Transcriptional gene silencing mediated by a plastid inner envelope phosphoenolpyruvate/phosphate translocator CUE1 in Arabidopsis

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Abstract

Mutations in ROS1 (REPRESSOR OF SILENCING 1) lead to the transcriptional gene silencing (TGS) of Pro$_{RD29A}$:LUC and Pro$_{35S}$:NPTII reporter genes. We performed a genetic screen to find suppressors of ros1 that identified two mutant alleles in the Arabidopsis chlorophyll a/b binding protein (CAB) gene underexpressed 1 (CUE1) gene, which encodes a plastid inner envelope phosphoenolpyruvate/phosphate translocator. The cue1 mutations released the TGS of Pro$_{35S}$:NPTII and the transcriptionally silent endogenous locus TSI (TRANSCRIPTIONAL SILENCING INFORMATION) in a manner that was independent on DNA methylation but dependent on chromatin modification. The cue1 mutations did not affect the TGS of Pro$_{RD29A}$:LUC in ros1, which was dependent on RNA-directed DNA methylation. It is possible that signals from chloroplasts help to regulate the epigenetic status of subset of genomic loci in the nucleus.

Key word, a plastid inner membrane protein, transcriptional gene silencing,

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INTRODUCTION

Heterochromatin is characterized by DNA methylation and/or histone modifications including histone H3K9, H3K27 methylation, and H3 as well as H4 hypoacetylation. Heterochromatin can lead to transcriptional gene silencing (TGS), which plays a central role in repressing transposon movement and mediating gene repression during developmental and cellular differentiation in plants (Martienssen and Colot, 2001; Henderson and Jacobsen, 2007; Matzke et al., 2007; Matzke et al., 2009). TGS relies on both DNA methylation-dependent and independent pathways in plants (Martienssen and Colot, 2001; Henderson and Jacobsen, 2007; Matzke et al., 2007). Various components in the RNA-directed DNA methylation pathway have been identified (Henderson and Jacobsen, 2007; Matzke et al., 2007; Matzke et al., 2009). The reversible removal of DNA methylation is catalyzed by ROS1 (REPRESSOR OF SILENCING 1), DEMETER, and two DEMETER-like proteins, DML2 and DML3, through a base excision repair mechanism (Gong et al., 2002; Agius et al., 2006; Morales-Ruiz et al., 2006). In the DNA methylation-independent pathway, several proteins involved in DNA replication and repair regulate the epigenetic status of transcriptionally silent endogenous locus TSI (TRANSCRIPTIONAL SILENCING INFORMATION) in Arabidopsis. These proteins include BRUSHY1 (BRU1) (a DNA repair related protein) (Takeda et al., 2004), ROR1/RPA2A (repressor of ros1/replication protein A2) (Xia et al., 2006), FAS1 and FAS2 [(FASCIATA1, FASCIATA2, two subunits of CHROMATIN ASSEMBLY FACTOR 1 (CAF1))] (Kaya et al., 2001; Takeda et al., 2004), MSI1 (Hennig et al., 2003), TSL (TOUSLED protein kinase) (Wang et al., 2007), NRP1/NRP2 (NUCLEOSOME ASSEMBLY PROTEIN1-RELATED proteins, histone chaperones) (Zhu et al., 2006), TSO2-1 and RNR2a-1 (two subunits of ribonucleotide reductase) (Wang and Liu, 2006), DNA polymerase epsilon (Yin et al., 2009) and others. These proteins participate in chromatin assembly, DNA recombination, DNA replication, and/or DNA repair, indicating crucial roles of DNA replication and repair in maintaining chromatin structures. Previous studies also indicate that TGS of some developmental genes is...
regulated by other chromatin remodeling proteins such as Polycomb group (PcG) proteins. A well-studied example is the regulation of *FLOWER LOCUS C* (FLC) expression by the PcG protein VERNALIZATION 2 in Arabidopsis (Dennis and Peacock, 2007) during extended cold treatment. The extended cold treatment suppresses *FLC* expression by increasing H3K27 methylation, which is mediated by the VER2 repressor complex (Bastow et al., 2004).

