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LEAF EPIDERMAL PROFILING AS A PHENOTYPING TOOL FOR DNA METHYLATION MUTANTS

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Summary: Phenotypic evaluation of epigenetic mutants is mainly based on the analysis of plant growth and morphological features. However, there are cellular level changes that are not visible to the naked eye and require analysis with higher resolution techniques.
In this study, we carried out a phenotypic characterisation of several *Arabidopsis thaliana* hypomethylation mutants by quantitative image analysis combined with flow cytometry. This phenotyping approach permitted identification of abnormalities at the cellular level in mutants with wild-type morphology at the organ level. Morphometry of adaxial leaf epidermis revealed variations in the size and number of pavement cells, and the density and distribution of stomata in the analysed second rosette leaves from the mutants studied. A direct correlation between DNA ploidy status and leaf pavement cell size in wild type and mutant leaves was observed. Recognition of hidden phenotypic variations could facilitate the identification of key genetic loci underlying the phenotypes caused by modifications of DNA methylation. Thus, this study outlines an easy and fast phenotyping strategy that can be used as a reliable tool for characterisation of epigenetic mutants at the cellular level.

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Abbreviations: CMT2 – CHROMOMETHYLASE 2; CMT3 – CHROMOMETHYLASE 3; DDM1 - DECREASE IN DNA METHYLATION; *DIC* - differential interference contrast; DRM1 - DOMAINS REARRANGED METHYLTRANSFERASES 1; DRM2 - DOMAINS REARRANGED METHYLTRANSFERASES 2; MET1 - METHYLTRANSFERASE 1; RdDM - RNA-directed DNA methylation.

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INTRODUCTION

Evaluation of plant growth is considered to be one of the main ways to rapidly screen and evaluate different mutants and responses to stress factors. It provides information on the plant status and enables following of the integrated response at the whole-plant level. However, this type of characterisation is not always accurate and sufficiently reproducible, and provides limited insight into potential mechanisms underlying the phenotypic differences. Molecular profiling techniques are another popular approach to identify specific aspects of the phenotypes associated with genetic and epigenetic modifications. Although very powerful, this approach restricts our understanding to the molecular level, showing rather indirectly which contribution a particular mutation makes with regard to altered plant morphology. A better understanding of the basis of phenotypic variations could be provided by a combination of growth and molecular analyses with studies at the cellular level. This approach integrates molecular-level regulation to the tissue, organ and whole-plant level (Nelissen et al. 2013).

In plant epigenetics, most of the phenotypic differences are categorised on the basis of a comparison of morphological features (Migicovsky et al. 2014; Virdi et al. 2015). Disruption of the DNA methylation machinery may affect growth, bolting rate, phenology and phenotypic plasticity of the model plant *Arabidopsis thaliana* (Bossdorf et al. 2010; Chan et al. 2006; Saze et al. 2003). However, many cellular level alterations are not visible to the naked eye and require analysis using special techniques with a higher resolution. Cell-scale analyses are often focused on the epidermal layer because it is more accessible and considered to be the major growth-driving tissue layer (Savaldi-Goldstein et al. 2007; Dhondt et al. 2014). Leaf epidermis affects cell division rate and plays an important role in regulating organ size (Marcotrigiano 2010).

The *Arabidopsis* genome is commonly methylated at cytosine bases in three sequence contexts, CG, CHG, and CHH (where H = A, T, or C). CG methylation is maintained by METHYLTRANSFERASE 1 (MET1), symmetrical DNA methylation in the CHG context is maintained by CHROMOMETHYLASE3 (CMT3), and *de novo* DOMAINS REARRANGED METHYLTRANSFERASES 1 (DRM1) and 2 (DRM2) are responsible for CHH methylation through the RNA-directed DNA methylation (RdDM) pathway. CHROMOMETHYLASE2 (CMT2) methylates both CHG and CHH sites at targets that are regulated by H3K9 methylation in *Arabidopsis* (Stroud et al. 2014). It has been shown that the chromatin remodeler DECREASE IN DNA METHYLATION 1 (DDM1) ensures to some extent access for MET1, CMT3 and CMT2 to heterochromatin (Zemach et al. 2013). Manipulation of these methylation systems in *Arabidopsis* leads to morphological abnormalities because key genes that regulate plant development are misregulated.

