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RESEARCH PAPER

Low relative humidity triggers RNA-directed *de novo* DNA methylation and suppression of genes controlling stomatal development

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Abstract

Environmental cues influence the development of stomata on the leaf epidermis, and allow plants to exert plasticity in leaf stomatal abundance in response to the prevailing growing conditions. It is reported that *Arabidopsis thaliana* 'Landsberg *erecta*' plants grown under low relative humidity have a reduced stomatal index and that two genes in the stomatal development pathway, *SPEECHLESS* and *FAMA*, become *de novo* cytosine methylated and transcriptionally repressed. These environmentally-induced epigenetic responses were abolished in mutants lacking the capacity for *de novo* DNA methylation, for the maintenance of CG methylation, and in mutants for the production of short-interfering non-coding RNAs (siRNAs) in the RNA-directed DNA methylation pathway. Induction of methylation was quantitatively related to the induction of local siRNAs under low relative humidity. Our results indicate the involvement of both transcriptional and post-transcriptional gene suppression at these loci in response to environmental stress. Thus, in a physiologically important pathway, a targeted epigenetic response to a specific environmental stress is reported and several of its molecular, mechanistic components are described, providing a tractable platform for future epigenetics experiments. Our findings suggest epigenetic regulation of stomatal development that allows for anatomical and phenotypic plasticity, and may help to explain at least some of the plant's resilience to fluctuating relative humidity.

Key words: DNA methylation, environmental stress, humidity, plasticity, siRNAs, stomatal index.

Introduction

The density and operation (opening) of stomatal pores on leaf surfaces are both heavily influenced by environmental cues. Together, they control leaf stomatal conductance to water vapour over short (minute to hour) and long (seasonal to lifetime) timescales (Casson and Hetherington, 2010) and enable the plant to balance the conflicting needs to capture atmospheric carbon dioxide for photosynthesis and to minimize water loss through transpiration. Plants maintain plasticity in their capacity to moderate stomatal density during leaf growth and, although stomatal density correlates with the macro-environment over geological timescales (Hetherington and Woodward, 2003), there is also a strong inverse correlation with water use efficiency (WUE) during growth and development (Miyazawa *et al.*, 2006; Lake and Woodward, 2008; Sekiya and Yano, 2008). The frequency of stomata on the leaf epidermis (Stomatal Index, SI: stomata as a percentage of epidermal cells) responds to light, CO_2 concentration, drought, and evaporative demand—relative humidity—(Royer, 2001; Hetherington and Woodward, 2003; Casson *et al.*, 2009), indicating a strong environmental

influence on the developmental pathway forming stomatal guard cells.

The pathway governing stomatal development involves a 'default' fate of protoderm epidermal cells to form stomata. Stomatal development is determined jointly by the expression of a series of patterning genes that block entry into the stomatal lineage and by positive regulators that control lineage progression to form stomatal guard cells via cell divisions (Nadeau, 2009). The master regulators of this differentiation pathway in *Arabidopsis thaliana*, the subgroup Ia basic helix-loop-helix (bHLH) proteins, are well-conserved across divergent plant taxa and evolutionary lineages, and their role as transcription factors driving divisions in stomatal guard cell development is likely to be a specialized function (MacAlister and Bergmann, 2011).

The molecular mechanisms by which environmental change influences this pathway are unclear. The bHLH genes in the stomatal pathway are required to initiate stomatal cell fate lineage in the developing leaves of many species (Liu *et al.*, 2009) and their expression is finely co-ordinated in both time and space. In *A. thaliana*, SPEECHLESS (SPCH) is the controlling transcription factor that regulates the first asymmetric division in the stomatal pathway (MacAlister *et al.*, 2007). SPCH is functionally repressed by phosphorylation (Lampard *et al.*, 2008) at the end of a mitogen-activated protein kinase (MAPK) cascade that includes several environmentally-responsive kinases (Wang *et al.*, 2007). Such an interaction could link regulation of *SPCH* expression (and therefore stomatal development) with the response to environmental cues, although there is currently no experimental support for this theory. Nevertheless, transcriptional regulation (expression pattern) of these transcriptional regulators does appear to be a common theme in the regulation of this pathway (Hunt and Gray, 2009; Lampard *et al.*, 2009; Hunt *et al.*, 2010; Sugano *et al.*, 2010; Yoo *et al.*, 2010).

This is not, however, the whole story. Within the same species, different accessions respond variously to environmental cues such as CO_2 and relative humidity (Gray *et al.*, 2000; Lake and Woodward, 2008). There is a genotypic component to this relative sensitivity (Gray *et al.*, 2000), and a genetic component also evidenced by QTL for WUE and both stomatal density and SI (Ferris *et al.*, 2002; Masle *et al.*, 2005; Nilson and Assmann, 2010). Comparing the fossil with the short-term experimental records, Royer (2001) found a stronger and more consistent correlation between atmospheric CO_2 levels and stomatal density and SI of fossil samples. He suggested that plants are capable of a short-term plastic response and a longer-term genetic adaptation but, crucially, that both are determined by the prevailing conditions during growth. There is also a systemic signal from mature to developing leaves that controls stomatal development in response to environment (Lake *et al.*, 2001) and an intriguing coincidence between genome size and stomatal guard cell density: size ratios that appears to be largely independent of environmental conditions (Beaulieu *et al.*, 2008; Lomax *et al.*, 2009).

It is hypothesized that short-term, environmentally-induced plasticity in SI is mediated by epigenetic regulation of the expression of genes in the stomatal development pathway. DNA cytosine methylation is one of the best-characterized epigenetic control mechanisms of transcription in plants and can alter when a plant is exposed to stress. Boyko *et al.* (2010) in *Arabidopsis* and Verhoeven *et al.* (2010) in *Taraxacum officinale* have both demonstrated that environmental stress can induce changes in DNA methylation on a genome-wide scale and shown that such changes can be heritable. Furthermore, several genome-wide surveys have reported on the methylation status of stomatal pathway gene loci in *A. thaliana* plants grown in one environment (Zhang *et al.*, 2006; Zilberman *et al.*, 2007; Cokus *et al.*, 2008; Lister *et al.*, 2008). This study examined whether environmentally-induced plasticity in SI is linked to the DNA methylation status and expression of genes implicated in the stomatal development pathway.

It was found that growth under low relative humidity (LRH) induced additional methylation in two stomatal development gene loci, *SPCH* and *FAMA*. The pattern of DNA methylation in *A. thaliana* is complex with frequent methylation found throughout the genome (Lister *et al.*, 2008). *De novo* DNA methylation is induced in all sequence contexts in regions of RNA–DNA complementarity by the production of small, non-coding, interfering, 21~25 nt RNAs (sRNAs). sRNAs recruit DNA methylation to silence or suppress transcription in the process known as RNA-directed DNA methylation (RdDM) (Wassenegger *et al.*, 1994; Chan *et al.*, 2004). RdDM is readily lost in asymmetric sequence contexts without the continued presence of the sRNA triggers (Aufsatz *et al.*, 2002) and may result in reversible methylation. It was proposed that the differential methylation of gene loci with environmental treatment in our plants must be reversible, and hypothesized that it might, therefore, involve RdDM. If sRNAs recruited the observed methylation, double-stranded short-interfering RNA transcripts (siRNAs) should be inducible by the treatment and positively correlated with increased methylation, as well as associated with the loci. Environmentally-induced increases in the concentration of siRNAs were found to be associated with additional methylation at the *SPCH* and *FAMA* loci.

Materials and methods

Plants and growth environment

Arabidopsis thaliana (L.) Heynh. ecotypes Landsberg *erecta* (L*er*) and Columbia (Col-0), methyltransferase mutants for MET1 ((Decreased Methylation 2DNA), *met1*), Chromomethylase (*cmt3*) and Domains rearranged methyltransferase 1/2 (*drm1*/*drm2*), and RNA-directed DNA methylation (RdDM) mutants Argonaute, glabra (*ago4-1*, *gl1-1*), RNA dependent rna polymerase 2 (*rdr2*), RNA dependent rna polymerase 6 (*rdr6-11*), Dicer-like 3 (*dcl3-1*), and Dicer-like 4 (*dcl4*) were supplied by NASC (Nottingham, UK). Sixty seeds of each type were sieved to standardize the size of seeds sown in seedling compost (Sinclair, Lincoln, UK), germinated, and grown in controlled environment growth cabinets (Saxcil, R.K. Saxton, Bredbury, Cheshire, UK) until harvest according to the Arabidopsis Biological Resource Center guidelines, with constant temperature of 22±1 °C and 16 h day length from fluorescent lighting and PAR of ~450 μmol m^{-2} s^{-1}. The

relative humidity of one cabinet was controlled at 45±5% (low relative humidity; LRH) whilst the other was maintained at 65±5% (control). After 64 d, stage 9.70 (Boyes *et al.*, 2001), seeds were harvested from each individual. For subsequent experiments, harvested seeds and supplied seeds (sourced as before) were sown, germinated and grown under identical conditions. This replicate experiment was repeated four times with self-pollinated plants grown under identical control conditions to create three-generation lines. Different (rotated) growth chambers (Saxcil, as before, and Sanyo Gallenkamp, Loughborough, UK) were used in all four repeated experiments to accommodate for growth chamber effects. The complete dry biomass and seed mass of individual harvested plants were weighed and the seeds counted and measured, following threshing through a series of graded meshes, by capturing a digital image of collected seeds using an Epson Perfection 3170 scanner (Epson (UK), Hemel Hempstead, UK) then subjected to particle size analysis using ImageJ software version 1.37 (freeware NIH, USA). The effect of changing RH during development was examined in two subsets of 12 wild-type (WT) plants each (first and second generation control progeny) by moving plants between treatments during rosette leaf growth (stage 3.50; Boyes *et al.*, 2001).