*ROS1* encodes a DNA glycosylase/lyase, and its mutations lead to TGS of originally active Pro<sub>RD29A:LUC</sub> and Pro<sub>35S:NPTII</sub> reporter genes in a T-DNA region. Screening for suppressors of *ros1* mutant using silenced Pro<sub>35S:NPTII</sub> as a selection marker has identified two genes encoding RPA2A/ROR1 and TSL in previous studies (Kapoor et al., 2005; Xia et al., 2006; Wang et al., 2007). An independent screening for *ros1* suppressors using silenced Pro<sub>RD29A:LUC</sub> as a selection marker has identified several genes including *NRPD1/NRPD1a* (*NUCLEAR RNA POLYMERASE D*), *NRPE1/NRPD1b* (*NUCLEAR RNA POLYMERASE E*), *NRPD2a* (*NUCLEAR RNA POLYMERASE D 2A*), *RDM2* (*RNA-directed DNA methylation*), *AGO4* (*ARGONAUTE 4*), *HEN1* (*HUA ENHANCER 1*), *DRD1* (*DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1*), and *HDA6* (*HISTONE DEACETYLASE 6*) (He et al., 2009). Mutations in RDM2, NRPD1, NRPE1, NRPD2a, HEN1, and DRD1 release the TGS of only Pro<sub>RD29A:LUC</sub>, but not Pro<sub>35S:NPTII</sub>, suggesting the biological roles of these genes in the siRNA-directed DNA methylation pathway. Mutations in HDA6 and AGO4 reactivate the expression of both silenced Pro<sub>RD29A:LUC</sub> and Pro<sub>35S:NPTII</sub>, suggesting that the two proteins mediate TGS in both siRNA-directed DNA methylation dependent and -independent pathways (He et al., 2009). Here we characterized two new alleles of the Arabidopsis *chlorophyll a/b binding protein* (CAB) gene underexpressed 1 (*cue1*) gene encoding a plastid inner envelope phosphoenolpyruvate/phosphate translocator (Li et al., 1995; Streatfield et al., 1999). *cue1* mutations release the TGS of *TSI* and silenced and Pro<sub>35S:NPTII</sub> in *ros1*, but not Pro<sub>RD29A:LUC</sub>, suggesting that the possible signals from chloroplasts not only regulate nuclear gene expression but also modulate nuclear epigenetic status in
an siRNA-directed DNA methylation independent manner in Arabidopsis.

