

Cell Reports

Supplemental Information

**Involvement of Multiple Gene-Silencing Pathways
in a Paramutation-like Phenomenon in *Arabidopsis***

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Figure S1

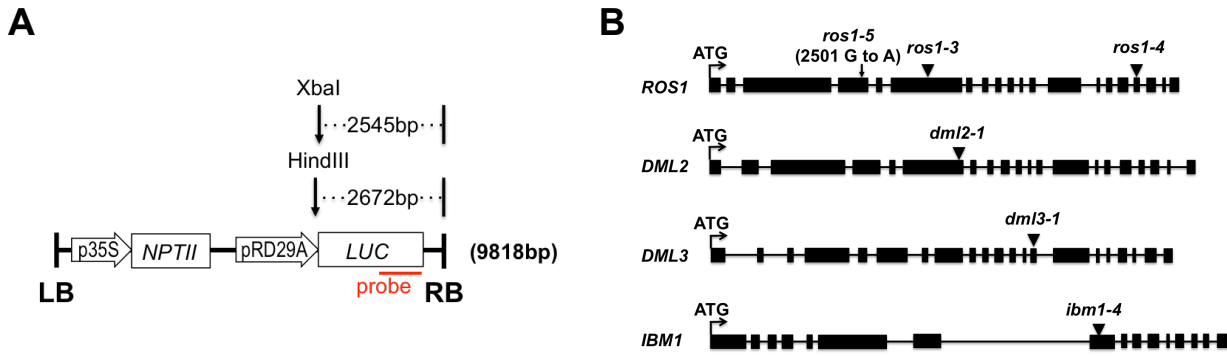


Figure S1. Information on the vector and mutants used in Figure 1. (A) Diagram showing the gene structure of T-DNA that was integrated into the Col-0 and C24 genomes. The distance between Xba I or Hind III site and the right border of the T-DNA is indicated above the diagram. The probe used for southern blotting analyses in Figure 6 is indicated by a red line below the diagram. (B) Diagram showing the gene structure and mutation sites of the mutants used in Figure 1. Point mutations are marked by an arrow and T-DNA insertions are marked by triangles.

