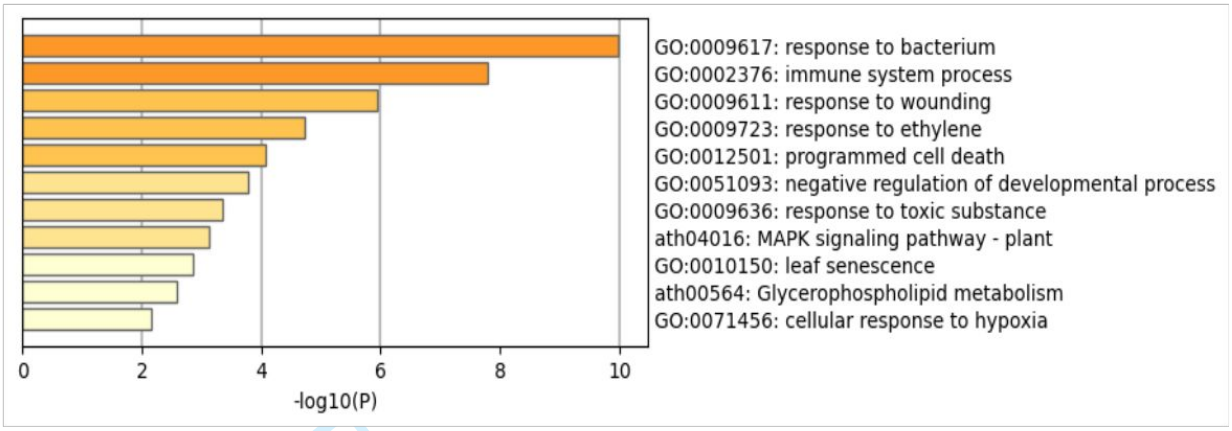
(a)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GO:0002376: immune system process
GO:0009611: response to wounding
GO:0009723: response to ethylene
GO:0012501: programmed cell death
GO:0051093: negative regulation of developmental process
GO:0009636: response to toxic substance
ath04016: MAPK signaling pathway - plant
GO:0010150: leaf senescence
ath00564: Glycerophospholipid metabolism
GO:0071456: cellular response to hypoxia
0
2
4
6
8
10
-log10(P)

(b)