In this study, we performed a phenotypic characterisation of several hypomethylation mutants of *A. thaliana*, combining image analysis with flow cytometry approaches. Some of these mutants are known to display a wild-type phenotype at the whole-plant level (Bartee et al. 2001; Ito et al. 2015). By quantitative

phenotyping of the mutant rosette leaves, we determined specific changes in the size and number of adaxial epidermal (pavement) cells and stomata that could not be seen unaided. Pavement cells are the most abundant epidermal cell type, typically showing endopolyploidy (Ramsay and Glover 2005). In *Arabidopsis*, ploidy levels range from 2C to 64C (Melaragno et al. 1993; Barow 2006). We determined the DNA ploidy pattern of the wild type and mutant leaves, and found consistency between the observed variations in the leaf morphology and ploidy levels.

MATERIAL AND METHODS

Plant material

Seeds of *Arabidopsis thaliana* ecotype Columbia (Col-0) and the homozygous methylation mutants in Col-0 ecotype background: *met1-1* (Kankel et al. 2003), *ddm1-10* (SALK_0930095), *cmt3* (Lindroth et al. 2001), *cmt2* (SALK_012874C) and *drm2-2* (SALK_150863), were sterilised for 2 min in 70% ethanol and 15 minutes in 30% sodium hypochlorite (NaOCl) in 1.5 ml Eppendorf tubes, then plated onto ½ MS medium (Murashige and Skoog 1962), solidified with 8 g/L plant tissue culture agar. After a stratification period of 48 h in the dark at 4°C, the plates were transferred into a growth chamber under continuous light (light intensity 250 μmol m^{-2} s^{-1}) at 21°C. For leaf phenotypic analyses, 7-day-old seedlings were transferred into round plates (Greiner Labortechnik) with ½ MS medium and regularly spaced about 2.0 cm apart. After two weeks, the fully developed second rosette leaves were used for clearing and assessment of ploidy level.

Leaf size and cell morphology

Digital images of the second rosette leaves from 21-d-old plants were taken by a Stereo microscope BMS 140 Bino Zoom (http://www.breukhoven.nl). For the preparation of microscopy slides, the leaves were collected in 2 mL Eppendorf tubes and incubated in absolute ethanol for at least 48 hours to clear off chlorophyll, then transferred into 1.25M NaOH : EtOH (1 : 1, v/v) solution for 2 h at 60°C, and finally mounted in lactic acid (Acros Organics) on microscopic slides with the adaxial side upwards. The samples were photographed with with a HIGH CONTRAST DIC and XC50 digital microscope camera connected to an Olympus BX51 upright microscope.

Morphometric analysis

The leaf blade area was measured using the image processing software ImageJ 1.48 (National Institutes of Health, Bethesda, USA). Microscopic examination of adaxial epidermal cells was carried out in the middle region of the leaf blade and approximately midway between the leaf midvein and margin. Size and number of individual pavement cells and number of stomata were analysed by the imaging software Cell B (Olympus, Germany). Rosette leaves from at least 35 plants per mutant line and Col-0 control were examined to determine the consistency of leaf epidermal features, and three independent experiments were performed.

DNA ploidy analysis

Assessment of nuclear DNA content was performed using flow cytometry. To release cell nuclei, leaves were chopped with a sharp razor blade in 200 mL of Cystain UV Precise P Nuclei extraction

buffer (Partec), then stained with 800 mL of staining buffer. The mix was filtered through a 50-mm green filter and read through the CyflowMB flow cytometer (Partec). The data were analyzed with the Cyflogic v.1.2.1 software (CyFlo, Turku, Finland). Rosette leaves from at least twenty plants per mutant line and Col-0 control were analysed and three independent experiments were performed.

Statistical analysis

Data were evaluated by analysis of variance (ANOVA) using STATGRAPHICS PLUS 5.1 software (Statistical Graphics, Warrenton, VA). A P-value of less than 0.05 was considered statistically significant.