Figure S2

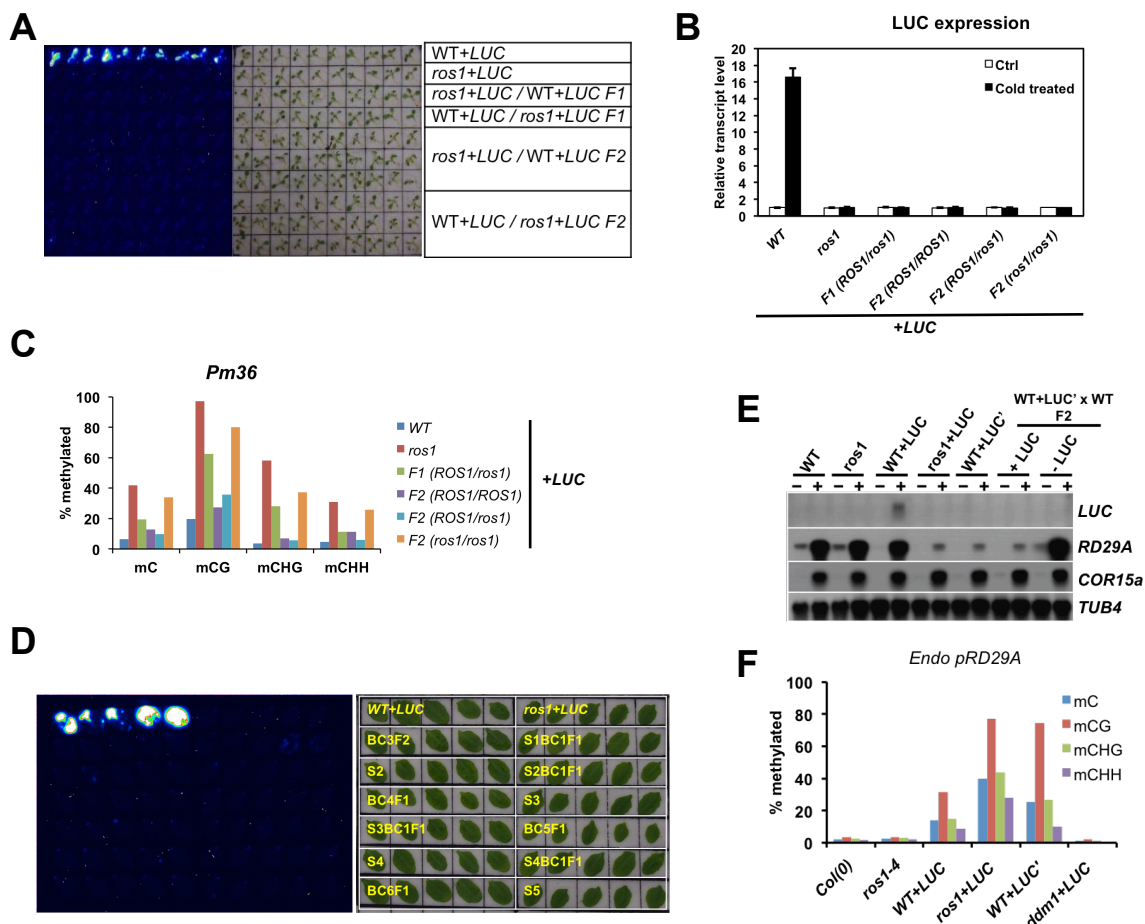


Figure S2. The *pRD29A-LUC* transgene exhibit paramutation-like phenotype while the endogenous *RD29A* gene does not, related to Figure 2. (A) Luminescence phenotype of F1 and F2 plants with indicated genotype derived from reciprocal *WT+LUC* and *ros1+LUC* crosses. Two-week-old seedlings were subjected to cold treatment (4°C, 24 hr) followed by salt treatment (2% NaCl, 4 hr) before imaging. (B) *LUC* transcript levels in the F1 and F2 plants examined by qRT-PCR. (C) *ros1-4* is a recessive mutation that positively affects DNA methylation levels at the Pm36 locus. DNA methylation levels are measured by bisulfite sequencing. (D) Luminescence phenotype of selective plants from Figure 2D & E. Genotype or the generation number of plants was indicated by yellow texts on the plants. Cauline leaves from indicated 3-week-old plants were subjected to cold treatment (4°C, 24 hr) followed by salt treatment (2% NaCl, 4 hr) before imaging. (E) Northern blotting analyses of the transgenic *pRD29A-LUC* gene and the endogenous *RD29A* gene. Two-week-old seedlings before (indicated by -) and after (indicated by +) stress treatment (4°C 48 hrs) were used for the analyses. *TUB4* and *COR15A* each serve as the loading control and the plant stress responsive control. (F) DNA methylation levels of the endogenous *RD29A* promoter in plant with the indicated genotype/phenotype as examined by bisulfite sequencing.