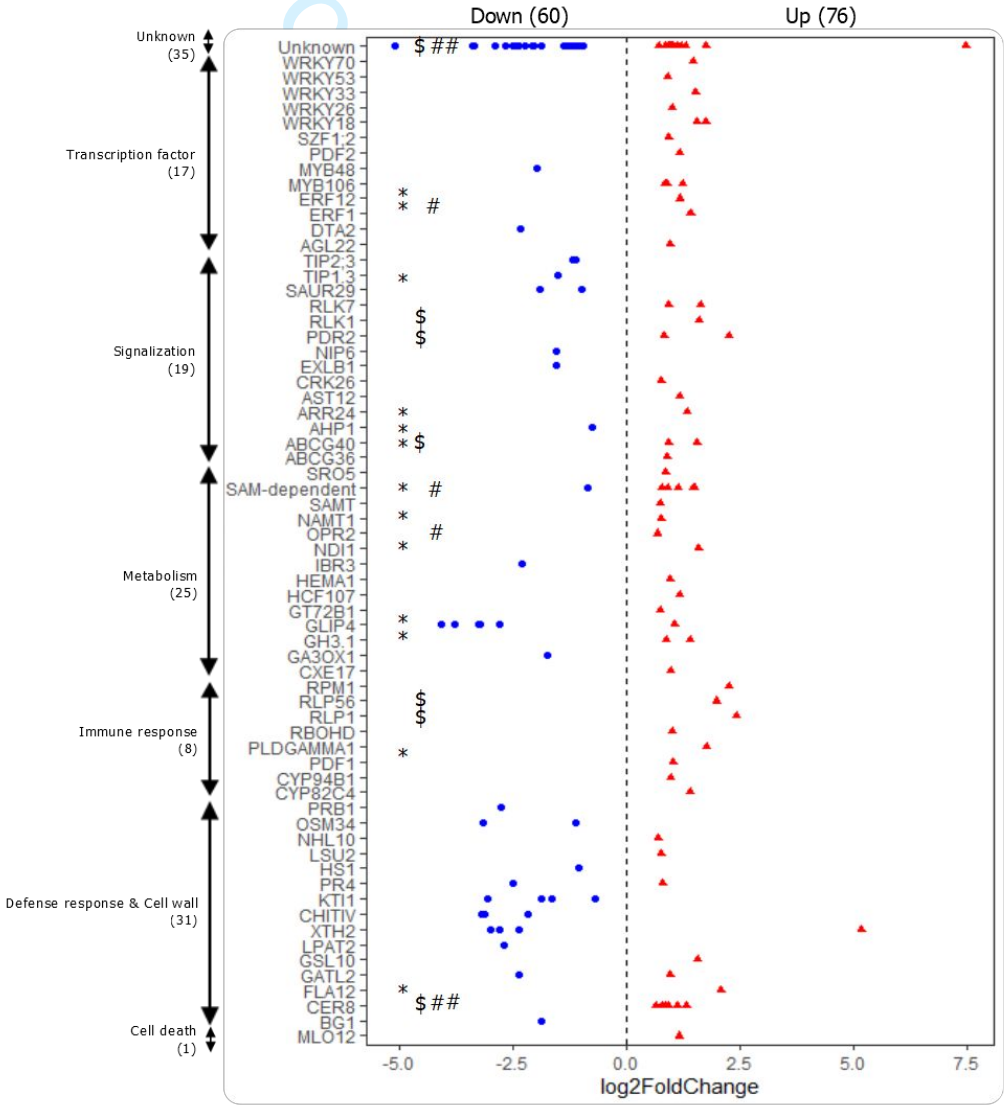
Down (60)
Up (76)
Unknown (35)
Transcription factor (17)
Signalization (19)
Metabolism (25)
Immune response (8)
Defense response & Cell wall (31)
Cell death (1)
Unknown
WRKY70
WRKY53
WRKY33
WRKY26
WRKY18
SZF1-2
PDF2
MYB48
MYB106
ERF12
ERF1
DTA2
AGL22
TIP2;3
TIP1;3
SAUR29
RLK7
RLK1
PDR2
NIP6
EXLB1
CRK26
AST12
ARR24
AHP1
ABCG40
ABCG36
SRO5
SAM-dependent
SAMT
NAMT1
OPR2
NDI1
IBR3
HEMA1
HCF107
GT72B1
GLIP4
GH3.1
GA3OX1
CXE17
RPM1
RLP56
RLP1
RBOHD
PLDGAMMA1
PDF1
CYP94B1
CYP82C4
PRB1
OSM34
NHL10
LSU2
HS1
PR4
KTI1
CHITIV
XTH2
LPAT2
GSL10
GATL2
FLA12
CER8
BG1
MLO12
-5.0
-2.5
0.0
2.5
5.0
7.5
log2FoldChange

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Fig. 7 Gene expression variations in RNAi-*ddm1-23 vs.* WT line in Water Deficit/ReWatering condition (WD-RW) in shoot apical meristems collected one week after rewatering (t_2). (a) GO annotation of the differentially expressed genes (genes with an adjusted p-value by FDR, false discovery rate < 0.05 = 136 DEGs identified) between RNAi-*ddm1-23* and the WT. GO labels were retrieved from popgenie and heatmap realized with Metascape. (b) Annotation of DEGs with expression variation values (log2FoldChange) using GO labels retrieved from popgenie. Blue dots mark downregulated genes and red triangles upregulated genes. The * indicates hormone related genes found among DEGs; The $, DEGs overlapping with DMRs; The #, DEGs that overlap with TEs; and the numbers (1), (31), (8), (25), (19), (17), (35) represent the number of DEGs found in Cell death, Defense & Cell wall, Immune response, Metabolism, Signalization, Transcription factors and Unknown processes, respectively. Log2FoldChange = log-ratio of normalized mean read counts in RNAi-*ddm1-23 vs*. WT lines (n = 3 biological replicates for each line).

