

SUPPLEMENTARY INFORMATION

RESULTS

Sorting the sperm and vegetative cell nuclei of mature *Arabidopsis* pollen. DNA cytosine methylation represents another layer of repressive epigenetic mark for constitutive heterochromatin. To separately analyze DNA from the vegetative and sperm nuclei, we developed a method to fractionate each type of nucleus using fluorescence-activated cell-sorting (FACS) based on differences in their DNA content and granularity (**supplementary Fig 1A**) To verify the purity of each sorted nuclear population, we took advantage of a double homozygous transgenic line expressing GFP-tagged CenH3 under the control of its own promoter and RFP-tagged histone H2B driven by a vegetative nucleus-specific promoter (**supplementary Fig 1B**) to positively identify both nuclei types under the fluorescence microscope after cell sorting (**supplementary Fig 1C**). CenH3-GFP decorates centromere spots in the sperm nuclei, but not in the vegetative nucleus, which has undergone decondensation of centromeric heterochromatin, whereas H2B-RFP is expressed specifically in the vegetative nucleus in a mature pollen grain of the transgenic plant. This method allows the direct sorting of vegetative and sperm cell nuclei from various plant lines without the need to generate transgenic lines that express nucleus type-specific fluorescence tags.

Bulk *180CEN* repeats remain transcriptionally silent in pollen

To investigate whether heterochromatic silencing is maintained in the vegetative nucleus, we tested for the presence of *180CEN* transcripts in wild-type or *ddm1* seedlings and pollen using a pair of primers that amplifies bulk *180CEN* repeats (May et al, 2005). Elevated transcript levels were detected in *ddm1* seedlings as previously described and in *ddm1* pollen, whereas *180CEN* transcripts were hardly detectable in the wild-type seedlings and pollen (**supplementary Fig 3**). The results indicate that *180CEN* repeats remain silenced nearly fully in the sperm and vegetative cell nuclei of the wild-type pollen.

METHODS

Generation of a transgenic *Arabidopsis* plant carrying *pCenH3::CenH3::GFP*. A DNA fragment containing the open reading frame and 1519 bp of 5'-flanking

sequences of *HTR12* (accession#AF465800), encoding CenH3, was amplified from *Arabidopsis* genomic DNA using primers, HTR12 FWD 5'CAAACACATCAAAGCTCCTCTTCATCT3' and HTR12 REV2 5'GCCCATGGTAGCAACTGGTCTTGACCAAGGTCTGCCTTTTCCTCCAAGC3', which contains a synthetic *NcoI* site (**bold**) and 6 codons (*italicized*) encoding the linker polypeptide SRPVAT at its 5'-end. The resulting PCR product was digested with *HindIII* and *NcoI* and cloned into a GFP-expression vector psGFP(S65T) (Chiu et al, 1996). A *HindIII-EcoRI* fragment from the resulting plasmid pHT1, consisting of 1489 bp upstream of *CenH3*, *CenH3::GFP*, and nos3' terminator sequences, was then cloned into a binary expression vector pHGW0 (Plant System Biology), yielding pHT2 and used for *Agrobacterium*-mediated transformation of a wild-type (Columbia ecotype) *Arabidopsis* line. The transformed plants were selfed and the resulting seeds carrying the transgene were selected on hygromycin-containing (20µg/ml) medium. F1 transformant plants were grown on soil and selfed. F2 plants homozygous for the transgene, in which 100% of pollen showed CenH3-GFP-positive sperm cell nuclei, were verified by fluorescence microscopy.

Generation of a transgenic *Arabidopsis* plant carrying *pLAT52::H2B::RFP*. The vegetative cell-specific LAT52 promoter (Twell et al., 1989; Eady et al., 1994), Histone H2B and mRFP1 (Campbell et al., 2002) were amplified from plasmid preparations using high fidelity Phusion DNA polymerase (Finnzymes) and primers with suitable 15 bp attachment site (*attB*) adapters. Full-length *attB* adapters were added to each fragment in a second high fidelity PCR. The fragments were cloned into pDONRP4-P1R, pDONR221 and pDONRP2RP3 (Invitrogen) respectively in a Gateway BP reaction using BP Clonase II (Invitrogen) and verified by sequencing. A multipart Gateway LR reaction using LR Clonase plus (Invitrogen) and the destination vector pB7m34GW (Karimi et al., 2005) were then used to generate *pLAT52::H2B::RFP* which was transformed into wild-type *Arabidopsis* (Columbia ecotype) by *Agrobacterium*-mediated transformation. Transformed seeds were selected on soil with 30 µg/ml BASTA (glufosinate ammonium, DHAI PROCIDA) fed by sub-irrigation, and pollen from primary transformants was analysed by fluorescence microscopy. A line in which 50% of pollen showed a bright RFP signal, indicating a single insertion, was selfed and pollen of the offspring screened to identify a homozygous line.