Results

cue1 mutants show a release of TGS of Pro_{35S}\text{-}\text{NPTII}

The ros1-1 mutant was isolated in a previous study in the C24 background, which carries a T-DNA insertion with silenced both Pro_{RD29A}\text{-}\text{LUC} and Pro_{35S}\text{-}\text{NPTII} genes. Two allelic mutants, named cue1-101 and cue1-102, were isolated during a genetic screening for suppressors of ros1-1 on MS-agar medium containing 40 mg/L kanamycin. Both mutants showed a similar reticulate leaf phenotype. We used map-based cloning to identify the CUE1 gene encoding a plastid inner envelope phosphoenolpyruvate/phosphate translocator (PPT) with six transmembrane domains which imports phosphoenolpyruvate (PEP) into plastids (Fischer et al., 1997; Streatfield et al., 1999; Knappe et al., 2003). After sequencing the CUE1/AT5G33320 gene, we found a G_{1639} to A_{1639} point mutation which changed Gly_{251} to Glu_{251} in ros1-1 cue1-101. Gly_{251} lies between transmembrane domain III and IV in a region which is highly conserved in different PPTs from Arabidopsis, cauliflower, tobacco, and maize, and also in other chloroplast triose phosphate/phosphate translocator (cTPT) (Fischer et al., 1997). In cue1-102 ros1-1, a G_{886} to A_{886} point mutation that disrupts the acceptor splicing site in the first intron of CUE1 gene was found. This mutation is expected to produce an abnormal mRNA which would be translated into a truncated protein due to a putative early stop codon. As shown in Figure 1B, cue1-101 ros1-1 and cue1-102 ros1-1 seedlings were more resistant than ros1-1 seedlings to kanamycin but less resistant than the wild type, suggesting that the cue1 mutations only partially released the TGS of Pro_{35S}\text{-}\text{NPTII}. Northern blot analysis indicated that both cue1-101 ros1-1 and cue1-102 ros1-1 expressed more NPTII transcripts than the ros1-1, but less than wild type (Figure 1E), which is consistent with the phenotypes of the seedling growth on kanamycin. However, a ddm1 mutant allele (named ddm1-101) isolated during our ros1 suppressor screening increased the NPTII expression at a much higher level even than wild type C24 (Figure 1E). Because Pro_{RD29A}\text{-}\text{LUC} and endogenous RD29A are silenced in the ros1 mutant, we
also analyzed their gene expression. COR47 and RD29A are two stress inducible genes with similar inducible patterns under ABA or other abiotic stress treatments (Xia et al., 2006). Under 100 μM ABA treatment for 3 h, the silenced Pro_{RD29A}:LUC or endogenous RD29A were reactivated in the C24 wild type, but not in ros1-1 or cue1-101 ros1-1, cue1-102 ros1-1 mutant based on bioluminescence analysis (Figure 1D) and Northern blot (cue1-101 ros1-1) (Figure 2F). As a control, the COR47 gene was expressed at similar levels in the wild type, ros1-1 and cue1-101 ros1-1 (Figure 1F).

**cue1 mutations increase the transcripts of TSIs but do not change the DNA methylation levels**

TSIs are silenced markers frequently used in TGS analysis (Steimer et al., 2000). The expression of TSIs was barely detected in the wild type or ros1-1. TSI transcripts were expressed at higher levels in cue1-101 ros1-1 and cue1-102 ros1-1 than in the wild type or ros1-1 single mutant, although the expression in the suppressor mutants was much lower than in the DDM1-101 (Figure 2A). TSI transcripts were increased slightly more in cue1-101 or cue1-102 single mutants than in cue1-101 ros1-1 or cue1-102 ros1-1 double mutants (Figure 2A). These results indicate that cue1 mutations release the TGS of not only a transgene but also endogenous genes in Arabidopsis.

A previous study indicated that cue1 mutations reduce the key aromatic amino acid metabolites in the shikimate pathway, and feeding aromatic amino acids could rescue the reticulate leaf phenotype of cue1, which was confirmed by our experiments (data not shown) (Streatfield et al., 1999). We further tested whether feeding aromatic amino acids could rescue the TGS phenotype of cue1 by examining the expression of TSI and NPTII gene. cue1-101 ros1-1 seedlings were grown on MS medium or MS medium containing 1 mM phenylalanine (Phe), 1 mM tryptophan (Trp), 1 mM tyrosine (Tyr), or all three aromatic acids (1 mM for each) together for 10 days. Interestingly, TSI expression was reduced to different levels in cue1-101 ros1-1 by feeding different aromatic acids with the highest reduction by feeding Tyr when compared with no feeding control (Figure 2B). NPTII expression was noticeably
reduced by feeding Tyr or combined three aromatic amino acids, but seems not affected by feeding Phe or Trp in cue1-101 ros1-1 (Figure 2B). Here C24 wild type and ros1-1 were used as control without detection of TSI expression. NPTII transcripts were clearly detected in the C24 wild type (Figure 2B). These results indicate that feeding aromatic amino acid(s) to cue1 mutant can rescue or partially rescue the gene silencing phenotype in ros1 background. These results further suggest that the mutated CUE1 or wild type CUE1 itself does not directly interfere with the TGS in nucleus; instead, the key role of CUE1 is to translocate PEP (a necessary substrate for the shikimate pathway) in plastids where unknown signals that might mediate the TGS in nucleus are produced.

