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Title: Growth, Morphology, and *in Vitro* Development of Transgenic Poplars with RNAi-inhibited *PtDDM1-1/2* Gene Expression

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Steven H. Strauss

Epigenetic mechanisms are important for control of plant development, and may play a particularly important role in trees given their long life cycles and distinctive and stable tissue types. To help understand the role of epigenetics in tree development, we produced transgenic poplars with reduced activity of the *DDM1* genes, whose activity are known to be critical for the maintenance of DNA methylation in plant genomes. DNA methylation is widely recognized as a major element of epigenetic variation, where its presence is usually associated with loss of gene expression. The *DDM1*

gene is necessary for the maintenance of DNA methylation in the extensive heterochromatic fractions of the genome.

We identified two highly similar *DDM1* homologs in poplar (*PtDDM1*) and used RNAi to suppress both of their transcripts with a single construct. *PtDDM*- RNAi transgenic poplars showed a wide range of suppression efficiency, with the most strongly suppressed gene insertion events (lines) having a reduction in RNA expression of 70% based on combined stem and leaf *in vitro* materials and real time RT-PCR. Six transgenic lines were analyzed for their total cellular cytosine DNA methylation by HPLC (High Performance Liquid Chromatography). The DNA methylation percentages were generally correlated with *PtDDM1* expression. A greenhouse study identified variation in growth rate associated with events, but these were not associated with *PtDDM1* gene expression. There were also no visible differences in morphology of the transgenic lines. However, after dormancy, transgenic trees with strong *PtDDM1* suppression that were growing out of doors in a covered "lathouse" showed a severe mottled leaf phenotype in all of its ramets, and two other transgenic events with strong *PtDDM1* suppression showed similar but less severe symptoms in some of their ramets. A study of *in vitro* callogenesis of stem explants showed an inverse correlation between *DDM1* expression and the percentage of rapidly growing callus; however, these results were not repeated with leaf explants or in a second experiment with a different experimental design. The second study showed a positive correlation between *PtDDM1* expression and callus size. We speculated that the reduced methylation promoted tissue dedifferentiation,

redifferentiation, and cell division, and that the mottled leaf phenotype was a result of DNA methylation change in the poplar genome. Our results suggest that transgenic demethylation may be a useful tool for promoting *in vitro* regeneration, but requires considerably more study of different target genes and suppression methods.

Growth, Morphology, and *in Vitro* Development of Transgenic Poplars with RNAi-inhibited *PtDDM1-1/2* Gene Expression

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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CONTRIBUTIONS OF AUTHORS

Former Senior Research Assistant Olga Shevchenko designed and assembled the RNAi construct. Cathleen Ma and Elizabeth Etherington transformed it into poplar clone *717-1B4*. Cathleen gave extensive advice and help for maintaining *in vitro* materials and related studies. Stephane Verger made a major contribution to make greenhouse measurements and the *in vitro* regeneration study, and performed many DNA extraction. Dr. Christopher K. Mathews provided reagents, HPLC facilities, the nucleoside hydrolysis protocol, and invaluable advice in studying genomic cytosine methylation. Dr. Michael Freitag helped to design the overall project. Dr. Steven H. Strauss was the primary creator and designer of the project, provided funding, and gave frequent advice on experimental design, data analysis, communication of results, and the structure and writing of the thesis.

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CHAPTER 1: Introduction and literature review

Defining epigenetics

An often quoted definition of epigenetics is “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequences” (Allis et al. 2005). "epi-", as a prefix, means "upon, besides, attached to" (Merriam Webster, 2011). An array of epigenetic marks, such as DNA methylation, histone modification, and small RNAs, form intricate networks of interaction and regulate genome activity. Changes in epigenetic marks in a specific region are heritable but potentially reversible, and likewise so are the associated changes in gene expression and phenotypes. By combinations of their presence or absence, they can determine whether a gene is turned on or off; or whether a large chromosomal region is accessible to transcription or not.

The word epigenetics was first used in late 19th century; however, it was only in the mid 20th century that it began to be recognized as a distinct scientific field. In 1941 the *Drosophila* geneticist H.J. Muller described the phenomenon of PEV (Position Effect Varigation) based on a *Drosophila* mutant – the mosaic colored eye phenotype. He demonstrated that in this mutation the *white* eye gene was placed near a pericentrimeric region by an inversion on the X chromosome, consequently the white gene was turned on in some cells and off in others, resulting in the mosaic phenotype (Büchner et al. 2000).

In 1950, Barbara McClintock speculated that the “mutable loci in maize” described in her work and many others could have the same mechanism as described for PEV (McClintock 1950). Later in 1956, she developed the idea of a “Spm controlling elements system” and transacting factors that could suppress a gene without mutating it (McClintock 1956). These “controlling elements” was later recognized as “transposable elements,” shown to be major parts of genomes.

DNA methylation was the first epigenetic mark shown to correlate with gene expression (Razin and Riggs 1980). Other epigenetic marks such as histone modifications, small RNA, and chromatin remodeling have also been vigorously researched in recent years.

Epigenetic modifications in plant genomes

The development and the life cycle of plants are regulated by different epigenomes, which the plants adopt at appropriate times. In addition, because of its sessile life style, plants employ epigenetic changes to cope with biotic or abiotic stress. In plants, homologs of mammal or fungi epigenetic regulatory proteins have been identified, such as DNA methyltransferase 1 and histone methyltransferases. However, plants contain the largest number of chromatin regulatory proteins (Alberts et al. 2007) and display unique features in some epigenetic marks and pathways.

DNA, nucleosome, and chromatin

In 1944, DNA was discovered to be carrying the heritable information of living organisms (Steinman and Moberg 1994). It was proposed in 1953 to be a double helix

of complementary sequences of deoxycytodine (dC), deoxyadenosine (dA), thymine (T) and deoxyguanosine (dG) (Schindler 2008). As the material to store heritable information of development and maintenance of a living organism, the double helix of DNA is packed in each cell nucleus. The tight and ordered packaging is assisted by specialized proteins. There are traditionally two classes of such proteins: the histones and the non-histone chromosomal proteins. The complex of both classes of proteins and the DNA is called chromatin (Alberts et al. 2007).

Nucleosomes, discovered in 1974 by electron microscopy as "beads on a string," are involved in the first level of DNA packaging (Olins and Olins 1974). The observed "beads on a string" is DNA wound around a nucleosome core particle in a periodic manner. A nucleosome core particle consists of a histone octomer (two molecules each of histone H2A, H2B, H3, and H4) and about 146bp of double-stranded DNA. The histones have extensive interactions with DNA molecules: hydrogen bonds, hydrophobic interactions, and salt linkages. The histone molecules in a nucleosome core complex are contained with the wound DNA. However, they all have long N-termini extending out of the DNA histone core. These "tails" often have covalent modifications that regulate local gene expression and chromatin states.

Heterochromatin and euchromatin

During interphase in eukaryotic cells, chromatin is greatly extended and fills the nucleus. Under the light microscope, however, two types of chromatin can be observed: a highly condensed form which is called heterochromatin, and the less

condensed form which is called euchromatin (Alberts et al. 2007). In heterochromatin, chromatin is packed in a higher order structure and highly condensed. Therefore, it acts as a barrier to prevent regulatory proteins and RNA polymerases from approaching DNA. As a result, expression of genes in heterochromatin is repressed (Reyes et al. 2002). In euchromatin, however, DNA is readily accessed and thus can be actively transcribed. Another distinguishing feature of heterochromatin is that it has much lower gene density and contains large regions of repetitive DNA and transposable elements (Richards and Elgin 2002). Despite the abundance of those elements, heterochromatin has a low recombination rate and transposable elements are rarely mobile because of its condensed feature (Grewal and Rice 2004). In addition, centromeres and telomeres are always heterochromatic.

Heterochromatin is associated with a special group of epigenetic marks. Some are repressive marks because they are related to gene silencing and heterochromatin formation. Modification of histones may be the cause of nuclear compartmentalization, in which chromatin is arranged differently in the interphase nuclei, and as a consequence different parts of the chromatin obtain either active or silent states. In mammals, gene-rich and active euchromatin resides in the internal part of the interphase nuclei, whereas gene-poor and silent heterochromatin resides in the periphery (Bártová et al. 2008). In plants, co-localization of transcription machinery and nuclear actin in subnuclear compartments suggest specialized components and function in nuclear compartmentalization (Cruz and Moreno 2008).

Transposable elements

Transposons are mobile genetic elements that can insert themselves into other DNA sequences by a specific enzyme, usually encoded by the transposon and called transposase (Alberts et al. 2007). No homology is required at the site of insertion with the ends of the transposable element. Transposons and repetitive elements are enriched in heterochromatin and usually silenced. But there are rare cases, particularly when plant is under environmental stress, that movements of the transposons are triggered (Tittel-Elmer et al. 2010).

Transposons are divided into three categories as reviewed by Alberts et al. 2007: DNA-only transposons, retroviral-like retrotransposons, and nonretroviral retrotransposons. The first two classes share a similar mechanism that both utilize a transposase to integrate DNA into the genome. In DNA-only transposons, the DNA sequences are replicated and inserted into another place without an RNA intermediate. In retroviral-like retrotransoposons, however, first an RNA molecule is transcribed from the retrotransposon, and then a special polymerase (viral RNA-dependent DNA polymerase, or vRdDP) that can use either DNA or RNA as a template is used to produce a cDNA which is inserted into the genome by integrase (similar to the transposase using DNA-only transposons). A retrovirus encodes not only an integrase and a vRdDP, but also a protein capsid that can carry it to transport from cell to cell. It has been reported some retrotransposons in plants have retained the protein capsid encoding sequence, and thus are in effect dormant retroviruses that still have potential

for infection (Vicient et al. 2001; Wright and Voytas 2002; Marco and Marín 2008). The third class is non-retroviral retrotransposons. They move by different mechanism, in which reverse transcription has a more direct role in the recombination event that leads to integration.

Chromatin remodeling

Chromatin contains histone and DNA in a nucleosomal complex that has ordered "packing" (Cooper and Hausman 1996), and is regulated by ATP-dependent complexes called chromatin remodelers. They affect the spacing of nucleosomes and the accessibility of DNA in a particular region to various machineries such as transcription factors, RNA polymerases, and complexes involved in DNA repair. Therefore, DNA-dependent activities, such as replication, transcription, repair, and recombination, are influenced by chromatin remodeling (Tsukiyama and Wu 1997). Remodeling within the nucleosome core might involve movement of the H2A-H2B dimers, since the H3-H4 tetramers are very stable and hard to rearrange. Chromatin remodeling can provide easier accessibility to nucleosomal DNA, or changes in nucleosome position along DNA (Albert et al. 2002). A recent finding suggests that A-type lamins interact with nuclear actin during chromatin remodeling (Ivorra et al. 2006; Bártová et al. 2008).

The various remodelers identified have been categorized based on their ATPase subunits. The major classes according to Allis et al. (2005) are: SWI/SNF complexes (contain ATPases related to SWI2/SNF2), ISWI complexes (contain ATPases related

to Imitation-SWI), and CHD complexes (contain ATPases related to CHD1 and Mi2). In addition to the ATPase subunit, remodelers also have other subunits that carry out specific functions. ACF and CHRAC maintain even spacing; SWI/SNF is considered to be opening a region of DNA by remodeling the nucleosomes to be further apart; and ISWI has an opposite role to that of SWI/SNF. Among the various chromatin remodeling complexes, the group SWI/SNF is of particular interest in this paper, because it is prevalent in the trithorax proteins group to which *DDM1* belongs.

The Polycomb (PcG) and trithorax (trxG) groups

The PcG was identified in the 1940s in *Drosophila*, when the mutation of a gene that spatially controls the homeotic HOX gene resulted in the spreading of the first leg identity to the second and the third legs (Lewis 1978). Regulatory genes, such as PcG, have been classified into two antagonistic groups according to Allis et al. (2005): PcG proteins, which are required to maintain the silenced state of developmental genes, and trxG proteins, which are required to maintain the activated state of developmental genes.

PcG proteins were categorized into two groups according to their roles in maintaining gene repression (Allis et al. 2005): Polycomb repressive complexes1 (PRC1) and Polycomb repressive complexes2 (PRC2). These two groups of proteins work consecutively. First, PRC2 methylates H3K27 and/or H3K9 at the target loci, and secondly, PRC1 recognizes and binds to the modified histone and brings about chromatin structural change required for gene repression. A typical PRC2 complex has

four core proteins, one of which is the SET histone methyltransferase Enhancer of Zeste (E(Z)). E(Z) is a 760 amino acid protein, which has a SET domain that has histone lysine methyltransferase (HKMT) activity.

In contrast to PcG, trxG group maintains the active state of target gene expression, but the mechanism is not as clear as that is for PcG group (Allis et al. 2005). However, a considerable subset of trxG group proteins is related to chromatin modification and remodeling. One example is the common presence of the SET domain in trxG proteins TRX and ASH1, presumably to maintain the high gene expression by active histone methylation (e.g., H3K4). Another connection is the common presence of SWI/SNF. SWI/SNF was identified in screens of mating-type switch and sucrose fermentation mutants and later found to be required for expression of numerous developmental genes. Biochemical experiments show that the range of sites within the nucleosome which SWI/SNF complexes able to remodel is unusually large compared to other ATP-dependent chromatin remodeling complexes (Brzeski and Jerzmanowski 2003). Although chromatin modification and remodeling are probably among the mechanisms by which trxG maintains a high level of target gene expression, they are not the only ways for trxG's to work; any gene involved in the numerous steps of activation of developmental genes can be termed as a trxG protein.

DNA methylation

DNA methylation refers to a class of modifications of nucleotide bases, specifically, the addition of a methyl group at cytosine or adenine. Cytosine methylation has been

more thoroughly studied than adenine methylation. Our study focuses on cytosine methylation.

Cytosine methylation has been found in a variety of eukaryotes, but the methylation levels differ markedly: mammals and plants have high cytosine methylation levels, while some invertebrates, such as *Drosophila* and *Caenorhabditis elegans*, have undetectable to low methylation levels (Allis et al. 2005). In mammalian genomes, cytosine methylation is mostly found in a symmetrical CpG context, with the exception of methylation in the CHH context (where H is A, C, or T) in stem cells. In contrast, DNA methylation in plant genomes is more diverse, including CpG, CHG, and CHH sequences (where H is any nucleotide) (Chan et al. 2005).

De novo and maintenance DNA methylation

Once established, cytosine methylation is replicated with DNA duplication and passed to daughter cells. Traditionally, there are two ways by which cytosine becomes methylated.

De novo cytosine methylation is the establishment of cytosine methylation at a new genomic position. *De novo* cytosine methylation usually occurs when cell is changing genome function as a response to developmental need or to changes in the environment. Domain rearranged methyltransferase (DRM1) and DRM2 are two recognized *de novo* methyltransferases in plants (Allis et al. 2005). As a response to internal or external stimuli, demethylation can occur in selective regions. This is accomplished by DNA glycosylases.

Maintenance methylation refers to the preservation of the methylation pattern during DNA replication. Methyltransferase 1 (*MET1*) is the major maintenance methylation enzyme at CpG sites, whereas chromomethyltransferase (*CMT*), which is unique in plants, is responsible for maintenance of cytosine methylation at non-CpG sites. The *met1* mutant has strongly reduced CpG methylation and exhibits morphological defects such as changes in meristem identity and prolonged phases of development. *cmt3* mutants show strong reduction in CpHpG methylation but do not exhibit morphological defects, indicating that CpG methylation is the primary source of methylation, while non-CpG methylation patterns appears to provide a secondary level of regulation (Mahfouz 2010).

The role of DNA methylation

DNA hypermethylation is one of the most prominent epigenetic marks in heterochromatin. Heavy cytosine methylation level is essential for silencing of transposable elements located in heterochromatin. Drastic loss of DNA methylation often results in reactivation of transposable elements (Hirochika et al. 2000; Singer et al. 2001; Wright and Voytas 2002; Lippman et al. 2004). In addition, cytosine methylation may influence the establishment of other epigenetic marks, such as repressive histone methylation and deacetylated isoforms of H3 and H4 (Richards and Elgin 2002). A surprising role for DNA methylation is in the demarcation of centromeric chromatin in *Arabidopsis*. Zhang et al. (2008) suggested this when they found that the 178 bp repeats in the centromeric region were hypomethylated, while

the flanking pericentromeric with the same repeats were hypermethylated (Zhang et al. 2008).

In euchromatin, DNA methylation shows a slightly different function. Methylation profiling of *Arabidopsis* has shown that cytosine methylation of gene promoter regions is usually associated with transcription inhibition, but genes with cytosine methylation in coding regions (gene-body methylation) are often expressed at moderate to high levels, and have little tissue specificity (Chan et al. 2005; Mahfouz 2010).

Histone modification

The N-terminus of the four core histones are highly conserved among all eukaryotes. The post-translational modifications of specific residues on the histone tails play major roles in regulating genome function, presumably by recruiting of transcription factors or other protein complexes that affect chromatin structure and state (Allis et al. 2005). The modifications include acetylation of lysine, phosphorylation of serine, methylation of lysine, and ubiquitination (Alberts 2002). Of these modifications of histones, acetylation and methylation have been widely studied and have demonstrated functions in control of chromatin structure.

Histone acetylation

Histone acetylation is an active chromatin mark, which means that most of the time it is associated with euchromatin and actively transcribed regions. Histone deacetylation shows the opposite function, marking regions of heterochromatin and silenced regions. The acquisition of H3K9 acetylation results in chromatin decondensation and

separation of actively transcribed genes from heterochromatic regions (Chambeyron and Bickmore 2004; Bártová et al. 2008). Acetylation of histone lysine residues is catalyzed by histone acetyltransferases (HATs), and the reverse process is carried out by histone deacetylases (HDACs), which removes the acetyl groups. Compared to mammals and fungi, plant HAT and HDAC proteins show considerable conservation as well as specialization. For instance, *Arabidopsis* has 18 putative HDAC and 12 putative HATs. Fourteen of the putative HDACs found in Arabidopsis are conserved in other eukaryotes, but four of them belong to a plant-specific family, *HD2* (Tanaka, Kikuchi, and Kamada 2008). Three of the HD2 family proteins in *Arabidopsis* have been studied: the *HD6* deficient mutant exhibits a substantial decrease in C(N)G methylation except centromeric region and rDNA repeats, and HD6 appears to play a role in maintenance of Cp(N)G methylation in the RNA-directed pathway (Aufsatz et al. 2002). Interestingly, an HD6 deficient hybrid lost nucleolar dominance, the phenomenon of specifically silencing of one parental set of ribosomal genes in a hybrid (Earley et al. 2006). However, HD6 mutants did not show any obvious defects in growth or development. In contrast, *HDA1* deficiency and *HDA19* deficiency both produced in pleiotropic mutant phenotypes (Allis et al. 2005; Tanaka, Kikuchi, and Kamada 2008). Plant HATs and HDACs appear to have substantial functional redundancy as well as specialized functions.