Stomatal analyses

SI was determined by making impressions on clear nail varnish of the entire abaxial surface of one mature rosette leaf (insertion 6–8, length approximately 40 mm) and one cauline leaf (insertion 13–15, length approximately 15 mm) collected from 48 plants (each of 16 replicate plants from each of three individual parents in the descent lines; except *ago4-1*; see the Results) at the same physiological stage (6.50; Boyes *et al.*, 2001) in each of the sequence of experiments. Digital images were captured from these impressions on an Axioskop 2 microscope with an Axiocam camera attached (Carl Zeiss Ltd), using Axio Vision 3.1 (Image Associates, Oxfordshire, UK) software. The number of stomata and other epidermal cells per unit area was then counted using ImageJ software (as above).

DNA methylation analyses

WT whole seedlings (first true leaf stage), mature and immature leaves from ⩾12 replicate plants were snap frozen in liquid nitrogen and stored at –80 °C. DNA was extracted using the DNeasy plant mini-kit (Qiagen, UK) according to the manufacturer's instructions. Genomic DNA (2 µg) was bisulphite-modified using the EZ DNA methylation kit (Zymo Research, Orange, CA) according to the manufacturer's protocol. Desulphonated DNA was diluted 1 in 5. High Resolution Melting (HRM) analysis was used to analyse differential methylation with treatment as in Wojdacz and Dobrovic (2007), except that each 20 µl reaction mix contained 1× Biomix (Bioline, London, UK), 25 µM Syto9 dye (Invitrogen, Carlsbad, CA), and 300 nM of each bisulphite-specific primer for the gene of interest. PCR conditions used were: 2 min at 95 °C, then 50 cycles of 95 °C for 15 s and 50 °C for 30s, 60 °C hold for 1 min, and HRM from 58–80 °C at 0.5 °C s^{-1}. For each gene, untreated genomic DNA (diluted 1 in 1000) was included as a positive control using the equivalent, but not bisulphite-specific, primer. Differential methylation with treatment was initially identified using the RotorGene™ 6000 Series Software version 1.7 (Qiagen, UK), with the confidence level set at 80% (White *et al.*, 2007). Assays were repeated 6–8 times for genes putatively identified as differentially methylated.

Positive results indicating differential methylation in the *SPCH* and *FAMA* genes were validated by capturing the methylated portion of genomic DNA using the Methylamp Methylated DNA Capture kit (Epigentek, Cambridge Bioscience, Cambridge, UK) and performing comparative qPCR analysis using negative controls provided in the kit (Ig mouse antibody) and mock-treated genomic DNA as a positive control. Subsequently, primers were designed to target every 300 bp of the coding regions, for 600 bp of (5') upstream regions of the *SPCH* and *FAMA* genes and for the 2.3 kb upstream genomic region 3' of *SPCH*, and assayed in WT and mutant mature leaf and whole seedling samples. A 51 bp fragment of *SPCH* found to be hypomethylated in all the amplicons examined was re-assayed separately. qPCR and HRM conditions were as described above except that 15 ng of template DNA were used, T_a was 56 °C and an extension phase of 66 °C for 6 min replaced the 1 min hold; HRM was performed from 68–90 °C. 'Epigenotype' (methylated/unmethylated) for each primer pair region was identified using the RotorGene™ 6000 Series Software version 1.7 (as above).

Base-pair resolution methylation profiles of target fragments were obtained by sequencing ⩾32 cloned amplicons (vector pCR2.1; Invitrogen, Carlsbad, CA) per sample of three, pooled, replicate plants (Geneservice, Source Bioscience PLC, Nottingham, UK) following bisulphite treatment and PCR, as described above, except that 5 nM of *de novo* synthesized, labelled DNA (Sigma-Aldrich Ltd., Gillingham, UK) containing methylated and unmethylated cytosines in defined positions (see Supplementary Table S1 at *JXB* online) was added to the 2 µg sample gDNA prior to bisulphite treatment as a positive control to ensure complete bisulphite conversion. Assays were repeated with samples from two of the repeated experiments. Differential methylation was assessed by reference to the unmodified genomic DNA sequence, the positive control, and by comparison with the cytosine to thymine conversion between treatments. Sequences were aligned using ClustalW2 (Larkin *et al.*, 2007) and presented using BioEdit v. 7.0.9.0 (Hall, 1999). Following each round of HRM, qPCR, and PCR, a sample of products was analysed for size accuracy and purity using the Agilent Bioanalyzer Series II DNA 1000 chip (Agilent, Winnersh, UK).

RNA expression analyses

Total RNA was isolated from frozen leaf material using the RNeasy Plant Mini kit (Qiagen, UK) using the manufacturer's protocol. Primers for Multiplexed Tandem PCR (MT-PCR; Stanley and Szewczuk, 2005) were designed for the target genes *SPCH* and *FAMA* and for the internal control genes *PP2A* and *SAND* (Czechowski *et al.*, 2005). MT-qPCR was performed as in Stanley and Szewczuk (2005) using 500 ng starting RNA, except that Sensimix (Quantace, London, UK) reverse transcriptase and buffer were used, and reverse transcription was executed at 45 °C for 15 min followed by 70 °C for 15 min. First round multiplexed amplification was performed in the ABI9700 thermal cycler using Sybr Premix Ex Taq polymerase (Takara Bio Europe, Saint-Germain-en-Laye, France) and final volumes of 200 nM for each primer. PCRs used the following conditions: 1 min at 95 °C, 10–15 cycles of 95 °C for 15 s, 58 °C for 20 s and 72 °C for 15 s then 72 °C for 7 min. Pre-amplification products were diluted 1:1 (v/v) and second-round PCRs prepared using Sybr Premix Ex Taq (as before), internal primers, and 1 µl template cDNA. qPCR was performed in the RotorGene™ 6000 thermal cycler (Qiagen, as before) as follows: 95 °C for 1 min, then 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 8 s, and HRM from 70–96 °C at 0.5 °C s^{-1}. All reactions were prepared in triplicate, with three biological replicates and serial dilutions completed for genes of interest and controls. MT-qPCR data were analysed by comparison with reference genes of equal efficiencies to target genes by two standard curve analysis. RotorGene™ 6000 Series software version 1.7 was used to determine gene amplification efficiencies and RNA quantification (as before). The data presented are mean calculated expression from samples in three of the repeated experiments and at least three separate assays. A subset of PCR and qPCR products from each round was analysed for size accuracy and purity using the Agilent Bioanalyzer DNA 1000 chip and kit.

(si)RNA analyses

Total RNA was isolated from seedling samples using the mirVana miRNA isolation kit (Ambion, Warrington, UK) as per the

manufacturer's instructions, checked and quantified using the Agilent Bioanalyzer RNA 6000 Nano chip and bioanalyzer. Small RNAs (sRNAs) in each equalized total RNA sample ($n \geqslant 3$ in each experiment) were quantified using the Small RNA chips and kits (Agilent, as before) and the smear analysis software function of the Agilent 2100 Bioanalyzer against small dsRNA standards (New England Biolabs, Hitchin, UK) and the quantification standard ladder provided in the kit.

Multiple siRNAs expression was analysed by in solution hybridization and RNase digestion of both the total and the small RNA fractions with custom synthesized probes, followed by electrophoretic separation and quantification of the protected probes. Antisense probes for siRNA analyses were designed for the small RNA sequences downloaded from the *Arabidopsis* Small RNA Project (ASRP) database (http://asrp.cgrb.oregonstate.edu/) and Lister *et al.* (2008) for the region 3:8714.3k..8721k for *FAMA* and 5:21601k..21611.4k for *SPCH*. In the ASRP database *SPCH* is currently located at 5:21603.8k. Additional (A) bases were added to create different length probes artificially. The antisense probe for *FAMA* RNA was CUUCUGCCGUAAACCUCGUUUCACUUGaaaa and for *SPCH* was UUAAGUGCUCGUUCAUUUGCUUUCUCCGaaaa.

Total RNA from each sample (200 ng) was enriched for the small RNA fraction using the mirVana miRNA isolation kit (as before). Unlabelled antisense RNA probes of differing nt lengths were designed and constructed using the mirVana probe construction kit (Ambion, as before) according to the manufacturer's instructions for *SPCH*, *FAMA*, and local sRNAs; up to four probes were detected in each reaction using the mirVana detection kit (Ambion) according to the manufacturer's instructions. Probes were post-labelled and visualized and quantified fluorescently using the Small RNA chip, standards and the bioanalyzer software.

