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Research article

Functional analyses of *PtRDM1* gene overexpression in poplars and evaluation of its effect on DNA methylation and response to salt stress

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ABSTRACT

Epigenetic modification by DNA methylation is necessary for all cellular processes, including genetic expression events, DNA repair, genomic imprinting and regulation of tissue development. It occurs almost exclusively at the C5 position of symmetric CpG and asymmetric CpHpG and CpHpH sites in genomic DNA. The RNA-directed DNA methylation (*RDM1*) gene is crucial for heterochromatin and DNA methylation. We overexpressed *PtRDM1* gene from *Populus trichocarpa* to amplify transcripts of orthologous RDM1 in 'Nanlin895' (*P. deltoides* × *P. euramericana* 'Nanlin895'). This overexpression resulted in increasing *RDM1* transcript levels: by ~150% at 0 mM NaCl treatment and by ~300% at 60 mM NaCl treatment compared to WT (control) poplars. Genomic cytosine methylation was monitored within *5.8S* rDNA and histone *H3* loci by bisulfite sequencing. In total, transgenic poplars revealed more DNA methylation than WT plants. In our results, roots revealed more methylated CG contexts than stems and leaves whereas, histone *H3* presented more DNA methylation than *5.8S* rDNA in both WT and transgenic poplars. The NaCl stresses enhanced more DNA methylation in transgenic poplars than WT plants through histone *H3* and *5.8* rDNA loci. Also, the overexpression of *PtRDM1* resulted in hyper-methylation, which affected plant phenotype. Transgenic poplars revealed significantly more regeneration of roots than WT poplars via NaCl treatments. Our results proved that RDM1 protein enhanced the DNA methylation by chromatin remodeling (e.g. histone *H3*) more than repetitive DNA sequences (e.g. *5.8S* rDNA).

1. Introduction

Studies have shown that plants must adjust to environmental conditions to survive (Santner and Estelle, 2009). Numerous environmental-stress-responsive genes have been used to improve plant genomes and proteomes using genomics, proteomics and transcriptomics approaches (Gu et al., 2017). Introduction of environmental stresses can cause damage to plants by reducing gene expression, remodeling chromatin and altering chromosomes, resulting in suppression of growth and productivity (Hirakawa et al., 2017). Heritable epigenetic resistance exists in plants. One mechanism is chromatin remodeling, which regulates gene expression in the presence of stresses (Feng et al., 2010). Epigenetic marks such as histone modifications and DNA methylation are distributed along four states of chromatins: intergenic regions, repressed genes, active genes and silent repeat elements (Roudier et al., 2011). Histone *H3* methylation accompanied by DNA methylation will be associated with gene expression to promote or/and maintain the differentiation status of plant cells (Ikeuchi et al., 2015). Already, lots of researches have been done on methylation of histone *H3* at lysine 9 (H3K9me), but to date there is no research on DNA methylation of histone *H3* gene body. This study is the first research on investigation of DNA methylation of histone *H3* gene body in comparing with *5.8S* rDNA, impacted by abiotic stress.

The RNA-directed DNA methylation (RdDM) pathway involves several important genes that regulate DNA methylation, which is a mechanism of epigenetic regulation in eukaryotes (Movahedi et al., 2015a,b,c). One such gene is RNA-directed DNA methylation 1 (*RDM1*) (Gao et al., 2010; Movahedi et al., 2015a,b,c), some of mutations in RDM1 cause loss-of-function in the *rdm1* gene, which suppresses accumulation of 24 nt siRNAs, resulting in decreased DNA methylation (Gao et al., 2010). RDM1 is a small nuclear protein that associates with ARGONAUTE (AGO 4/6/9), KOW domain-containing transcription

Abbreviations: RDM1, RNA-directed DNA methylation 1; ROS1, Repressor of Silencing; qPCR, quantitative Polymerase Chain Reaction; RdDM, RNA-directed DNA methylation