Histone methylation

Histone methylation can be associated with heterochromatin or euchromatin, according to which lysine residue is methylated and to what extent it is methylated (e.g. mono-, di-, or tri-) (Richards and Elgin 2002). Some well studied examples are H3K4 di-methylation and H3K9 di-methylation. H3K4 di-methylation often associates with euchromatic regions, whereas the H3K9 di-methylation tends to be located in heterochromatic regions. Proteins that methylate histone lysine residues are called histone lysine methyltransferases (HKMTs). HKMTs usually have a common SET domain (SU(VAR)/E(Z)/TRX), with the exception of *DOT1* (Disruptor of telomeric silencing1). Some of the SET domain proteins belong to PcG or trxG families, and they presumably maintain the active or repressed state of the target genes by establishing or maintaining histone methylation by SET domain (Allis et al. 2005).

The "histone code"

Other histone modifications, such as phosphorylation, ubiquitylation, sumoylation, and histone variant replacement, are of no less importance than the previously introduced histone acetylation and methylation. However, they have not been well studied. Taken together, with various modifications at diverse positions of histone tails, the "histone code" provides a major epigenetic language for control of chromatin state and gene expression (Loidl 2004).

Small RNA related silencing pathways

Small RNAs have gained special attention in recent decades because of their importance in genome regulation. The transcription and/or processing of small RNAs are controlled in space and time to produce 21-26 nt sequences that target specific DNA or RNA substrates. Analysis of small RNA targets has shown that a majority (70%) of small RNAs have transcription factors as targets, though transcription factors only comprise a small fraction (6%) of the genome (Steimer et al. 2004).

The mechanism of small RNA silencing (Allis et al. 2005)

In order to produce 21-26 nt small RNAs, a double stranded RNA is first needed as a substrate. The dsRNA can come from many sources, including complimentary mRNAs transcribed from endogenous genes and transgenes, viral RNA replicated by viral RNA dependent RNA polymerases (vRdRP), mRNA transcribed from a pair of inverted repeats, and mRNA replicated by endogenous RNA dependent RNA polymerases (RdRP). The dsRNA is captured and processed into 21-26nt small RNAs by proteins including DCL and HYL1. The processed small RNAs direct post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). In general, processed small RNAs direct regulatory proteins to either homologous DNA sequences or homologous mRNA transcripts. The former is epigenetically modified to form heterochromatin and the latter is either cleaved or retained in translation.

Categories of small RNAs

According to Allis et al. (2005), there are two categories of small RNAs in terms of function: The 21nt small RNA brings the RNA-induced silencing complex (RISC) and a member of the Argonaute family to mRNA for its cleavage, and 24-26 nt small RNAs direct a set of proteins for epigenetic modification of the homologous genomic sequences. These proteins include plant specific RNA polymerase IVb which works together with *DRD1* (a SWI2/SNF2-like chromatin remodeling protein) to achieve *de novo* DNA methylation.

According to the origin of the small RNA and the target of the silencing pathway, three functional categories were suggested by Matzke and Scheid (Allis et al. 2005): The first category describes a host defense system in which transgenes and viruses are silenced by RNAi-mediated pathway; the second category is regulation of plant development by small RNA inhibition of endogenous gene expression; and the third group is RNA directed DNA methylation (RdDM), heterochromatin formation, and transcriptional silencing (Matzke et al. 2009).

The role of small RNAs

Small non-coding RNAs are essential for establishment of DNA methylation in all sequence contexts, as well as for maintenance of non-CG DNA methylation (Huettel et al. 2007). Interestingly, in the RdDM pathway, small RNA interacts with *DRD1* (a putative SWI2/SNF2-like chromatin remodeling protein) and with *DRD2* and *DRD3* (subunits of a putative plant specific polymerase Pol IVb) (Chan et al. 2006). It has

been reported in the *drd1* mutant, which shows decondensation of the major pericentromeric repeats and loss of repressive mark H3K9 dimethylation at chromocenters (Pontes et al. 2009). Although the putative function of chromatin-remodeling or polymerases including *DRD1*, *DRD2* and *DRD3* have not yet been validated, it suggests the coordination of small RNA, chromatin-remodeling, chromatin modification, and the transcription machinery (Kanno et al. 2004).

Breakthrough in biotechnology: RNA interference

The discovery of small RNA mediated silencing has been considered a great science and technology breakthrough. The RNAi technique won the Nobel Prize for Andrew Fire and Craig Mello in 2006 based on their work in *C. elegans*. This introduces into the genome inverted DNA sequence constructs which, when transcribed, form double-stranded RNAs. The double-stranded RNAs are recognized and processed into small RNAs by the machinery in the small RNA silencing pathway. Consequently, the small RNAs direct silencing or inhibition expression of target genes (Fire et al. 1998). This was used in our study by suppressing the *DDM1* gene in *Populus*.

***DDM1* and DNA methylation in plants**

DDM1 was first identified in *Arabidopsis* as a mutant with a decrease in DNA methylation (Vongs et al. 1993). It was thus named *DDM1* (Decrease in DNA methylation). *DDM1* is well conserved among fungi, animals, and plants, but no homologs of *DDM1* were found invertebrate genomes (Lippman et al. 2004).

Mutation of DDM1 leads to many epigenetic changes

In *Arabidopsis*, *ddm1* mutation leads to loss of cytosine methylation, changes in histone modification and small RNAs, and reactivation of transposons especially at heterochromatic regions. The *Arabidopsis* homozygous mutation *ddm1* causes a 70% reduction in cytosine methylation (Kakutani et al. 1999). In *Arabidopsis*, DDM1 is involved in maintenance of CpG methylation, and in maintenance of CHG and CHH methylation in heterochromatic regions (Chan et al. 2005). In addition, DDM1 seems to affect histone H3 modification and the large-scale organization of heterochromatin in the nucleus. Nuclei of *ddm1* mutants showed a reduction in heterochromatin content relative to wild type (Blevins et al. 2009). Microarray analysis of *ddm1* mutants in *Arabidopsis* by Lippman et al. (2004) showed that a strong reactivation of transposable element such as Gypsy-like and CACTA, and that the heterochromatic repeats and transposable elements that are influenced by *DDM1* coincide with small RNAs. Cytosine methylation and gene expression in euchromatin, however, was unaffected (Hudson et al. 2011).

DDM1, a possible chromatin remodeling factor

Arabidopsis DDM1 is a SWI/SNF family chromatin remodeling factor, and shows ATP-dependent chromatin remodeling capability both with naked and nucleosomal DNA. Specifically, it induces nucleosome repositioning on small DNA fragments *in vitro* and the activity is not affected by DNA methylation (Jeddeloh et al. 1999; Brzeski and Jerzmanowski 2003). However, DDM1 has been suggested to be closely

related to the mammalian LSH protein which is required for genome-wide methylation (Dennis et al. 2001; Reyes et al. 2002). DDM1 protein co-localizes with MBDs (Methyl-CpG binding domain protein), which target methylated cytosines in a CpG context (Zemach et al. 2005). This suggests a possible role in which DDM1 remodels chromatin to provide access for methylated cytosine.

Mutation in DDM1 resulted in various developmental abnormalities

A variety of developmental abnormalities has been observed in *ddm1* mutants. These include late flowering, small stature, and excessive sensitivity to UV radiation (Jacobsen et al. 2000; Soppe et al. 2000; Shaked et al. 2006; Saze and Kakutani 2007). They can result either from the change in epigenetic state in certain loci or because of activated transposon insertions into functional genes (Miura et al. 2001; Singer et al. 2001). Mutant phenotypes are not manifest for several generations. In *Arabidopsis*, the bonsai phenotype was observed in homozygous *ddm1* mutants in the eighth generation (Saze and Kakutani 2007).

Characteristics of DDM1

To conclude, *ddm1* mutants show six basic features: **1.** *ddm1* mutants show a large decrease in DNA methylation (Vongs et al. 1993). **2.** *ddm1* mutation can promote the expansion of DNA methylation at specific loci. This phenomenon, termed specific hyper-methylation, suggests that the role of DDM1 in maintaining DNA methylation is complex (Saze and Kakutani 2007). Similar effects are also found in certain mammalian tumor cell lines (Reyes et al. 2002). **3.** *DDM1* is recessive. Kakutani et al.

(1999) observed no obvious difference in *ddm1* heterozygous mutants. **4.** *ddm1* mutants do not show an immediate mutant phenotype, but can still "deterministically induce certain phenotypes generations later" (Saze and Kakutani 2007). This is likely because of the accumulation of the gradual decrease in DNA methylation resulting from *ddm1* loss of function and the partial restoration by the RdDM pathway. **5.** Remethylation of sequences hypomethylated by the *ddm1* mutation is extremely slow, even in wild-type *DDM1* backgrounds. Unlike *met1* and *cmt3* mutants which show immediate recovery of DNA methylation, the demethylated loci in the *ddm1* mutants remain hypomethylated two generations after crossing with wildtype (Kakutani et al. 1999; Mahfouz 2010). **6.** Despite the presence of *DDM1* transcripts, DDM1 protein is absent in the vegetative nucleus of pollen. In pollen vegetative nucleus, transposable elements showed reduced DNA methylation, reactivation, transposition, and changes in small RNAs at speficic loci, similar to that in *ddm1* mutants (Slotkin et al. 2009).

How does DDM1 work?

Despite the remarkable losses of cytosine methylation in the heterochromatin of *ddm1* mutants, there appear to be some regions in which cytosine methylation is unaffected. This includes PAI1-PAI4 and NOSpro inverted repeats. Mahfouz (2010) suggested that DNA methylation in those regions might be maintained in a pathway that does not require DDM1. He further suggested that in *ddm1* mutants, constitutive heterochromatin regions are unaffected while facultative heterochromatins can either be repressed or activated, and that those two distinct regions are under different

methylation maintenance pathways. Facultative chromatin might be under the RdDM pathway where both methylation and demethylation take place and where DDM1 is involved; however, constitutive heterchromatins might not be subject to pathways involving DNA glycosylases (DNA demethylase) and DDM1 (Mahfouz 2010).

Blevins et al. (2009), however, suggested that DDM1 might not be involved in RdDM, but work separately in an overlapping pathway. This was based on the deficiencies of PolIV (a component of the RdDM pathway) and that DDM1 has additive effects with it in silencing. They also found increased small RNAs in 5s rDNA arrays in *ddm1* mutants, suggesting that DDM1 "attenuates" small RNA production and the RdDM pathway.

Schoft et al. (2009) also argued that DDM1 might be involved in a pathway overlapping RdDM. They observed that mutations in the components of the RdDM pathway do not affect DNA methylation in constitutive centromeric heterochromatin, despite the presence of 24 nt siRNAs in those regions; but mutations in pollen vegetative nuclei showed a remarkable increase in DNA methylation. They suggested that constitutive heterochromatin is protected from the RdDM pathway because of their condensed state, but are exposed in pollen vegetative nuclei where they are decondensed and where DDM1 proteins are absent. The hypothesis that DDM1-dependent maintenance of silent chromatin and the RdDM pathway are overlapping can explain, to a certain degree, why loss of DNA methylation is not immediate but

rather progressive and why in the context of genome-wide hypomethyation, region specific hypermethylation could occur (Saze and Kakutani 2007).

It also has been suggested, because of the strong effect of the *ddm1* mutation on normally hypermethylated heterochromatic regions, that DDM1 functions as a chromatin remodeler and increases the accessibility of hemimethylated DNA in newly replicated chromatin to DNA methyltransferases (Reyest et al. 2002). The mechanism by which DDM1 interacts with RdDM to maintain DNA methylation in heterochromatic regions is still a topic of considerable research.

5-azacytidine (5-AC) and *MET1*

5-AC is a cytidine analogue in which carbon 5 of the primidine ring is replaced with nitrogen. 5-AC substitution of cytidine can interfere with DNA methyltransferase (mammalian *Dnmt1* and Arabidopsis *MET1*) and form irreversible methyltransferase-DNA adducts, resulting in degradation of the methyltransferase and therefore a reduction in genome DNA methylation (Kuo et al. 2007).

5-AC is a DNA methyltransferase inhibitor and transiently reduces DNA methylation

5-AC is used in many studies as a DNA demethylating reagent. Demethylation occurred progressively after treatment of 5-AC, and reached to a maximum after 48 hrs in human colon cancer cultures (Stresemann and Lyko 2008). In *in vitro* cultured cells of tobacco, variations in genome methylation were observed as well as significantly lower level of DNA methylation in repetitive regions. The 5-AC treated

cells also showed hormone-independent autonomous proliferation that was termed habituation, possibly as a result of the change in genome methylation (Durante et al. 1989).

Since degradation of DNA methyltransferases can be replaced by newly synthesized enzymes, the treatment effect of 5-AC is transient. Durante et al. (1989) has shown that the effect of 5-AC on subculturing disappears after 5 days in cultured cells. Also, DNA methylation is reported to recover 2 days after 5-AC treatment. However, CCGG sites (target of the HpaII DNA methylation sensitive enzyme) in human and mouse globin gene regions still remain hypomethylated four days after 5-AC treatment (Ley et al. 1984). Globin genes in mammals often reside in gene clusters associated with repeats, but whether repetitive elements are the cause of this delay in remethylation unknown.

DDM1 vs. MET1 function

Although both are related to maintenance of DNA methylation, *MET1* differs from *DDM1* in many aspects:

1. MET1 is a cytosine methyltransferase which is homolog of mammalian DNMT1 (Allis et al. 2005). DDM1, on the other hand, is a SWI/SNF family chromatin remodeling factor, and has ATP-dependent chromatin remodeling ability (Jeddeloh et al. 1999; Brzeski and Jerzmanowski 2003).

2. MET1 is considered to maintain CpG methylation, but also has been reported to be associated with RdDM *de novo* methylation (Allis et al. 2005). In contrast, DDM1 takes part in maintenance of CpG methylation as well as CHG and CHH methylation in heterochromatin (Chan et al. 2005).

3. Suppression of *MET1* by complimentary RNA expression in *Arabidopsis* resulted in a strong decrease (from 34 to 71%) in genome cytosine methylation (Ronemus et al. 1996), whereas the effect of demethylation on cytosine methylation seemed inconsistent with RNAi *DDM1* suppression efficiency. Fujimoto et al. (2008) observed by Southern blot that one transgenic *Brassica rapa* with 82% RNAi suppression of *DDM1* mRNA transcripts showed strong demethylation in 18s rDNA region; however, another transgenic events with 88% RNAi *DDM1* suppression showed only a small decrease in DNA methylation in this region. No genomic DNA methylation level has been analyzed with RNAi *DDM1* suppression in transgenic plants.

4. *met1* mutants displayed a range of immediate developmental defects (Ronemus et al. 1996), but *ddm1* homozygous mutants didn't show any observable phenotypic change until being inbred for eight generations (Saze and Kakutani 2007).

5. Loss of DNA methylation was observed both in heterochromatin and euchromatin in *met1* mutants, while only heterochromatin was demethylated in *ddm1* mutants.

6. When crossed back to wild type, *met1* mutants were partially remethylated in centromeric repeat arrays, in contrast to the extremely slow recovery in DNA methylation when *ddm1* was crossed with *DDM1* (Kankel et al. 2003).

What is the relation between the functions of MET1 and DDM1?

Arabidopsis ddm1met1 double mutants exhibited a more severe mutant phenotype than *met1* mutants in that, in addition to late flowering in *met1*, it also had a darker color and curled leaf phenotype (Kankel et al. 2003). Transcription profiling of *ddm1* mutants and 5-AC treated seedlings revealed that transposable elements were activated preferably in centromeric and pericentromeric regions in *ddm1* mutants, while in 5-AC treated seedlings transposable elements were activated without a preference in chromatin context (Hudson et al. 2011). It is clear that MET1 is essential for maintenance of global cytosine methylation patterns and normal development in *Arabidopsis*, while DDM1 seems to be in an additional pathway for maintaining DNA methylation in the heterochromatin region.

Callogenesis and *in vitro* regeneration

Compared to animals, many plants show greater totipotency (the capability to generate a complete new plant) and pluripotency (the capability to generate an organ such as a root or a shoot) (Smulders and Klerk 2010). Both require that somatic cells from mature plants undergo de-differentiation and re-differentiation and become an entire organism (Grafi 2004). This process is usually induced *in vitro*. The dedifferentiation

process is called callogenesis, and subsequent shoot, root, or embryo developments are called regeneration.

In vitro culture often induces genetic and/or epigenetic changes

Genetic and/or epigenetic changes often occur during *in vitro* development. Sometimes plants generated from *in vitro* processes show aberrant phenotypes that were not visible in the original plant. For instance, *in vitro* propagation of *Zantedeschia* gave rise to a bushy cultivar, with the bushy trait persisting for years. *In vitro* culture also has led to abnormal, tumor-like growths at or near the crowns of *Rhododendron, Azaleas,* and *Kalmia*, and oil palms with flowers that developed a second whorl of carpals instead of stamens (Smulders and Klerk 2010).

Habituation

Habituation is defined as the acquisition of the capacity of autonomous growth in a hormone-free medium by plant cells which originally require exogenous hormones (Smulders and Klerk 2010). In tissue culture, plant cell proliferation usually requires auxin and cytokinin. However, it was discovered in 1942 by Gautheret that strains of carrot tissues gradually lost the requirement for exogenous auxin, which was then called auxin habituation. Later cytokinin habituation also was discovered. Transcriptome analysis revealed 800 differentially expressed genes between habituated and non-habituated *Arabidopsis* cells. In habituated cells, hormone metabolites, transposon elements, chromatin modifying enzymes were up-regulated (Pischke et al. 2006). The mechanism of habituation is unknown. Triggering of

habituation after 5-AC treatment was also reported, but poorly understood (Durante et al. 1989).