RESULTS

Wild-type *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) plants and mutants that are deficient in key enzymes involved in DNA methylation were characterized for phenotypic differences by analyzing leaf size and epidermal cell morphology (Fig.1A-D). A detailed analysis was performed on the second

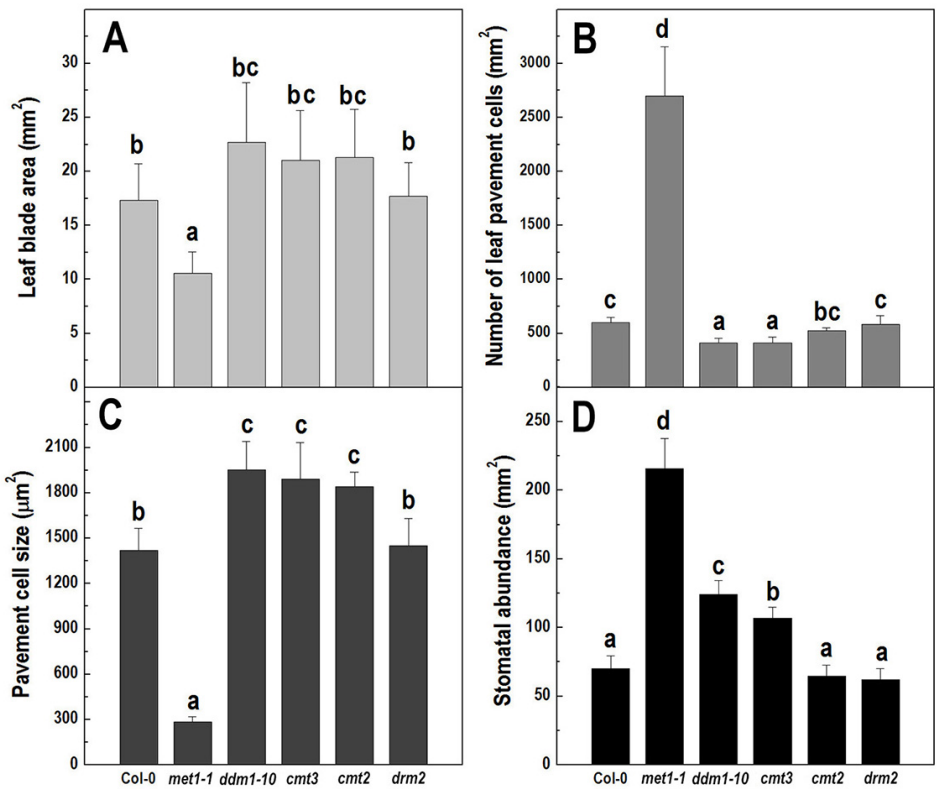

Figure 1. Quantification of morphology of the second rosette leaves of the *Arabidopsis thaliana* wild type Col-0, and the hypomethylation mutants *met1-1*, *ddm1-10*, *cmt3*, *cmt2* and *drm2*: (**A**) leaf blade area (mm^2); (**B**) number of leaf pavement cells per mm^2; (**C**) size of leaf pavement cells per µm^2; (**D**) stomatal abundance in the adaxial epidermis per mm^2. Rosette leaves from at least 35 plants per mutant line and control were examined and three independent experiments were performed. Data represent average ± standard error (SE). Different letters indicate statistically significant differences ($P < 0.05$) for multiple comparisons.

rosette leaf, which we consider as a representative of the adult vegetative phase (Asl et al. 2011). Reduced levels of DNA methylation in *met1-1* plants resulted in significantly smaller rosette leaves compared to other methylation mutants and the wild type (Fig. 1A). Rosette leaves of *ddm1-10*, *cmt2* and *cmt3* mutants had a slightly larger size but the difference was not statistically significant when compared to the wild type Col-0. *drm2* mutant leaves did not show any deviations from the wild type.

To gain cellular-level insights into the changed leaf size, the adaxial epidermis of the second rosette leaf of the methylation mutants was examined using differential interference contrast (DIC) microscopy. Representative images of leaf epidermal morphology are shown in Fig. 2A-D. Apart from *met1-1* (Fig. 2B), the leaves of Col-0 (*Fig. 2A)* and other methylation mutants *(Fig. 2C, D)* had pavement cells with the characteristic *Arabidopsis* jigsaw puzzle cell shape *(*Guerriero et al. 2014). In the *met1-1* mutant, these cells were of highly variable shapes and alignments (Fig. 2B). The average number of *met1-1* epidermal cells (per mm^2) was approximately 4.5 times higher, but their size was 5.0 times reduced, compared to Col-0 (Fig. 1B). This mutant also differed from the wild type in stomatal density and distribution. Stomata in *met1-1* leaves were 3.0 times more abundant than in Col-0 leaves (Fig. 1D). The mutants *ddm1-10*, *cmt3* and *cmt2* developed fewer but larger