Generation of a double homozygous transgenic *Arabidopsis* plant carrying *pCenH3::CenH3::GFP* and *pLAT52::H2B::RFP*. A plant line homozygous for both *pCenH3::CenH3::GFP* and *pLAT52::H2B::RFP* was derived from a cross between homozygous parent lines.

Harvesting *Arabidopsis* pollen. We provided pure pollen samples as described previously with modifications (Johnson-Brousseau et al, 2004). Inflorescences from approximately 1000 plants were cut and collected in a beaker. 300 ml of 9% sucrose was added and the beaker was vigorously shaken for 1 min. The pollen suspension was then filtered through a 100 μ m nylon mesh. Pollen grains were precipitated in 250 ml centrifuge tubes using a Beckman Coulter Avanti J-26XP centrifuge with the J-10 rotor (2800 rpm, 10 min, 4°C). To purify pollen, the pellet was resuspended in buffer A (1 M sorbitol, 7% ficol PM 400, 20% glycerol, 5 mM MgAc, 3 mM CaCl₂, 5 mM EGTA, 50 mM Tris-HCl pH7.5, 2% Triton X-100) and filtered through a 40 μ m nylon mesh. The supernatant was added back to the beaker containing the inflorescences, and the pollen harvesting procedure was repeated once again. The resulting two fractions of pollen suspension were pooled, concentrated in a 15 ml Falcon tube by centrifugation (800 g, 10 min, 4°C), and then precipitated in a 1.5 ml centrifuge tube (7300 rpm, 5 min). The pollen pellet was resuspended in approximately 4 times the volume of buffer A.

Preparation of crude pollen nuclei. The rigid cell wall of mature pollen grains complicates cytological studies on pollen nuclei. To overcome this problem, we established conditions under which vortexing in the presence of glass beads disrupts pollen grains but keeps the nuclei intact. 50 μ l aliquots of pollen suspension were loaded onto 1.8 g of acid-washed glass beads (0.4-0.6 mm, Sartorius) in 2 ml centrifuge tubes, and subjected to vortexing using a Retsch MM301 bead-beater for 1.5 min at a frequency of 15 Hz. Subsequently, holes were cut at the bottom of the tubes using a needle (\varnothing 0.45 mm), and the crude free pollen nuclei were collected in 1.5 ml centrifuge tubes by centrifugation (800 g, 10 min, 4°C). The pollen nuclei suspensions were pooled, and a DAPI-stained aliquot was inspected by fluorescence microscopy to evaluate the efficiency of pollen extraction and the quality of the nuclei.

Slide preparation of pollen nuclei. Pollen nuclei were fixed in 3.7% formaldehyde for 20 minutes. 5 μ l of the fixed nuclei were dropped onto a poly-L-lysine-coated slide for immunostaining or onto a sulfocromic solution-washed slide for FISH. The

nuclei were then squashed with a cover slip, frozen in liquid nitrogen and washed in 96% EtOH for 2 minutes, followed by air dry for FISH or 3 x 5 min washes in 1x PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄) for immunostaining.

Immunostaining. The following antibodies were used in this study: anti-bulk histone H3, 1:100 (Abcam: ab-1791); anti-CenH3, 1:500 (Abcam: ab-72001); anti-H3K9me₂, 1: 75 (Abcam: ab-1220); anti-H3K27me, 1:250 (gift from T. Jenuwein); anti-rabbit-DyLight547, 1:200 (Pierce: 31020); anti mouse-FITC, 1:200 (Abcam: ab-6785).

Immunostaining was performed according to Soppe et al, 2002, except that before antibody reaction, slides were incubated in 1x DAKO Target Retrieval Solution (DAKO, S1699) for 15 minutes at 97-99°C and then immediately dipped into a jar containing 1x PBS at room temperature. For double immunostaining, the slides were incubated simultaneously with two different primary antibodies and separately with different secondary antibodies (1 h each, washed in between with 1x PBST 3 x 5 min). Finally, the slides were mounted in 10 µl of Vectashield with DAPI (2 µg/ml) and analysed by Zeiss Axio Imager M1 and MetaVue Version 7.0 software.