Rejuvenation

In vitro regeneration of trees is related to rejuvenation, which is also termed reinvigoration. Trees have two phases during their lifetimes. In the juvenile phase, trees are unable to flower, and their cuttings are easiest to root. In the adult phase, trees are capable of flowering, and their cuttings become more recalcitrant to rooting. In normal development, juvenile trees undergo maturation and become adult trees. When cultured *in vitro*, mature explants from adult trees often undergo a reverse change (from adult to juvenile), and *in vitro* regenerated trees often show characteristics of juvenile plants, especially improvements in the ability to root. However, this reverse change of phase is often incomplete. For example, *in vitro* regenerated trees may flower sooner than normal seedlings (Smulders and Klerk 2010).

Epigenetics and plant development and adaptation

In vitro rejuvernation may be the results of epigenetic reprogramming. Many studies have been done to compare the DNA methylation in different developmental stages, and they suggested that total cytosine methylation levels increase with maturation, decrease with rejuvenation, and decrease during dedifferentiation in cell cultures (Fraga et al. 2002; Valledor et al. 2007; Noceda et al. 2009).

Differential DNA methylation in developmental stages

A transient DNA methylation decrease in fertilized *Castanea sativa* was found necessary for subsequent development, indicating a reprogramming of DNA methylation pattern during natural embryogenesis (Viejo et al. 2010). Fraga et al. (2002) reported that a reverse correlation was observed with the level of DNA methylation and the degree of reinvigoration of *Pinus radiate* and that the difference in DNA methylation between juvenile and adult trees was small in differentiated tissues, however, was large in meristems. Tanurdzic et al. (2008) compared differentiated tissues and cell cultures in *Arabidopsis* and found large changes in many epigenetic marks. From mature tissues to cell cultures, euchromatin became hypermethylated while heterochromatin became hypomethylated; some transposable elements were activated. They also found that the activated or silent transposable elements were enriched with different small RNA populations in cell cultures, the former being enriched with the 21 nt class, and the latter with the 24 nt class. This finding indicates that genome wide epigenetic changes occurred during *in vitro* dedifferentiation. It also suggests that small RNA groups are differentially regulating transposable elements during the process.

Responses to environment are related to DNA methylation

DNA methylation were reported to correlate with embryogenetic potential in pine (Noceda et al. 2009), heterosis in cotton (Zhao et al. 2008) and drought tolerance in poplar (Gourcilleau et al. 2010). Noceda et al. (2009) suggested that DNA methylation might be associated with embryogenetic potential. The methylation levels had a

reverse correlation with the ability to regenerate plants in pine cell cultures. Zhao et al. (2008) showed that highly heterotic cotton hybrids were less methylated than less heterotic hybrids by the methylation-sensitive-amplified-polymorphism (MSAP) method. Gourcilleau et al. (2010) found a positive correlation between DNA methylation level and productivity for six hybrid poplars under well watered conditions, as well as large variations in the changes in DNA methylation in meristems in response to water deficit conditions among those hybrids. The functional significance of all of the correlations, however, is unclear.

Rationale for this study

Black cottonwood (*Populus trichocarpa*) is a deciduous broadleaf tree native to western North America. Its genome is about 403Mbp and divided into 19 chromosomes. It was chosen to be the first tree species to have its genome sequenced because of its value and many experimental advantages as a model tree; it has small genome, extensive expressed sequence resources, is easily cloned and transformed, and is fast growing compared to other tree species (Tuskan et al. 2006).

Black cottonwood is also a species of economic and environmental importance (Niemie et al. 1995). It is one of the fastest growing temperate zone tree species, taking only 2 to 15 years to reach harvestable size, depending on the product (e.g., energy, pulp, solid wood). It is also ecologically important; it provides food for many kinds of wildlife, is a major component of riparian and wetland areas, and is often planted for in windbreaks. The wood is light in color and light in weight, and has an

even texture. It has good nailing properties and is ideal for making boxes and crates. It's widely used to make facial tissues and paper for high-quality books and magazines.

Epigenetic studies, such as mapping of DNA methylation in the genome or analysis of transgenic perturbations to the distribution of methylation, may have a number of possible applications in poplars: One possibility is improving stability of transgenic traits by selecting for insertions in euchromatic, non-repetitive parts of the genome to reduce the risks of gene silencing. Transgenes inserted near to transposable element rich regions may be silenced or activated in unpredictable ways, such as in response to environmental stress (Wu et al. 2003; Arnholdt-Schmitt 2004; Chinnusamy and Zhu 2009; Tittel-Elmer et al. 2010). The study of epigenetics may also improve *in vitro* regeneration and rejuvenation of poplar trees. Partial rejuvenation during propagation, a common result of *in vitro* regeneration in forest trees, often results in early flowering propagules (Smulders and Klerk 2010). If the presumably epigenetic phenomena could be controlled and understood, it might be possible to develop methods to regenerate poplar trees with desired flowering behavior, both in its advancement and postponement. What's more, epigenetic studies have the potential to aid reverse genetic studies in poplar trees. Currently, selfing is impossible for most species and genotypes, thus the phenotypes of homozygous mutants are invisible (Kumar and Fladung 2004). This difficult is further compounded by its long time to flowering. If we can identify and manipulate the presumably epigenetic mechanisms of gender determination and maturation in poplars, it may be possible to induce precocious, bisexual flowers—enabling selfing. Fortunately, the amenability of poplars to

transformation, combined with RNAi methods, provides another and more immediate route to reverse genetic studies in poplar, as this thesis shows.

DNA methylation has been extensively studied in the model plant *Arabidopsis,* but very little in other plant species. The poplar genome is about four times larger than the genome of *Arabidopsis*. It contains more repeats and redundant intergenic areas, which are enriched with transposable and repetitive elements. It also has a distinctive phlyogenetic history and life cycle. Genomic DNA methylation studies will help to broaden our understanding of the mechanisms of epigenetic phenomena, such as maintenance of centromeres and telomeres, cell and tissue type determination, de- and re-differentiation, physiological maturation and onset of flowering, and response to stress and environmental adaptation.

The focus of this study is on the function of DDM1 in *Populus*, the first such study that we are aware of in any tree or perennial plant. *Populus trichocarpa* has two predicted homologs of *AtDDM1* whereas *Arabidopsis* has a single gene; however, because of the similarity of the two poplar genes and the power of RNAi, we could simultaneously inhibit the expression of both of them with a single transgenic construct. The main effect of the *ddm1* mutant in *Arabidopsis* is to cause a decrease of DNA methylation that is restricted to heterochromatic and repetitive regions. Thus, we hypothesized that suppression of both transcripts of *DDM1* in *Populus* will have a similar effect, but because of the much larger extent of heterochromatic DNA in poplar might have different genomic or phenotypic consequences.

CHAPTER 2: RNAi *PtDDM1* transgenic poplars--production, molecular analysis, and phenotypic variation

ABSTRACT

DDM1 is necessary for the maintenance of DNA methylation and heterochromatin assembly in *Arabidopsis*. We used double-stranded DNA to induce RNA interference (RNAi) to suppress both transcripts of the orthologous *DDM1* genes in transgenic *Populus (PtDDM1)*. The RNAi-*PtDDM1* transgenic poplars showed a wide range of suppression efficiency. The strongest suppression events had 30% of the *PtDDM1* expression of the non-transgenic control. The cytosine DNA methylation percentages were correlated with *PtDDM1* expression; the event with the strongest reduction of DNA methylation had 10% that of the non-transgenic control. Two *in vitro* studies of the effect of reduced *PtDDM1* expression on callogenesis and shoot induction showed contrasing results and therefore were inconclusive. No developmental or growth rate abnormalities that were associated with *PtDDM1* expression were found during a six week greenhouse study. However, after a dormancy cycle and growth out of doors, a severe mottled leaf phenotype appeared in some of the events with reduced *PtDDM1* expression and DNA methylation. We speculate that reduced methylation is likely to have developmental consequences in poplar but require further studyto confirm and characterize them.

INTRODUCTION

Epigenetic modeling of chromatin plays a major role in genome regulation during plant development. In interphase nuclei, two distinct types of chromatin are present:

heterochromatin, where chromatin is condensed and repressed, and euchromatin, where chromatin is relaxed and actively transcribed. Heterochromatin is often enriched with repeats and transposable elements (Alberts et al. 2007). Epigenetic agents include DNA methylation, histone modifications and small RNAs. DNA methylation and histone modifications are the most commonly assessed epigenetic marks and are associated with formation and maintenance of either heterochromatin or euchromatin. Small RNAs are thought to direct epigenetic marks to specific chromatin regions (Chan et al. 2006). DNA methylation is important to genome regulation at many levels. Locus-specific DNA methylation, especially at promoter regions, is associated with reduced expression of the corresponding genes (Zhang et al. 2006). DNA methylation at extended chromatin regions is related to heterochromatin vs. euchromatin formation (Soppe et al. 2002). DNA methylation at the genome level has been suggested to relate to dedifferentiation and habituation during *in vitro* propagation of various plant species, and also to maturation and rejuvenation in forest trees (Fraga et al. 2002; Baurens et al. 2004).

Epigenetic marks are modified by a group of enzymes that are conserved in fungi, plants, and animals. These enzymes include the maintenance methyltransferase *MET1*, a homolog of mammalian *DNMT1*, which maintains symmetrical cytosine methylation, and *DDM1*, a *SNF/SWI* chromatin remodeling factor. There are also plant-specific enzymes. For instance, *CMT1* is a plant specific methyltransferase which maintains non-symmetrical cytosine methylation (Allis et al. 2005).

DDM1 was discovered in *Arabidopsis thaliana*. The mutant *ddm1* showed a decrease in DNA methylation, especially in heterochromatin (Vongs et al. 1993; Jeddeloh et al. 1999). *Arabidopsis* homozygous *ddm1* mutants show many mutant phenotypes; they are caused by a combination of epigenetic changes at functional genes and insertion of reactivated transposable elements into novel gene-associated locations. The exact mechanism of *DDM1* action is unknown; however, it appears to be involved in a pathway different from, and yet overlapping with, the RdDM silencing pathway (Blevins et al. 2009).

While epigenetic perturbations have been studied extensively in *Arabidopsis* and rice, forest trees remain unexplored. This is the first study on the function of *DDM1* in *Populus trichocarpa.* By RNAi suppression of the *DDM1* homologs in *Populus trichocarpa*, we studied the phenotypic consequences of DNA methylation in poplar. We report that RNAi-based suppression of *PtDDM1* did cause a decrease of genomic methylation, but its effects on gross plant phenotypes and *in vitro* development, though apparent during this study, were highly variable at the early stages of expression studied.

MATERIALS AND METHODS

Materials

A female hybrid clone developed by INRA, France named *717-1B4 (Populus tremula* × *P. alba*) that has been used extensively for transgenic studies (e.g., Durand et al.

2010; Han et al. 2011) was used for plant transformation and as a non-transgenic control.

Identification of two *AtDDM1* homologs in *Populus trichocarpa*

AtDDM1 homologs in *Populus trichocarpa* genomes were identified by NCBI blast and Psi-blast (Altschul et al. 1990). They were confirmed by the Phytozome gene search tool (Phytozome v7.0, 2011) and by a search for conserved domains (Marchler-Bauer et al. 2010). Blast alignments were done for genomic sequences, predicted transcripts/ESTs, and protein sequences of *PtDDM1-1*, *PtDDM1-2*, and *AtDDM1*. The *Populus trichocarpa* genome, like *Oliva sativa* and *Brassica rapa*, has two homologs to *Arabidopsis thaliana DDM1* (Tuskan et al. 2006) (**S Table 1**). Alignment of genomic sequences of *PtDDM1-1* and *PtDDM1-2* showed 82% maximum identity and 61% coverage. The genomic sequence of *PtDDM1-1* was about 800bp longer than that of *PtDDM1-2* (**Figure 1A**), but cDNA of their transcripts were both 2.2 kb long and showed 83% coverage and 88% maxim identity. They also showed high similarity at the protein level (94% coverage and 87% identity) (**Figure 1B**). *PtDDM1*genes were conserved relative to *AtDDM1*, especially at two functional domains (>90% coverage, 71% identity for overall alignment, 78% and 87% identities at two major conserved domains) **(Figure 1C)**.

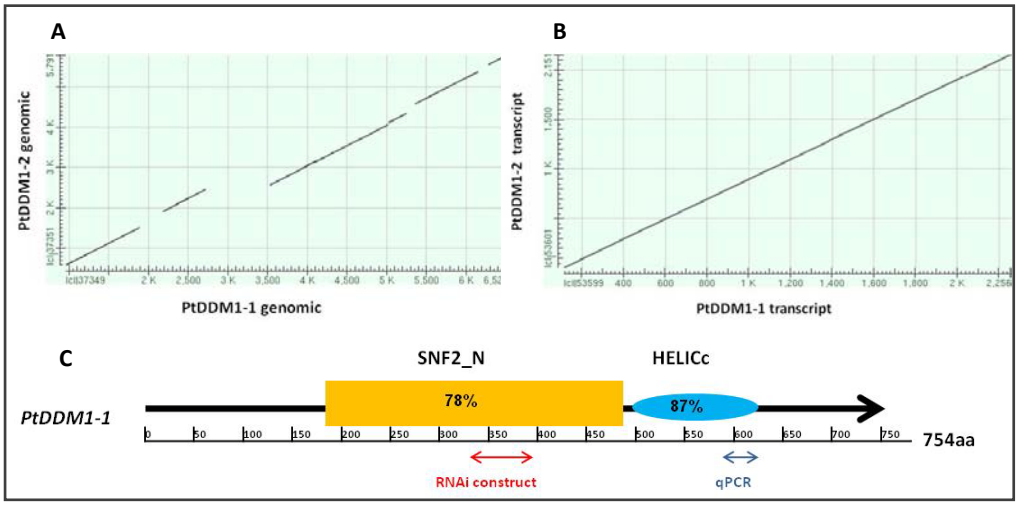

Figure 1 Allignment and structure of *PtDDM1 genes*. **A.** Dot matrix of genomic *PtDDM1-1* and *PtDDM1-2*. X- and Y-axes represent genomic sequences of *PtDDM1-1 and PtDDM1-2*, respectively, and the numbers on the axes represent positions of the residues. Matched residues are shown as dots, and long stretches of matched sequences are shown as lines. Only large fragments of mismatches/matches are visible; small matches/mismatches are not visible at the resolution shown. The *PtDDM1-1* has an intron of about 700 bp, thus is longer than *PtDDM1-2*. **B.** Dot matrix of transcripts of *PtDDM1-1* and transcripts of *PtDDM1-2*. The cDNA sequences of both transcripts were highly similar in length and sequence identity. **C.** The *Ptddm1-1* protein is similar to *Atddm1* (71% identity for overall alignment, identities of 78% and 87% at two major conserved domains). The positions of the region targeted by the RNAi construct and quantitative real time RT-PCR (qPCR) are labeled.

Construct assembly

The construct was designed to suppress both *DDM1* transcripts in *717-1B4*. Primers (DDM1-F01 and DDM1-R02) were designed to amplify the DNA fragments of both genes based on the BLAST results (**S Table 2**). We amplified a 500 bp product from *717-1B4* shoot tip cDNA using Platinum Taq HiFi Polymerase (Invitrogen). The 500 bp product was then cloned into the pCR-4 Topo vector (Invitrogen) and the sequence verified. We blasted the 500 bp 717-1B4 *DDM1* sequence against *P. trichocarpa DDM1* transcripts (Altschul et al. 1990), to design new primers (NDDM-F01 and NDDM-R01, **S Table 2**); they amplified 190 bp of DNA sequence for use in the RNAi

construct. Using Pfx Polymerase (Invitrogen), the 190 bp PCR product was amplified from *P.trichocarpa* shoot tip cDNA, cloned into the pENTRY vector (Invitrogen) and the sequence verified. The LR recombination reaction 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 (**S Figure 2**) were confirmed by restriction analysis and PCR. The resulting *PtDDM1* RNAi construct was transformed into *Agrobacterium tumefaciens* strain AGL1 by electroporation and verified by PCR. The RNAi construct is shown in **Figure 2**.

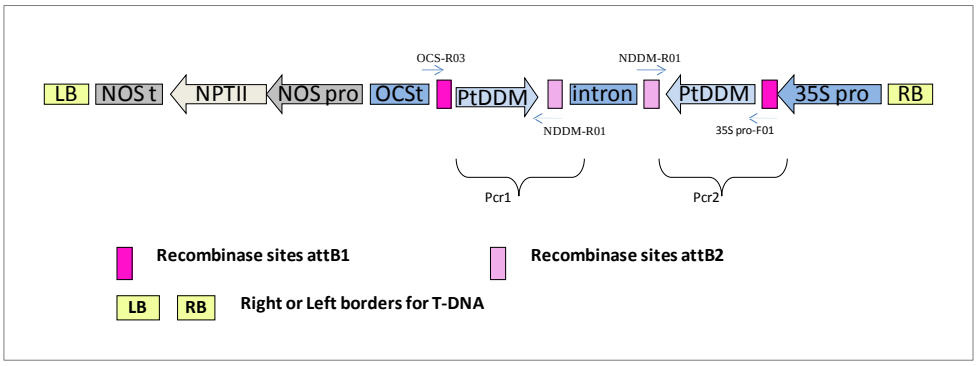

Figure 2 The T-DNA structure and primer sites for the *PtDDM1* RNAi construct within the pCAPD (pART27) binary vector backbone. The positions of primers used for PCR confirmation of the construct are labeled.

Transformation, *in vitro* regeneration, and confirmation of transgenic poplars by PCR

The *PtDDM1 RNAi* construct was transformed into *A. tumefaciens* strain *AGL1* and then transformed into *717-1B4* using the method described by Ma et al. (2004). All of the culture media contain kanamycin for transgenic tissue selection, and timentin for selection of *Agrobacterium* free tissue.

After two months in root induction medium, *in vitro* propagated candidates of transgenic poplars were harvested for DNA extraction and PCR confirmation. Primer pairs were designed to test the presence of the intact construct in the genome of transgenic poplars, and its absence on control non-transgenic poplars. The primer positions are shown in **Figure 2**, and sequences in **S Table3**. Only events that had both the sense and antisense copies of the 190bp *PtDDM1* sequence were selected. There were 22 confirmed transgenic events.

