A DNA 3' Phosphatase Functions in Active DNA Demethylation in *Arabidopsis*

María Isabel Martínez-Macías,[1] Weiqiang Qian,[2] Daisuke Miki,[2] Olga Pontes,[3,5] Yunhua Liu,[2,6] Kai Tang*,[2] Renyi Liu,[4] Teresa Morales-Ruiz,[1] Rafael R. Ariza,[1] Teresa Roldán-Arjona,[1] and Jian-Kang Zhu[2]

[1]Department of Genetics, University of Córdoba, 14071 Córdoba, Spain; [2]Department of Horticulture & Landscape Architecture, Purdue University, West Lafayette, IN 47906, USA; [3]Biology Department, Washington University, St. Louis, MO 63130, USA; [4]Department of Botany and Plant Science, University of California, Riverside, CA 92521, USA; [5]Present Address: Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA; [6]Present Address: College of Plant Sciences & Technology, Huazhong Agricultural University, Wuhan 430070, China, and Shanghai Agrobiological Gene Center, Shanghai 201106, China

INTRODUCTION

In *Arabidopsis thaliana*, active DNA demethylation requires a subfamily of DNA glycosylases whose founding members are ROS1 (repressor of silencing 1), DME (Dmeter), DML2, and DML3 (Demeter-like 2 and 3). ROS1 and its homologs are bifunctional DNA glycosylases/lyases that cleave the phosphodiester backbone at the 5-methylcytosine (5-meC) removal site by β-elimination, generating a 3' phospho α,β-unsaturated aldehyde at the strand break. In ROS1, a significant amount of β-elimination incisions proceed to β,δ-elimination products. Thus, part of the final reaction product generated by ROS1 is a single-nucleotide gap flanked by 3' and 5'-phosphate (3'-P and 5'-P) termini. A yet unknown DNA polymerase must fill this gap with an unmethylated cytosine before a DNA ligase can seal the remaining nick. However, all DNA polymerases characterized to date require a 3'-hydroxyl terminus to initiate synthesis. Therefore, the phosphate group present at the 3' end of the single-nucleotide gap generated by ROS1 must be removed prior to the polymerization and ligation steps that complete the DNA demethylation pathway. We hypothesized that 3' phosphates generated by ROS1 are putative substrates for the 3'-phosphatase activity of ZDP (zinc finger DNA 3' phosphoesterase), and thus ZDP may be important for epigenetic regulation through participation in active DNA demethylation. In this work, we report biochemical, genetic, and cell biological evidence that ZDP functions with ROS1 in active DNA demethylation and is an important player in shaping the DNA patterns of hundreds of genomic loci.

RESULTS

ZDP Removes the 3'-Blocking Phosphate from the Gapped Product Generated by ROS1 and Increases the Processivity of ROS1

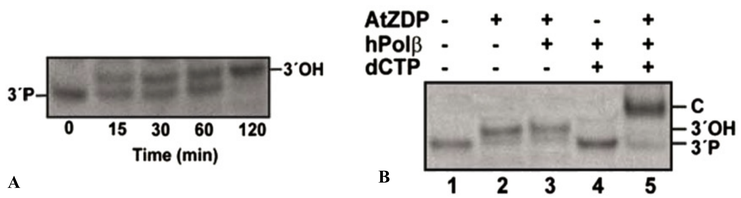

Figure 1. **Characterization of ZDP Biochemical Activity**
(A) Purified ROS1 (37.5 nM) was incubated at 30°C for 16 hr with a double-stranded oligonucleotide substrate (40 nM) containing a 5-meC:G pair . Reaction products were incubated with purified ZDP (1.5 nM) at 30°C . Reactions were stopped at the indicated times, products were separated in a 15% denaturing polyacrylamide gel and visualized by fluorescence scanning.
(B) Reaction products of ROS1 were incubated with purified ZDP (6 nM) and human DNA polymerase β (hPol β, 0.5 U) during 30 min at 37°C , in the absence (lane 3) or presence (lane 5) of dCTP (0.2 mM). Lane 1: 30-phosphate (30P) marker. Lane 2: control reaction without hPol β. Lane 4: control reaction without ZDP.