Figure S3

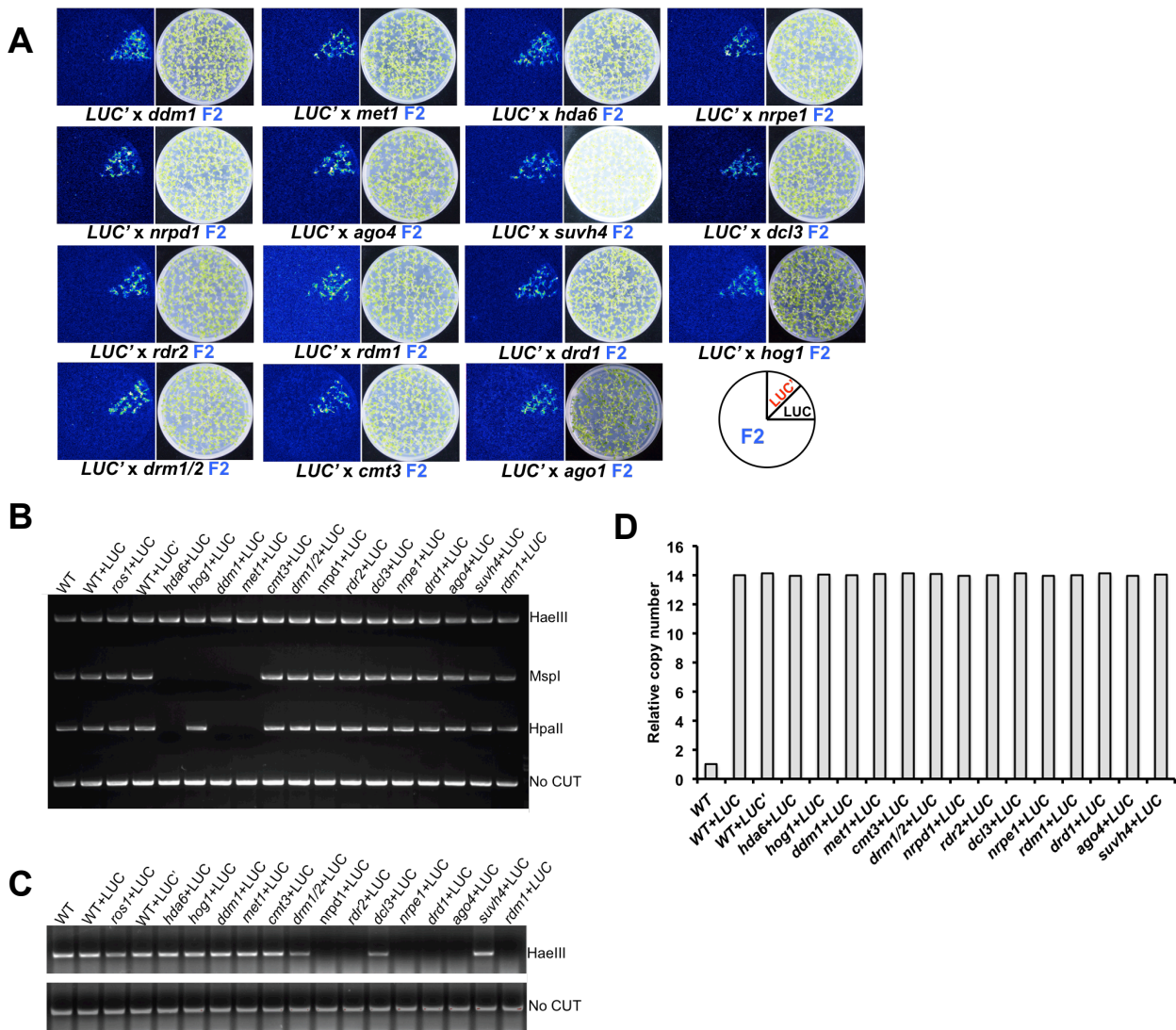


Figure S3. Characterization of the epigenetic mutants used in Figure 3. (A) Luminescence phenotype of F2 populations generated from *mutant*/WT+LUC' crosses. WT+LUC (*LUC*) and WT+LUC' (*LUC'*) each serves as positive and negative controls on the plate. (B and C) DNA methylation levels at (B) AtSN1 and (C) 5S rDNA repeats were examined by chop-PCR, confirming the phenotype of mutants used. (D) Descendants of the plants in Figure 4A all contain 13 copies of transgenic *RD29A* promoter sequences as measured by qPCR using genomic DNA as templates. The native *RD29A* promoter sequence in Col-0 (WT) plants serve as the reference of one.