Endogenous *PtDDM1* expression by real time RT-PCR

In order to select strongly *PtDDM1*-suppressed transgenic events, we performed real time RT-PCR, also termed qRT-PCR, to estimate endogenous *PtDDM1* gene expression. Shoot tips from 2-month *in vitro* propagated plants were used to extract RNA. We completed three independent extractions for each of the 22 events and non-transgenic control. RNA was extracted using freshly made extraction buffer (4M guanidine, 0.2M sodium acetate [pH 5.0], 25mM EDTA, 2.5% polyvinylpyrrolidine, 1% [v/v] β-mercaptoethanol) and purified by Qiashredder columns and a Qiagen RNeasy Mini Kit. RNA quality was checked by electrophoresis for clear 28s and 18s ribosomal bands, and quantified by ND-1000 UV-Vis Spectrophotometer. From each sample, 10 μg RNA was treated by TURBO DNase I kit (Ambion), and quantified by ND-1000 UV-Vis Spectrophotometer. 260/280 nm readings after DNAse treatment were 2.17 ± 0.003. One μg of DNAse treated RNA was used to synthesize cDNA (SuperScript III First-Strand Synthesis System for RT-PCR, Invitrogen). The quality of the cDNA and the presence of genomic DNA were checked by a pair of primers

which spans an intron (PtDDM1_C and PtDDM1_3R, **S Table 4**). Using this pair of primers, PCR products from genomic DNA template and cDNA template were 200bp different in length. The reference gene was polyubiquitin in *P. trichocarpa* as suggested for poplar by Brunner et al. (2004). The primer pairs used to amplify the 107 bp-fragment of endogenous *PtDDM1* transcripts for qRT-PCR were PtDDM1_C and PtDDM1-NR01 (**S Table 4**). We used three 96-well plates and followed a randomized block design on each plate. Two technical replicates were performed for each reaction and placed in adjacent wells in plates. Dissociation curves were checked for single peaks for all products.

PtDDM1 expression was calculated using the following formulas:

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

$$R_0 = \frac{1}{e^{\Delta CT}}$$

$$Fold\_decrease = e^{\Delta\Delta CT}$$

$$\Delta\Delta CT = \Delta CT_{control} - \Delta CT_{event}$$

Where ΔCT is the difference in cycle threshold between the target gene and the reference gene; R_0 is the normalized starting amount of the target gene transcripts, or normalized *PtDDM1* expression; *e* is the amplification efficiency of each reaction; and fold decrease is the percentage of target gene expression compared to the control. The efficiency was calculated by real-time PCR Miner (Zhao and Fernald 2005).We used one-way ANOVA to test the null hypothesis of homogeneity of *PtDDM1* expression among transgenic events and control *717-1B4*.

Total cellular cytosine methylation by HPLC

We measured five selected events and the non-transgenic control for their total cellular cytosine methylation by HPLC. 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 to the control. For DNA extraction, we used young expanded leaves from an outdoors covered growth area (lathhouse), employing a CTAB extraction method (Porebski et al. 1997). The quality of DNA was checked by gel electrophoresis and quantified by ND-1000 UV-Vis Spectrophotometer. About 500 ng/μL of DNA in 1mL was treated with 1μL of RNase A (100mg/mL) and 1 μL Ribonuclease T1 (0.5mg/mL), chloroform:octanol (24:1) extracted and ethanol precipitated, and then washed twice by 70% ethanol, centrifuged, and air-dried. The sample was resuspended with 170 μL 1x Turbo DNase I buffer (Ambion) overnight, and incubated with Turbo DNaseI for 12 hours at 37℃. At the end of the incubation period, 20 μL of 100mM Tris-HCl (pH 10.2) and 10 μL snake venom phosphodiesterase I (10mg/mL, Sigma, Lot#001m2048v) were added to the mixture, and incubated 8 hours at 37℃.

For the separation of nucleosides, the HPLC elution program followed was: First, 0-35 minute, mobile phase change from 10% buffer B and 90% buffer A to 25% buffer B and 75% buffer A; second, 35-40 minute, from 25% buffer B and 75% buffer A back to 10% buffer B and 90% buffer A (Buffer A: 8mM TBA-OH [tributylammonium hydroxide], 0.01M KH_2PO_4; Buffer B: 2mM TBA-OH, 0.10 M KH_2PO_4). The column used was an AlltimaTM C18 5u (lot # 50413817), nucleosides were detected

at 280nm, and nucleoside standards were processed together with the hydrolysate. The amount of deoxycytosine and methyl-deoxycytosine were calculated from the standard curve. Separation of a typical hydrolysate is shown in **Figure 3**. DNA methylation level was calculated as: $\%^{m}dC=^{m}dC/(^{m}dC+dC)x100\%$. One-way ANOVA was conducted to compare the effect on total cellular methyl-cytosine level in event*15*, *23*, *13*, *102*, *21*, *and 717-1B4*. We also did a two-sample t-test to compare each transgenic event and *717-1B.*

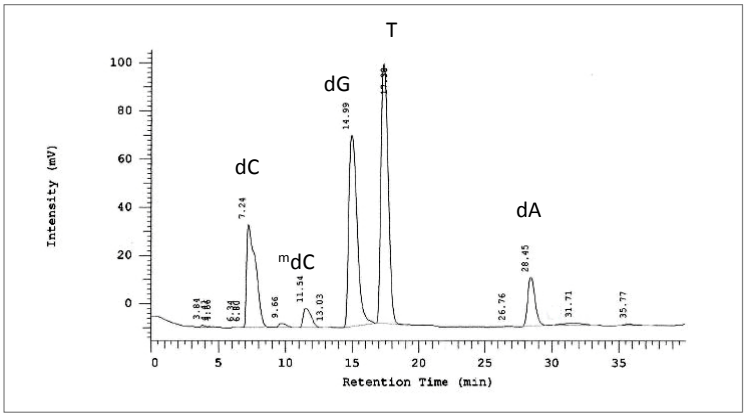

Figure 3 Chromatograms of hydrolyzed sample on HPLC. Total DNA from expanding leaf materials in the lath house were hydrolyzed into deoxycytidine (dC), methyl-deoxycytosine (^{m}dC), deoxygranosine (dG), thymidine (T), and deoxyadenosine (dA), which were separated using High Performance Liquid Chromatography (HPLC). Peaks corresponding to each nucleoside were labeled. Peak areas were used for calculation of dC and ^{m}dC quantity.

5-AC dose-response experiment

We carried out the 5-AC dose-response experiment to determine the effect of 5-AC on the rate of survival, callus induction, root induction, and shoot induction of *717-1B4* stem and leaf explants. The data from this study was used to determine the 5-AC concentration for the first *in vitro* study. Explants were put on Murashige and Skoog

(MS) medium supplemented with 2, 4-D and NAA. The concentrations of 5-AC were: 0 µM, 150 µM, 300 µM, 600 µM, 1200 µM. Stem and leaf explants were collected from 2-month-old tissue culture *717-1B4* plants. Each of the 5-AC treatments for leaf and the stem explants had four replicate plates, and each plate had 25 explants. The percentage of survival and the formation of callus, root, or shoot were recorded at four, seven, and twelve weeks later.

Phenotypic studies

First *in vitro study on all 23 genotypes*

We conducted this study to see the effect of RNAi *PtDDM1* suppression when combined with 5-AC treatment on *in vitro* regeneration. Plant materials were cultivated in magenta boxes for two months before use. From each event, eight sets of two poplar plants were collected. Each set was used to produce approximately 25 leaf explants on one plate and 25 stem explants on another. In total, eight sets of two poplar plants produced eight plates of leaf explants and eight plates of stem explants. For the eight plates of leaf explants, four were assigned a 5-AC treatment (300µM); the other four were left untreated as controls. The same was done for the plates of stem explants. Thus, for each event, there were four replicate plates for the following groups: leaf/5AC+, leaf/5AC-, Stem/5AC+, and Stem/5AC- (**Figure 4 A**). The plates were arranged in order on a large shelf and their positions were changed every two weeks. The environmental effects in the culture room were assumed to be negligible.

Data was collected six weeks after the explants were put into medium. Explants were scored “0” (dead/no callus), “1” (slowly growing callus), “2” (rapidly growing callus) according to the callus size that was induced. In the same way, explants were scored for shoot induction. The presence or absence of roots for each explant was also recorded. The scoring criteria are shown in **Figure 4 C** & **D**. Ideally there should be 184 plates for stem explants and 184 plates for leaf explants. However, due to contamination, some plates were discarded. The analysis was carried out with the data from the remaining plates (147 for stem explants and 157 for leaf explants). Plates were assumed to be independent of each other for statistical analysis.

Second in vitro study on four selected events

To examine the effect of reduced *PtDDM1*expression as well as 5-AC treatment on *in vitro* regeneration, we selected four events based on qPCR results. Because of the reduced experimental size, and the intention to minimize the plate-to-plate variation, we designed the second *in vitro* study in a different way. The plants included two low *PtDDM1* expression events (event 15 and 23), and a pair of high *PtDDM1* expression genotypes (event 21 and control *717-1B4*). The plant materials were cultivated in tissue culture in magenta boxes for two months before use, then for each event 16 *in vitro* propagated plants were used. They were divided into four sets of four plants, and every four plants were used to produce ten leaf explants and ten stem explants derived from the upper part of the plant; ten leaf explants and ten stem explants were also derived from the lower part of the plant. The ten explants were divided to two parts

and put on 5AC+ and 5AC- callus induction medium separately. Therefore, on each plate (either 5AC+ or 5AC-), each event took a quarter of the space (with leaf and stem explants each 1/8 of the plate) (**Figure 4 B)**. Photographs of each plate were taken every two weeks, and the area of explants was measured using ImageJ image processing software (Abramoff et al. 2004) as an index of the amount of callus produced. Shoot and root induction data was also collected. Data for leaf explants and stem explants were analyzed separately by ANOVA. The response variable percentage of rapidly growing callus (%RGC) was square-root transformed for closer approximation to a normal distribution.

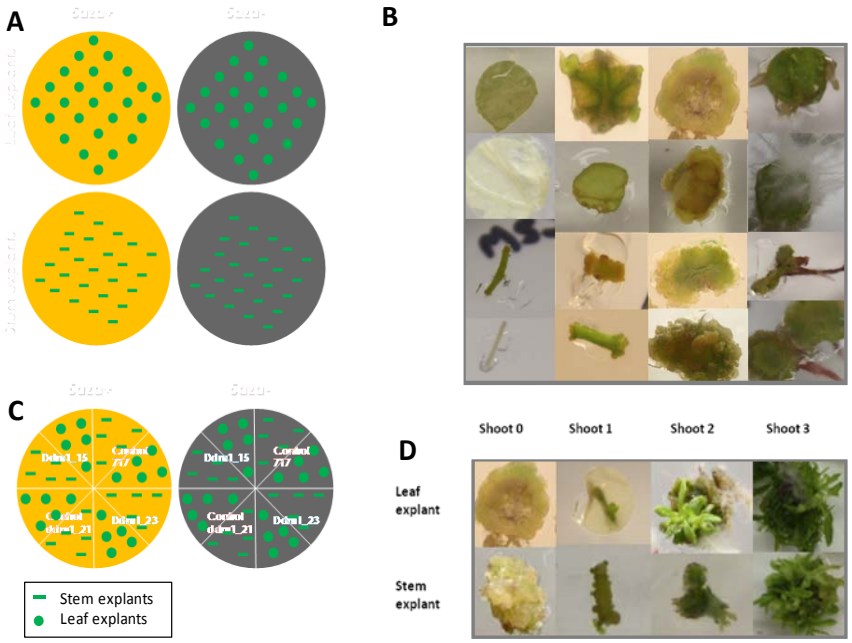

Figure 4 *In vitro* study experimental design and scoring criteria. **A.** The design of first *in vitro* study. Leaf and stem explants were collected and put on petri dishes (25 explants per plate). One replicate consisted of 5-AC treated leaf or stem plates and untreated leaf or stem plates. Each event had four replicates. **B.** Scoring criteria for callus formation. **C.** The design of second *in vitro* study. **D.** Scoring criteria for shoot induction.

Greenhouse study

We moved the two-month-old *in vitro* propagated transgenic poplars from the *in vitro* sterile environment gradually to become acclimated to an open environment starting in early January 2010. By the end of January 2010, we moved the plants to the greenhouse (**Figure 5 A**). 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. Height (cm) and diameter (cm^2) were collected every week for the first six weeks once the plants were moved into the greenhouse. With the height and diameter data, we calculated volume index ($diameter^2 \times height$, cm^3). We compared transgenic trees and controls carefully regarding leaf color, leaf shape, and leaf pubescence but were not able to find any difference. Thus no data was taken. Because we observed a high rate of sylleptic branching in the transgenic population compared to 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 recorded sylleptic branching frequency and length of the longest sylleptic branch of each tree. We also observed a large variation among the color of the leaves. Therefore, chlorophyll content of fully expanded leaves was measured by a chlorophyllmeter. At the end of the greenhouse study, we moved the plants to a cold chamber, in October 2010, where the plants went through a six month dormancy induction period at a

constant temperature of 4℃ under continuous darkness. In March 2011, the plants were moved to the lath house but not randomized there (**Figure 5 B**). Environmental variation was considered to be negligible for statistical analysis.

Figure 5 The greenhouse study of RNAi *PtDDM1* transgenic plants. **A.** May 2010, three months after moving to the greenhouse. **B.** June 2011, three months after moving to the lathhouseor

Statistical analysis

Data obtained from real time RT-PCR, *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). 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 (**S Figure 3**). Bonferroni corrections were made when more than one comparison of means were examined. In analysis of the correlation between *PtDDM1* expression and DNA methylation, one tailed p-values were reported because we hypothesized that DNA methylation was negatively correlated with *PtDDM1*

expression based on its known function in *Arabidopsis*. One-tailed tests were also used when testing the relationship of qPCR-determined expression to DNA methylation.

RESULTS

5-AC inhibition of *in vitro* regeneration

In 5-AC dose response experiment, seven weeks after explants were put on Petri dishes, we observed an inverse relation between percentage of surviving explants and 5-AC concentration. The percentage of survival was 100% in non-treated control plates, and was about 20% in plates with 1200μM of 5-AC (**Figure 6A**). Callus growth was delayed in the 5-AC treated explants, however, these explants showed callus growth after the four-week 5-AC treatment was completed, and then they were moved to cultures without 5-AC. At seventh week, 5-AC-treated explants showed a sharp decrease in callus formation compared to the untreated explants (**Figure 6 B**). Based on the seventh week data, leaf explants often formed hairy roots. However, regeneration of roots and rhizogenesis was strongly depressed in 5-AC-treated leaf explants (**Figure 6C**). Shoot regeneration, however, showed a modest increase and was highest at concentrations of 300 and 600 μM for stem and leaf explants, respectively (**Figure 6D**). There was also a rebound of callus and root formation at concentrations of 300 and 600 μM. In addition, we noticed that the leaf and stem explants response profiles were different. They showed similar and decreasing surviving rate as 5-AC concentration increased. While root and callus formation were severely inhibited in stem explants, the leaf explants showed increased root, callus,

and shoot formation from 150µM to 600µM. Stem explants also showed a similar rebound for shoot formation.

From the *in vitro* study involving 22 transgenic events and the non-transgenic control, both stem and leaf explants showed less growth under 5-AC treatment (P-values <0.05, **S Figure 4**). Stem and leaf explants showed different rates of callus formation, measured by percentage of rapidly growing callus (%RGC), among events. In stem explants, transgenic events showed both less and more %RGC than the control. In leaf explants, transgenic events generally showed greater %RGC than the control (**S Figure 5 C and D**). Shoot regeneration (percentage of explants which produced shoots) for stem explants, whether with or without 5-AC treatment, showed no obvious correlation with *PtDDM1* expression (**S Figure 5 A and B**).

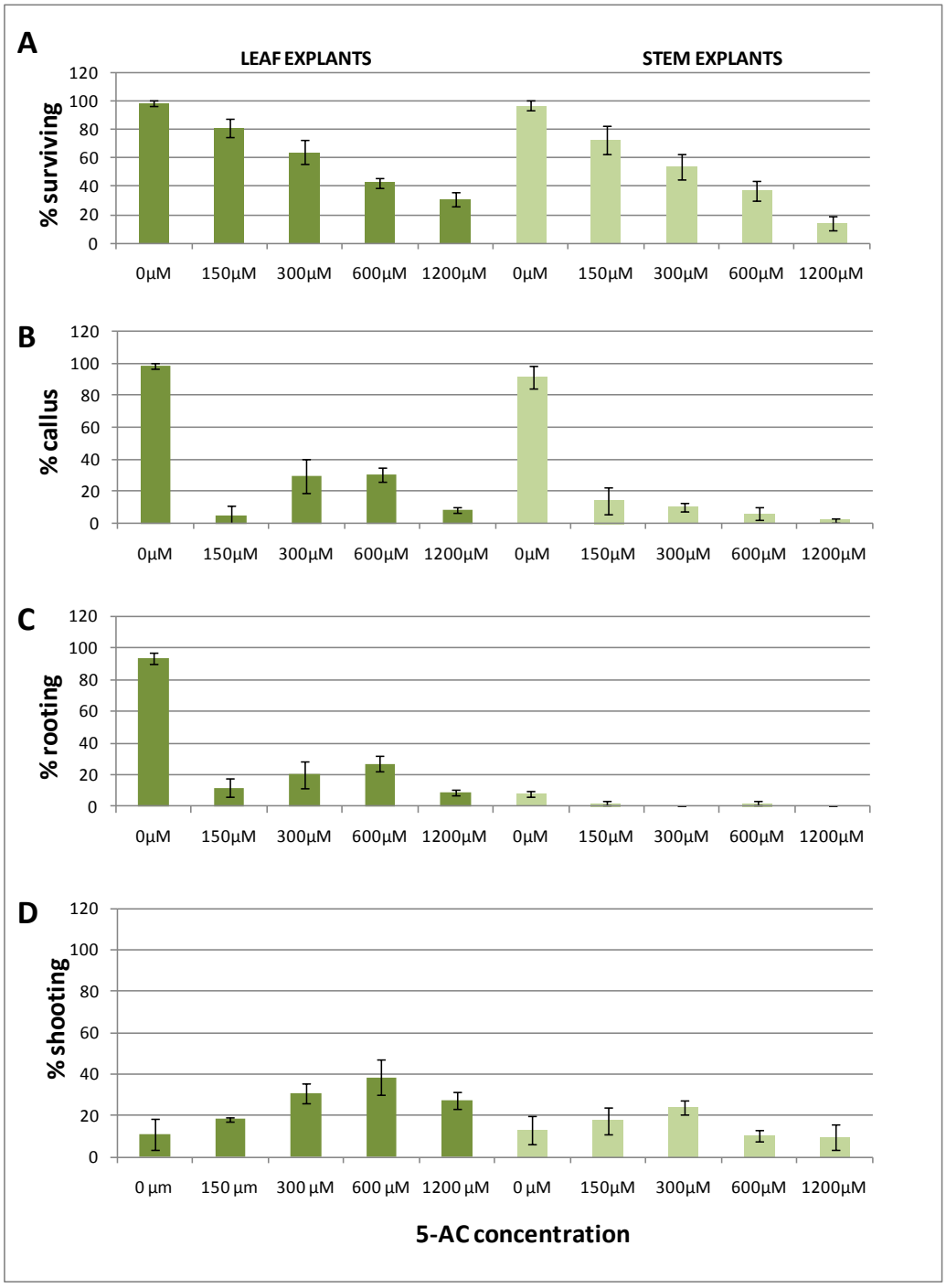

Figure 6 Responses of stem and leaf explants in survival (**A**), callus formation (**B**), root formation(**C**), and shoot formation (**D**) with 5-AC concentrations of 0,150, 300, 600,1200 µM.