TGS is mediated by both DNA methylation and/or histone modifications. We analyzed the DNA methylation status of RD29A promoters in both the T-DNA region and in the endogenous gene by bisulfite sequencing. There is no evidence for a DNA methylation difference between cue1-101 ros1-1 or cue1-102 ros1-1 and ros1-1 (Figure 2C). We also analyzed the centromeric DNA and 180-bp rDNA regions by using DNA methylation sensitive restrictive enzymes, but found no difference in DNA methylation (Figure 2D). These results suggest that the cue1 mutation releases TGS in a siRNA-directed DNA methylation independent pathway.

cue1 mutations decrease H3K9m2 but do not change the overall heterochromatin status

We then performed a chromatin immunoprecipitation (ChIP) assay to determine the effect of cue1 mutations on histone modifications in different genomic regions. Histone H3 acetylation (H3Ac) is a marker for transcriptional activation, while histone H3 Lys 9 dimethylation (H3K9m2) is a transcriptional silencing marker. We chose the ACTIN promoter as a control for active genes. When no antibodies were added, no PCR products were amplified (Figure 3A). Consistent with previous results, the ros1 mutation increased H3K9m2 at the 35S promoter, TSI, and LUC regions compared with the wild type (Xia et al., 2006). H3K9m2 was reduced at both the TSI and 35S promoter regions but was not changed at the ProRD29A::LUC locus in cue1-101
For histone H3Ac, we observed a clear decrease at 35S and LUC in ros1, and the similar level at 35S in wild type and cue1-101 ros1-1, but no difference at TSI among the three genotypes. Although a minor change in H3Ac levels at LUC in cue1-101 ros1-1 was detected, we did not detect a change for RD29A expression in our experimental conditions. Mono- and dimethylated histone H3K9 are enriched around Arabidopsis pericentromeric heterochromatin (Naumann et al., 2005). We performed an immunostaining assay to detect the distribution of H3K9m2 in nuclei using anti-H3K9m2 antibodies. As shown in Figure 3B, no apparent difference was found for immunostaining of H3K9m2 among wild-type, ros1-1, and cue1-101 ros1-1. The above results indicate that the cue1 mutation affects TGS of some loci by changing chromatin modifications but does not influence the global H3K9m2.

**cue1 mutants are more resistant than wild-type to MMS**

Previous studies indicated that plants with mutant alleles for several genes (BRU1, FAS1, RPA2A/ROR1, TOUSLED, and others) affecting TGS are also sensitive to the DNA-alkylating agent methylmethane sulfonate (MMS) (Takeda et al., 2004; Xia et al., 2006; Wang et al., 2007). These genes are all thought to be involved in DNA repair. In order to determine whether cue1 mutations influence DNA repair, we examined the sensitivity of cue1 seedlings to different concentrations of MMS. Five-day-old seedlings were transferred to MS medium containing different concentrations of MMS, and cultured for seven additional days. As shown in Figure 4, cue1 seedlings were smaller than the wild type under normal growth conditions (Li et al., 1995; Streatfield et al., 1999). MMS treatment inhibited the growth of all plants and caused yellowing of the leaves. However, the yellowing of leaves was more severe in the C24 wild type and ros1-1 than in cue1-101 ros1-1 or cue1-101 mutants, suggesting that cue1 seedlings are more resistant to MMS. The results suggest that the biological role of CUE1 in the DNA repair is different from other known proteins (Takeda et al., 2004; Xia et al., 2006; Wang et al., 2007).