Fluorescence *in situ* hybridisation (FISH). Slide pre-treatment, hybridisation and subsequent washes were performed as described previously (Pedrosa A. et al 2002). The slides were analysed as described above for immunostaining. The DNA probe used was pAL1, a cloned fragment of the centromeric repeats (Martinez-Zapater et al, 1986). Probes were labelled with Cy3-dUTP using a nick-translation mix (Roche) according to the manufacture's protocol.

Fractionation of the vegetative and sperm nuclei by FACS. The free pollen nuclei provided above were diluted with an equal volume of buffer B (15 mM Tris-HCl pH7.5, 2 mM Na₂-EDTA, 0.5 mM spermine·4 HCl, 80 mM KCl, 20 mM NaCl, 2% Triton X-100). The sample was filtered through a 35 µm nylon mesh and aliquoted into 3 FACS tubes. To each tube, 5 µl of SYBR Green I (Roche 11988131001) or 1 µl of propidium iodide was added. We sorted the vegetative and sperm cell nuclei using FACSAria (Becton & Dickinson), based on differences in their granularity and DNA content (supplementary Fig. 1A). The sperm- and vegetative-nuclear fractions were collected, digested with proteinase K, and DNA was purified by phenol extraction. DNA samples from several nucleus sorting experiments were pooled and concentrated with Amicon Ultra-4 (Ultracel-50k) centrifugal filter devices (Millipore).

Bisulphite genomic sequencing analysis of cytosine methylation. Genomic DNA,

extracted from the sorted pollen nuclei or seedlings, was treated with bisulphite using the EpiTect bisulfite kit (Qiagen). A single unique allele of the *180CEN* repeat and the *Athila* sequence was amplified by PCR from the bisulphite-treated genomic DNA. PCR products were cloned into the pGEMT easy vector (Promega), sequenced and analysed by CyMATE (Hetzl et al, 2007). The following primer pairs and thermo-cycling conditions were used for bisulphite genomic sequencing experiments:

| analysed site | oligos | cycling protocols |
|----------------------------------|---------------------------------------|---|
| <i>DDMI</i> , top strand | F: GAYGGGAAAAYGGAGAAAGATGY | 94°C 5', 35 x (94°C 30", 52°C 30", 72°C 1'), 72°C 10' |
| | R: CTTTTTRRCTCTCACTTTCTATCCCAT | |
| <i>180CEN</i> , top strand | F: ACTCCCACTCATRTATTTCTATCATARCR | 94°C 5', 35 x (94°C 30", 52°C 30", 72°C 1'), 72°C 10' |
| | R: GTTATYTGTYYTAAAAGATAATAGTGTTY | |
| <i>Athila</i> , bottom strand | F: TTTCTCRTCTATCTTATTTCTCTRTTTTATCRT | 94°C 5', 35 x (94°C 30", 56°C 30", 72°C 1'), 72°C 10' |
| | R: GATGGTGATAGTAGGTTTTTGTAGTYAATTATYY | |

Southern hybridisation analysis of cytosine methylation. Southern hybridisations were carried out according to standard methods (Sambrook & Russell, 2001). DNA was isolated from the sorted vegetative and the sperm nuclei (see above) or seedlings using the PHYTOPURE DNA extraction kit, GE Healthcare and digested with *HpaII*, *MspI*, *NlaIII* or *CviAII*. To verify that the digestions were complete, digested DNA was subjected to PCR to amplify a region of the unmethylated *Arabidopsis DDMI* gene, which contains *MspI/HpaII* and *CviAII/NlaIII* sites, using the primers, *DDMI(MspI, NlaIII)*-F 5'TCCACCTTTCCTTTTCATTTTCGTTATTT3' and *DDMI(MspI, NlaIII)*-R 5'GAGCAGCTGCTCTTCCTCCTGAGC3'. 10 ng of the DNA digests were separated on 1% agarose gels, and transferred to a membrane (Zeta-Probe Blotting Membranes, Bio-Rad) by standard capillary transfer (Sambrook J.). A probe for 180-bp centromeric repeats was generated by PCR with the primers, pAL-U 5'AGTCTTTGGCTTTGTGTCTT3' and pAL-R 5'TGGACTTTGGCTACACCATG3' and labelled using the Amersham Gene Images AlkPhos Direct labelling and detection system (GE-Healthcare). Probe hybridisation and washes were performed according to the manufacture's instructions. Probe detection was carried out using CPD-StarTM Detection Reagent (GE-Healthcare) with subsequent exposure to X-ray film (Amersham HyperfilmTM ECL).