The 136 DEGs (RNAi-*ddm1 vs*. WT in WD-RW condition) were grouped into main classes (Fig. 7b). Genes related to immune response were systematically up-regulated in RNAi-*ddm1* (*RBOHD, CYP94B1, RLP1, RLP56, RPM1, PLDGAMMA1, PDF1*; Fig. 7b). Most genes related to transcription factors (15/17) were also up-regulated (*WRKY, MYB106, ERF, SZF2, PDF2, SVP/AGL22*), with only two genes down-regulated (*MYB48* and *DTA2*). Defense and cell wall related genes were both up- (18, including *CHITIV*, *KTI1*, and *PR4* involved in plant pathogen-interaction) and down-regulated (13). Phytohormone pathways were also over-represented in distinct classes with 13 DEGs (8 up- and 5 down-regulated) directly involved in defense responsive hormone biosynthetic pathways such as salicylic acid (*SAMTs*), jasmonic acid (*OPR2*, *CYP94B1*), ethylene (*ERF1*, *ERF12*), auxin responsive genes (*SAUR29, GH3.1, IBR3, BG1, ABCG36*), gibberellic acid synthesis (*GA3OX1*) and cytokinins (*AHP1*) (Fig. 7b).

Although only 7 DEGs overlapped strictly with the DMR genomic locations (previously identified DMGs) (Fig. 7b), 53 were located in the direct vicinity of a DMR (+/- 10 kb) and 98 at +/- 25 kbp (Fig. S8a). Methylation in the three contexts correlated negatively with expression values when considering at least a +/- 10 kb window for DMRs (Spearman's *rho* = -0.32 at *P* = 0.0004) (Fig. S8b).

In summary, the analysis of the SAM stress-transcriptome suggests that, in the stress conditions tested, DDM1 plays a role in repressing genes involved in stress and defense response including hormonal pathways (Table 1 and Fig. S6b).

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460 **Mobilome analysis and TE genomic integration in SAM**

461 The mobilome-seq approach, consisting in the sequencing of intermediate extrachromosomal
462 circular forms (eccDNA) of TEs (Lanciano *et al*., 2017), allowed the identification of both active
463 DNA transposons and retrotransposons belonging to 44 to169 TE families depending on lines and
464 conditions (RNAi-*ddm1-23* in Fig. 8a, RNAi-*ddm1-15* in Fig. S9a). This number of identified
465 active TE families was always higher under water deficit conditions regardless of the line. Most
466 of the eccDNAs identified belonged to the annotated *Gypsy*, *Copia*, *ENSPM*, *L1*, *Ogre*, *POPGY*
467 and *SAT* families of TEs and repeats. We used the Depth of coverage (DOC) ranging from 4X to
468 51,000X to assign TEs into four categories. TEs identified in RNAi-*ddm1* and WT lines belonged
469 to the four groups under either condition (Figs. 8a, S9a, Table S3) with split-read (SR) coverage
470 ranging from 3X to 4,600X suggesting the presence of reads spanning the junction of eccDNAs.