Statistical analyses

Differences in mean growth and stomatal traits between treated plants and the wild-type controls were tested for statistical significance by Student's t tests in single experiments, and the effect sizes in each subsequent experiment calculated and compared with Cohen's d (Cohen, 1992) using the pooled standard deviation of the means from the treatment and control. Genotype×treatment interactions for mutants and their respective background ecotypes were tested with two-way ANOVA using Minitab v. 15.1.30. Maternal environmental effects were evaluated in descent lines by two-way ANOVA with growth cabinet and parent as fixed factors in the model. Effects of the treatment on gene expression were assessed for significance by Student's t test comparisons of the calculated mRNA concentrations from two-standard curve analysis (reference and gene of interest standard curves) of Ct values in MT-qPCR. Differences in [sRNAs] in total RNA with treatment were also analysed by two-way ANOVA for genotype×treatment interactions. The relationship between SI and [mRNA] of each gene of interest was analysed by linear regression of the means for each biological replicate of genotype (WT and mutant) and P-value of the ANOVA reported. Correlations of *SPCH* and of *FAMA* expression with siRNAs were assessed by linear regression of the concentration of the protected probes with adjusted r^2 value reported.

Primer designs

Primers for DNA methylation and RNA assays are listed in Supplementary Tables S2–S5 at *JXB* online. All primers were designed using Primer3 software. Bisulphite-specific primers were based on the returned, bisulphite-specific sequence from MethPrimer software (Li and Dahiya, 2002). Primers to genotype methyltransferase mutants were as in Cao *et al.* (2003) and Lindroth *et al.* (2001), flanking the insertion AAGTGGCACTTCATCGTCTCCCAATCAAAATGAAGCT (GenBank accession CC887813) for DRM2. Primers for RT-qPCR of RDR2 were RDR2F (5'-GGGTCCAGAGCTTGAGACTG-3') and RDR2R (5'-CCCTTCTCCAAGGATTGACA-3'). Primers for RT-qPCR of DCL3-1 were DCL3F (5'-GTCTTTGAGCCGTTGCTTTC-3') and DCL3R (5'-GTGAAGCTGCTTTTCCCAAG-3').

Results

Low relative humidity (LRH) reduces leaf stomatal frequency

First, the effect of low humidity on stomatal development was assessed. *A. thaliana* Landsberg *erecta* grown under constant LRH (45±5%) exhibited a consistently reduced SI compared with the control plants across all four repeated experiments (Fig. 1A; P <0.001; Cohen's d >0.80). The reduction in SI was similar in both developing cauline and mature rosette leaves of the same plant at the same physiological stage. There was also a decrease in SI (25% decline, P=0.028) when plants at the rosette leaf stage were moved between humidity conditions in either direction (i.e. LRH to control or control to LRH), implying that the response was not maturity-dependent and persisted after the removal of the environmental stress. The effect was also reproducible among descent lines from self-pollinated plants grown under identical control conditions. In all cases, SI was consistently reduced when offspring from plants grown under control conditions were grown in LRH rather than control conditions (27% decline, P <0.002; Cohen's d >0.80 in three successive generations). All sample plants were affected similarly. There were no significant influences of seed weight or size (see Supplementary Table S6 at *JXB* online), germination date or flowering time on the effect (data not shown), with a maximum recorded 2 d delay in flowering in the Control parent-LRH progeny lines in only one of the repeated experiments.

Low relative humidity induces DNA methylation of two genes in the stomatal development pathway

An initial screen was performed for differences in DNA cytosine methylation under LRH compared with the control environment around transcription start sites in 11 of the genes controlling stomata patterning and formation. The following putative upstream regulators of the MAPK cascade were assayed: the activating peptidase *STOMATAL DENSITY AND DISTRIBUTION1* (*SDD1*), the receptors *ERECTA* (*ER*), *ERECTA-LIKE1* (*ERL1*), *ERL2,* and *TOO MANY MOUTHS* (*TMM*) and, at the other end of this negative signalling pathway, the MAPKKK *YODA*. In the differentiation pathway, the positive regulating transcription factors *SPCH*, *MUTE*, and *FAMA* and their co-factors *ICE1* and *SCREAM2* were screened. Differential methylation associated with RH treatment was evident as divergent melt curve profiles only in the *SPCH* and *FAMA* genes (Fig. 1). Using affinity-based capture of the methylated portion of the genome and qPCR, the differential methylation assay was extended to ~5 kb surrounding the *SPCH* locus and ~2.5 kb around *FAMA*. This revealed LRH-induced DNA methylation throughout the whole 5 kb

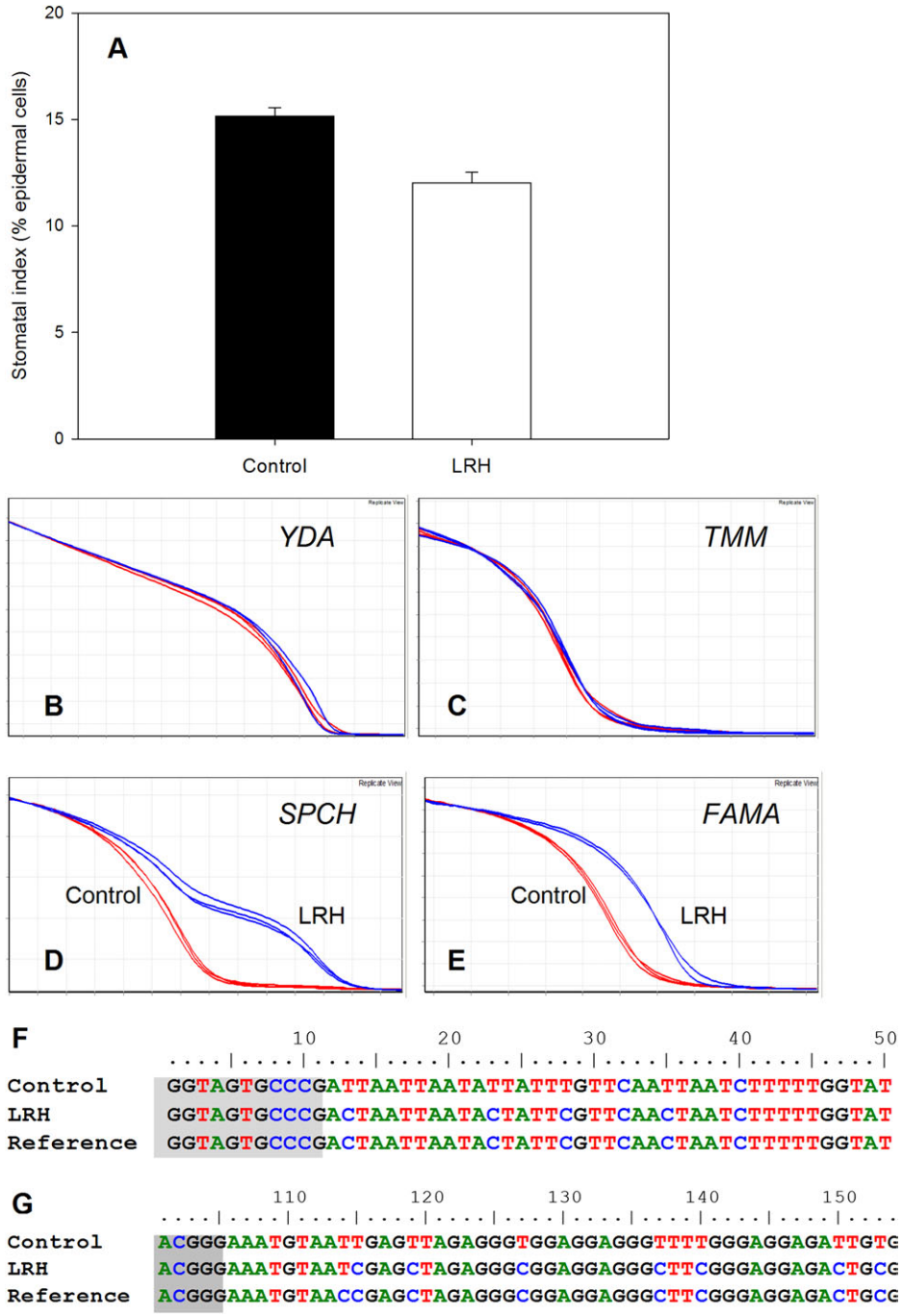

Fig. 1. Stomatal index and differential methylation of genes in the stomatal development pathway under low relative humidity (LRH) treatment. (A) Mean (±SE) stomatal index (stomata as a percentage of epidermal cells) of mature, wild-type (*Arabidopsis thaliana* Landsberg *erecta*) leaves in the control (solid bar) and under LRH treatment (open bar). B–E) High resolution melting curves [*y*=normalized fluorescence (0–100)/ *x*=degrees Celsius (61–79)] of amplified fragments of the (B) *YODA* (*YDA*, At1g63700), (C) *TOO MANY MOUTHS* (*TMM*, At1g80080), (D) *SPEECHLESS* (*SPCH*, At5g53210.1), and (E) *FAMA* (At3g24140.1) gene loci from bisulphite-treated sample DNA following PCR in the control (red lines) and LRH treatment (blue lines). *YDA* shows the typical high melting temperature and arc of cytosine-methylated DNA and *TMM* is typical of unmethylated DNA; *SPCH* and *FAMA* are differentially methylated with LRH treatment. (F, G) Comparison of sequence fragments from cloned amplicons of (F) *SPCH* and (G) *FAMA* following bisulphite treatment and PCR of samples from the Control and LRH treatment and in unmodified DNA (Reference). Following bisulphite treatment and PCR, unmethylated 'C' bases are converted to 'T' whereas methylated C's are not. These examples show the methylation in both symmetric (CG) and asymmetric (CHH) sequence contexts in the LRH samples at these loci. Shaded areas are primer bases.