Transcript analysis. RNAs were isolated from mature pollen or seedlings using peqGOLD RNAPure (PEQLAB). DNA contaminants were removed by DNase I

(Roche). Reverse transcription (RT) was carried out with 1 µg of RNA using RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas). Negative controls were performed in the absence of RT. Transcripts were semi-quantified by PCR. The primers used for reverse transcription (RT) of *UBIQUITIN10* (AT4G05320), *PECTINESTERASE* (AT5G07430) and 180-bp centromeric repeats (cen180) (May et al, 2005) were:

| | |
|-------------------------|--------------------------------------|
| <i>UBIQUITIN10</i> R | 5'CGACTTGTCATTAGAAAGAAAGAGATAACAGG3' |
| <i>PECTINESTERASE</i> R | 5'GTTGCCCTTGTCGTCACAGAGAGTA3' |
| cen180Fc | 5'GGTTAGTGTTTTGGAGTCGAATATG3' |
| cen180Rc | 5'TTGCTTCTCAAAGATTTTCATGGT3' |

Negative controls for detecting contaminated DNA lacked RTase. The primers used for semi-quantitative PCR amplification were the same as the primers used for RT as described above and the following corresponding primers:

| | |
|-------------------------|------------------------------------|
| <i>UBIQUITIN10</i> F | 5'GATCTTTGCCGAAAACAATTGGAGGATGGT3' |
| <i>PECTINESTERASE</i> F | 5'TGCGGAAACGGTCTTGACTIONAC3' |

The thermo-cycling conditions were: 96°C 3 min, 30 x (96°C 20", 60°C 20", 72°C 1 min), 72°C 3 min. PCR products were analysed on a 1.5% agarose gel.

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SUPPLEMENTARY FIGURE LEGENDS

Supplementary Fig. 1: Sorting the sperm and vegetative cell nuclei of mature *Arabidopsis* pollen. (A) Sybrgreen-activated cell-sorting of pollen nuclei from wild-type. The sperm and the vegetative cell nuclei were sorted according to the difference in their DNA content (FITC-channel) and in nuclei granularity (side scatter) as displayed in the dot plot. Nuclear populations within the gates (indicated in box) were collected. (B) Fluorescence microscopy on a mature pollen grain from a transgenic *Arabidopsis* line carrying *pCenH3::CenH3::GFP* and *pLAT52::H2B::RFP*. CenH3-GFP decorates five centromeric foci in the sperm cell nuclei, but not in the vegetative cell nucleus in which CenH3 chromatin is disrupted, whereas H2B-RFP is expressed preferentially in the vegetative cell nucleus. (C) Validation of the identity of each FACS-sorted nuclear cloud using a fluorescence microscope. We determined the purity of both nuclear clouds (left: the sperm cell nuclei, right: the vegetative cell nuclei) to be >95%.