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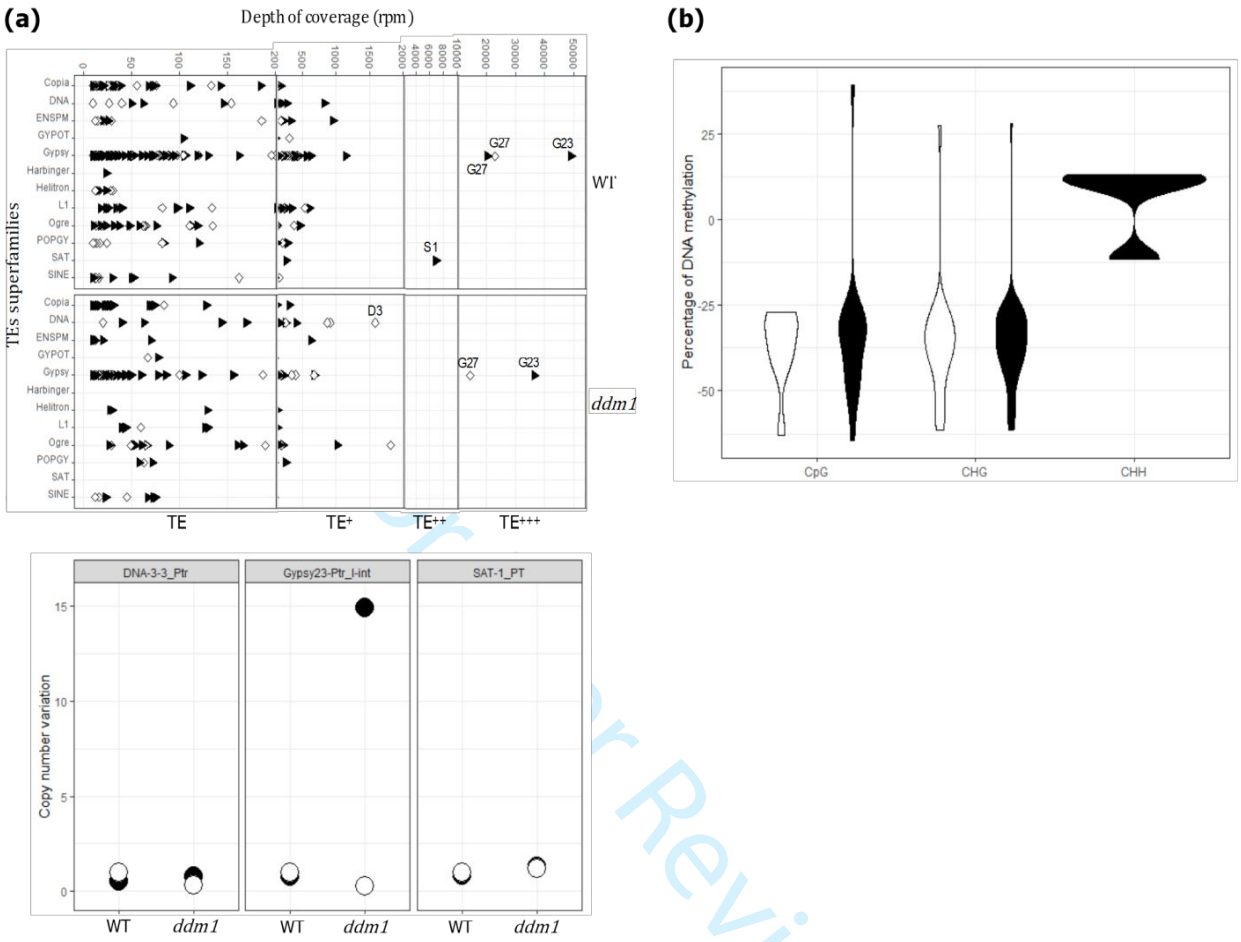

Fig. 8 Transposable elements activity in RNAi-*ddm1* and WT lines in shoot apical meristem collected one week after rewatering (t_2). (a) Mobilome-seq depth of coverage (read per million, rpm) of different TE families in WT and RNAi-*ddm1*-23 lines and in both Well-Watered (WW, white diamonds, 115 and 44 families for WT and RNAi-*ddm1-23,* respectively) and Water Deficit ReWatering (WD-RW, black triangles, 149 and 109 for WT and RNAi-*ddm1-23,* respectively) conditions. The coverage was calculated for each TE family and families are represented according to their superfamily. The four most active TE families are annotated: *Gyspy23* (*G23*), *Gypsy27* (*G27*), *DNA-3-3* (*D3*), *SAT-1* (*S1*). TE activity was classified based on the sequencing coverage of eccDNA forms, as follows: TE (0 to 200X); TE+ (200X to 2,000X); TE++(2,000X to10,000X); TE+++ (10,000X to 55,000X). (b) DNA methylation variations at active TEs in RNAi-*ddm1* versus WT, represented for each context of methylation under WW (white) and WD-RW (black) conditions. Note that no active TE could be detected inside a CHH DMR under WW conditions. (c) DNA copy number variations analyzed by qPCR on SAM genomic DNA for three different TE families (*DNA-3-3*, *Gypsy23* and *SAT-1*) in the WT and RNAi-*ddm1-23* lines in both WW (white circles) and WD-RW (black circles) conditions.

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Constitutive (WW) and stress-induced (WD-RW) specific TEs could be identified (414 TE families in all lines and conditions; Fig. 8a, Table S3). The two most active TEs (*Gypsy23* and *Gypsy27*) were detected in both WT and RNAi-*ddm1* lines, but *Gypsy23* was specifically activated upon stress (WD-RW condition) (Figs. 8a, S9a). Active TEs only detected in RNAi-*ddm1* lines were found under WW (28 TEs) or WD-RW (89 TEs) conditions. About 21% of the active TE detected by mobilome-seq strictly co-localized with RNAi-*ddm1* DMRs. This percentage reached more than 50% when considering the presence of a DMR at +/- 25 kb (Fig. S9b). These active TE families were mainly hypomethylated (*ca*. 92% of the TE families) in RNAi-*ddm1* lines (Fig. 8b) and the most active TEs (TE+++) co-localized with DMRs in the CHG context (data not shown). Under stress conditions, active TEs were also hypomethylated in both CG and CHG contexts but hypermethylated in the CHH context.