Transformation efficiency

We confirmed by PCR that 22 events had an intact RNAi construct (**Table 1**). Compared to the number of starting explants, the transformation efficiency was 2.5% for leaf explants and 1.6% for stem explants.

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%

Real time RT-PCR of *PtDDM1* suppression

RNAi *PtDDM1* suppression efficiency varied widely among the transgenic events (**Figure 7**). Box-plots of ΔCT values of all events are shown in **S Figure 6**. The high *PtDDM1* expression events included the control and events *155, 114, 139, 21* and *134*. These events, except the control and event *21*, had a large amount of variance among replicates. The lowest *PtDDM1* expression events included event *3, 23,* and *15,* and these events had low variance among replicates. A repeated study for selected events showed similar relative expression levels (**S Figure 7**). One-way ANOVA analysis suggested a possible but inconclusive effect of event on *PtDDM1* expression for the 23 genotypes [$F(22,74)=1.60$, $p=0.07$]. Two sample t-tests suggest that *PtDDM1* expression of event *3, 15, 23, and 113* were significantly different from control *717-1B4* (P-values<0.05 after Bonferroni correction). Six genotypes were then selected as

low (*15*, *23*), intermediate (*13*, *102*), and high groups (*717* control, *21*) *PtDDM1* expression for further molecular analysis. These selections were based both on mean and the standard error values for each event.

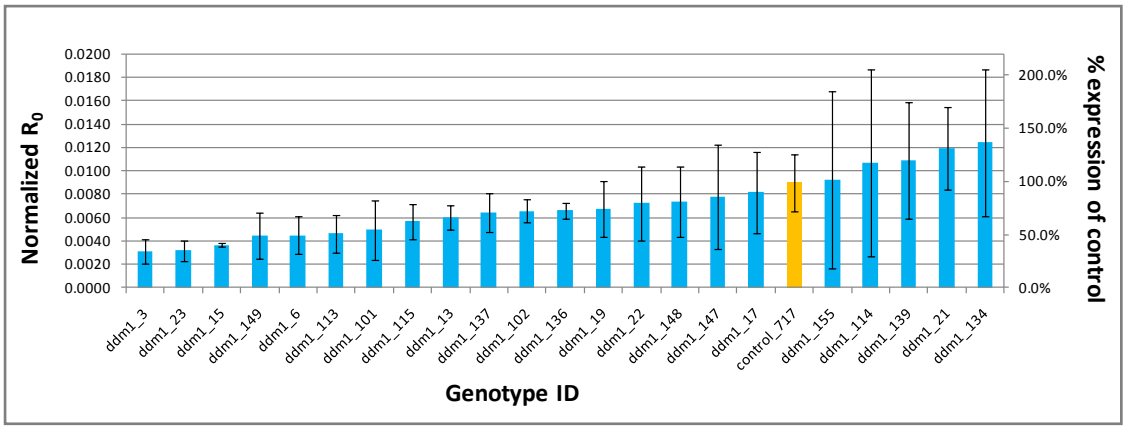

Figure 7 Endogenous *DDM1* expression in transgenic events (Blue) vs. control *717-1B4* (Yellow) calculated by real time PCR miner (Zhao and Fernald 2005). RNA was extracted from combined *in vitro* leaf and stem tissues. Standard error bars show variation among biological replicates.

Genomic cytosine methylation

The regression of nucleoside standards for ^{m}dC and dC showed an R^2 of 0.99 and 0.99, respectively. The low and intermediate *PtDDM1* expression events showed a moderate decrease (3% ~ 10%) in percentage of methyl-deoxycytidine (^{m}dC%) (**Figure 8**). Boxplots of genomic cytosine methylation level are shown in **S Figure 8**. One-way ANOVA analysis between events suggested a significant effect of events on genomic methyl-cytosine level for the six genotypes [$F(5,10)=7.78$, $p=0.003$]. Two sample t-tests suggested that *PtDDM1* expression of event *13* and *15* were significantly different from the non-transgenic control *717-1B4* (P-values<0.05). Event *15*, which had low *PtDDM1* expression, showed the greatest decrease (10%). Despite having

similar *PtDDM1* expression with event *15*, event *23* showed a very small decrease (3%) in ^{m}dC%. This was inconsistent with other events. Linear regression analysis of *PtDDM1* expression and ^{m}dC% showed an adjusted R^2 of 0.53 and a one sided P-value of 0.03. Excluding event *23* as an outlier from the otherwise strongly linear relationship, the linear regression had an adjusted R^2 of 0.99 and a one sided P-value of 0.00015.

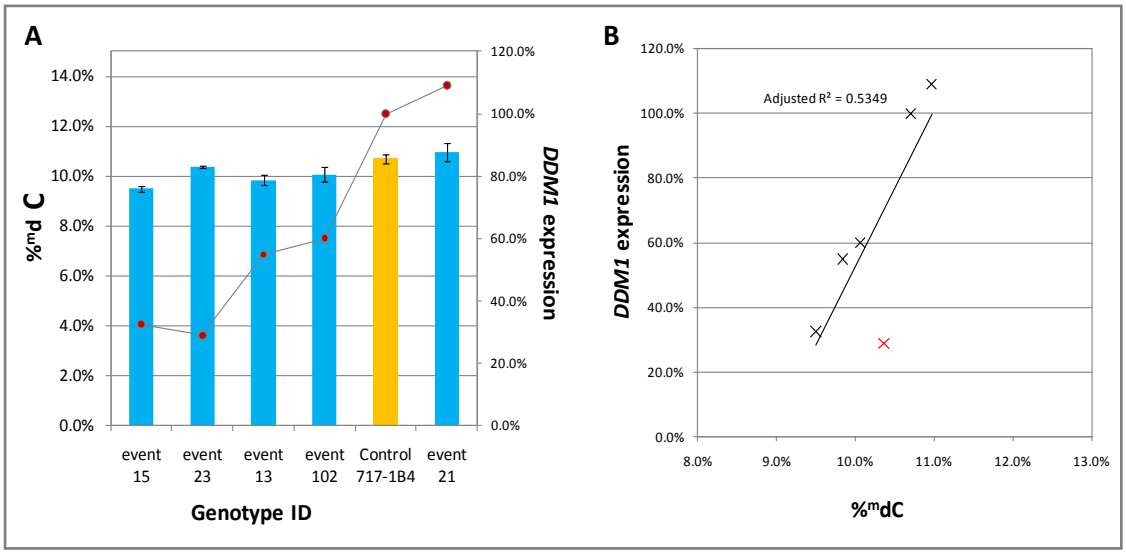

Figure 8 Total cellular cytosine methylation for selected events measured by HPLC. **A.** The association between methyl-deoxycytidine level (^{m}dC%, bars) and *PtDDM1* expression (dotted line) for selected six events. **B.** Linear regression of *PtDDM1* expression and ^{m}dC%.

PtDDM1 expression in relation to *in vitro* development

In vitro study on all 23 events showed extensive variation in callus formation and shoot formation within and among treatments. For stem explants without 5-AC treatment, the percentages of rapidly growing calli were larger for low *PtDDM1* expression events than those for high *PtDDM1* events (**Figure 9 and 10**). Linear

regression analysis of *PtDDM1* expression and %RGC showed an adjusted R-square of 0.19 and a P-value of 0.02 (**Figure 11**). The percentage of rapidly growing calli for leaf explants and the percentage of shoot regeneration for leaf/stem explants showed no correlation with *PtDDM1* expression (**S Figure 9 and 10**).

Callus formation rate differed strongly among explants within treatments and events (**Figure 12 A**), however, explants from the same plate were often strongly correlated in size and appearance (**Figure 12 B and C**). Replicate plates often showed very different callus, morphology, which contributed to the large variation seen in **Figure 10**. We therefore designed a new experiment which compared the performance of four selected events on the same plate in order to reduce the influence of plate-to-plate variation.

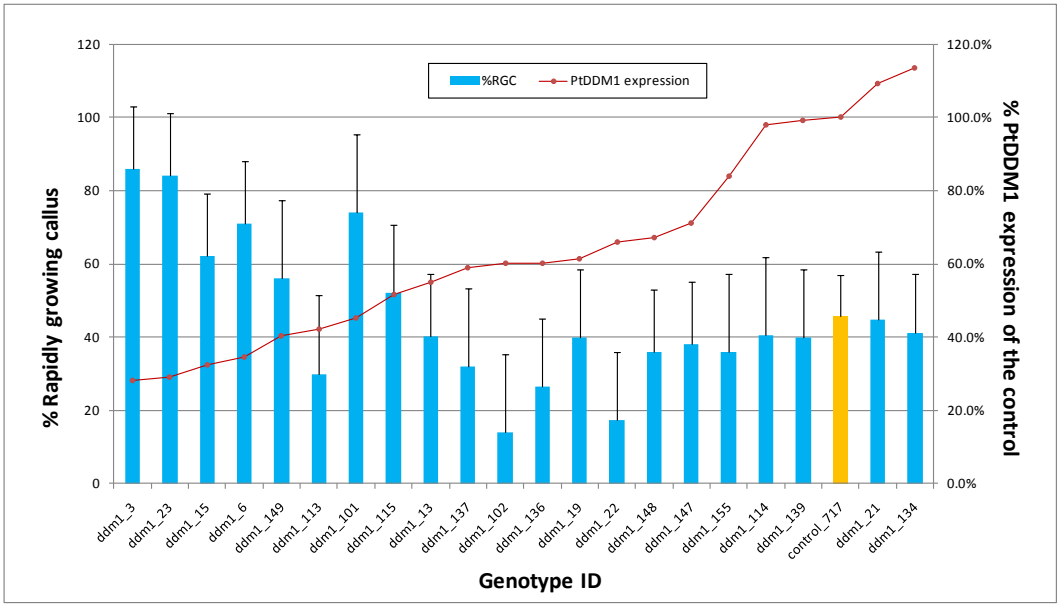

Figure 9 The percentage of rapidly growing callus (%RGC) of each event and corresponding *DDM1* expression. From the 25 explants of each plate, the number of rapidly growing calli was analyzed to calculate %RGC.

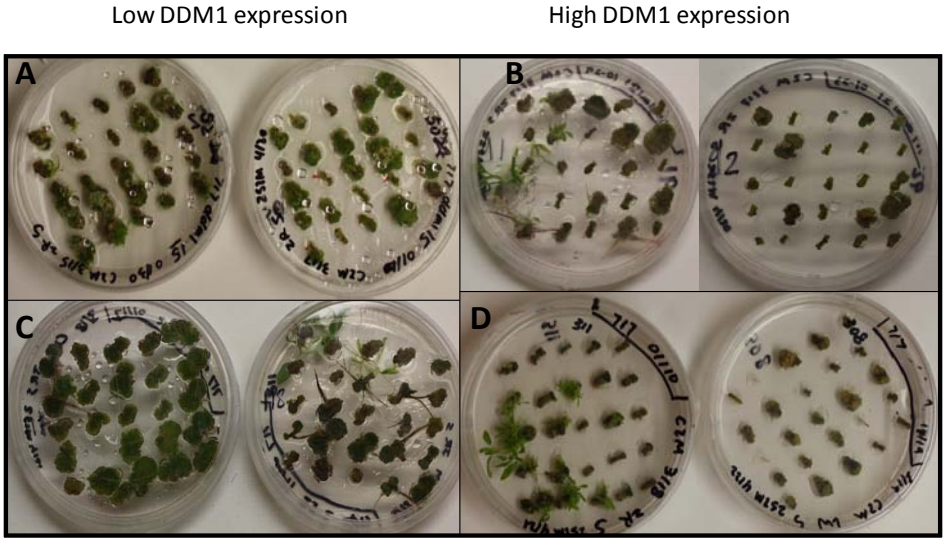

Figure 10 Examples of the difference of %RGC for low *DDM1* expression events and high *DDM1* expression events in stem explants without 5-AC treatment. **A.** Stem explants from event 15 on medium without 5-AC. **B.** Stem explants from event 21 on medium without 5-AC. **C.** Stem explants from event 23 on medium without 5-AC. **D.** Stem explants from control 717 on medium without 5-AC. Event 15 and 23 were low in *DDM1* expression; Event 21 and control 717 were high in *DDM1* expression.

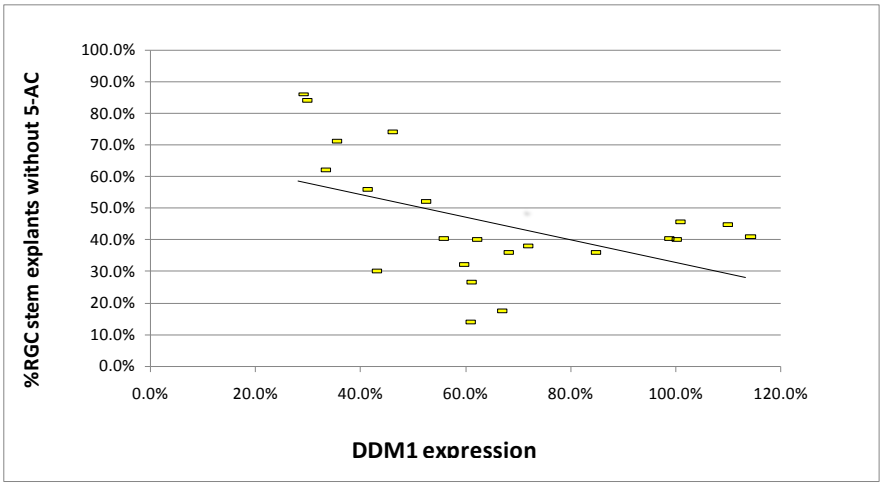

Figure 11 Regression analysis of %RGC and *DDM1* expression. Each dot corresponds to one event; the horizontal axis represents the *DDM1* expression of that event; the vertical axis represents the %RGC. Linear regression of %RGC and %*PtDDM1* expression showed an adjusted R^2 of 0.19, and a P-value of 0.02.

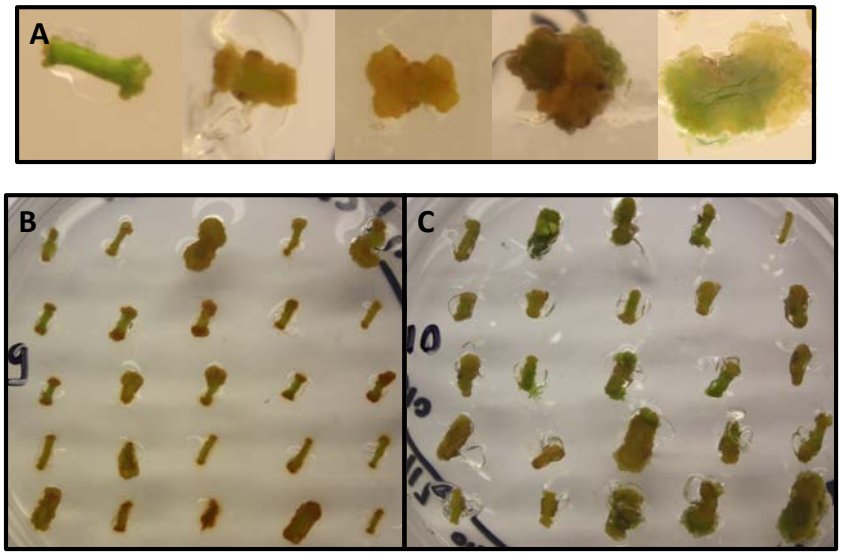

Figure 12 Variation in callus formation among plates. **A.** Various types of callus formed from stem explants. **B.** and **C.** Two plates of stem explants from control 717-1B4, though on identical culture medium, showed distinct modes of callus formation.

The second *in vitro* study placed the selected events on the same *in vitro* culture plates in order to reduce plate-to-plate variation. These were event *15* and event *23* (low *PtDDM1* expression) and event *21* and control *717-1B4* (high *PtDDM1* expression). We observed that explants from the same plate shared similar characteristics, and plate-to-plate variation was large, which was similar to the first *in vitro* study. After accounting for plate variation, only modest differences were found between the low *PtDDM1* expression events and the high *PtDDM1* expression events. For leaf explants without 5-AC treatment, no difference was found between low *PtDDM1* expression events and high *PtDDM1* expression events (**Figure 13 A**). However, for leaf explants with 5-AC treatment, low *PtDDM1* expression events showed a smaller area index than high *PtDDM1* expression events (P-value=0.003 from two sample t-test) (**Figure 13 B**). For stem explants without 5-AC treatment and with 5-AC treatment, modest

differences were observed from some plates, but no statistical significant difference was found (**Figure 13 C and D**). By visual observation, differences in callus formation in stem explants were not obvious, but differences in leaf explants seemed substantive, especially for event *15* (**Figure 14**).