**Discussion**

Previous studies suggest that several retrograde signaling pathways transducing
signals from chloroplasts to the nucleus regulate the expression of genes which function within the chloroplast (Nott et al., 2006; Koussevitzky et al., 2007; Pesaresi et al., 2007). However, these signals are not well characterized (Koussevitzky et al., 2007). \textit{CUE1} was originally isolated in a genetic screen to identify mutants with reduced expression of \textit{chlorophyll a/b binding proteins (CAB)} (Li et al., 1995). \textit{CUE1} is a nuclear gene encoding a plastid inner envelope phosphoenolpyruvate/phosphate translocator (PPT), which plays crucial roles in positively regulating the expression of nuclear genes encoding plastid proteins (Streatfield et al., 1999). In contrast to reduced expression of \textit{CAB} genes, \textit{cue1} mutations reactivated the silenced Pro\textsubscript{35S}:NPTII and TSI in \textit{ros1}. Interestingly, the reactivated Pro\textsubscript{35S}:NPTII or TSI could be re-silenced by feeding aromatic amino acids which also rescues the reticulate leaf phenotype of \textit{cue1}. It is possible that \textit{cue1} mutations interfere with the shikimate pathway. The metabolic products such as aromatic amino acids produced from shikimate pathway could possibly act as signals or participate in the synthesis of important mediators to regulate TGS in nucleus. It is unlikely that CUE1 or its mutant isoforms would function in the nucleus to directly mediate the chromatin status.

The TGS mechanism at the Pro\textsubscript{35S}:NPTII locus differs from that at Pro\textsubscript{RD29A}:LUC (Xia et al., 2006; He et al., 2009). The TGS at the Pro\textsubscript{RD29A}:LUC locus is caused by small RNA-directed DNA methylation (Gong et al., 2002), while the TGS of Pro\textsubscript{35S}:NPTII is not related to siRNA-directed DNA methylation but likely results from a heterochromatin-spaying pathway (Kapoor et al., 2005; Xia et al., 2006; Wang et al., 2007; He et al., 2009). Blocking small RNA production in \textit{ros1} releases the TGS of Pro\textsubscript{RD29A}:LUC but not of Pro\textsubscript{35S}:NPTII, suggesting that these two loci possess different TGS silencing mechanisms (Kapoor et al., 2005; Xia et al., 2006; Wang et al., 2007; He et al., 2009). Mutations in \textit{CUE1} influenced the histone H3K9 dimethylation and H3 acetylation only at some loci, suggesting that these signals produced by \textit{CUE1} mutations influence TGS independent on DNA methylation probably by modulating histone modifications in the affected loci, but did not affect global histone modifications.
The proteins such as BRU1, FAS1, FAS2, RPA2A/ROR1, ABO4/DNA Polymerase ε, TSL and MOM1 are required for maintenance of TGS at TSI independent on DNA methylation (Takeda et al., 2004; Elmayan et al., 2005; Kapoor et al., 2005; Shaked et al., 2006; Xia et al., 2006; Wang et al., 2007). Except for MOM1, all other proteins are also functioning in DNA repair as plants with mutations in these genes are sensitive to DNA damage regents (Takeda et al., 2004; Elmayan et al., 2005; Kapoor et al., 2005; Shaked et al., 2006; Xia et al., 2006; Wang et al., 2007). cue1 mutants were more resistant to MMS than the wild type. Clearly, CUE1 affects nuclear genome stability through a different new pathway. Our studies suggest that signals from chloroplasts might be essential for coordinating both gene expression and chromatin structures; such coordination may be required for appropriate short-term responses to environmental changes and possibly also for long-term, evolutionary adaptation to environmental stresses by eliciting epigenetic changes (Yin et al., 2009).