The number of genes identified in the vicinity of TEs varied from 45 (TEs inside genes) to 1,788 (when considering TEs at +/-25kb of genes) (Fig. S9b, Table S3). GO annotation of the genes co-localizing with TEs (+/- 10 kb of genes) revealed an enrichment for function in hormone response, immune/defense/abiotic responses and development (Fig. S9c). However, only seven of these genes were previously detected as DEGs (Fig. 7b) when considering the vicinity of +/- 25kb.

In order to test for new genomic integrations of the detected active TEs, we assessed copy number variation for three highly active TEs (*DNA-3-3_1*, *Gypsy23* and *SAT-1*) localized in or near DMRs by qPCR analysis (Figs. 8c, S9d). For *DNA-3-3_1*, there was no significant variation in the copy number regardless of the lines and conditions. For the other TEs, an increase in copy number was observed in RNAi-*ddm1* lines under water deficit only; 15 copies of *Gypsy23* in RNAi-*ddm1-23* (Fig. 8c) and 18 copies of *SAT-1* in RNAi-*ddm1-15* (Fig. S9d). In the RNAi-*ddm1* background, *Gypsy23* is located inside a CHG hypomethylated DMR, while *SAT-1* is found near (2kb) to a CG and CHG hypomethylated DMR.

In summary, hundreds of active TEs were detected in SAM and several of them were stress-induced. *DDM1* knock-down had only a limited impact on the active TE population. However, genomic copy number increase of two TE families could only be detected in RNAi-*ddm1* lines showing a role for DDM1 in protecting meristem genome integrity upon stress condition (Figs 8c, S6b).

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Discussion

Relevance of RNAi-*ddm1* poplar lines to investigate stress response in trees

Decreased DNA Methylation I (*DDM1*) is a plant gene encoding a nucleosome remodeler which facilitates DNA methylation (Zemach *et al.,* 2013; Zhang *et al.,* 2016; Osakabe *et al*., 2021). While studies on *ddm1* mutants have revealed a major role in silencing TEs and a minor role on some heterochromatic genes depending on annuals plants (Arabidopsis, tomato, maize, rice), its role in trees and the effect of the environment remain largely unknown (Zemach *et al.,* 2013; Tan *et al.,* 2016; Zhang *et al.,* 2016; Corem *et al.,* 2018; Long *et al.,* 2019). Here, we investigated the role of *DDM1* in a perennial tree (*i.e.* poplar) under drought conditions in the shoot apical meristem, the center of morphogenesis and differentiation of reproductive cells. We previously characterized stress-induced DMRs in the SAM of various poplars, including the wild type *P. tremula* × *P. alba* 717-1B4, revealing that epigenome and transcriptome remodeling following post-drought recovery preferentially affects genes in hormonal pathways (Lafon-Placette *et al.,* 2018). DNA methylation and other epigenetic marks are supposed to interact with hormone signaling to control developmental plasticity (Ojolo *et al.,* 2018) especially in meristems (Maury *et al.,* 2019; Amaral *et al.,* 2020), but functional evidences in meristems have so far been lacking. Thus, we used a reverse genetic approach and investigated the drought response of RNAi-*ddm1* poplar lines already stabilized *in vitro* for several years (Zhu *et al.,* 2013).

The global hypomethylation observed in the two poplar RNAi-*ddm1* lines indicated that *PtDDM1* knock-down (Zhu *et al.,* 2013) was effective in the shoot apical meristem. Whole-genome bisulfite sequencing further confirmed this hypomethylation at the genomic level in the three contexts (CpG, CHG and CHH). However, methylation levels in CHG were drastically reduced suggesting that poplar DDM1 preferentially targeted methylation in this context. This agrees with reports in rice and maize (Li *et al.,* 2014; Long *et al.,* 2019, Tan *et al.,* 2016) but differs from those in Arabidopsis and tomato (Vongs *et al.,* 1993; Kakutani *et al.,* 1995; Kakutani, 1997; Lippman *et al.,* 2004; Zemach *et al.,* 2013; Corem *et al.,* 2018). In addition, DDM1-dependent methylation in the SAM of poplar was shown to affect active TEs but also many genes, and to be remodeled by the stress conditions. This confirms that the effects of

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546 DDM1 on euchromatic and heterochromatic DNA methylation patterns are different depending
547 on species (Zemach *et al.,* 2013; Tan *et al.,* 2016; Long *et al.,* 2019) and environmental
548 conditions.