zdp Mutants Are Deficient in DNA 3'-Phosphatase Activity

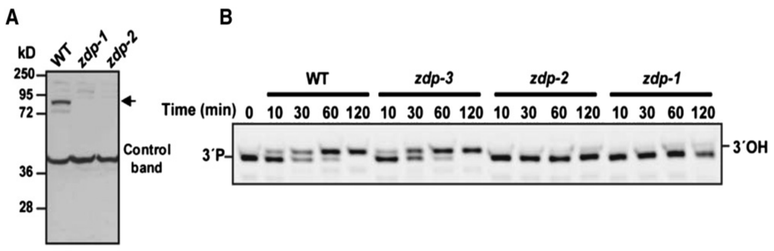

Figure 2. *zdp* Mutant Plants Are Unable to Process DNA Demethylation Intermediates Containing a Gap Flanked by 3'- and 5'-Phosphate Groups
(A) Detection of ZDP protein levels in wild-type and mutant plants by western blotting using an antibody against ZDP. A prominent nonspecific band from the western blot serves as a control for loading. Arrow points to the position of ZDP protein.
(B) A DNA duplex containing a single-nucleotide gap flanked by 3'-P and 5'-P termini was incubated at 30°C with cell extracts from wild-type and *zdp* mutants. Reactions were stopped at the indicated times, products were separated in a 15% denaturing polyacrylamide gel and visualized by fluorescence scanning.

Role of ZDP in Preventing Transcriptional Gene Silencing

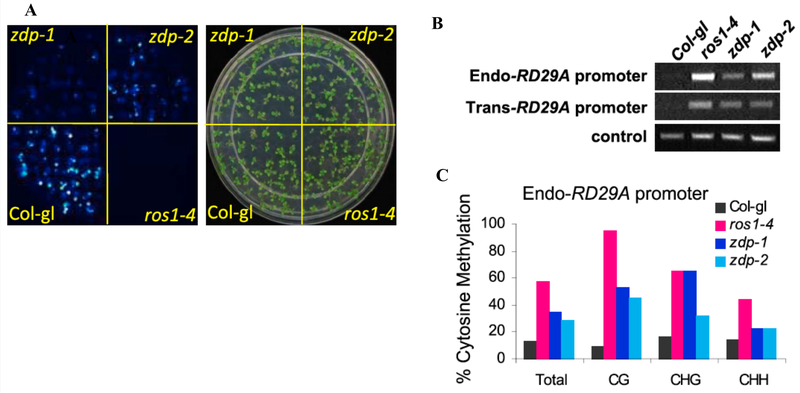

Figure 3. Partial Silencing of RD29A-LUC in *zdp* Mutant Plants
(A) Two-week-old plants grown on MS plates were imaged for luminescence after cold treatment at 4°C for 2 days. Luminescence imaging showed that RD29A-LUC expression was reduced in the *zdp-1* and *zdp-2* mutants compared to the wild-type.
(B) PCR analysis of the RD29A promoters that were digested with a methylation-sensitive restriction enzyme indicated that like *ros1-4*, both *zdp-1* and *zdp-2* mutations caused hypermethylation of the transgene and endogenous RD29A promoters.
(C) Bisulfite sequencing confirmed the hypermethylation and revealed increased levels of methylation in not only CG but also CHG and CHH (H is A, T, or C) sequence contexts in the endogenous RD29A promoter in ros1-4 as well as *zdp-1* and *zdp-2* mutants.