Methods

Plant materials and growth conditions

Arabidopsis seedlings were grown on MS (Sigma) medium supplemented with 3% sucrose and 0.8% agar in a growth chamber using a 16-h-light/8-h-dark cycle at 22 ºC with light intensity of 50 μmol m⁻² s⁻¹ and 70% RH. For aromatic amino acid feeding assay, seeds were sown on MS agar medium or MS agar medium supplemented with 1 mM phenylalanine, 1 mM tryptophan, 1 mM tyrosine, or all three aromatic acids (1 mM for each) together, respectively, and cultured in a growth chamber for 10 days.

Mutant screen, genetic analysis, and position cloning

Ethyl methanesulfonate mutagenesis and screening of mutants were performed as described before (Xia et al., 2006). We identified two allelic mutants that showed similar kanamycin sensitivity and exhibited reticulate leaf phenotypes. Each of the two mutants was backcrossed to rosl-1, and the F2 population showed 1:3 segregation of kanamycin resistant to sensitivity phenotype. All kanamycin resistant mutants also had the reticulate leaf phenotype, suggesting that the kanamycin resistant
phenotype was linked to the reticulate leaf phenotype. The reticulate leaf phenotype was used to isolate the mutants. The simple sequence-length polymorphisms were used to map the gene. We narrowed the mutation around CUE1/AT5G33320. The mutations were found by sequencing the full length AT5G33320 from two mutants.

**Nucleic acid isolation and gel blot analysis**
Total genomic DNA was digested by methylation-sensitive restriction enzymes HpaII and MspI. DNA methylation analysis was performed as described previously using rDNA and 180 bp-centromere DNA as probes by Southern blot analysis (Xia et al., 2006). For Northern blot analysis, 20 μg of total RNAs isolated from 2-week-old seedlings (treated or not with 100 μM ABA for 3 h) were resolved on 2% formaldehyde gels and blotted onto Hybond-N+ membranes (Amersham). NPTII, TSI, rDNA, and the 180-bp centromeric repeat were labeled with α-32P-dCTP using a random priming labeled kit (Takara) as described previously (Xia et al., 2006).

**Bisulfite sequencing**
Genomic DNA (2 μg) was digested with EcoRI, EcoRV, and HindIII. Digested DNA was used for bisulfite treatment as described previously (Wang et al., 2007). Bisulfite-treated DNA was amplified using endogenous and transgenic RD29A promoter primer pairs (Xia et al., 2006). Amplified PCR fragments were cloned into pMD18-T vector (Takara). About 15 randomly selected clones were sequenced for each genotype. The sequences of the different genotypes were compared, and the percentage of methylation at endogenous and transgenic RD29A promoters was calculated.

**Chromatin immunoprecipitation (ChIP) assay**
ChIP assays were performed using a previously described method (Xia et al., 2006). Chromatins were immunoprecipitated with anti-H3 dimethyl K9 antibodies (Upstate), and anti-H3 Acetyl antibodies (Upstate). Immunoprecipitated DNA was purified and analyzed with PCR using primer pairs TSI, LUC, 35S promoter, and ACTIN promoter as described (Xia et al., 2006). At least three independent experiments were done and showed similar results.
Methyl Methanesulphonate (MMS) treatment

Five-day-old seedlings were transferred to MS-agar medium supplemented with different concentration of MMS. Seedlings were photographed 7 days later.

Immunostaining

Preparation of plant nuclei and immunostaining experiments were performed as described before (Probst et al., 2004). Anti-lysine9-di-methylated H3 (upstate, 07-441) antibody was diluted to 1:100 and incubated with the blocked slides in the solution of 1% BSA in PBS (overnight at 4 ºC). An anti-rabbit FITC-coupled antibody was applied to detect the signals (Molecular Probes, 1:100, 37 ºC, 40 min) in 0.5% BSA in PBS, and DNA was counterstained with DAPI.