549 The necrotic spots on RNAi-*ddm1* leaves reported by Zhu *et al*. (2013) were consistently
550 observed in our experiment mainly on mature leaves and this has previously been associated to
551 *ddm1* mutation in Arabidopsis (Kakutani *et al.,* 1995; Qüesta *et al.,* 2013; Kooke *et al.,* 2015) and
552 hypomethylated *drm1 drm2 cmt3* mutants (Forgione *et al.,* 2019). This phenotype has been
553 suggested to be associated with either the demethylation-induced hyperactivation of disease
554 resistance genes or the demethylation-induced reactivation of a retrotransposon and virus-like
555 elements under stressful conditions (Zhu *et al.,* 2013). In our case, the higher endogenous levels
556 of SA observed in the SAM of RNAi-*ddm1* lines would support the first hypothesis, since the
557 accumulation of SA is known to be associated with *DDM1* mutations and is related to the
558 activation of hypersensitive response cell death or systemic acquired resistance (Dong, 2004;
559 Song *et al.,* 2004; Liu *et al.,* 2010; Zhang *et al.,* 2016; Badmi *et al.,* 2019). However, further
560 study in leaves will be needed to confirm this hypothetical SA accumulation in mature leaves in
561 relation to the appearance of necrotic spots during the time-course of the experiment.

562 Apart from leaf phenotypic alterations, we did not observe other striking phenotypes for
563 RNAi-*ddm1* lines, growth and water relations under control conditions being mostly comparable
564 to the WT. As gas exchanges were measured on young developing leaves without necrotic spots,
565 we do not take into account their potential effect. However, the moderate water deficit revealed
566 subtle differences in growth susceptibility, which, combined with the intrinsic higher cavitation
567 resistance, suggest that alterations of the DDM1-dependent DNA methylation in the two RNAi-
568 *ddm1* lines translated into increased tolerance to water-stress.

569 **Hypomethylated RNAi-*ddm1* poplar lines are more tolerant to water deficit**

570 Tolerance to water deficit is a complex trait encompassing multiple physiological determinants
571 that relate to diverse processes such as growth maintenance, survival, or recovery, depending on
572 the context of drought intensity and duration (McDowell *et al.,* 2008; Volaire *et al.,* 2018). The
573 moderate water deficit we imposed avoided a rapid growth cessation, and promoted steady-state
574 acclimation. The time course of soil REW was similar between the RNAi-*ddm1* and the WT lines

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during the whole experiment, indicating that all lines actually faced the same level of water deficit. However, while growth was progressively slowed down in the WT as REW dropped below 40%, as commonly observed in poplars (Bogeat-Triboulot *et al.,* 2007), RNAi-*ddm1* lines remained unaffected. This, combined with the fact that RNAi-*ddm1* lines exhibited a growth similar to the WT under control conditions, suggested increased tolerance to moderate water deficit in the RNAi-*ddm1* lines. Furthermore, the RNAi-*ddm1* lines also exhibited higher xylem resistance to drought-induced cavitation suggesting improved stress resilience under severe water deficit (Brodribb & Cochard, 2009; Barigah *et al.,* 2013). How modifications of the DDM1 machinery can affect xylem resistance to cavitation remains unknown at this stage. Interestingly, xylem morphometric features, xylem density and xylem biochemical composition were not significantly different from the WT. However, given the mechanistic understanding of drought-induced cavitation in angiosperms, it is likely that the increased resistance observed in the RNAi-*ddm1* lines was primarily linked to modifications in the ultrastructure of vessel-vessel bordered pits (Plavcová & Hacke, 2011; Fichot *et al.,* 2015). Whether the slight gain in intrinsic cavitation resistance (*i.e.* a few tenth of MPa) does promote increased survival under severe water deficit, and whether epigenetics might be exploited as such for increasing drought tolerance, remains to be purposely tested.