surrounding *SPCH* that extended upstream, throughout the gene locus and downstream but with a short (51 bp) break at a simple sequence repeat in the first exon (see Supplementary Fig. S1 at *JXB* online). This finding showed that LRH-induced DNA methylation extends throughout *SPCH* and into surrounding non-coding regions. At *FAMA,* there

was similar evidence of LRH-induced methylation in 5' untranslated regions that extended through the first three coding regions of the gene (see Supplementary Fig. S1 at *JXB* online). Bisulphite sequencing was performed on cloned fragments from the same (presumed hypermethylated) regions of *SPCH* and *FAMA* to generate a single base resolution image of the extent and distribution of methylated cytosine at these loci. The data recovered indicated that LRH had induced methylation in both symmetric (CG) and asymmetric (CHH, where H is A, C or T) sequence contexts at both gene loci (Fig. 1F, G).

Expression of SPCH *and* FAMA *is reduced under low relative humidity*

RT-qPCR assays of *SPCH* and *FAMA* expression indicated consistent and substantial suppression of both genes in LRH-grown plants (by 31–58%; Fig. 2). These reductions correlated with reduced leaf stomatal frequency (*SPCH* r=0.82, P=0.012; *FAMA* r=0.67, P=0.029).

Stomatal frequency is unaffected by low relative humidity in two methyltransferase mutants

A series of methyltransferase mutants were used to investigate a possible link between cytosine methylation and the regulation of stomatal frequency in the face of reduced humidity. SI was not reduced by LRH in the double mutant for Domains Rearranged Methyltransferases 1 and 2 (*drm1/drm2*) (Fig. 3A). *FAMA* expression was not reduced by LRH in the mutant and *SPCH* expression actually increased (Fig. 3B, C). Methyl capture followed by qPCR showed that while there was differential methylation under LRH in the WT plants, this divergence was not apparent in the *drm1/2* plants at either locus (Fig. 4A, B). This finding confirmed the essential role of DRM1/2 for the establishment of methylation associated with LRH treatment.

The *met1* mutation for the maintenance of CG methylation methyltransferase, MET1 (Decreased Methylation 2DNA), had no effect on SI under control conditions (relative to its background WT, Col-0), but SI was no longer significantly reduced under LRH (Fig. 3A). Furthermore, RT-qPCR showed that expression of *SPCH* was not reduced by LRH treatment and that *FAMA* expression was actually increased under these conditions (Fig. 3B, C). Similarly, methyl capture followed by qPCR showed that the LRH-induced methylation of *SPCH* and *FAMA* was reduced but not abolished in the *met1* plants (Fig. 4A, B).

Close examination of the leaves of the non-functional mutant of CHROMOMETHYLASE 3 (*cmt3*) revealed that it responded to LRH in the same manner as the WT plants, with reduced SI. Use of RT-qPCR on total RNA isolated from these plants revealed reduced expression of *SPCH* and *FAMA* (Fig. 3). Furthermore, methyl capture followed by qPCR showed that methylation of *SPCH* and *FAMA* had sharply increased in these plants, in common with the WT controls (Fig. 4). Thus, CMT3 appeared not to be essential for the differential methylation associated with LRH.

There were no significant differences in *FAMA* expression or in SI between any of these single methyltransferase mutants and WT genotypes when grown under control conditions (Fig. 3). Expression of *SPCH* was increased in *drm1/2* and in *cmt3* (P <0.001) but showed no associated increase in SI. Both cell and stomatal density (mm^{-2}), however, were higher than WT in *cmt3* (see Supplementary Fig. S2 at *JXB* online). Compared with each other, and under control conditions, there was a significant increase in *SPCH* expression and in cell and stomatal density in *cmt3* compared with *drm1/2*.

Biomass is unaffected by low relative humidity in the methyltransferase mutants drm1/2 *and* met1

WT plants exhibited a significant reduction in final dry biomass at harvest under LRH (P=0.003). There were no significant changes in the sizes of *met1* and *drm1/2* in the control environment compared with each background WT ($P \geqslant 0.071$; Fig. 5A), suggesting that the mutations do not significantly impact on the biomass under control conditions. However, in contrast with the WT plants, the imposition of LRH stress had no effect on the biomass of these two mutant lines (Fig. 5B, C).

Additional LRH-induced DNA methylation is not reproduced in siRNA biogenesis mutants

In order to investigate the potential role of RdDM in LRH-induced methylation and the SI, mutants were grown for RNA-directed DNA methylation (RdDM) and siRNA biogenesis in the control and LRH treatment. In primary RdDM, DRM2 is incorporated into an ARGONAUTE4 (AGO4)-containing effector complex. In downstream transcriptional gene silencing (TGS), RDR2 (rna dependent rna polymerase

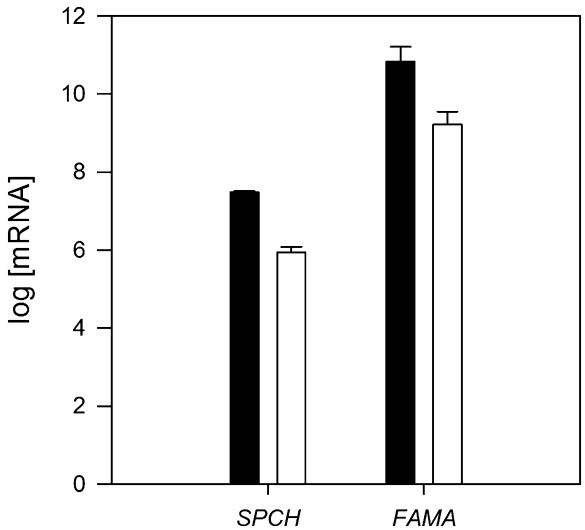

Fig. 2. *SPCH* and *FAMA* gene expression in the control (solid bar) and low relative humidity (LRH) treatment (open bar). Results are the log transformed calculated mRNA concentrations (±SD) following reverse transcription and MT-qPCR (see methods) of sample RNA (n=9). Data have been $\log_e$ transformed for presentation at the same scale.