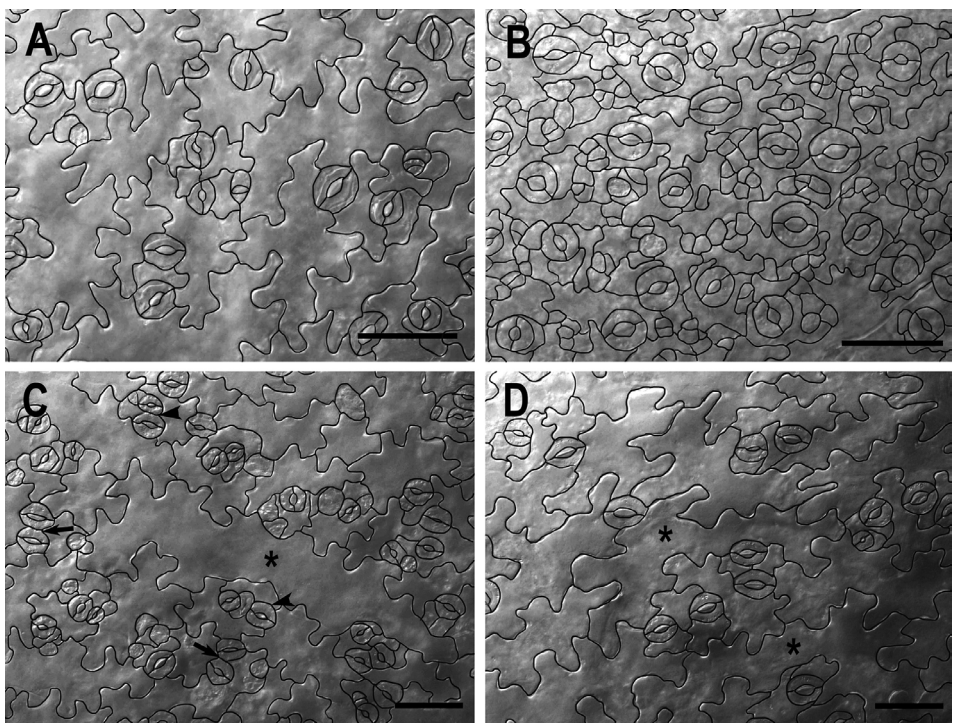

Figure 2. Representative DIC images of adaxial epidermis from the second rosette leaves of 21-d-old plants: (**A**) Col-0; (**B**) *met1-1*; (**C**) *ddm1-10*; (**D**) *cmt3*. Labels: **asterisks**, polyploid cells; **arrowheads**, stomatal clusters; **arrows**, paired stomata. Scale bar = 50 μm.

pavement cells (Fig. 1B, C). In *ddm1-10*, giant pavement cells could frequently be seen (Fig. 2C). Mutations in *DDM1* and *CMT3* resulted in a 1.8-fold and 1.5-fold increase in stomatal density in rosette leaves, respectively, compared to the control (Fig. 1D). It should also be noted that leaves of *ddm1-10* and *met1-1* displayed an abnormal stomatal patterning, as manifested by the frequent presence of a number of closely located meristemoids in *met1-1* (Fig. 3A-C), and paired and clustered stomata in *ddm1-10* (Fig. 3D-F).

The effects of aberrant DNA methylation on leaf cell morphology of methylation mutants were compared with the wild-type by measuring the DNA ploidy level in leaves using flow cytometry analysis (Fig. 4). In *met1-1,* over 80% of the leaf cells had ploidy levels of 2C and 4C, whereas cells with 32C ploidy were not detected. By contrast, loss of *DDM1* and *CMT3* gene functions increased the number of polyploidy cells. Proportions of 32C cells in rosette leaves of *ddm1-10* were 11%, and in *cmt3* about 8%, as compared to 2% for Col-0. In the rosette leaves of *cmt2* and *drm2*, overall DNA ploidy patterns were not significantly different, compared to Col-0.