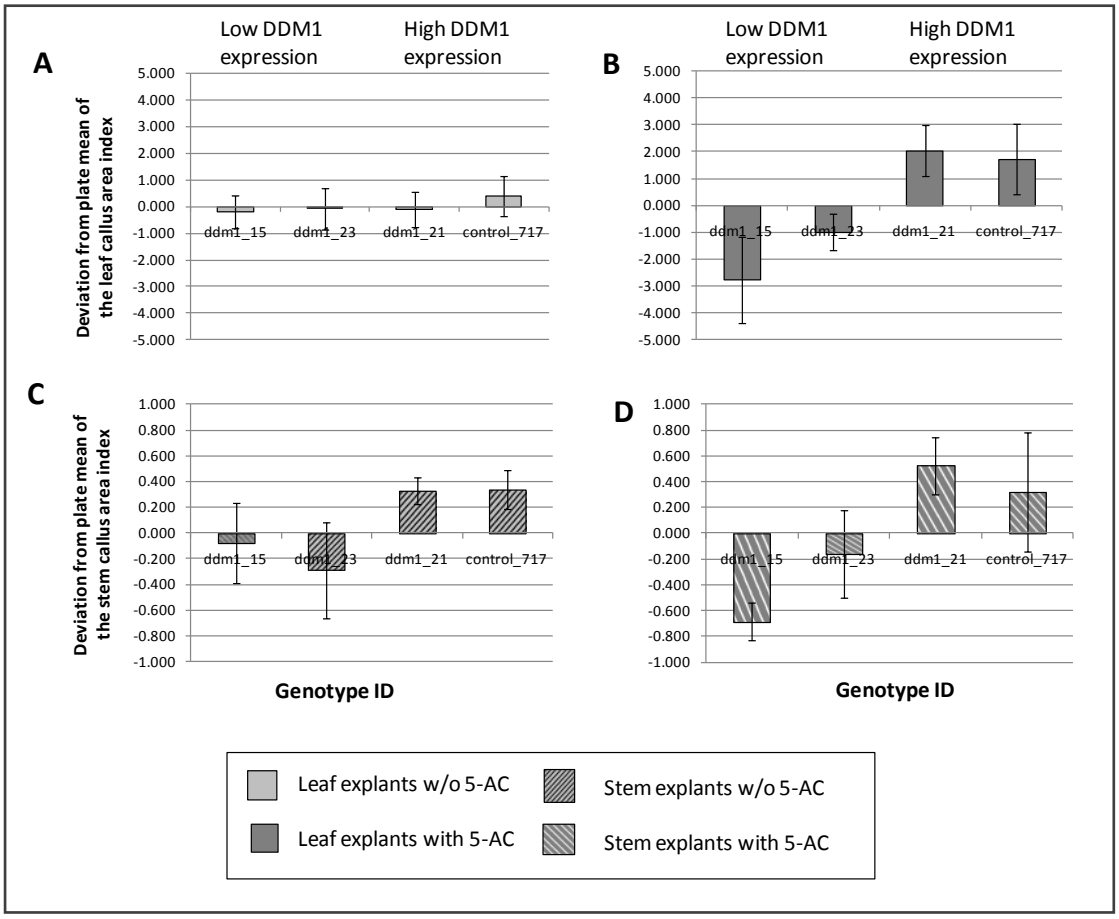

Figure 13 Callus formation for event *15*, *23*, *21*, and control *717-1B4*. Event *15* and 23 were low in *DDM1* expression; Event *21* and control *717-1B4* were high in *DDM1* expression. **A.** Deviation from plate mean of the leaf callus area index for leaf explants without 5-AC treatment. **B.** Deviation from plate mean of the leaf callus area index for leaf explants with 5-AC treatment. **C.** Deviation from plate mean of the stem callus area index for leaf explants without 5-AC treatment. **D.** Deviation from plate mean of the stem callus area index for leaf explants with 5-AC treatment.

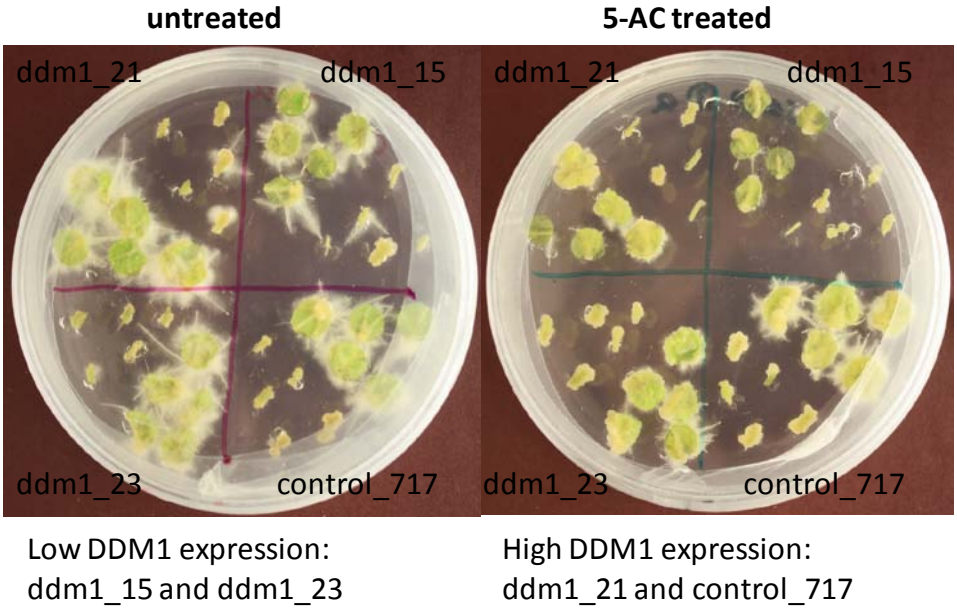

Figure 14 Example plates from the second *in vitro* study. The picture on the left is a plate without 5-AC treatment. The picture on the right is a plate with 5-AC treatment. Event names are shown on the corners of the pictures. Event 15 and 23 were low in *DDM1* expression; Event 21 and control 717 were high in *DDM1* expression.

***PtDDM1* expression and greenhouse growth**

ANOVA suggested a strong evidence in differences among events in diameter [$F(21,106)=2.57$, $P=0.0008$] (**S Figure 11 A**) and height [$F(21,106)=2.88$, $P=0.0002$] (**S Figure 11 B**) and in volume index [$F(21,106)=3.11$, $P<0.0001$] (**Figure 15**). 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 differences between transgenic poplars and the non-transgenic control 717-1B4 in height ($P=0.16$), diameter ($P=0.32$), number of sylleptic branches ($P=0.13$), and chlorophyll content ($P=0.62$) (**S Figure 12**). The correlation of endogenous *PtDDM1* expression and diameter was not statistically significant for diameter ($P=0.13$) or height (two tailed

P=0.58), e.g., **S Figure 11 A and B**). The same was true for sylleptic branching (P=0.92, **S Figure 11 C**). During the greenhouse study, transgenic poplars were also inspected carefully for abnormal development or stem/leaf morphology, but we found no obvious differences thus no detailed measurements were taken.

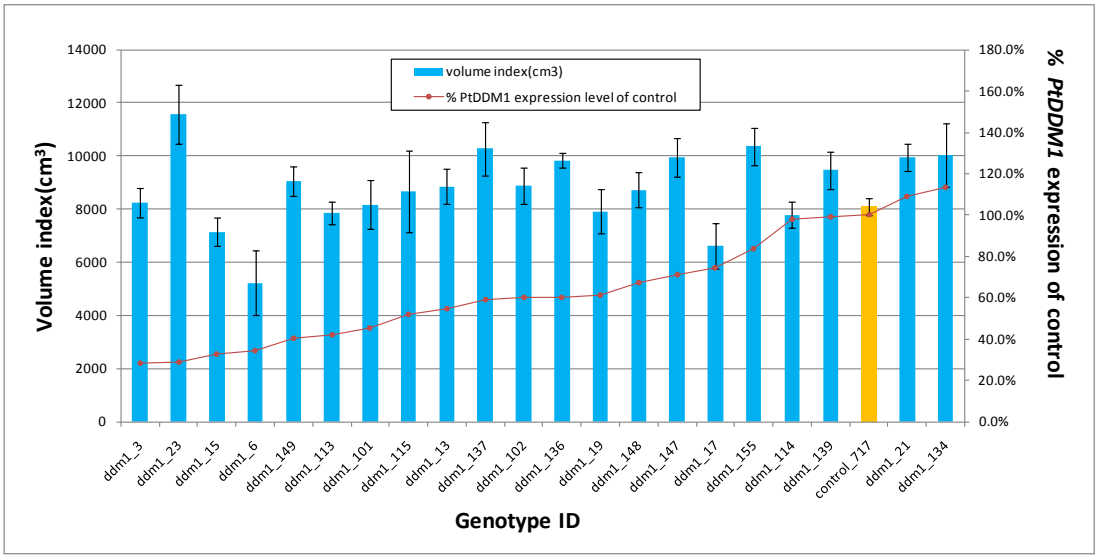

Figure 15 Volume index from greenhouse study showed no obvious correlation with *PtDDM1* expression. Height and diameter data was collected after three months' growth in greenhouse. Volume index = height x diameter^2 (cm^3).

Outdoor leaf phenotypes

After being in a cold chamber to induce dormancy for five months and subsequently in a lathhouse for three months where growth resumed, a severe mottled leaf phenotype was observed in all the ramets of event *15* (**Figure 16 A**). 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. For event *13*, *21*, and control showed no mutant phenotype (**Figure 16 and S Table 5**). A summary of the mottled leaf phenotype data is shown in **Table 2**. A Fisher-Freeman-Halton exact test of the *PtDDM1* expression of events

and the presence of mottling among ramets showed a P value of 0.067. After identifying the mottled leaf phenotype, we reduced the watering frequency to see if it could be further induced by stress. Control *717-1B4* and event *13* showed signs of wilt because of drought, but no symptoms of the mottled leaf phenotype. In addition, in the already mottled ramets, symptons did not expand due to drought.

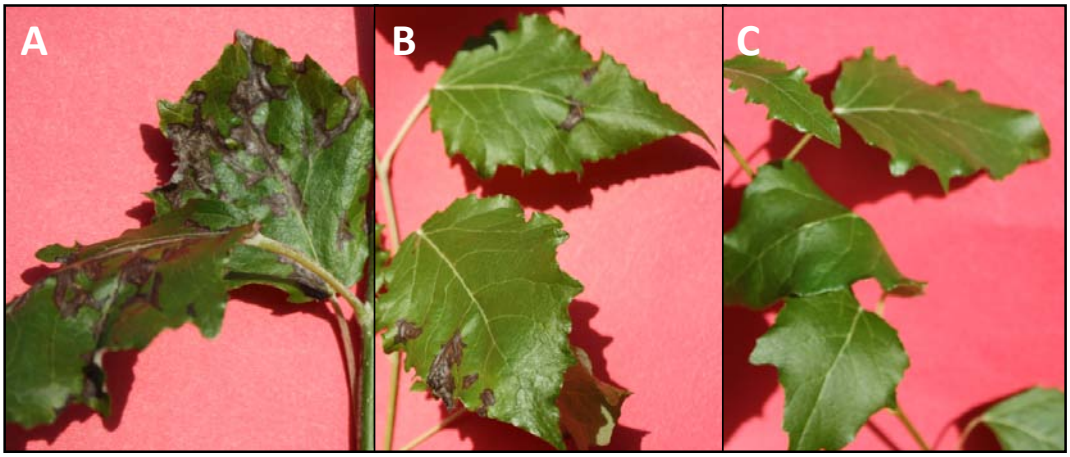

Figure 16 Examples of mottled leaf phenotype observed in the lath house. **A.** Mutant leaves of event 15. Event 15 showed a severe mottled leaf phenotype in all of its ramets. **B.** Mutant leaves of event 23. Event 23 and 102 showed a moderate mottled phenotype. **C.** Wild type leaves of control 717-1B4. No mottling was found in any of the ramets of event 13 nor in the control *717-1B4*.

Table 2 Mottled leaf phenotype distribution among different *PtDDM1* expression events. Event 15 and 23 have low *PtDDM1* expression; event 13 and 102 have intermediate *PtDDM1* expression; event 21 and the 717-1B4 control have high *PtDDM1* expression. Severity was scored according to the following criteria: "+", only 1-5 leaves are mottled, and mottled area is less than 1/3 of the whole leaf; "++", 5-10 leaves are mottled, and mottled area on average is more than 1/3 of the whole leaf, and less than 2/3 of the whole leaf. "+++", over 10 leaves are mottled, and mottled area on average is more than 2/3 of the whole leaf.

PtDDM1 expression	Event ID	Severity	# 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

DISCUSSION

Transformation efficiency

The transformation efficiencies of 1.5% and 2.1% are comparatively lower than the reported 6-8% by Ma et al. (2004) and that is common in our laboratory. We believe that this is because after we have obtained enough PCR confirmed transgenic events, we didn't continue to select additional transgenic, regenerated plants that were present. Additionally, the rate of transformation can vary widely among experiments due to uncontrolled factors. Alternatively, it is possible that the *DDM1* construct inhibited

transformation rate; however, because we did not use a control plasmid in our transformations we are unable to evaluate this hypothesis.

5-AC inhibition of callogenesis, rhizogenesis, and shoot regeneration

The dramatic drop of survival in 5-AC treated leaf and stem explants appeared to be related to the toxicity of 5-AC, which has been well documented in animals as well as in plants. Ueno et al. (2002) reported cell cycle arrest and apoptosis in the rat fetal brain after injecting 5-AC in pregnant mother rats. Brown et al. (1989) reported 5-AC inhibition of growth in plant tissue cultures of maize and tobacco. The 5-AC treated explants showed less callus, root, and shoot formation. A similar inhibitory effect was also reported in epidermal carrot cells and *Petunia* cell cultures (Yamamoto et al. 2005; Prakash and Kumar 1997). The inhibitory effect is likely the result of toxicity of 5-AC, which triggers cell cycle arrest and generally inhibitsgrowth. We observed a gradual increase in root and callus formation for leaf explants at higher 5-AC concentrations, and the rate of shoot formation for both leaf and stem explants also increased; however, we are not aware of similar observations in other studies. Perhaps small amounts of 5-AC stimulated de-differentiation and re-differentiation, but this effect was overcome by 5-AC toxicity as concentrations were increased. The distinct response profiles of stem vs. leaf explants might be a result of their very different populations of cell types.

RNAi suppression efficiency

RNAi *DDM1* suppression efficiency varied widely among transgenic events, similar to observations made in other studies. Fujimoto et al. (2008) used RNAi to downregulate DDM1 in *Brassica rapa* and they observed strong and variable suppression, with a maximum of 88% based on real-time RT-PCR. This is similar to the strongest suppression observed in our study of 70% for event *15*. This similarity occurred in spite of some significant differences in RNAi construct design. In our construct, 190 bp sequence homologous to both *PtDDM1-1* and *PtDDM1-2* were used for the inverted repeats; Fujimoto et al. (2008) used a 300bp sequence for their inverted repeats. It has been suggested that the suppression efficiency and frequency increase with the length of the inverted repeats (Bleys et al. 2006). Second, the 300bp sequence used in Fujimoto's study was 100% identical between the two *DDM1* homologs in *Brassica rapa* (*BrDDM1a* and *BrDDM1b*). However, *PtDDM1-1* and *PtDDM1-2* had no long stretches of DNA with 100% homology; the 190bp sequence had 96% homology between transcripts of two gene models.

Variation in total cellular methyl-cytosine

We observed only a moderate loss (3%-10%) of genomic DNA methylation in leaves of the most strongly *PtDDM1*-suppressed events in the RNAi transgenic poplars. The reduction in cytosine methylation was much less than the 70% loss observed in homozygous, loss of function *Arabidopsis ddm1* mutants (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 further decreases in ^{m}dC% after several cycles of dormancy or *in vitro* propagation.

Association of *PtDDM1* expression and cytosine methylation

DDM1 is required in the maintenance of DNA methylation in *Arabidopsis* (Jeddeloh et al. 1998). However, the quantitative relationship between *DDM1* and total cellular DNA methylation has not been reported previously. In our study, we produced transgenic poplars with a range of *PtDDM1* expression by RNAi and selected six representative events to measure total cell cytosine methylation by HPLC. Our result showed a positive correlation in the *PtDDM1* expression and DNA methylation in five out of six events. The outlier (event *23*) had low *PtDDM1* expression but high DNA methylation. The reason for this discrepancy was not clear. Fujimoto et al. (2008) showed a similar lack of correlation between *PtDDM1* expression and DNA methylation in their study of Brassica *Pt*DDM1 suppression.

One possible explanation for the outlier we observed is that the RNAi construct for event 23 lost its suppressive effect during propagation or acclimation to the greenhouse. The real time RT-PCR used materials from *in vitro* propagated plants, and our HPLC studies used leaf material from the lathhouse. The different material sources could have different expression and methylation patterns.

Another explanation for the outlier could be the effect of mutation in another gene in a pathway that counters the action of *DDM1*. Although *DDM1* is required for maintaining DNA methylation, functional analysis suggested that it is not a DNA

methyltransferase, but rather a chromatin remodeling factor. It has been suggested that *DDM1* could be maintaining cytosine methylation not by providing access to DNA methyltransferase, but by inhibiting DNA demethylase (Mahfouz 2010). If this is the case, mutation in a DNA demethylase or any protein upstream of the demethylation pathway could counter the effect of suppression of *DDM1*. Obviously, given the small number of transgenic events studied, this explanation is improbable.

PtDDM1 and *in vitro* growth

The two *in vitro* experiments used different approaches to study the relation of *PtDDM1* expression and *in vitro* regeneration ability, and gave contrasting results. The first *in vitro* experiment included all 23 events and each plate contained 25 explants that belonged to one type of explant from one event (**Figure 4 A**). The second *in vitro* experiment included only three selected events and the control, two of which had low *PtDDM1* expression and the other two had high *PtDDM1* expression (**Figure 4 C**). Each plate had both leaf and stem explants from all four selected events. But for each explant-type/event combination, there were only five explants on the plate. While the first experiment had more replications of explants most were in the same plates and thus shared environmental sources of variance, whereas in the second experiment the explants were fewer but by sharing the same plate environments with other treatments they shared less environmental, plate-to-plate variation.