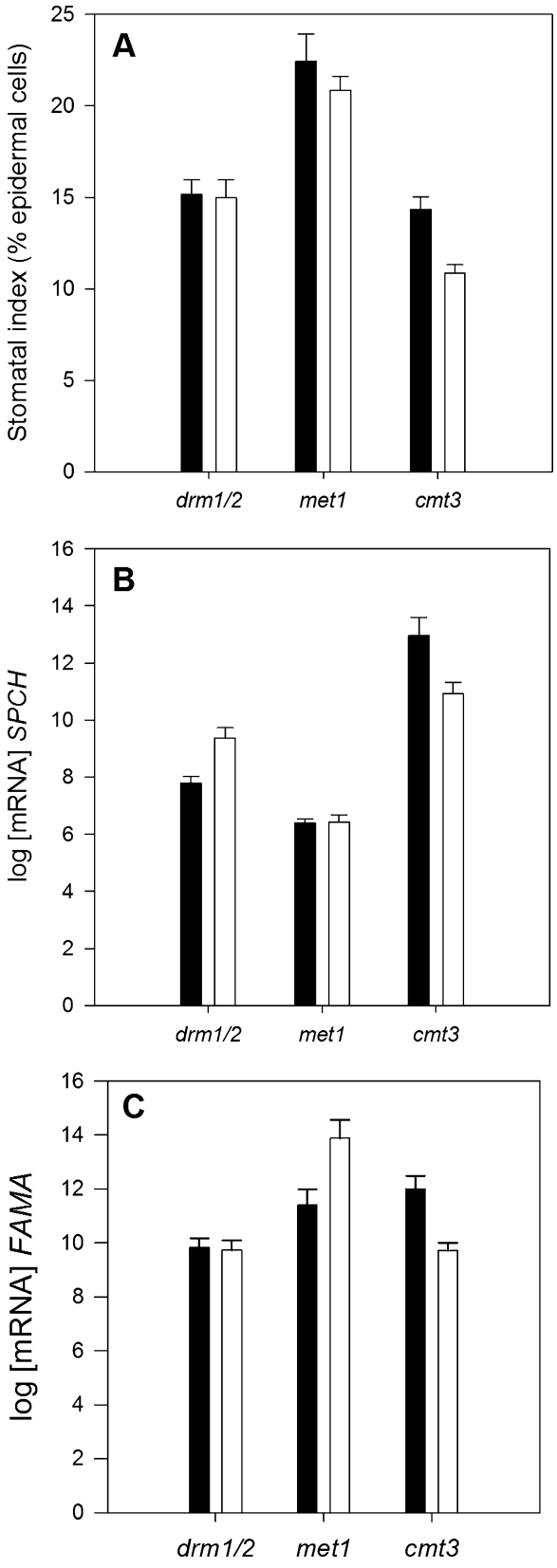

Fig. 3. Stomatal index and expression of the *SPCH* and *FAMA* genes under low relative humidity (LRH) treatment in the methyltransferase mutants *drm1/2*, *met1*, and *cmt3*. (A) Mean (±SE) stomatal index (stomata as a percentage of epidermal cells) of mature leaves, (B, C) calculated and log transformed [mRNA] (±SD) of (B) *SPCH* and (C) *FAMA* (n=9) in the control (solid bar) and under LRH treatment (open bar). *drm1/2* and *cmt3* are in the L*er* background (WT shown in Fig. 1), *met1* is in the Col-0 background (Col0 SI: control=23.27±3, LRH=18.18±1).

2) is required for the synthesis of secondary siRNAs (Xie *et al.*, 2004). Both maintenance and transitivity—the spreading of methylation downstream of the original target RNA-DNA site (Voinnet, 2008)—of post-transcriptional gene silencing (PTGS) require RDR6 and transcription of the target gene (Vaistij *et al.*, 2002). Dicer-like RNA III proteins process dsRNA or hairpin RNAs with DICER-LIKE 3 (DCL3), primarily acting on RDR2-produced RNAs and DCL4 on RDR6-produced RNAs. There is, however, some overlap and compensatory processing by the four *Arabidopsis* DCLs in single *dcl* mutants (Gasciolli *et al.*, 2005).

Methylation, induced by LRH treatment in WT plants at *SPCH* and at *FAMA* was not reproduced in the siRNA biogenesis mutants *rdr2* or *dcl3* (Fig. 6A, B). Under LRH, both *SPCH* and *FAMA* remained comparatively unmethylated at asymmetric bases in *rdr2* and *dcl3*, as in *drm1/2*. Methylation in the *ago4-1* plants was highly variable and not completely abolished at either gene locus in either the control (nascent methylation) or LRH environment. In bisulphite-sequenced fragments there were fewer asymmetric methylated sites and, in general, there was more methylation in control than LRH-grown *ago4*-1 plants, but the pattern was inconsistent and at unpredictable sites (data not shown). LRH-induced methylation was reduced overall in *rdr6* but not abolished in either symmetrical or asymmetrical contexts in *SPCH* or *FAMA*. These data indicated that both *SPCH* and *FAMA* were targets of RdDM under LRH and implied that both loci are probably subject to TGS and PTGS.

sRNAs concentration is increased under low relative humidity and affects SI

siRNAs in different size classes and at different upstream, genic and downstream locations of the target genes were compared for correlation with LRH-induced methylation. TGS by unidirectional methylation of gene promoter sequences in *Arabidopsis* is directed by ~24 nt siRNAs (Wassenegger *et al.*, 1994; Wassenegger and Pélissier, 1998; Chan *et al.*, 2004; Daxinger *et al.*, 2009; Naumann *et al.*, 2011) and the spread of methylation is associated with 24 nt secondary siRNAs (Daxinger *et al.*, 2009). PTGS by 21–22 nt secondary siRNAs has also been associated with bi-directional methylation of transcribed regions (Vaistij *et al.*, 2002; Eamens *et al.*, 2008) and transitivity. Total sRNAs found in all size classes (17–25 nt) were increased in LRH-grown WT plants compared with the control environment (Fig. 7A), with no one size class affected significantly more than any other (Fig. 7B).

Only a small number of *ago4-1* mutant plants grew in LRH. In the control environment, *ago4-1* SI was increased compared with the WT and expression of both *SPCH* and *FAMA* was increased, implying that RdDM could

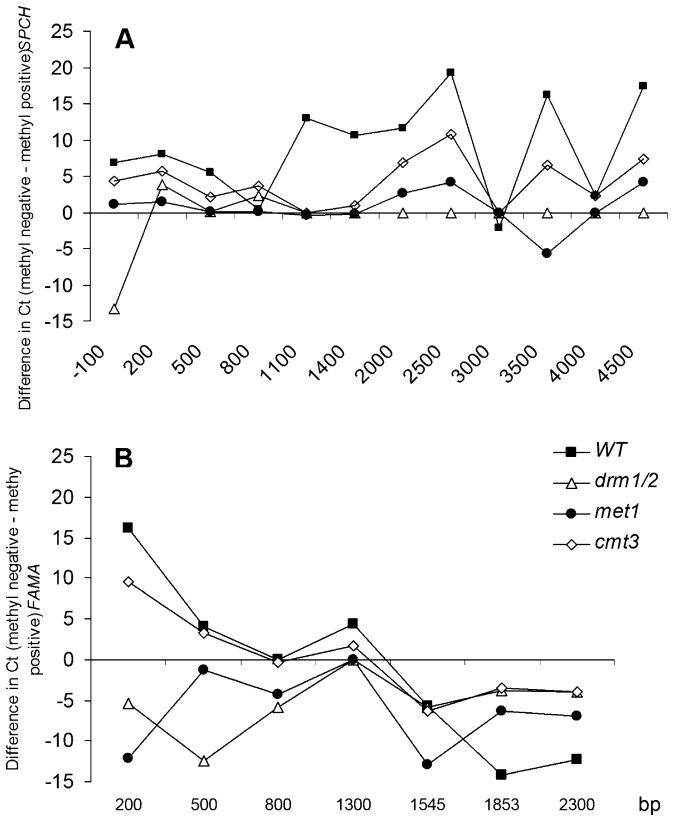

Fig. 4. The abolition of methylation in the *drm1/2* and *met1* methyltransferase mutants under low relative humidity (LRH). DNA methylation spread through (A) ~5 kb surrounding the *SPCH* locus and (B) ~2.3 kb surrounding the *FAMA* locus under LRH in wild-type (WT, solid square) compared with *drm1/2* (open triangles), *met1* (solid circles), and *cmt3* (open diamonds) samples (n=9). Data points show the difference in Ct values in qPCR for methylation negative or methylation positive DNA following affinity capture, so that a positive value indicates methylation. Where there was no amplification signal following 50 cycles of PCR (in contrast with the positive control) a Ct value of 50 has been assumed.

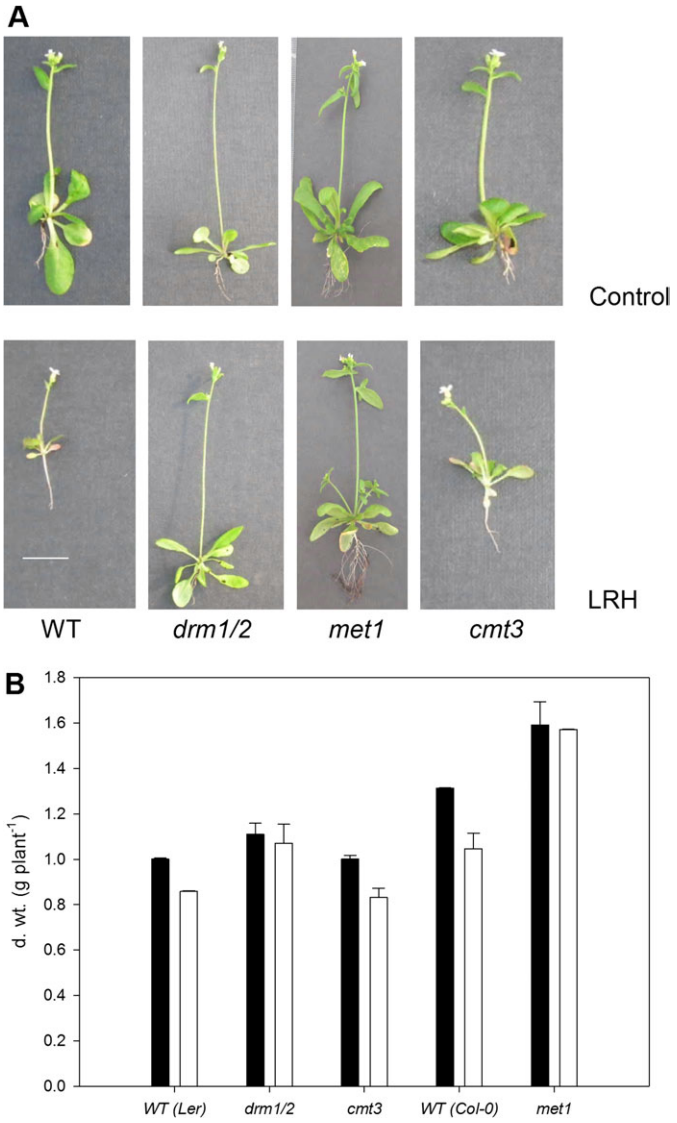

Fig. 5. The reduction in dry weight (d. wt., g plant^{-1}) under low relative humidity (LRH) in the wild type (WT) is not replicated in the methyltransferase mutants *drm1/2* and *met1*. (A) Images of plants in the control (top row) and LRH treatment (bottom row), from left to right: WT, *drm1/2*, *met1*, and *cmt3*. (B) Mean d. wt. (±SE) at harvest of the background ecotypes Landsberg *erecta* (*Ler*) and Columbia (*Col-0*) and mutants, *drm1/2, cmt3*, and *met1* in the control (solid bar) and LRH treatment (open bar).

suppress the formation of stomata by down-regulating these genes. However, all size classes of sRNAs were present in whole seedling samples in both the control and the LRH-treated plants, with no reduction in the 24 or 25 nt sRNAs in tested samples compared with the WT in either environment (n=6) and with an apparent and still significant induction in 24 nt sRNAs in some tested LRH-grown plants (n=3) (Fig. 8A).