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Figure legends,

Figure 1. cue1 mutations released transcriptional gene silencing (TGS) of 35S-NPTII but did not affect the expression of RD29A-LUC or endogenous RD29A gene

(A) Seedling growth on MS-agar medium. WT: C24 wild type, which carried a T-DNA insertion locus. ros1-1: A mutation in the ROS1 gene led TGS of ProRD29A:LUC and Pro35S:NPTII in the T-DNA region as well as to TGS of the endogenous RD29A gene. cue1-101 ros1-1, cue1-102 ros1-1: two mutant alleles isolated in this study. (B) WT, ros1-1, cue1-101 ros1-1, and cue1-102 ros1-1 seedlings grown for 2 weeks on MS-agar medium supplemented with 40 mg/L Kanamycin. (C) and (D) Seedlings grown on MS-agar medium were treated with 100 μM ABA for 3 h, and then (D) the expression of ProRD29A:LUC was imaged by luminescence analysis. Luminescence was not detected in ros1-1, cue1-101 ros1-1, or cue1-102 ros1-1 but was detected in C24 wild type. (E) NPTII expression in WT (lane 1), ros1-1 (lane 2), cue1-101 ros1-1 (lane 3), cue1-102 ros1-1 (lane 4), and ddm1-101 ros1-1 (lane 5). The ddm1-101 ros1-1 mutant was isolated during our ros1 suppressor screening. rRNAs were used as loading control. (F) RD29A and LUC expression under no stress
conditions [wild type (lane 1) ros1-1 (lane 2), cue1-101 ros1-1 (lane 3)] or after 100 μM ABA treatment for 3 h [wild type (lane 4) ros1-1 (lane 5), cue1-101 ros1-1 (lane 5)]. COR47 was used as a control for ABA inducible gene expression, and Tubulin was used as a loading control.

**Figure 2. cue1 mutations released the TGS of TSI (transcriptionally silent information) but did not affect DNA methylation.**

(A) TSI expression in different plants. ACTIN was used as a loading control. cue1 single mutations tended to increase TSI transcripts more than cue1 ros1-1 double mutation. (B) The expression of TSI and NPTII gene in cue1-101 ros1-1 seedlings were partially rescued by feeding different aromatic amino acids. cue1-101 ros1-1 seedlings were grown on MS agar medium or MS agar medium containing 1 mM phenylalanine (Phe), 1 mM tryptophan (Trp), 1 mM tyrosine (Tyr), or all three aromatic acids (1 mM for each) together, respectively, for 10 days. Total RNAs were isolated and used for Northern blot with 35P-labeled NPTII or TSI probes. rRNAs were used as loading controls. (C) Bisulfite sequencing analysis of DNA methylation in endogenous RD29A promoter and transgenic RD29A promoter. No DNA methylation difference was found between ros1-1 and cue1-101 ros1-1. WT: C24 wild type. (D) DNA methylation analysis in WT, ros1-1, cue1-101 ros1-1, and cue1-102 ros1-1 at rDNA and centromeric DNA (cen-DNA) digested with HpaII or MspI.

**Figure 3. Chromatin immunoprecipitation (ChIP) analysis of TSI, 35S promoter, and ProRD29A:LUC region, and dimethylated H3K9 immunostaining in nuclei.**

(A) ChIP analysis in partial TSI as well as in LUC and 35S promoter region using specific antibodies against dimethylated H3K9 (anti-H3K9m2) and Histone H3 acetylation (anti-H3Ac). PCR amplification of ACTIN was used as controls. No antibody precipitation was used as a mock control. WT, wild type. (B) Immunostaining with anti-dimethylated H3K9 antibody and DAPI counterstaining in nuclei. No heterochromatin difference was found among wild type, ros1-1, and cue1-101 ros1-1.

**Figure 4. cue1 seedlings were more resistant to methyl methanesulphonate (MMS) treatment than wild type and ros1-1.**
Five-day-old seedlings were transferred to MS-agar medium containing different concentrations of MMS, and were photographed 7 days later. The different seedlings treated with the same concentration of MMS were grown in the same plate for comparison.