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factor 1 (KTF1) and DNA-dependent RNA polymerase V (PolV) to localize domain rearranged methyltransferase 2 (DRM2), leading to DNA methylation (Gao et al., 2010; Movahedi et al., 2015a,b,c). The repressor of silencing 1 (*ROS1*) gene family, which mediates demethylation activities, and the RdDM pathway are maintained in equilibrium (Gao et al., 2010). Biologically, ROS1 is a DNA glycosylase/lyase that activates DNA demethylation, which is critical for demethylation of promoter sequences (Wang et al., 2017). This dynamic epigenetic regulation may be necessary for efficient responses to environmental and developmental stresses. Questa et al. (2013) showed that during the initial stages of development, germination or post-germination in plants, ROS1 binds with pol IV to remodel chromatin in *5.8S* rDNA.

Gao et al. (2010) reported that in the presence of *ros1*, heavy methylation occurs in all sequences, including CpG, CpHpG and CpHpH, where H represents A, T or C. The plant RDM1 protein has a highly conserved sequence, homologs of which are present in both dicots and monocots (Allard et al., 2005; Ma et al., 2015). According to Gao et al. (2010), suppression of RDM1 or loss-of-function caused by mutation results in defective DNA methylation. These authors also showed that a mutant RDM1 that contained alanine in place of methionine exhibited decreased DNA methylation due to the suppression of binding of RDM1 to $_{m}$CpHpH sequences. Gao et al. (2010) reported that the mutation of *rdm1-1* blocked asymmetric cytosine methylation CpHpH sites, but had no impact on symmetric cytosine methylation CpG sites. That is why the *rdm1-1* genes cause a decrease in *de novo* methylation; thus, RDM1 is an essential factor for DNA methylation. Also, it has been proved that RDM1 is involved in a complex associated with DRM2 and AGO4 that locally catalyzes CG, CHG, and CHH methylation in plants (Gao et al., 2010). In total, the role of *RDM1* gene in development of DNA methylation and also, the role of DNA methylation in response to environmental stresses, led the authors to assess the role of *PtRDM1* gene overexpressing in improving resistant poplars against salt stress.

2. Materials and methods

2.1. PtRDM1 identification, RNA isolation, plasmid construction and assembly

A BLASTp search was carried out for detecting homologs of RDM1 domain from *Arabidopsis thaliana* using Uniprot database (http://www.uniprot.org/blast/). Species containing characterized RDM1 were isolated to identify consensus sequences. The putative *PtRDM1* was amplified from *P. trichocarpa* using degenerate primers (Supplementary 1; DGF and DGR) (Geneious version 10.3 created by Biomatters development team. Available from https://www.geneious.com) and introduced into the pEASY-T3 cloning vector (pEASY®-T3 cloning kit) via the TA cloning technique. The linked putative *PtRDM1* was then sequenced (GeneScript Company). Finally, the analyzed sequence was submitted to the National Center for Biotechnology Information (NCBI) under accession number KT633998.

Total RNA was extracted from *P. trichocarpa* using TRIzol (Tiangen Biotech, Beijing, China) reagent according to the manufacturer's instructions. RNA was treated with DNaseI (NEB, USA) and the RNA concentration was determined using a BioDrop spectrophotometer (UK). Total RNA (2 μg) and oligo-dT primers were used to synthesize cDNA using a Prime Script One Step RT-PCR ver. 2 kit (Takara Biotechnology, Dalian, China) according to the manufacturer's instructions. Continuously, the full-length cDNA of *PtRDM1* was amplified from cDNA of *P. trichocarpa*, corresponding to the positions 48–575 in XM-006379786 with a length of 528 bp (SnapGene™ 1.1.3 software, Chicago, USA). The amplified fragment was then introduced into the pGWB9 expression vector (accession number AB289772) via the infusion gateway technique in the sense orientation. The expression of *PtRDM1* was driven by the *35S* promoter and terminated by the *NOS* terminator.