In *rdr2* and *dcl3* plants grown in LRH, total sRNAs content was increased compared with the WT but there was no increase in LRH compared with the control environment. At high sensitivity, a tiny fraction of 24 nt sRNAs was detected in seedlings although at much reduced levels (Fig. 8A). SI of these mutants was higher than WT in LRH (P=0.041) but with no significant effect of the treatment on SI for *rdr2* compared with its own control (Fig. 8B). In *dcl3,* SI was increased by LRH compared with its own control (P=0.003). In these two mutants, expression of both *SPCH* and *FAMA* increased in comparison with the WT.

The 21–22 nt class of sRNAs was present in *dcl4* plants in both treatments but not at measurable levels in *rdr6* (Fig. 8A). Expression of both *SPCH* and *FAMA* increased in *rdr6*. Furthermore, SI increased both compared with the WT control, and under LRH compared with the *rdr6* control-grown plants (Fig. 8B).

siRNA transcripts are up-regulated in low relative humidity at the FAMA *and* SPCH *loci*

Small RNA reads from high-throughput sequencing of the reference *A. thaliana* 'Columbia' plant show sRNAs associated with the *FAMA* gene locus (Kasschau *et al.*, 2007;

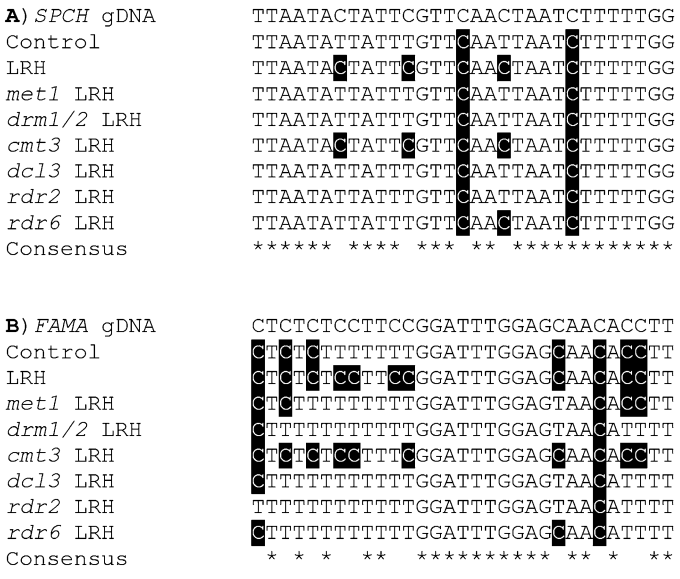

Fig. 6. Comparison of sequence fragments from sub-cloned amplicons of (A) *SPCH* and (B) *FAMA* following bisulphite treatment and PCR of samples from the control and low relative humidity (LRH) treatment in the wild type and in methyltransferase (*met1*, *drm1/2*, and *cmt3*) mutants and siRNA biogenesis (*dcl3*, *rdr2*, *rdr6*) mutants grown in LRH. Following bisulphite treatment and PCR, unmethylated 'C' bases are converted to 'T' whereas methylated C's are not.

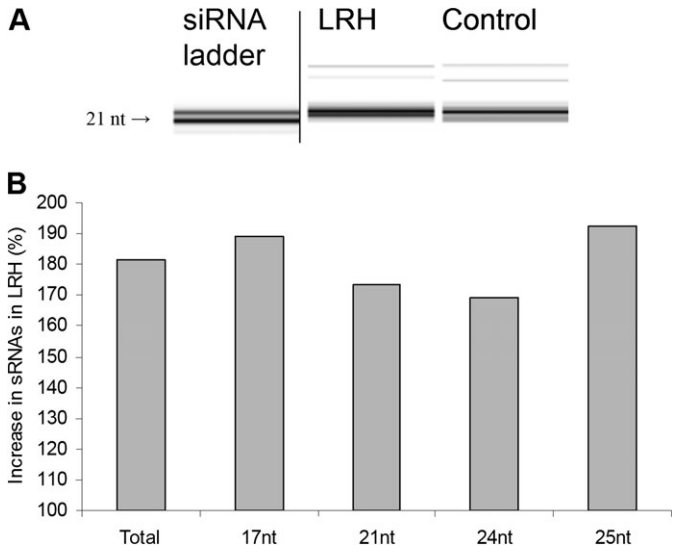

Fig. 7. Small RNAs (sRNAs) concentration in total RNA in the control and low relative humidity (LRH) treatment. (A) Fragment of a bioanalyser gel image for small RNAs in equalized total RNA in an example control and an example LRH sample, showing the increase under LRH in the 19–25 nt range. The ladder (double-stranded sRNAs) is from the same gel; the vertical line indicates lanes not shown. (B) The percentage increase in [sRNAs] under LRH compared with the control within RNA size class (data are means for n=6).

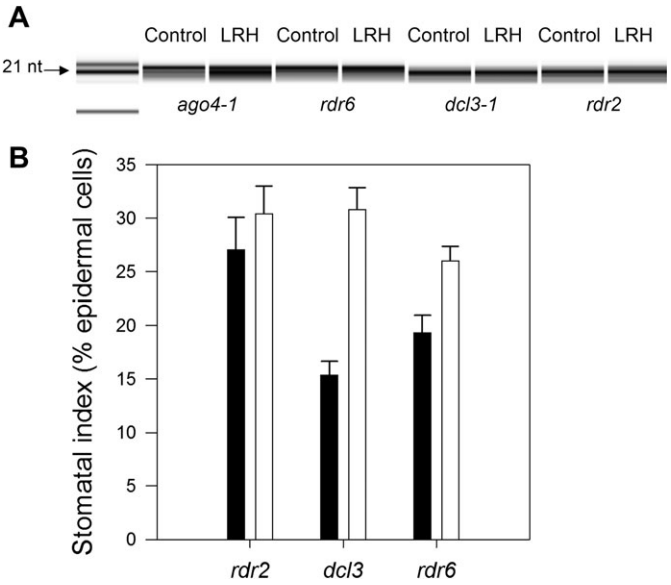

Fig. 8. Small RNAs (sRNAs) concentration and stomatal index in plants mutant for components of the RNA-directed DNA methylation (RdDM) pathway in the control and low relative humidity treatment (LRH). (A) Fragment of a bioanalyser gel image for small (6–150 nt) RNAs in 200 ng equalized total RNA in sample control and sample LRH-grown *ago 4-1*, *rdr6*, *dcl3-1*, and *rdr2* plants. (B) Mean (±SE) stomatal index (stomata as a percentage of epidermal cells) of mature leaves of siRNA mutants *rdr2*, *dcl3*, and *rdr6* in the control (solid bar) and under LRH treatment (open bar).

Lister *et al.*, 2008) (accessed in TAIR 9 http://gbrowse.arabidopsis.org) (Swarbreck *et al.*, 2008): These include a genic 21 nt sRNA, one unique upstream 23 nt sRNA and a cluster of 21–25 nt sRNAs 3' within 300 bp of the gene locus. This cluster is also detected in genome-wide sequencing of the C24 ecotype but not in Landsberg *erecta* (with detection stringency as defined by Groszmann *et al.*, 2011). Expression of these siRNAs was quantitatively assayed in our sample plants together with *FAMA*. Surprisingly, given the increased expression of *FAMA* in *rdr2* plants, there was no significant induction of the upstream siRNA transcript in LRH, and the downstream 24 nt siRNAs were not present (data not shown). However, the 21 nt genic siRNA was strongly (>2.3-fold) induced by LRH and abolished by the *drm1/2*, *rdr2*, and *dcl3* mutations in both control and LRH conditions in our sample plants. This siRNA aligned with the sequence start/ end point of LRH-induced DNA methylation.