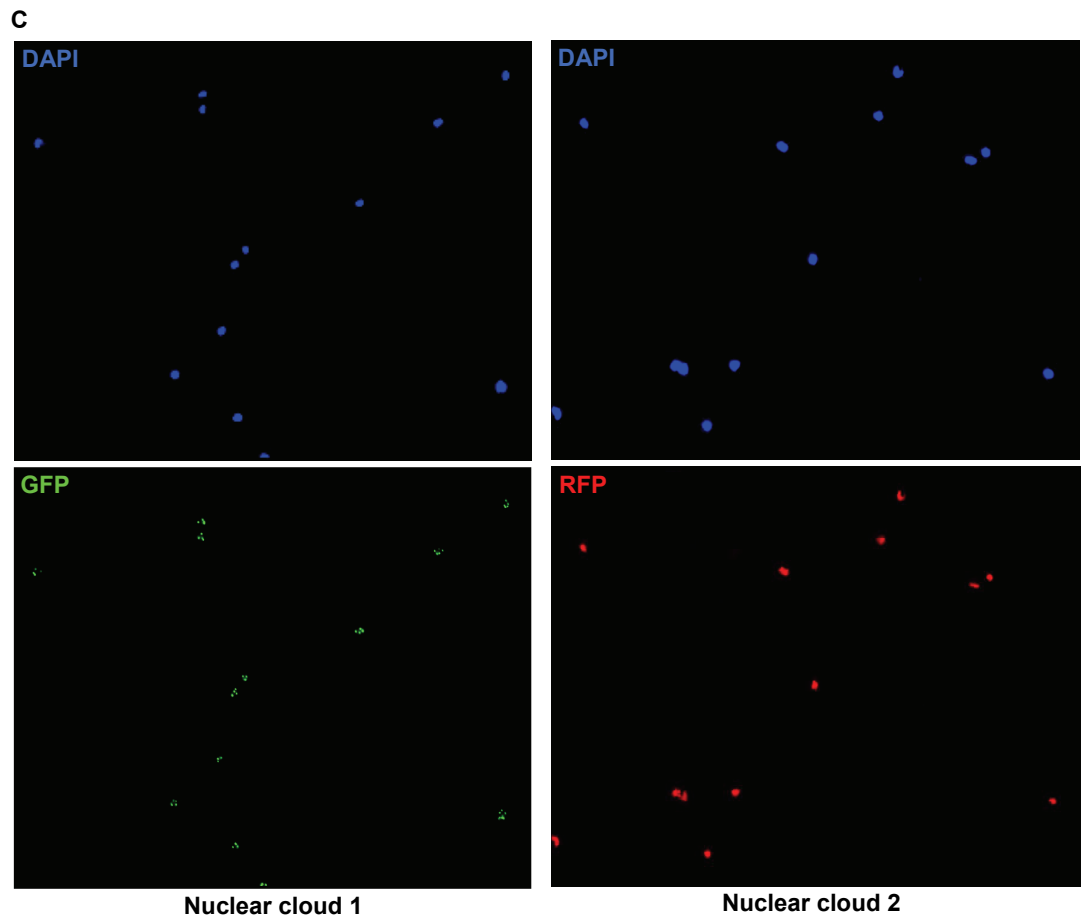
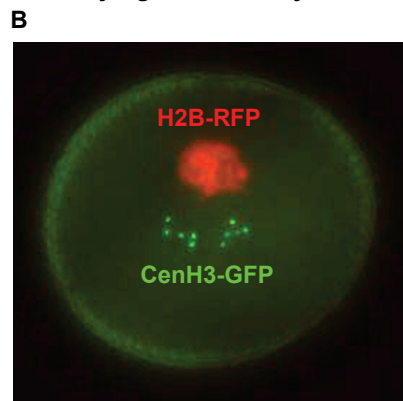
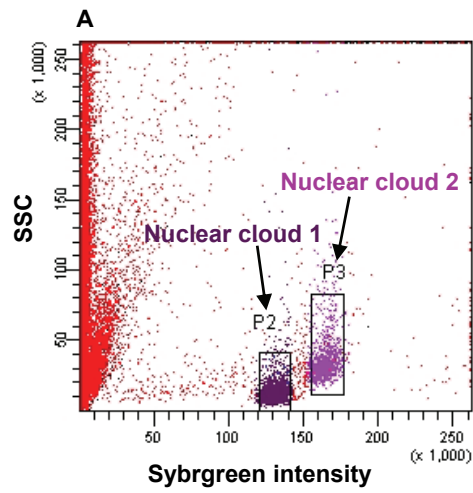
Supplementary Fig. 2: (A) FISH image of vegetative (VN) cell nuclei of wild-type and *drm1 drm2 Arabidopsis* (WS) mature pollen with a probe for *180CEN* repeats. Nuclei were counterstained with DAPI. ~100 vegetative nuclei were examined and all of them showed extensively dispersed FISH signals. (B, C) Bisulphite sequencing analysis of cytosine methylation at a single unique allele of the *180CEN* repeat and of the *Athila* region from pollen nuclei of wild-type and *drm1 drm2 Arabidopsis* (WS) plants. The graphs show the percent methylation (%^mC) at individual cytosines in the *180CEN* (left) and *Athila* (right) sequences from the sorted pollen vegetative (VN, top) and sperm (SN, bottom) nuclei of (B) wild-type and (C) *drm1 drm2* plants. Black lines: CG methylation; blue lines: CHG methylation; red lines: CHH methylation (where H is A, T or C). We define “hypermethylated non-CG sites in the vegetative nucleus in pollen” as the CHH or CHG sites at which more cloned sequences from vegetative nuclei show methylation than those from sperm nuclei. Red and blue arrows indicate the CHH and CHG sites, respectively, which show loss of hypermethylation in mutant in comparison to wild-type vegetative nuclei. Red and blue asterisks mark the CHH and CHG sites, respectively, showing more than 50% reduction in %^mC in mutant in comparison to wild-type vegetative nuclei. Cytosine