DDM1-dependent DNA methylation and hormones signaling interact in SAM to orchestrate stress tolerance

The improved stress-tolerance in the two RNAi-*ddm1* lines was not associated with sharp differences in water relations or more general phenotypes. Another possibility could be physiological differences related to the phytohormone balance under water deficit. Epigenetics has been linked to phytohormone pathways (Latzel *et al.,* 2012; Yamamuro *et al.,* 2016; Ojolo *et al.,* 2018; Raju *et al.,* 2018; Kooke *et al.,* 2019), which could play a major role in meristems for developmental plasticity (Maury *et al.,* 2019; Amaral *et al.,* 2020). Interestingly, although poplar RNAi-*ddm1* lines displayed hormone profiles in the shoot apex comparable to the WT under control conditions, differences were visible under the water deficit conditions especially for cytokinins (CKs) and salicylic acid (SA). RNAi-*ddm1* lines also displayed decreased expression of histidine-containing phosphotransfer protein 1 (AHP1) which plays a role in propagating cytokinin signal transduction. CKs are known to be negative regulators of stress signaling and CK-deficient plants with reduced levels of various CKs are generally more tolerant to drought

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606 and salt stress (Havlovà *et al.,* 2008; Nishiyama *et al.,* 2011; Ha *et al.,* 2012). Higher endogenous
607 levels of SA, as observed for the RNAi-*ddm1* lines compared to WT, have also been shown to
608 promote tolerance to stresses including drought (Munné-Bosch & Penuelas, 2003; Bandurska &
609 Stroi ski, 2005; Azooz & Youssef 2010; Pandey & Chakraborty, 2015; Sedaghat *et al.,* 2017).
610 Accordingly, *SAMT1*, a salicylic acid methyltransferase gene, was up-regulated in stressed
611 RNAi-*ddm1* lines compared to the WT (Mofatto *et al.,* 2016).

612 Although *PtDDM1* knock-down affected the methylation of about 900 genes common to
613 both RNAi-*ddm1* lines, only a limited number of DEGs was found under water deficit with seven
614 DEGs strictly overlapping with DMRs (53 for genes at +/-10 kb of DMRs). While no clear
615 correlation between DNA methylation and expression changes may exist at the genomic level,
616 the transcriptional activity of a subset of genes still might be regulated, both directly and
617 indirectly, by DNA methylation in response to abiotic stress (Karan *et al.,* 2012; Garg *et al.,*
618 2015; Chwialkowska *et al.,* 2016, Lafon-Placette *et al.,* 2018). Indeed, we found genes such as
619 transcription factors and hormone-related pathways that are likely to explain, at least in part, the
620 improved performance of poplar *RNAi-ddm1* lines under water deficit. Several transcription
621 factors such as *SVP, MYB48, ERF1* acting in phytohormone pathways and involved in biotic and
622 abiotic stresses were also upregulated in RNAi-*ddm1* lines in comparison to WT under water
623 deficit (Bechtold *et al.,* 2016; Sun & Yu, 2015; He *et al.,* 2016; Wang *et al.,* 2017; Heyman *et al.,*
624 2018; Wang *et al.,* 2018; Guo *et al.,* 2019). Other DEGs including genes involved in the leaf
625 cuticle and waxes (*MYB106*, *CER8*, *FLA12*) could also partly explain the improved tolerance to
626 moderate water deficit in RNAi-*ddm1* lines by preventing uncontrolled water loss (Chen *et al.,*
627 2011; Wettstein-Knowles, 2016; Oshima & Mitsuda, 2013, 2016).

628 Altogether, these findings confirm that common changes in DDM1-dependent DNA
629 methylation found in both RNAi-*ddm1* lines, especially in the shoot apical meristem, can alter
630 hormonal balance and pathways under stress conditions possibly leading to improved
631 physiological performance (Fig. S6b). This demonstrates the complex connections between
632 chromatin, hormones in meristems and plasticity (Lafon-Placette *et al.,* 2018; Maury *et al.,*
633 2019).