3' of *SPCH* (and a predicted 177 bp gene At5g53205 for an unknown protein) is a cluster of rolling-curve-type helitron family transposable elements (TEs (Repeat Masker annotation ATREP3)) corresponding to 42 sRNAs and a 40 bp tandem repeat within a 427 bp dispersed repeat region. Expression of *SPCH* was found to be inversely correlated with expression of a measured subset of these siRNAs (P=0.004, r^2=0.87) such that *SPCH* was methylated in LRH and also down-regulated when expression of these siRNAs was up-regulated (Fig. 9). In plants under LRH stress, siRNAs corresponding to the TEs at the *SPCH* locus were induced and assayed DNA methylation had spread into the regulatory and genic regions of *SPCH*.

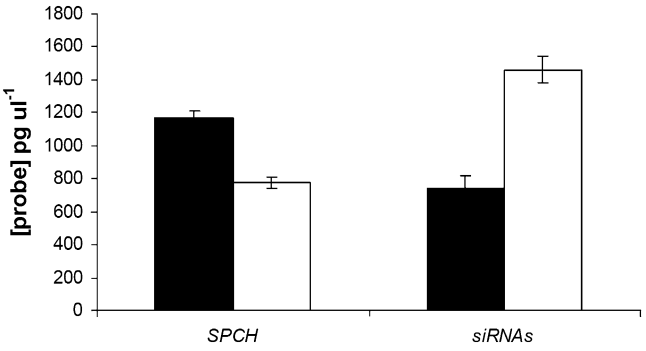

Fig. 9. The concentration (pg μl^{-1}) of probes for *SPCH* mRNA and local siRNAs corresponding the transposable elements downstream of *SPCH* in the same samples in the control (solid bar) and LRH treatment (open bar) (data are means of n=6 ±SE).

Discussion

The targeted methylation screen of plants grown under control conditions (Fig. 1) showed good concordance with the methylation status of the same genes reported previously in whole genome surveys. For example, the stomatal regulatory gene *YODA* was found to be hypermethylated whereas *SPCH, FAMA*, *TMM*, and most other genes examined were predominantly unmethylated (Cokus *et al.*, 2008; Lister *et al.*, 2008). The finding that two of these genes (*SPCH* and *FAMA*) changed their methylation status when the relative humidity was lowered (Figs 1, 4) naturally leads to the tempting supposition that these methylation changes are functionally implicated in the observed reduction in stomatal index associated with low relative humidity. This hypothesis requires that expression levels of both genes alter in response to their changed methylation status and that expression is tied to the observed phenotype. The expression of both *SPCH* and *FAMA* regulates the entry of protoderm cells into the stomatal lineage and subsequent cell divisions that culminate with the formation of stomatal guard cells (Nadeau, 2009). *SPCH* initiates the first asymmetric cell divisions to form meristemoids (Pilliteri *et al.*, 2007). *FAMA* terminates the pathway and regulates the final division of the guard mother cell (Ohashi-Ito and Bergmann, 2006). In the present study *SPCH* and *FAMA* were consistently *de novo* methylated when offspring from isogenic parents were grown under low relative humidity. When expression of these genes was assayed, both were significantly suppressed in the hypermethylated plants. The effect on SI and expression of these genes was consistent across experiments and all wild-type sample plants were similarly affected. However, it was difficult at this point to infer a causal relationship between the methylation of these genes and the change in SI since there may be several other routes by which either or both genes could be down-regulated, including interaction with other members of the pathway (Lampard *et al.*, 2008).

Reference to knock-out mutants of genes involved in the initiation and maintenance of DNA methylation in *Arabidopsis* helped shed some light on the issue. There were no differences in SI between WT plants and any of the methyltransferase mutants grown in control conditions. Nor was there any difference in the expression of *FAMA*. However, the finding that expression of *SPCH* was elevated in the *drm1/2* and *cmt3* mutants, but SI was no longer correlated with gene expression requires some consideration. There are several plausible causes of the uncoupling between expression and SI in these plants. The first relates to the use of single mutants. The individual methyltransferases interact with one another, with other histone and chromatin modifiers and with demethylation processes, in some cases redundantly (Cao *et al.*, 2003; Chan *et al.*, 2006; Penterman *et al.*, 2007). Non-CG methylation, for example, is maintained redundantly by DRM2 and the protein CHROMOMETHYLASE 3 (CMT3) (Lindroth *et al.*, 2001). It is possible that such functional redundancy could itself lead to a lack of correlation between expression and phenotype in single mutants and so compromise ability to link methylation-based expression to changes in phenotype. Some evidence was found of redundancy and compensation between the effects of *drm1/2* and *cmt3*, with these mutants showing more variability in gene expression, SI, cell density, and, in particular, stomatal density than occurred in the WT, and a reduction in cell densities in *drm1/2* compared with *cmt3*. More importantly, the increased cell and stomatal density in *cmt3* and the strong increase in *SPCH* expression beyond that in the WT controls is suggestive of CMT3-maintained methylation-based repression of other genes involved in the development of the leaf epidermis when plants are grown under normal conditions. Whilst no significant differences in *SPCH* or *FAMA* expression have been reported in previous, genome-wide screens of the *met1*, *ddm1* (Decreased DNA Methylation 1) or the *drm1 drm2 cmt3* triple mutants grown in one environment (Zhang *et al.*, 2006; Zilberman *et al.*, 2006; Lister *et al.*, 2008), Hudson *et al.* (2011) did report that *FAMA* alone was significantly *down*-regulated following the complete disruption of methylation by chemical intervention, again implying a complex control system for the pathway. The increased variability in epidermal cell numbers in the *drm1/2* and *cmt3* mutants and changes in gene expression seemed to indicate a misregulation of epidermal cell development pathways when non-CG methylation was not maintained, but the combined effects of the methylation machinery may be of most biological relevance.

Even based on current understanding, the relationship between methylation-induced down-regulation of *SPCH* and *FAMA* under LRH and the associated effect on SI is complex and difficult to dissect because of the genes' relationship in a dosage-dependent pathway with their ICE1.SCREAM2 heterodimer partners and with the intermediate gene in the pathway *MUTE* (Kanaoka *et al.*, 2008). Although the SI of both *drm1/2* and *met1* was unaffected by LRH, *SPCH* expression actually increased under the treatment in *drm1/2* and *FAMA* expression similarly increased in *met1*. These data also imply that both loci are normally subject to some degree of maintained, methylation-directed suppression. Intriguingly, *FAMA* is amongst a relatively unusual class of genes where upstream

genomic methylation increases in the triple mutant for demethylation activity (*ros1-3 dml2-1 dml3-1* triple mutant, termed *rdd*) compared with the WT (Lister *et al.*, 2008) suggesting a dynamic system of de/methylation. However, further careful dissection of the separate elements is required before this conclusion can be drawn.

Of special interest was the mutant for the methyltransferase DRM1/2. DRM2 is the only enzyme so far confirmed to methylate DNA *de novo* in *Arabidopsis* (Cao *et al.*, 2003) and its loss of function in the *drm1/2* genotype seemingly precluded the possibility of new methylation being triggered by low relative humidity in our plants. Thus, the finding that these genotypes showed no differential methylation between LRH and control conditions for either *SPCH* or *FAMA* supports the theory that the reduced humidity had catalysed the change in methylation status. Furthermore, this loss of differential methylation between growing treatments was matched by an associated lack of suppressed expression for both genes when grown under LRH, implying a causal link between the *de novo* methylation and suppressed expression of both genes in the WT plants grown in LRH. Finally, the loss in these mutants of the reduction in SI associated with LRH found in the WT plants suggests that the *de novo* methylation of *SPCH* and *FAMA* was responsible for their reduced expression under LRH and the corresponding reduction in stomatal frequency.

Reference to the *met1* mutant plants supported this view. The *met1* mutant is non-functional for the methyltransferase responsible for the maintenance of CG methylation, MET1 (Decreased Methylation 2DNA; Johnson *et al.*, 2007) and when these plants were grown under LRH, SI was no longer reduced (Fig. 3A), again implicating methylation in the process. A link between methylation and suppressed expression of *SPCH* and *FAMA* under LRH in WT plants could similarly be implied from the observation that the *met1* mutants showed no reduced expression for these genes under LRH. However, the WT-like response to LRH was unaffected in the *cmt3* plants, implying either that CHG methylation is not functionally important in the response, or that the considerable redundancy in the maintenance of asymmetric methylation (Cao *et al.*, 2003) is sufficient to mask any effect of CMT3 knock-out.

Zilberman *et al.* (2007) demonstrated the genome-wide relationship between methylation and transcription in *A. thaliana*. Methylation of short genes (<3 kb) clearly reduced expression and genes were generally depleted in methylation at the 3' and 5' ends, implying that methylation blocked transcript elongation. This relationship was quantitative, with more heavily methylated genes affected to a greater extent. Thus, heavy methylation of short, transiently expressed, transcription factor genes—as found here in *SPCH* and *FAMA* under LRH—is rare. Taken collectively, the extensive LRH-induced *de novo* methylation found, therefore, seems likely to be responsible for the down-regulation of *SPCH* and *FAMA* expression and thereby for the associated reduction in SI.