The most striking common feature between the results of the first and the second *in vitro* experiment was that both produced calli of widely different characteristics (**Fig**

13 A) and that there was strong plate-to-plate covariation in callus morphology (**Fig 13 B**). The different callus types possibly reflect different epigenetic states and associated physiological expressions. It is possible that hormones or other growth regulating substances played an important role in determination of which type of callus would form. However, those hormones were not exogenous hormones added to the medium, but rather may have been induced endogenously in some explants and then diffused in the medium or were volatilized, triggering the same mechanism in other explants contained in the same plates. The original trigger may have been environmental or biological, or an interaction of the two. This hypothesis could explain the large variation between plates, since the switch of hormones can produce great difference in development. However, this does not explain why the first *in vitro* experiment showed a negative correlation of *PtDDM1* expression and stem-derived callus growth, while the second *in vitro* experiment showed the opposite. There, only leaf (not stem) explants without 5-AC treatment showed a positive association of *PtDDM1* expression and callus growth. It could be that although both were statistically significant, one of the differences was observed by chance.

Growth and development in greenhouse

Although we found that the growth in diameter, height, and volume index did vary significantly among events in the greenhouse based on ANOVA, we did not observe any unusual morphological phenotypes. This is in line with observations in *Arabidopsis*, where *ddm1* mutants produced no mutant phenotypes until several generations later (Saze et al. 2007). However, the *Arabidopsis* studies did not include

a randomized quantitative assessment, thus it is unclear if the *Arabidopsis ddm1* mutants might have had early growth rate effects like we observed in poplar. Alternatively, the variation in we observed could have been the result of transgene insertion or somaclonal effects, which are not uncommon as a result of plant transformation.

Cause of the mottled phenotype

Unfortunately, because only a subset of plants was grown in the lathhouse, we were unable to statistically confirm a relationship of mottling to *PtDDM1* gene suppression. However, in the discussion below we assume a relationship exists and examine its possible causes. The mottled phenotype was visibly similar to the well-known "lesion mimics" (Wu et al. 2008). Such a phenotype could have resulted from demethylation and hyperactivation of disease resistance genes, which can be a trigger for programmed cell death (PCD) (Lorrain et al. 2003). Vining et al. (2011) showed by MeDIP-sequencing data that a cluster of leucine-rich repeat (LRR) disease resistance genes were hypermethylated in vegetative tissues and hypomethylated in male flowers; if their normally high methylation in leaves was disturbed by *PtDDM1* gene suppression they might have triggered PCD.

Another possibility is reactivation of a retrovirus. A striking feature of the mottled leaf phenotype is the vein-associated lesions with sharp boundaries, which are very similar to what is seen with many virus infections. It has been reported that in the *ddm1 Arabidopsis* mutant plant retrotransposons that had retained the envelope-like protein

coding regions were activated (Vicient et al. 2001). Many studies suggested that these retrotransposons in plants can be considered as retroviruses, and can possibly retain the potential to infect (Wright and Voytas 1998; Vicient, Kalendar et al. 2001; Wright and Voytas 2002). If this hypothesis is true, the mottled leaf phenotype could represent internal retrovirus activation.

The mottled leaf phenotype appeared after a long dormancy period and in an environment where trees were potbound and had limited water and nutrients, indicating that stress might be the triggering factor. Stressful environments, such as cold temperature, high temperature, and drought, was correlated with reactivation of transposable elements and epigenetic changes (Chua et al. 2003; Wu et al. 2003; Arnholdt-Schmitt 2004; Chinnusamy and Zhu 2009; Tittel-Elmer et al. 2010). For example, transposable elements were reactivated during cold treatment and temperature shifts in *Arabidopsis* (Tittel-Elmer et al. 2010). It is possible that in *PtDDM1*-suppressed events, environmental stress induced demethylation and reactivation of hypermethylated heterochromatic regions, which was avoided in events with normal *PtDDM1* activity and thus normal methylation.

Future research

DNA methylation assessment

Because of the gradual loss of DNA methylation in *Arabidopsis ddm1* mutants, it would be beneficial to measure the total cell DNA methylation after one or more cycles of *in vitro* propagation and dormancy. Given additional cell divisions, we

would expect further reductions in DNA methylation and associated phenotypes to be expressed.

Test for endogenous growth factors as causes of correlated growth in Petri dishes

We proposed above that growth factors, produced and diffused within plate, contribute to the high environmental variance seen in our *in vitro* studies. This could be tested and ameliorated in a number of ways. First, if true the autocorrelation among explants should be reduced if fewer explants per plate are employed. A study could examine the change in autocorrelation as a function of explants density. This hypothesis can also be tested by moving explants that show different kinds of morphology to plates with contrasting morphology to see if it affects their developmental outcomes. Fast growing calli, for example, should stimulate growth of plate with slow growing calli with such transfers. Finally, an effective means for studying the phenomenon and for avoiding the statistical problems it causes would be to re-randomize explants during *in vitro* experiments. For example, to collect, randomize, and reallocate explants to plates biweekly. This would be laborious and risk microbial contamination, but might greatly increase experimental precision.

Further study of the mottled leaf phenotype

It would be of interest to take the transgenic poplars through another dormancy cycle to see if the mottled leaf phenotype repeats next year and if the phenotype expands to other events. It would also be of interest to study effect under stressed and non-stressed conditions, for example by varying pot size and water/fertilizer frequency. If

the mottled phenotype persists, event *15* and at least one other high *PtDDM1* suppression and mottled event might be used to study the genome-scale methylation-gene expression landscape by MeDIP- or bisulfite-sequencing and RNAseq or microarrays. The data could be examined for reactivation of transposable elements and evidence for hyperexpression of genes related to predisposition to disease lesion mimic phenotype expression and PCD.

In order to test the hypothesis of retrovirus infection as a cause of leaf mottling, we first need evidence that retroviruses with envelope sequences actually exist and are activated during *PtDDM1* suppression in poplar. This can be done via searches of the poplar genome for all possible retrotransposons that retained an envelope sequence and their expression in the transgenic plants with high *PtDDM1* suppression. In addition, we would need to microscopically examine leaves for the presence of viral particles in lesion areas, and determine if they can be moved to other plants and cause infection, such as by grafting or physical inoculations.

CONCLUSIONS

We studied the role of *PtDDM1* in *Populus* by generating transgenic poplar lines with RNAi constructs that were expected to suppress *PtDDM1* gene expression and assessing their characteristics. The transgenic plants were analyzed for *PtDDM1* gene expression, cellular DNA methylation, in vitro development, and plant morphology and growth in a greenhouse and outdoor environment. The major findings from this study are:

1. RNAi-caused *PtDDM1* suppression of transcription had a wide range of efficiency based on 22 studied transgenic events. The mean suppression, based on real-time RT-PCR, was 37% and the highest suppression of RNA expression was 70%.
2. The maximum change in total cellular DNA methylation in leaves grown *in vitro*, based on analysis of the event with the most strongly reduced gene expression, was 10%. DNA methylation was correlated with *PtDDM1* suppression, though an outlier event was also observed.
3. We produced statistical evidence that *PtDDM1* suppression promoted callus growth and shoot regeneration *in vitro* under specific conditions. However, this could not be repeated in a subsequent experiment with a distinct design. Further studies are required to evaluate this observation.
4. No distinctive morphologies were observed during a greenhouse study. However, after the plants went through a dormancy cycle and were put in an outdoor shade lathhouse under stress due to being potbound, a striking mottled leaf phenotype was observed on some of the most strongly *PtDDM1*-suppressed events. This observation also requires further evaluation.

We conclude that our work suggests that perturbation of DNA methylation by RNAi are effective, and may be a valuable tool for modifying *in vitro* development. It also shows that DNA methylation may play an important role in maintaining plant homeostasis with respect to disease resistance/programmed cell death

responses. However, the observations made require considerable further research for confirmation and to understand their mechanisms.

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APPENDICES

Appendix A Supplementary tables

S Table 1 Two orthologs of *AtDDM1* in *Populus trichocarpa*

Given name	Position	Transcript name
PtDDM1-1 or POPTR_0007s12710	scaffold_7: 12951719 - 12958538	POPTR_0007s12710.1
PtDDM1-2 or POPTR_0019s15030	scaffold_19: 15655586 - 15661763	POPTR_0019s15030.1

S Table 2 Primers used for construct assembly

PCR target	Primer name	Sequence of primers from 5' to 3'
500bp from *717 DDM1*	DDM1-F01	TGGTTATTGCCCCTCTTTCCACTC
	DDM1-R02	CTGAGCCCTTCGCCTTTCTTCTAC
190bp from *Pt DDM1*	NDDM-F01	CACCGTGGACAATAAGCTC
	NDDM-R01	CCTGAGCCCTTCGCCTTTC

S Table 3 Primers used for PCR confirmation

PCR target	Primer name	Sequence of primers from 5' to 3'
Pcr1, sense *PtDDM1* sequence	NDDM-R01	CCTGAGCCCTTCGCCTTTC
	OCS-R03	CGTCTCGCATATCTCATTAAAGC
Pcr2, antisense *PtDDM1* sequence	NDDM-R01	CCTGAGCCCTTCGCCTTTC
	35S pro-F01	TCCAACCACGTCTTCAAAGC

S Table 4 Primers used for realtime-RT-PCR

PCR target	**Gene bank accession No.**	**Primer name**	**Sequence of primers from 5' to 3'**
PtDDM1-1 transcript position 1748-1855nt	NC_008473	PtDDM1_C	AAGAGCTTGGTGGACTGGGTA
		PtDDM1-NR01	ACAGGCTTGGTTTGCCCAATTC
PtDDM1-1 transcript position 1748-2268	NC_008473	PtDDM1_C	AAGAGCTTGGTGGACTGGGTA
		PtDDM1_3R	ACCGCATCCTACAAAGCAAA
Polyubiquitin	BU879229	Ubq L	GTTGATTTTTGCTGGGAAGC
		Ubq R	GATCTTGGCCTTCACGTTGT

S Table 5 Mottled mutant phenotype observed in DDM1 strong suppression events in lath house

Genotype	Clone	Mottled	Severity	Pictures
Event 15	2	Yes	+++	15.2
	3	Yes	+++	15.3
	6	Yes	+++	15.6
	7	Yes	+++	15.7
	8	Yes	+++	15.8
Event 23	1	Yes	+	23.1
	4	Yes	+	23.4
	5	No	N/A	23.5
	6	Yes	+	23.6
	8	Yes	+	23.8
Event 13	1	No	N/A	13.1
	3	No	N/A	13.3
	4	No	N/A	13.4
	5	No	N/A	13.5
	6	No	N/A	13.6
	7	No	N/A	13.7
Event 102	1	No	N/A	102.1
	2	Yes	+	102.2
	3	No	N/A	102.3
	6	No	N/A	102.6
	7	No	N/A	102.7
Event 21	3	No	N/A	21.3
	4	No	N/A	21.4
	5	No	N/A	21.5
	7	No	N/A	21.7
	8	No	N/A	21.8
Control 717	1	No	N/A	717.1
	2	No	N/A	717.2
	3	No	N/A	717.3
	4	No	N/A	717.4

	6	No	N/A	717.6
	14	No	N/A	717.14
	15	No	N/A	717.15
	17	No	N/A	717.17
	20	No	N/A	717.20
	22	No	N/A	717.22
	24	No	N/A	717.24
	25	No	N/A	717.25
	27	No	N/A	717.27

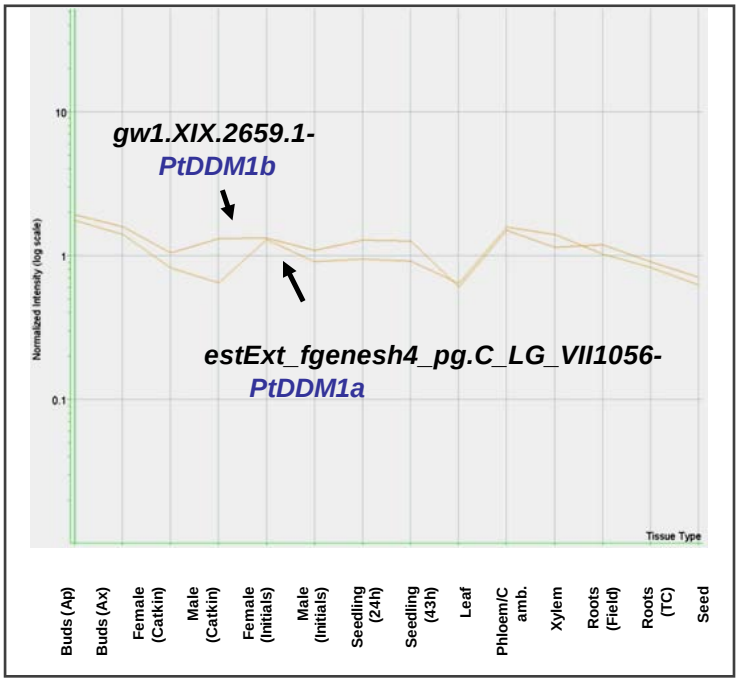
Severity

\+ only 1-5 leaves are mottled, and mottled area is less than 1/3 of the whole leaf.

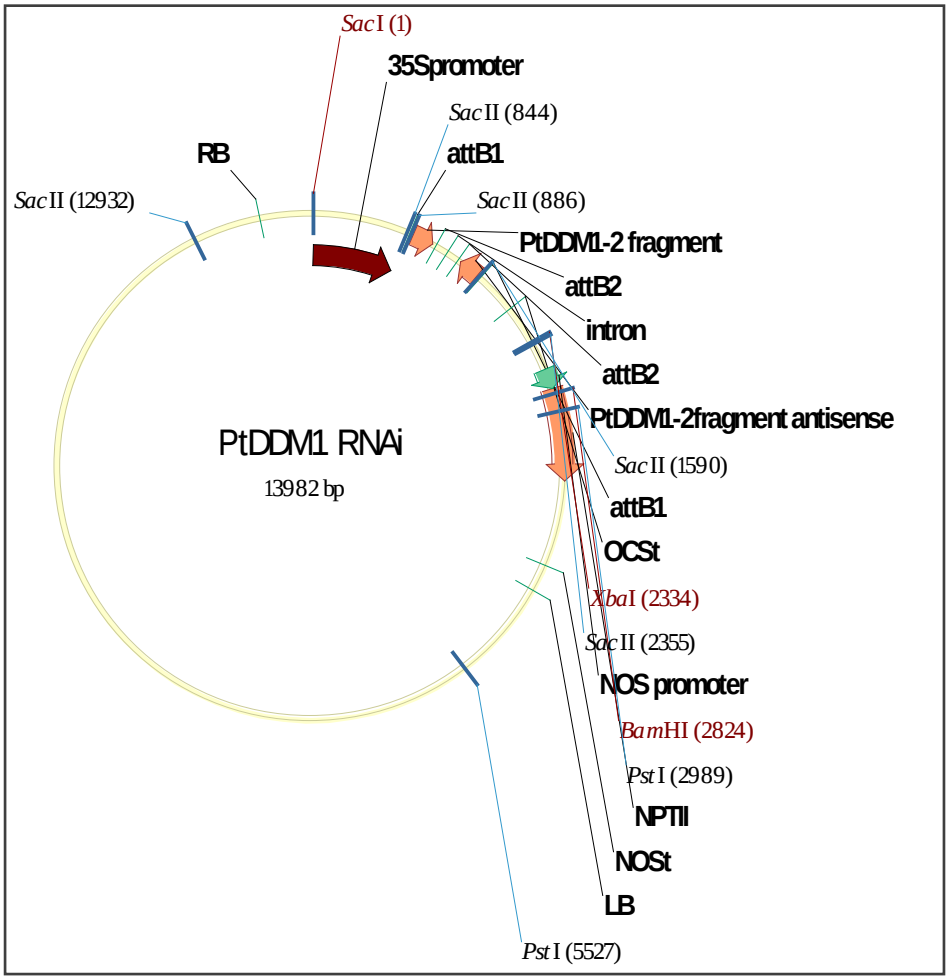
++ 5-10 leaves are mottled, and mottled area on average is more than 1/3 of the whole leaf, and less than 2/3 of the whole leaf

+++ over 10 leaves are mottled, and mottled area on average is more than 2/3 of the whole leaf

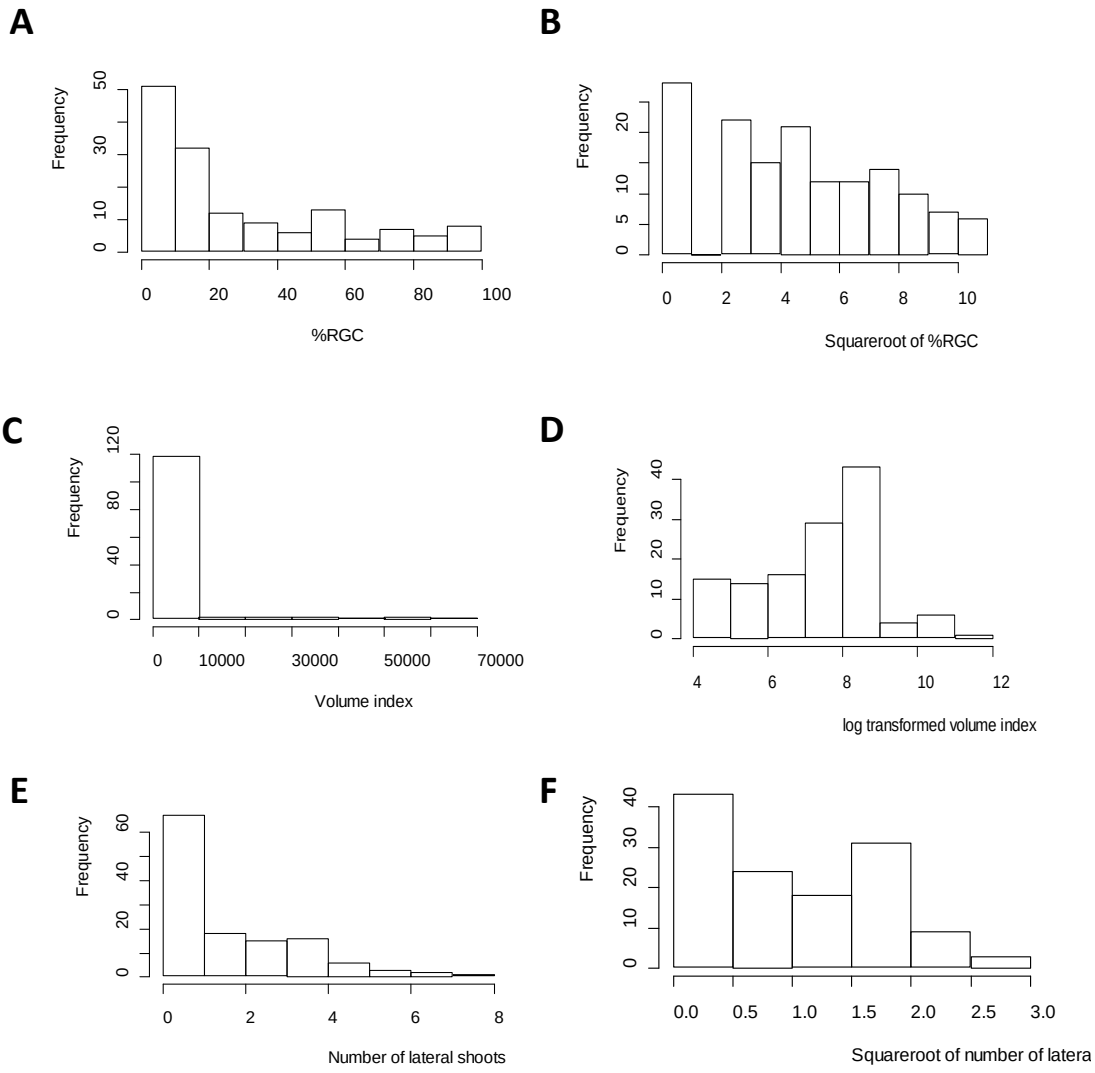
Appendix B Supplementary figures



S Figure 1 The expression pattern of *PtDDM1* genes at different kind of tissues based on microarray data.