Genome-wide Effects of ZDP on DNA Methylation

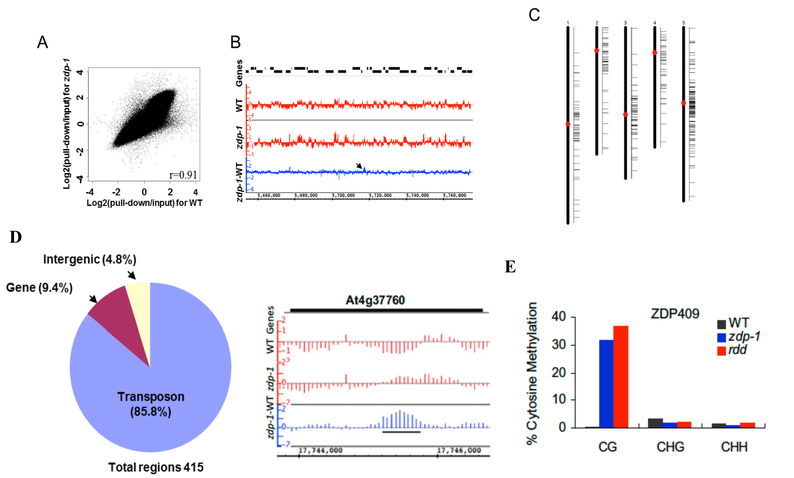

Figure 4 Whole Genome Methylation Profile in Wild-Type and *zdp-1* Mutant Plants
(A) Scatter plot showing the correlation between wild-type (Col-0) and *zdp-1* tiling array data. (r = 0.91)
(B) An example of tiling array result. Black boxes indicate genes, with oriented 5'-3' on the top and the reverse direction at the bottom. The bottom scale indicates chromosome positions in base pairs on chromosome 1. Each red bar represents the log2 ratio of the immunoprecipitated and input DNA signals, in wild type and *zdp-1*, respectively. Blue bars represent the difference between log2 signals in the *zdp-1* mutant and wild type samples. The black arrow points to an apparent hypermethylated region.
(C) Distribution of hypermethylated loci on the 5 chromosomes in *zdp-1* mutant plants.
(D) Pie chart showing the composition of hypermethylated loci in *zdp-1* mutant.
(E) Confirmation of a randomly chosen hypermethylated regions by bisulfite sequencing.

Genetic Interaction among *zdp*, *ros1*, and RNA-directed DNA Methylation (RdDM) Mutants

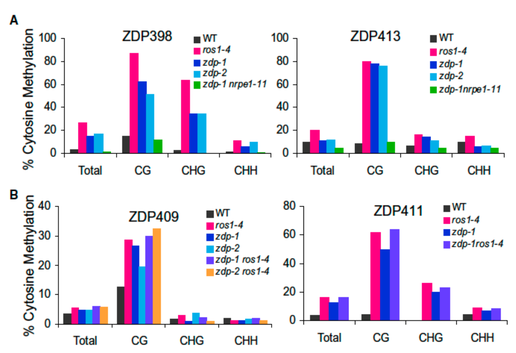

Figure 5. Genetic Interaction Analysis (A) Suppression of the hypermethylation phenotype of the *zdp-1* mutant at the ZDP398 and ZDP413 loci by the *nrpe1-11* mutation. This suggests that ZDP counteracts methylation that is dependent on the RdDM pathway.
(B) Absence of additive effects between *ros1-4* and *zdp-1* or *zdp-2* alleles on the hypermethylation phenotype at the ZDP409 and ZDP411 loci. This suggests that ZDP functions in the same genetic pathway as ROS1.

Subnuclear Colocalization of ZDP with ROS1 and ROS3

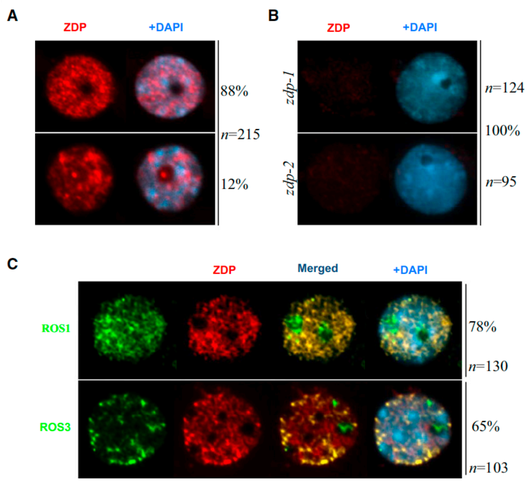

Figure 6. Subnuclear Localization of ZDP and Colocalization with ROS1 and ROS3 (A and B) The nuclear distribution of ZDP was analyzed by immunostaining using anti-ZDP (red) in wild-type(A) and *zdp* mutants (B).
(C) Dual immunolocalization using anti-ZDP (red) in transgenic lines expressing recombinant full-length epitope-tagged MYC-ROS1 and FLAG-ROS3 (green). In all panels, the DNA was stained with DAPI (blue). The frequency of nuclei displaying each interphase pattern is shown on the right. We found that ZDP colocalized with both ROS1 and ROS3 within nucleoplasmic foci, as shown by the strong yellow signals, which denoted overlapping of the green and red signals .

CONCLUSION

- ZDP and ROS1 interact in vitro and co-localize in vivo
- *zdp* mutants show increased promoter DNA methylation and TGS of a reporter gene
- Hundreds of endogenous loci are hypermethylated in zdp mutant plants
- ZDP functions downstream of ROS1 in one branch of the active DNA demethylation pathway. One of the final products of ROS1 is a single-nucleotide gap flanked by 3'-P and 5'-P termini and ZDP can convert the 3'-P blocking group into a free 3'-hydroxyl DNA terminus.

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