LRH treatment led to an increase in ds-sRNAs in all size classes. The role of micro-RNAs in the regulation of abiotic stress responses is better understood than that of siRNAs; nevertheless roles for siRNAs and RdDM have been proposed (Chinusammy and Zhu, 2009). Natural antisense siRNAs have been shown to regulate salt tolerance in *Arabidopsis* (Borsani *et al.*, 2005) and the up-regulated expression of other siRNA classes following abiotic stress treatments has also been observed (Sunkar and Zhu, 2004). Yao *et al.* (2010) found that mutants for siRNA biogenesis had different responses to genotoxic stress with *dcl4* and *rdr6* more sensitive to, and *dcl2*, *dcl3*, *d2d3* (double mutant) and *rdr2* more tolerant of stress. Ito *et al.* (2011) demonstrated a protective role for siRNAs in preventing the persistence and transposition of the *ONSEN copia*-type retrotransposons accumulated under heat stress. Our data show that LRH induces the accumulation of many siRNAs and that this has a measurable biological effect on stomatal pathway gene expression and the development of stomata.

In our plants, the stress-induced increase in siRNAs was positively correlated with increased DNA methylation of *SPCH* and *FAMA* under LRH and negatively correlated with gene expression and stomatal index. Mutations in RDR6, RDR2, and DCL3 blocked methylation, gene expression, and physiological responses similarly to *met1* and *drm1/2*. The results for these mutants showed a release of TGS methylation under stress when siRNA biogenesis was impaired. Surprisingly, despite the general accumulation of siRNAs under LRH and the co-involvement and dosage-dependent expression of *SPCH* and *FAMA* in the same genetic pathway, it was possible to separate siRNA-mediated methylation responses at the two loci.

For *SPCH,* we were able to identify local siRNAs associated with the downstream TEs/repeat region and methylation in the gene and its promoter. Strikingly, these siRNAs, and the genic siRNA for *FAMA*, collected under LRH, were co-incident with the start/end points for methylation. Daxinger *et al.* (2009) confirmed a stepwise pathway for the spread of RdDM where the Pol IV-RDR2-dependent secondary siRNAs caused downstream RdDM following primary siRNA initiation at the siRNA-generating sequence. Our findings for *SPCH* are consistent with an RDR2–DCL3-dependent spread of RdDM in this manner. They suggest that AGO4-dependent primary RdDM contributes to the response to LRH treatment but that it is not responsible for the consistent effect on methylation, gene expression, and stomatal phenotype which, instead, is correlated with the increased production of secondary siRNAs and spread of RdDM. Interestingly, AGO4 might be involved more generally in LRH stress responses as few *ago4* plants were able to germinate and grow successfully with this mild drying stress.

The TE/repeat region downstream of *SPCH* is normally methylated in WT (L*er* and Col-0) control plants and methylation in all sequence contexts is observed at this location in genome-wide bisulphite sequencing from different tissues (Lister *et al.*, 2008). The introduction of stress causes additional methylation at this location that starts, moreover, ~1.5 kb distant of the 3' end of *SPCH*. The spread of methylation from TEs flanking genes is considered deleterious for endogenous gene transcription

(Hollister and Gaut, 2009) and TEs close to genes (<500 bp) are, therefore, less likely to be methylated. Ahmed *et al.* (2011) have shown, recently, that this tendency is specific to the 5'-end of genes and proposed that there is a bi-directional spread of methylation within TE sequences and in siRNA-targeted flanking sequences. To our knowledge, RdDM spread from TE regions >1 kb downstream of genes has not been investigated. RdDM is generally thought to be a genome defence mechanism active in the suppression of transposons and other harmful elements (reviewed in Lippman and Martienssen, 2004) that may influence proximal genes in response to stress (Huettel *et al.*, 2006). In a vital gene like *SPCH*, reversible RdDM spread from neighbouring TEs could have been co-opted as a response conferring advantage under LRH stress.

FAMA was a target of RdDM following LRH and the increased expression of *FAMA* in *rdr2* and *dcl3*, accompanied by the release of methylation, strongly suggested the involvement of secondary siRNAs. It was not possible, however, to identify 24 nt siRNA transcripts associated with the locus. It may be that we have yet to identify 24 nt siRNAs specific to *FAMA* in the Landsberg *erecta* ecotype. Intriguingly, though, mutation of RDR6 prevented the accumulation of a genic 21 nt siRNA in *FAMA* and released genic methylation observed in the control WT. This increased *FAMA* expression and final stomatal index. The role of genic methylation in gene silencing or suppression is still controversial. Usually, genic methylation is considered to be a hallmark of previous PTGS which may have only a moderate effect on endogenous gene expression when it does not block 3' elongation (Aufsatz *et al.*, 2002). Our findings for *FAMA* were reminiscent of those of Fojtová *et al.* (2006) who found, using TGS'd and PTGS'd-epiallelic transgenes of *Nicotiana tabacum*, that while PTGS-induced methylation was faithfully propagated and capable of silencing, TGS-induced methylation was not, in itself, very effective but that silencing ability was restored after the locus was hypomethylated and transcribed and PTGS-generated sRNA signals spread re-silencing *in trans*. RDR6 is required for the production of secondary siRNAs following reception of an RNA signal (probably 24 nt siRNAs; Dunoyer *et al.*, 2010*a*; Molnar *et al.*, 2010) in systemic, long-distance silencing (Brosnan *et al.*, 2007). Our data imply a connection between stress-induced RDR2–DCL3-dependent and RDR6-dependent siRNAs in RdDM at *FAMA*. Certainly, the genic methylation pattern and its spread into the promoter region are remarkably similar, and DCL3 and RDR2 are both required for the production of the stress-sensitive 21 nt siRNA. Brosnan *et al.* (2007) proposed a model for the interaction between RDR6–DCL4/2 and AGO1, and PolIV–RDR2–DCL3 and AGO4 in long-distance signalling. Dunoyer and Voinnet (2008), reviewing the model, pointed out the remaining gaps in our knowledge. Much research has been carried out with silencing induced from both transgenic and endogenous inverted repeats (Fojtová *et al.*, 2006; Lunerová-Bedřichova *et al.*, 2009; Dunoyer *et al.*, 2010*b*). *FAMA*, however, could very well be an example of an endogenous locus whose expression is suppressed by the production and systemic movement of siRNAs and could provide a natural laboratory for investigating the pathway under stress.

It is tempting to speculate that the lack of biomass reduction under LRH in the methyltransferase mutants was connected to the lack of response in stomatal development; however, there is no reason to assume a stomatal limitation to growth in our experiments. Rather, our results suggest that epigenetic plasticity is likely to be a general mechanism in many stress-response pathways. Verhoeven *et al.* (2010) showed that methylation in stress-sensitive pathways was broadly heritable. Our results also imply that stress-induced methylation is reversible. The epigenetic control of phenotypic plasticity with environment and consequences for adaptation and evolution are of great interest (Richards, 2006). Molinier *et al.* (2006) reported transgenerational epigenetic effects leading to enhanced somatic recombination in response to two, contrasting stresses and potentially adaptive fitness benefits have also been observed (Whittle *et al.*, 2009; Paun *et al.*, 2010). The findings presented here add an extra dimension to this train of thought by demonstrating the epigenetic control of an important process that confers physiological plasticity to the plant. In this sense, the processes involved may have adaptive significance. Moreover, it is entirely possible that when this methylation-based control system is activated, it provides a means of accommodating for variation in the growing conditions that otherwise may lead to dramatic reductions in biomass and productivity or, alternatively, increase LRH stress-tolerance. It could be argued that the need to do this is greatest among extreme inbreeding species such as *Arabidopsis* that typically lack variation within a population. The capacity to evoke such epigenetic modifications has the potential to buffer adaptation and allow for the longer term assumption of DNA sequence-based changes (Parkinson *et al.*, 2007). It could, therefore, be speculated that DNA methylation in the stomatal developmental pathway could ultimately provide at least part of the explanation for the differences in control between short-term plasticity and fixed, genetic, adaptation of stomatal density.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. Synthetic oligo designs for *SPCH* and *FAMA* fragments with known methylated cytosines (Cm) and SNP labels in red.

Supplementary Table S2. Primer designs for bisulphite-specific PCR of genes in the stomatal pathway.

Supplementary Table S3. Primers for unmodified genomic DNA (equivalent to the bisulphite-specific region assayed).

Supplementary Table S4. Primers for qPCR of methylated genomic DNA following affinity capture.

Supplementary Table S5. Primers for MT-qPCR of stomatal developmental and endogenous control genes.

Supplementary Table S6. Seed weights (mg per 100 seeds) and seed sizes (μm^2) of *Arabidopsis thaliana* Landsberg *erecta* plants germinated and grown from stock centre supplied seeds in the control and LRH treatments and from collected seeds following exposure to the environments.

Supplementary Fig. S1. The genomic context of differential methylation with low relative humidity at the *SPCH* (A) and *FAMA* (B) loci.

Supplementary Fig. S2**.** Pavement cell and stomatal densities (mm^{-2}) of the abaxial leaf epidermis of the background types and methyltransferase mutants.

Acknowledgements

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