634 **DDM1-dependent DNA methylation regulates TE reactivation and insertion in SAM in**
635 **stress conditions**

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The repressive role of DNA methylation and DDM1 on TE proliferation is well-established in plants (Miura *et al.,* 2001; Mirouze *et al.,* 2009; Tsukahara *et al.,* 2009; Reinders *et al.,* 2009; Johannes *et al.,* 2009; Corem *et al.,* 2018; Quadrana *et al.,* 2019). We identified a large set of active TE families in SAM with up to 50% of them located at +/- 25 kb of one RNAi-*ddm1* common DMR and being mostly hypomethylated in CG and CHG contexts and hypermethylated in the CHH context under stress conditions. However, the activation of TEs was more affected by the stress conditions than the *DDM1* knock-down. We could nonetheless detect increased copy number of some of the most active TEs (*Gypsy-23* and *SAT-1*) only in RNAi-*ddm1* lines under water deficit, suggesting that DDM1 plays a role in limiting TE reactivation and integration under stressful conditions in the SAM.

Our findings provide evidence that a repression of DDM1-dependent DNA methylation can reactivate TEs especially under stressful conditions, potentially producing genetic variations. Quadrana *et al.* (2019) proposed that TEs are potent and episodic (epi)mutagens that increase the potential for rapid adaptation. Epigenetic regulations may act as a hub by which non-genetically inherited environmentally induced variation in traits can become genetically encoded over generations (Danchin *et al*., 2019). Recently, Baduel & Colot (2021) proposed that the adaptive contribution of TE-associated epivariations is mainly related to their ability to modulate TE mobilization in response to the environment. This definitely deserves further attention as genes found nearby our active TEs were involved in hormone and stress responses or development. Elucidating the evolutionary significance of both naturally occurring and environmentally induced variations in DNA methylation in a context of tree population dynamics will be of particular interest for long-living organisms such as trees in the age of rapid climate changes.

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Author Contribution

SM designed and coordinated the research. The plant experimental design was established by SM, SHS, RF, and FB Ecophysiological measurements were performed by ALLG, RF, AD, ILJ and HC; analysis was conducted by ALLG and RF. NIRS measurements and analysis were performed by ALLG and VS Phytohormones analysis was performed by SC DNA, RNA extractions were done by AD and ALLG and MDS. HPLC analysis was done by AD and SM. RNA-seq was conducted and analyzed by JC, VB, LST with MDS. JT and CD realized WGBS analysis. WGBS data analysis was done by SM, ALLG and MDS. Bioinformatics for WGBS was done with the help of CC and CG. Mobilome analysis was done under the supervision of MM with SL and ALLG. QPCR analyses were done by MCLD with AD and MDS. Data analysis was done by SM, ALLG and MDS. Statistical analyses were done by RF, ALLG and MDS. SM, RF, ALLG and MDS conceived and wrote the first draft of the manuscript, MM helped revising the manuscript and SHS edited and helped to direct final analyses and organization of the manuscript. All authors approved the final version of the manuscript.

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Supplementary Strategies

Data Availability

The data that support the findings of this study are openly available: The WGBS data have been deposited in SRA at https://www.ncbi.nlm.nih.gov/sra/PRJNA611484, reference number PRJNA611484; the RNA-Seq data in GEO (Gene Expression Omnibus) at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135313 reference number

696 GSE135313 and also in CATdb database at http://tools.ips2.u-psud.fr/CATdb/) reference number NGS2017-01-
697 DDM1.; the Mobilome-Seq data in GEO at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147934,
698 reference number GSE147934 and also in the supplementary material of this article (Table S3).

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Supporting information

Fig. S1 Comparison of growth kinetics over the experiment between both conditions

Fig. S2 Leaf phenotypes among lines under both conditions

Fig. S3 Physiological differences among lines under both conditions

Fig. S4 Variations in DNA methylation among RNAi-ddm1 and WT lines in shoot apical meristem one week after rewatering (t2)

Fig. S5 Strategies for bioinformatic analysis of the methylome

Fig. S6 Concepts and summary of differential methylation analyses: From ddm1 DMRs to phenotype

Fig. S7 Identification of differentially methylated genes.

Fig. S8 Relationship between variation in DNA methylation and gene expression

Fig. S9 Transposable elements activity among RNAi-*ddm1* and WT lines in shoot apical meristem collected one week after rewatering (t2)

Table S1 Xylem structural, functional and biochemical traits measured for the WT and the two RNAi-*ddm1* poplar lines

Table S2 Mean methylation levels in RNAi-*ddm1* and WT lines in both conditions

Table S3 Lists of DMRs, DMGs, DEGS, TEs and Genes near TEs

Methods S1 Additional detailed procedures for ecophysiological, genomic and bioinformatic analyses

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