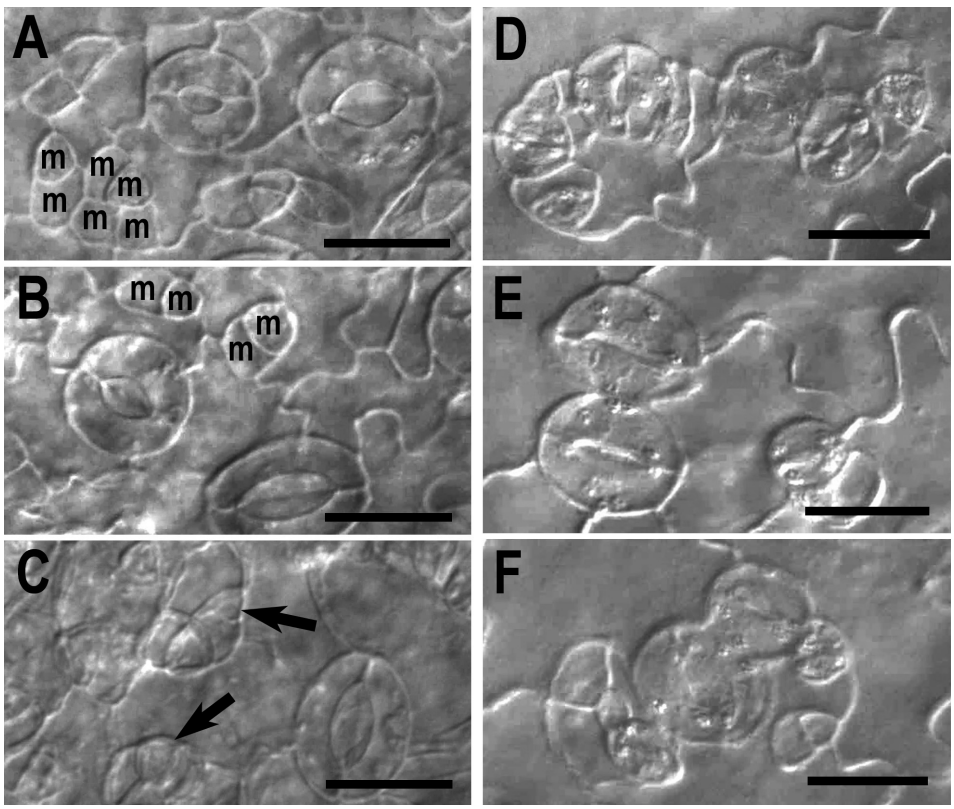

Figure 3. Aberrant stomatal patterning in adaxial rosette leaf epidermis of *met1-1* (**A-C**) and *ddm1-10* (**D-F**): (**A**) closely located meristemoids (**m**); (**B**) misplaced satellite meristemoids (**m**); (**C**) abnormal meristemoid divisions (**arrows**); (**D**) stomatal clusters in *ddm1-10*; (**E**) paired stomata in *ddm1-10*; (**F**) clustered and paired stomata in *ddm1-10*. Scale bar = 20 μm.

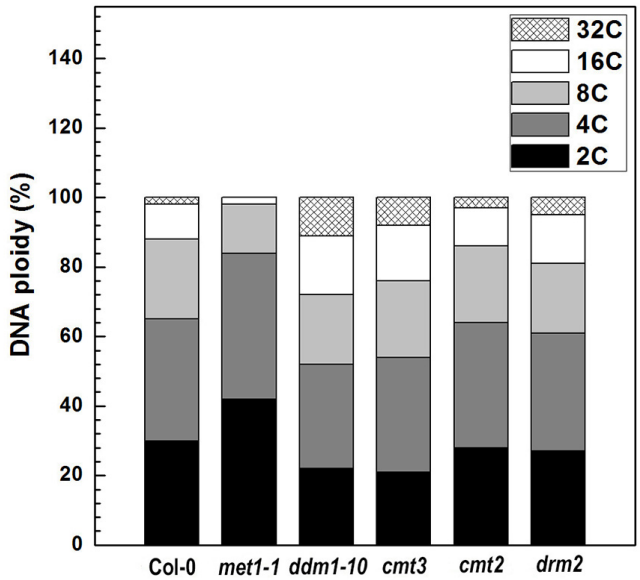

Figure 4. DNA ploidy levels of the second rosette leaves of the *A. thaliana* wild-type (Col-0) and the hypomethylation mutants *met1-1*, *ddm1-10*, *cmt3*, *cmt2* and *drm2*. The values represent average percentage of the observed ploidy levels of at least twenty biological repeats from three independent experiments.