positions in the amplified regions are indicated by numbers. The results are from 10 cloned sequences. Original data are shown in supplementary Fig. 4.

Supplementary Fig. 3: RT-PCR analysis of *180CEN* repeat transcripts in wild-type or *ddm1 Arabidopsis* (Col-0) seedlings and mature pollen. Reverse transcription (RT) was performed using the forward (**Fc**) or reverse (**Rc**) primer, which allows strand-specific amplification of bulk *180CEN* repeats (May et al, 2005). Each RT reaction mixture also contained primers for synthesizing cDNA of the ubiquitously transcribed *UBIQUITIN10* (AT4G05320) and the pollen up-regulated *PECTINESTERASE* (AT5G07430) as positive controls. The resultant cDNA was subjected to semi-quantitative PCR. Negative controls for testing DNA contamination lacked RT (**-RT**).

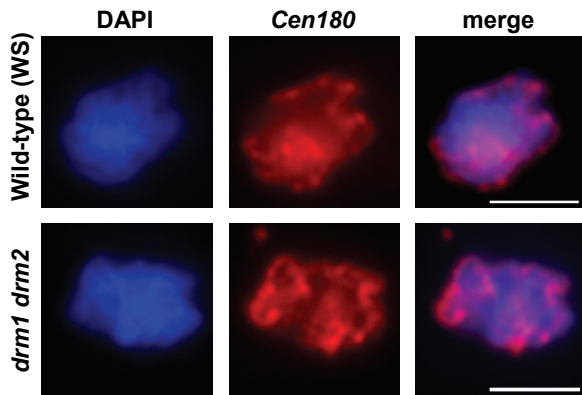
Supplementary Fig. 4: Original bisulphite sequencing data. The sequences of a single unique allele of the *180CEN* repeat (**A**) and the *Athila* (**B**) region were amplified by PCR from bisulphite-treated genomic DNA, cloned and sequenced. Sequences of individual PCR clones are shown using CyMATE (Hetzl et al, 2007). **wt_Se**: wild-type (Col-0) seedlings; **nrpd1_Sp**: *nrpd1* sperm nuclei; **nrpd1_Ve**: *nrpd1* vegetative nuclei; **nrpe1_Sp**: *nrpe1* sperm nuclei; **nrpe1_Ve**: *nrpe1* vegetative nuclei; **drm2_Sp**: *drm2* sperm nuclei; **drm2_Ve**: *drm2* vegetative nuclei; **cmt3_Sp**: *cmt3* sperm nuclei; **cmt3_Ve**: *cmt3* vegetative nuclei; **Ws_Sp**: wild-type (WS) sperm nuclei; **Ws_Ve**: wild-type (WS) vegetative nuclei; **drm_Sp**: *drm1 drm2* sperm nuclei; **drm_Ve**: *drm1 drm2* vegetative nuclei. CG, CHG and CHH sequence contexts are marked by **bead**, **rectangle** and **triangle** symbols, respectively. Methylated and unmethylated cytosine residues are represented by filled and open symbols, respectively. Cytosine positions in the amplified regions are indicated by numbers on the bottom.