S Figure 2 RNAi *PtDDM1* plasmid.

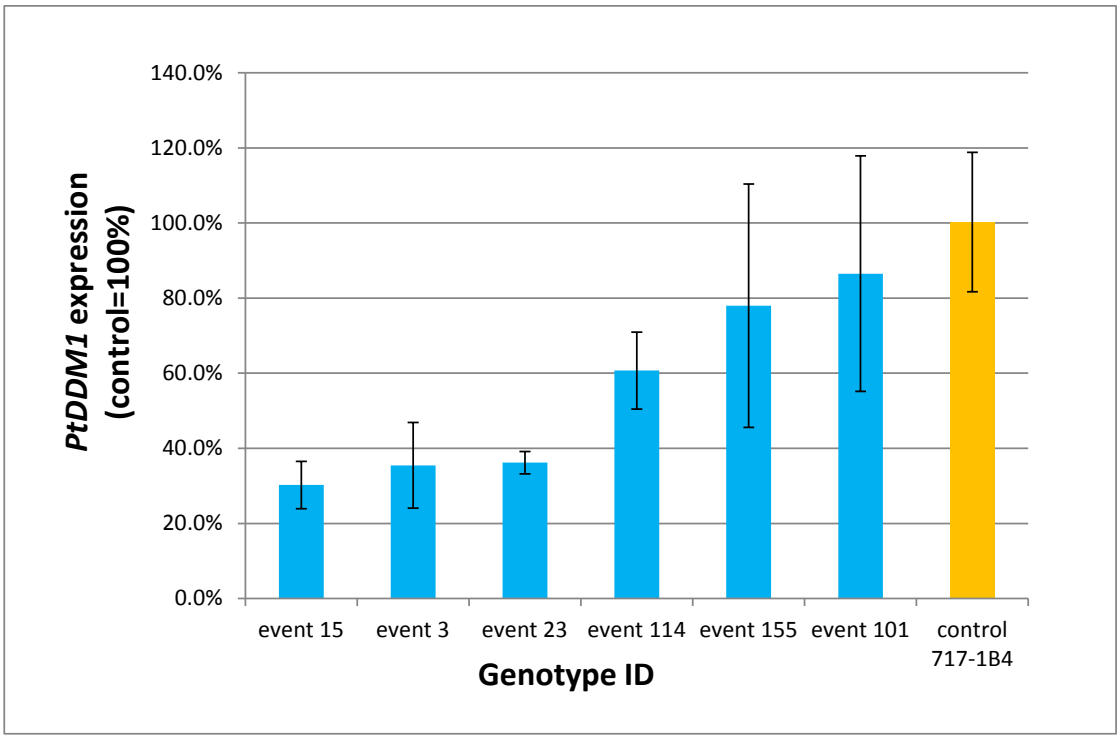


S Figure 3 Histograms of: Percent of rapidly growing callus before (**A**) and after squareroot transformation (**B**); volume index before (**C**) and after log transformation (**D**); number of lateral shoots before (**E**) and after squareroot transformation (**F**).

S Figure 4 Effect of 5-AC treatment on %RGC for stem (A) or leaf (B) explants from the first in vitro study.

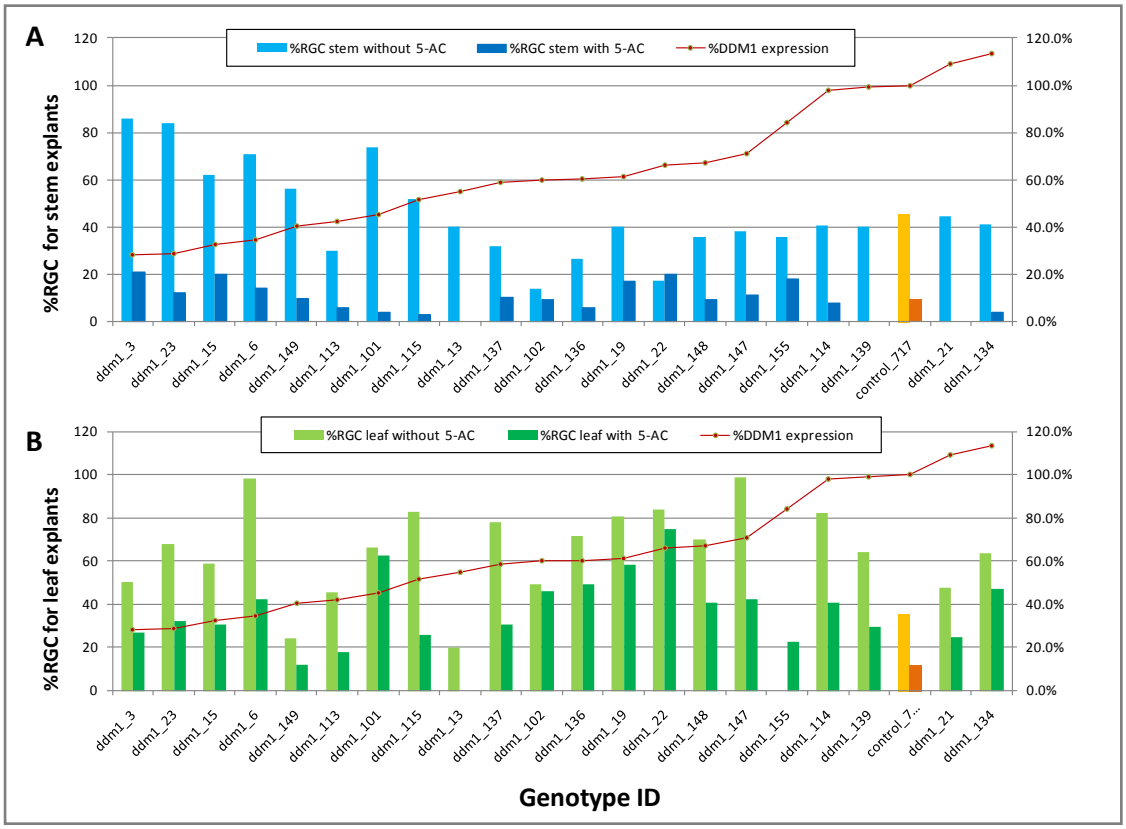
S Figure 5 %RGC in ascending order for stem explants without 5-AC treatment (**A**), with 5-AC treatment (**B**); for leaf explants without 5-AC treatment (**C**), with 5-AC treatment (**D**).

S Figure 6 ΔCT values from real time RT-PCR for 22 transgenic events and 717-1B4 control.

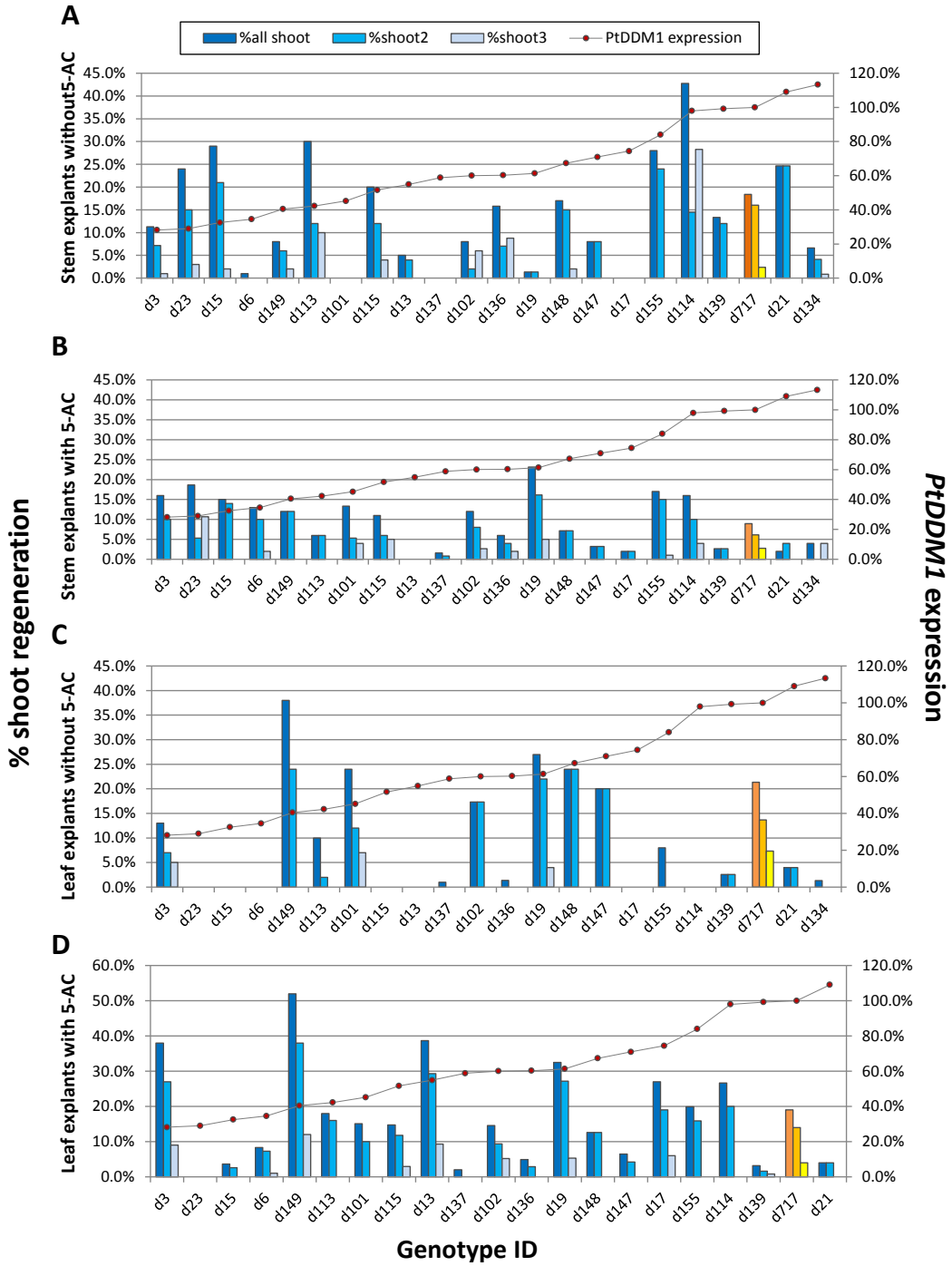


S Figure 7 qRT-PCR based on independent cDNA synthesis and qRT-PCR. Three biological replicates and two technical replicates were used for each event. This experiment repeated the first qRT-PCR experiment.

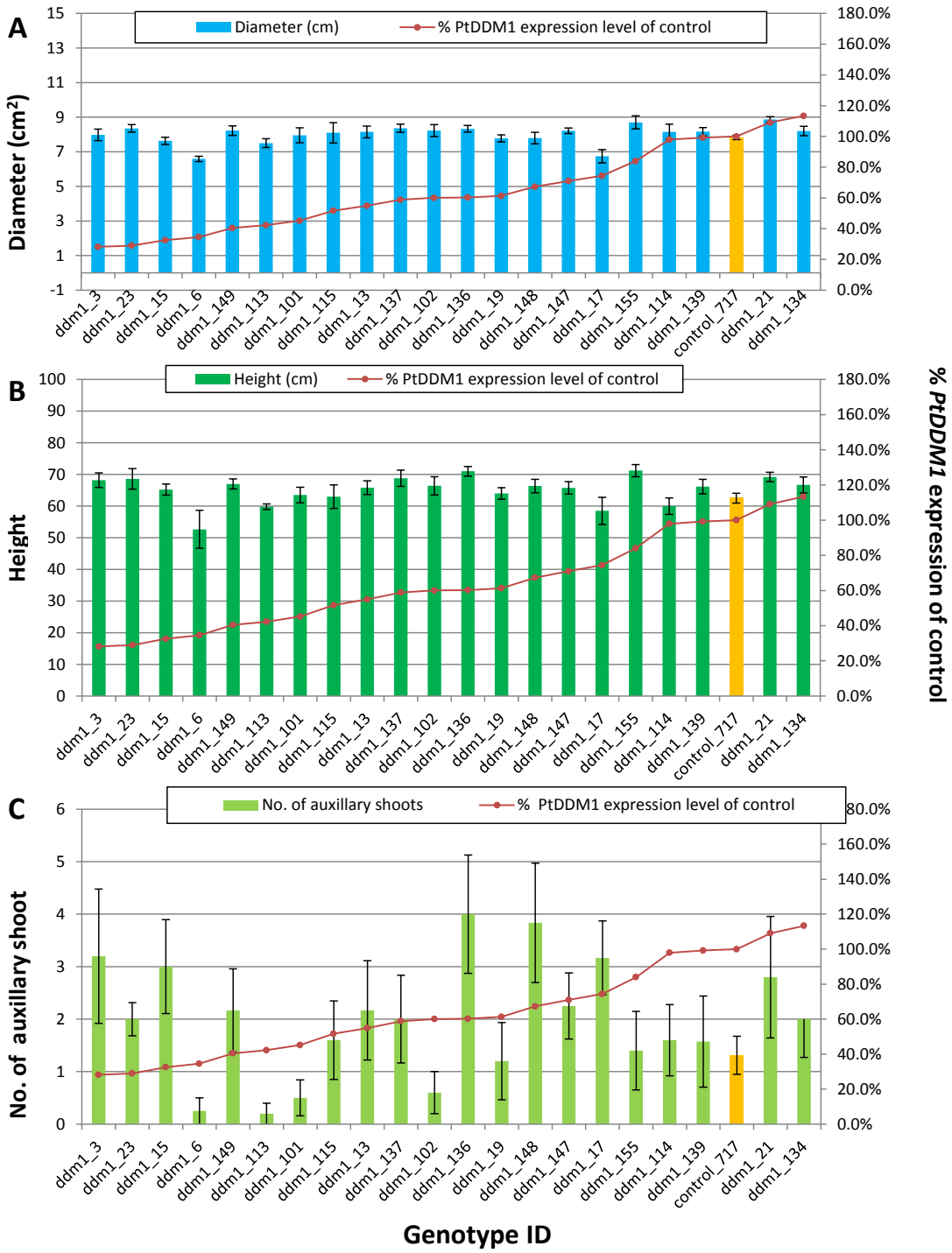
S Figure 8 Total cellular cytosine methylation of selected transgenic events (green) and control *717-1B4* (yellow) in ascending order.



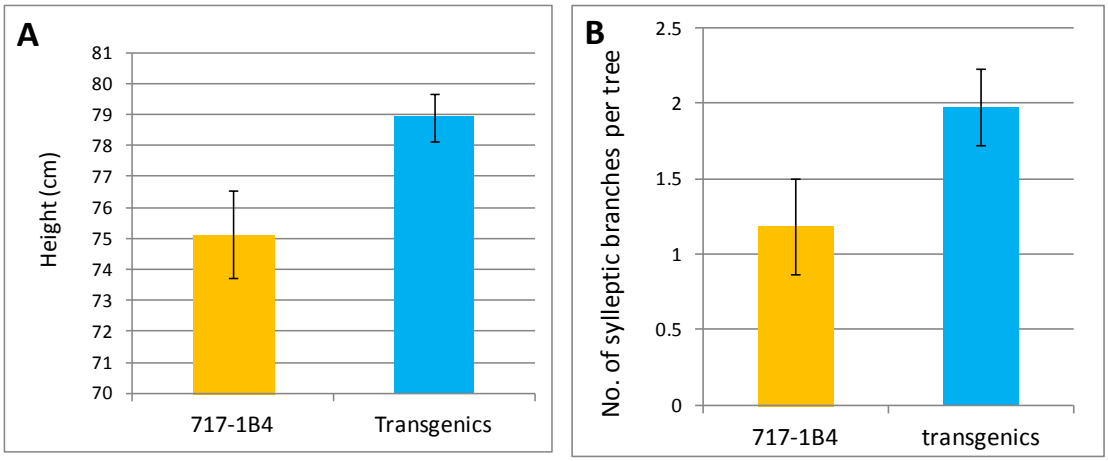
S Figure 9 *In vitro* observaton of the percentage of rapidly growing callus (%RGC) and its relationship with *PtDDM1* expression in stem explants (**A**) without 5-AC (light blue), with 5-AC(dark blue), in leaf explants (**B)** without 5-AC (light green), with 5-AC (dark green).



S Figure 10 *In vitro* observation of the percentage of shoot regeneration and its relationship with *DDM1* expression for stem explants without 5-AC treatment (**A**), with 5-AC treatment (**B**), for leaf explants without 5-AC treatment (**C**), with 5-AC treatment (**D**).



S Figure 11 Greenhouse observations of diameter (**A**), height (**B**), and lateral shoots (**C**) and their relationship to *PtDDM1* expression.



S Figure 12 A. Average height (cm) and standard deviation of transgenics vs. non-transgenic control 717-1B4. **B.** Average number and standard deviation of sylleptic branches per tree of transgenics vs. non-transgenic control 717-1B4.