2.2. Plant preparation, transformation and regeneration

According to Movahedi et al. (2015a,b,c), Murashige and Skoog (MS) media containing 0.5 mg/L N-6-benzyladenine (6-BA) and 0.004 mg L^{-1} thidiazuron (TDZ) were used to sub-culture 20 plants of hybrid clone 'Nanlin895' (*P. deltoides* × *P. euramericana* 'Nanlin895'). Then, two cm grown shoots were transferred to shoot elongation media supplemented with 0.25 mg L^{-1} 6-BA and 0.002 mg L^{-1} TDZ. Four cm grown shoots were subjected on half MS media to regenerate roots (Movahedi et al., 2015a,b,c). Two weeks old transferred plants (first expanded leaves) and four weeks old grown poplars (immature leaves and roots) were used to investigate the expression of *PtRDM1*, while two and three month old grown plants were assumed to be young and mature poplars, respectively.

Transformation of *P. deltoides* × *P. euramericana* 'Nanlin895' was carried out using *Agrobacterium tumefaciens* strain EHA105 according to (Movahedi et al., 2014). Briefly, cut leaves were immersed in *Agrobacterium* inducer liquid (MS) medium containing 5% sucrose and 200 μM acetosyringone (AS) and incubated for 120 min at 28 °C with shaking at 100 rpm. Explants were then transferred to semi-solid MS media supplemented with 200 μM AS and incubated for 2 days at 28 °C in a dark condition. The putative transformants were then transferred to selective MS media supplemented with 0.5 mg l^{-1} 6-BA, 0.004 mg l^{-1} TDZ, 200 mg l^{-1} cefotaxime and 50 mg l^{-1} kanamycin at 23 °C and a period of 16/8 h light/dark. One microgram of genomic DNA, as determined using a BioDrop spectrophotometer (UK), was extracted from young putative transformant leaves using the CTAB method. PCR amplification was performed using the specific primers of the neomycin phosphotransferase II (*NPT II*) gene (Supplementary 1; *NPTII*-F and *NPTII*-R). The 3546 bp amplified fragments were then resolved in a 1.5% agarose gel. Kanamycin-resistant and PCR positive plants were considered transgenic. In total, 9 lines of transgenic poplars (including ~135 individuals) were obtained.

2.3. Analysis of gene expression by RT-PCR

Total RNA was extracted from putative transformant plants using a Plant RNA Kit (Omega Biotech No: R6827-01, China) according to the manufacturer's instructions. Total RNAs from 9 independent putative transformant plants and 3 WT poplars were then treated with DNase I (Takara Biotechnology, China) and their concentrations were determined to be 1000 ng/μl using a BioDrop spectrophotometer (UK). The PrimeScript One Step RT-PCR ver. 2 kit (Takara Biotechnology, Dalian, China) was then used to synthesize cDNA with oligo-dT primers; the synthesized cDNA was stored in TE buffer. The *β-actin* gene (accession number: XM-006370951.1) was used to standardize the cDNA concentration from PCR using specific primers (Supplementary 1; *Actin*-F and *Actin*-R) with 2 min denaturation at 95 °C, 30 cycles of (5 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C) and 5 min extension at 72 °C. In addition, specific primers were designed to amplify the *PtRDM1* from synthesized cDNA (Supplementary 1; *PtRDM1*-F and *PtRDM1*-R). Equal amounts of amplified PCR products were resolved in a 1.5% TAE agarose gel and band intensity was quantified using the ImageJ 1.5b image-analysis software (USA).

2.4. qPCR analysis

Synthesized cDNA was used to quantify the expression of exogenous *PtRDM1* by quantitative real-time PCR (qPCR). Each NaCl treatment (0, 20, 40 and 60 mM) was carried out using three biological replicates from independent transgenic and one non-transgenic (wild type) poplars supplied with three repeats, and *β-actin* was used as an endogenous control (Movahedi et al., 2015a,b,c). An Applied Biosystems real-time PCR instrument (USA) and Fast Start Universal SYBR Green Master Mix (Rox; No. 04913914001: Roche, USA) were used to