Supplementary Figure 1

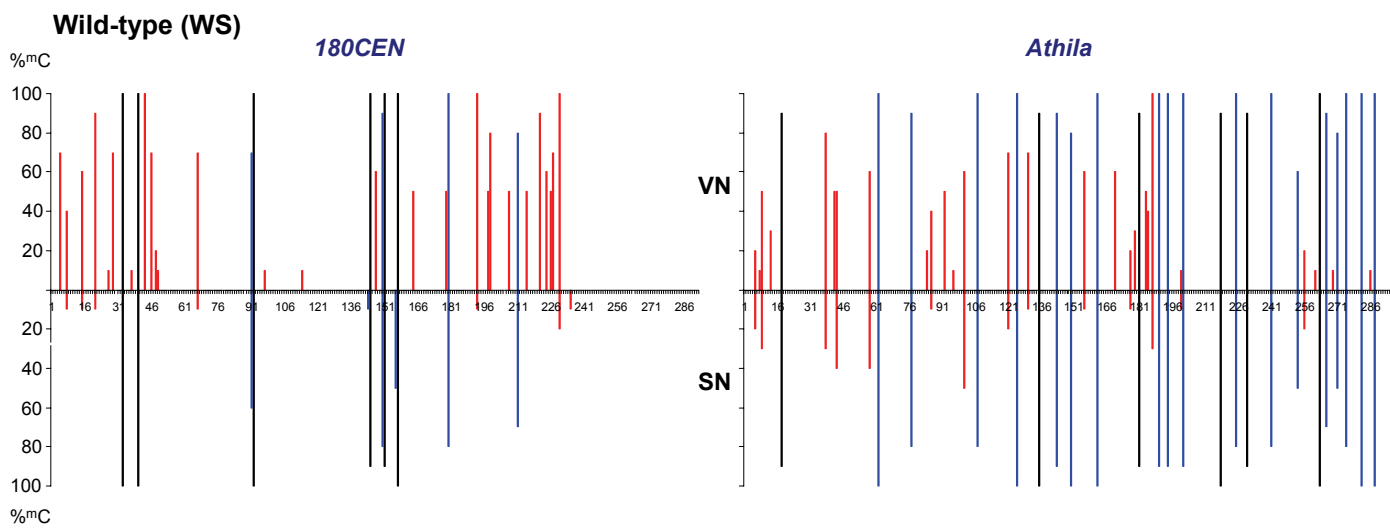


Supplementary Figure 2

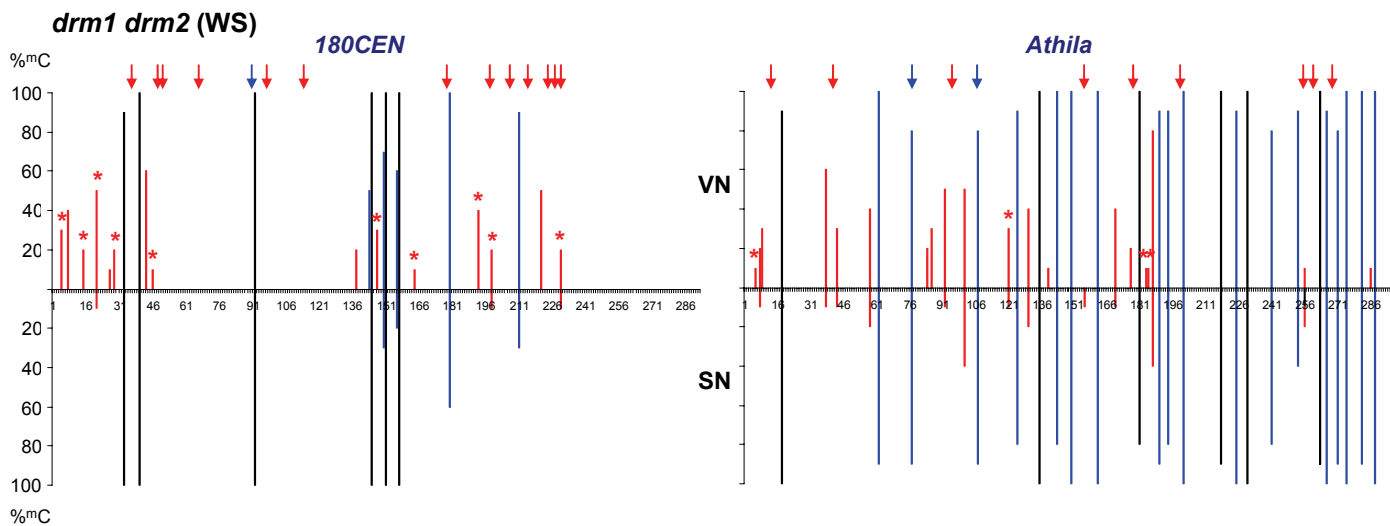
A



B



C



Black lines: CG methylation; Blue lines: CHG methylation; Red lines: CHH methylation

Supplementary Figure 3