DISCUSSION

Manipulation of DNA methylation levels can have phenotypic effects at the molecular, cellular, tissue, organ and organism levels. Current assessments of phenotypic variation of different methylation mutants rely mostly on visible changes in plant morphology and molecular identification of target genes. In the *met1-1* mutant, carrying a point mutation in the *MET1* gene, DNA methylation levels are highly reduced and plants display severe developmental defects that can be detected macroscopically (Kankel et al. 2003). These plants possess narrow leaves, short primary roots, altered flowering time and reduced fertility. Similarly, the *met1-3* mutant, where the *MET1* gene is tagged by a T-DNA insert, exhibits even more severe phenotypes, manifested by unusual development patterns and almost sterile plants (Saze et al. 2003). However, there are other types of methylation mutants, where phenotypic aberrancies cannot be seen by the naked eye. The *met1-2* mutant, with methylation levels reduced by 50%, displays normal development and morphology (Kankel et al. 2003). Despite decreased CHG methylation, *cmt3* mutants grow normally and exhibit wild-type morphology even after multiple generations (Bartee et al. 2001). Initial mutants of the *Arabidopsis* chromatin remodeler gene *DDM1* show a global reduction of DNA methylation in transposons and repeats, but grow relatively normally (Ito et al. 2015). Developmental abnormalities of the *ddm1* mutant arise after multiple rounds of self-pollination (Kakutani et

al. 1996; Kakutani 1997). While some methylation mutants display wild-type growth and morphology, they may have hidden abnormalities at the tissue and cellular level, associated with aberrant cell proliferation, expansion and differentiation changes that are harder to detect. In accordance, although the leaf size of *ddm1-10* and *cmt3* mutants shows no significant change from the wild-type, microscopic examination of the adaxial leaf epidermis of these mutants revealed substantial phenotypic variations. Both mutants displayed significant enlargement of pavement cell size and a decreased number of pavement cells, together with a higher stomatal abundance. The *ddm1-10* rosette leaves revealed the presence of individual giant cells that were at least three times the size of regular pavement cells. Flow cytometry analyses confirmed that leaf cells of the *ddm1-10* and *cmt3* mutants underwent extra rounds of endoreplication, resulting in increased levels of 16C and 32C cells. In addition, the *ddm1-10* mutant formed leaves with stomatal patterning defects, such as clustered and paired stomata. In *Arabidopsis* and most dicot leaves, stomatal distribution follows a pattern known as the "one-cell-spacing rule", meaning that two stomata are separated by at least one non-stomatal epidermal cell (Hara et al. 2007). Disruption of this pattern in *ddm1-10* could be associated with abnormal regulation of master genes involved in the stomatal development network (MacAlister et al. 2007; Pillitteri et al. 2007) that are likely to be under direct or indirect *DDM1*-dependent methylation control.

Surprisingly, the *met1-1* mutant showed the opposite trend of cell morphology changes observed in *ddm1-10*, manifested by the reduced pavement cell size and an increase in the number of pavement cells. In rosette leaves of *met1-1*, 80% of the pavement cells had ploidy levels of 2C and 4C, suggesting that leaf tissue is in an actively dividing state. The high population of 2C cells in *met1-1* could, in part, be explained by the threefold increase in the number of stomata, because stomatal guard cells have an exclusively 2C DNA content (Melaragno et al. 1993). Although we cannot exclude that these differences could be partly due to delays in *met1-1* development associated with the demethylation of the floral repressor FWA (Kankel et al. 2003), analysis of the phenotype of *met1-1* at later growth stages showed very similar defects in leaf morphology (data not presented), including an extreme reduction in leaf size compared with the wild type. It should also be noted that the observed increase in meristemoid divisions in *met1-1* leaves is very similar to the disrupted stomatal patterning associated with inactivation of the basic helix-loop-helix transcription factor SPEECHLESS (SPCH) (Lau et al. 2014). It is possible that misregulation of the methylation machinery could affect a number of genes involved in stomatal formation and patterning. It has already been shown that correct methylation is important for the size of the stomatal stem cell population in the leaf epidermis (Yamamuro et al. 2014).

This study outlines an easy and fast phenotyping strategy that can be used as a reliable tool for the characterisation of epigenetic mutants at the cellular level. Quantitative image analysis combined with flow cytometric assessment

revealed hidden phenotypic variations in hypomethylation mutants with wild-type morphology at the organ level. Detection of these variations and defects could trigger the identification of key genetic loci underlying the phenotypes caused by DNA methylation modifications.

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