Figure S4

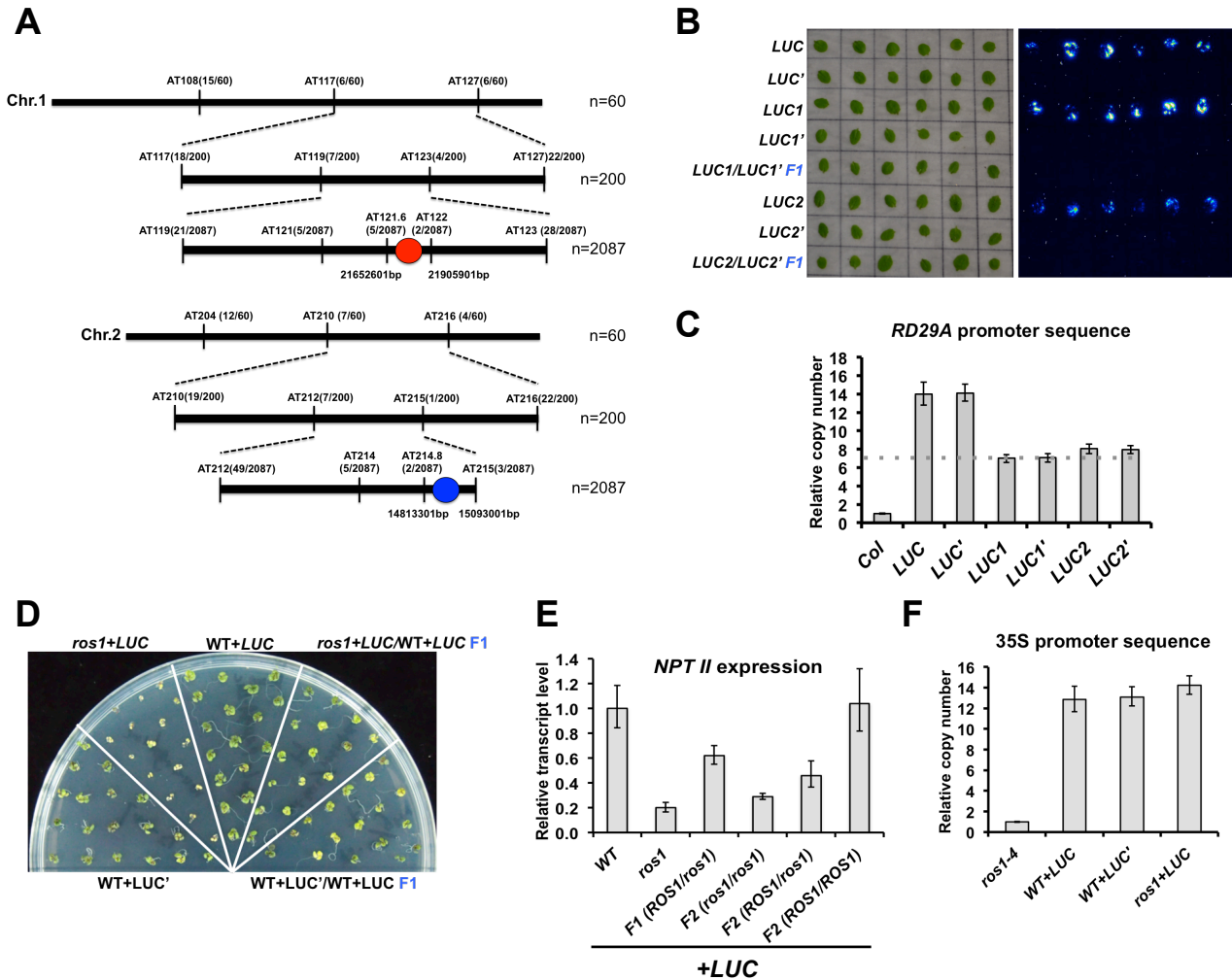


Figure S4. The *pRD29A-LUC* transgene are inserted in two locations of the genome, both exhibiting paramutation properties. The 35S promoter sequence exhibits no paramutation properties. Related to Figure 4. (A) Mapping the insertion site of the *pRD29A-LUC* transgene by using the F2 population generated from *WT+LUC'* (Col) x *WT+LUC* (C24). The two possible insertion sites of *pRD29A-LUC* are indicated by red and blue dots surrounded by specified markers. The numbers of “n” indicate numbers of individual plants used in each step of mapping. (B) Luminescence phenotype of *LUC1'* and *LUC2'* plants. Please refer to the text for the genetic processes used to generate *LUC1/LUC2* and *LUC1'/LUC2'*. Cauline leaves from indicated 3-week-old plants were subjected to cold treatment (4°C, 24 hr) followed by salt treatment (2% NaCl, 4 hr) before imaging. (C) Relative copy number of the *RD29A* promoter sequence in *LUC1* and *LUC2* plants was examined by qPCR using genomic DNA as templates. The native *RD29A* promoter sequence in Col-0 (WT) plants serve as the reference of one. The grey dashed line indicate a relative copy number of 7. (D) Kanamycin resistance phenotype of plants with indicated genotype. (E) *NPT II* transcript levels were examined using qRT-PCR in 2-week-old seedlings. (F) Relative copy number of the 35S promoter sequence in plants containing the *pRD29A-LUC* transgene. qPCR was performed using genomic DNA as templates and the *ros1-4* plant as a reference of one. (C, E and F) Error bars indicate standard deviation calculated from qPCR reactions of three technical replicates.

Table S1. List of primers and probes used in this study, related to Figure 1~4.

Table S2. List of mutants used in this study, related to Figure 1 & 3.

Mutant allele	Reference
<i>ros1-3</i>	(Penterman et al., 2007)
<i>ros1-4</i>	SALK_045303
<i>ros1-5</i>	(Lei et al., 2014)
<i>dml2-1</i>	(Penterman et al., 2007)
<i>dml3-1</i>	(Penterman et al., 2007)
<i>ibm1-1</i>	(Saze et al., 2008)
<i>ddm1-10</i>	SALK_093009
<i>met1-3</i>	(Johnson et al., 2007)
<i>hda6</i>	From Dr. Zhizhong Gong
<i>hog1</i>	SAHH1_121D7
<i>kyp-4</i>	SALK_044606
<i>cmt3-11</i>	(Henderson and Jacobsen, 2008)
<i>nrpd1-3</i>	(Onodera et al., 2005)
<i>nrpe1-11</i>	(Onodera et al., 2005)
<i>ago4</i>	SALK_071772
<i>rdm1-4</i>	(Gao et al., 2010)
<i>rdr2-2</i>	SALK_059661
<i>drd1-6</i>	(Kanno et al., 2005)
<i>dcl3-1</i>	(Xie et al., 2004)
<i>drm1/2</i>	(Henderson and Jacobsen, 2008)

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Saze, H., Shiraishi, A., Miura, A., and Kakutani, T. (2008). Control of genic DNA methylation by a jmjC domain-containing protein in Arabidopsis thaliana. *Science* 319, 462-465.

Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E., and Carrington, J.C. (2004). Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol* 2, E104.

Supplemental Experimental Procedures

Bioluminescence imaging of LUC transformed plants

Luciferase imaging was performed according to a published protocol (Chinnusamy et al., 2002). Typically two-week-old seedlings grown on ½ MS plates were subjected to cold treatment (4°C, 24 hr) followed by salt treatment (2% NaCl, 4 hr) before imaging. In the case where adult plants were used, rosette or cauline leaves were cut from individual plants and placed flat on the filter paper submerged in 2% NaCl solution for 4 hours before imaging.

DNA methylation analysis

For bisulfite sequencing, genomic DNA was extracted from 2-week-old seedlings using the Plant DNeasy Mini Kit (Qiagen). Bisulfite treatment was performed using the EpiTect Bisulfite Kit (Qiagen). For DNA methylation analyses of the transgenic RD29A promoter, bisulfite-treated DNA was amplified by nested PCR (Table S1). The 541-bp PCR fragment was cloned into pGEM-T Easy (Promega), and the ligation product was transformed into DH5α cells. At least 20 single colonies from the transformation were sequenced for each PCR product. The resulting sequences were aligned using clustalW and the methylation profile corresponding to the 343-bp RD29A promoter sequence within the PCR product was analyzed using CyMATE (<http://www.gmi.oeaw.ac.at>).

Chop-PCR was performed as previously described (He et al., 2009). In brief genomic DNA was extracted from seedlings using the Plant DNeasy Mini Kit (Qiagen). 500 ng of gDNA was digested using methylation-sensitive restriction endonucleases (HaeIII, HpaII, MspI and AluI). Equivalent amounts of digested and undigested DNA were

used as template for PCR using primers listed in the Table S1.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed according to a published protocol (Saleh et al., 2008). The anti-acetyl-histone H3 antibody (Millipore, 06-599) and anti-dimethyl-histone H3 (Lys 9) antibody (Millipore, 17-681) were used at 1:100 dilutions. After quantitative PCR, the amount of DNA in immunoprecipitated samples was normalized to the 1% input DNA sample and shown as percent input in the figure.

RNA and DNA blotting analysis

Arabidopsis seedlings grown on ½ MS plates at 23°C for 2 weeks were subjected to salt treatment (2%NaCl for 3 hours) before harvesting. Total RNA was extracted from the plants using the Trizol reagent (Life Technologies). Twenty micrograms of RNA for each sample was separated on 1.2% denaturing agarose gel, and transferred onto Hybond-N+ membranes (GE Life Sciences) for using standard northern blotting protocols. The PCR primer sequences used for generating [³²P]-labeled DNA probes are listed in Table S1.

For small RNA blotting, total RNA was extracted from floral tissues. Small RNA was enriched and following a modified northern blotting protocol as described previously (He et al., 2009a). The DNA oligos used for probe preparation are listed in Table S1.

For DNA blotting assays, 3 µg of CTAB-extracted genomic DNA was digested overnight with HindIII or SacI (NEB) and separated on 1% agarose gel. The separated

DNA was then transferred onto the Hybond-N+ membrane (GE Healthcare) and southern blotting was performed using a previously described protocol (Zheng et al., 2010). Primers used for generating [³²P]-labeled DNA probes was listed in Table S1.

Chemical Treatments

Arabidopsis seeds were surface-sterilized in 5% sodium hypochlorite (0.05% Trixton-100) for 5 min and washed with sterile water for 4 times before being plated on ½ MS medium plates or ½ MS plates supplemented with TSA (Sigma-Aldrich), ZEB (Sigma-Aldrich), and DHPA (donated by Ales Kovarik) at indicated concentrations. Plants were grown for two weeks at 23°C under long day conditions (16h light/8h dark) in the growth chamber (Percival Scientific, Inc., IA, USA).

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Reference:

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