The EMBO Journal (2005) 24, 1418–1429 | © 2005 European Molecular Biology Organization | All Rights Reserved 0261-4189/05
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THE EMBO JOURNAL

Pivotal role of AtSUVH2 in heterochromatic histone methylation and gene silencing in *Arabidopsis*

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SU(VAR)3–9 like histone methyltransferases control heterochromatic domains in eukaryotes. In *Arabidopsis*, 10 *SUVH* genes encode SU(VAR)3–9 homologues where *SUVH1*, *SUVH2* and *SUVH4* (*KRYPTONITE*) represent distinct subgroups of *SUVH* genes. Loss of SUVH1 and SUVH4 causes weak reduction of heterochromatic histone H3K9 dimethylation, whereas in *SUVH2* null plants mono- and dimethyl H3K9, mono- and dimethyl H3K27, and monomethyl H4K20, the histone methylation marks of *Arabidopsis* heterochromatin are significantly reduced. Like animal SU(VAR)3–9 proteins SUVH2 displays strong dosage-dependent effects. Loss of function suppresses, whereas overexpression enhances, gene silencing, causes ectopic heterochromatization and significant growth defects. Furthermore, modification of transgene silencing by SUVH2 is partially transmitted to the offspring plants. This epigenetic stability correlates with heritable changes in DNA methylation. Mutational dissection of *SUVH2* indicates an implication of its N-terminus and YDG domain in directing DNA methylation to target sequences, a prerequisite for consecutive histone methylation. Gene silencing by SUVH2 depends on MET1 and DDM1, but not CMT3. In *Arabidopsis*, SUVH2 with its histone H3K9 and H4K20 methylation activity has a central role in heterochromatic gene silencing.

The EMBO Journal (2005) **24,** 1418–1429. doi:10.1038/sj.emboj.7600604; Published online 17 March 2005
Subject Categories: chromatin & transcription; plant biology
Keywords: DNA methylation; gene silencing; heterochromatin; histone methylation

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Received: 20 July 2004; accepted: 8 February 2005; published online: 17 March 2005

Introduction

Epigenetically established changes in chromatin structure define the gene expression potential during development. These processes are controlled by complex DNA and histone modification systems. Modifications at the highly conserved N-terminal histone tails include acetylation, methylation, phosphorylation and ubiquitination, and show characteristic differences between active and repressed chromatin states (Stahl and Allis, 2000; Jenuwein and Allis, 2001). Lysine methylation at H3K4, H3K36 and H3K79 marks transcriptionally active chromatin, whereas methylation of H3K9, H3K27 and H4K20 defines repressed chromatin domains (Lachner *et al*, 2001; Fischle *et al*, 2003). Mono-, di- and trimethylation states of histone lysine residues extend the coding potential of the 'epigenetic histone code' and specific states of H3K9, H3K27 and H4K20 methylation define repressed heterochromatic domains in mouse and *Drosophila* (Peters *et al*, 2003; Schotta *et al*, 2004). In mouse H3K9 trimethylation, H3K27 monomethylation and H4K20 trimethylation index pericentric heterochromatin, whereas in *Drosophila* these regions show H3K9 di- and tri-, H3K27 mono-, di- and tri-, and H4K20 trimethylation (Ebert *et al*, 2004).

Plant development in contrast to animals is rather plastic and considerably affected by environmental factors. Therefore, subtle changes in chromatin structure might be required for fine-tuning of gene expression and probably for this reason multi-gene families for DNA and histone modification systems are found in plants. In *Arabidopsis*, three different classes of DNA methylases (Cao and Jacobsen, 2002), 12 putative methylcytosine-binding proteins (Zemach and Grafi, 2003), 37 SET domain proteins (Baumbusch *et al*, 2001), 18 putative histone deacetylases and 12 putative histone acetylases have been identified (*Arabidopsis* Genome Initiative, 2000; Pandey *et al*, 2002).

A predominant role in establishment of epigenetically stable active or repressed chromatin domains is attributed to histone lysine methylation by SET domain proteins. These proteins can be assigned to four groups typified by their *Drosophila* homologues E(Z), TRX, ASH1 and SU(VAR)3–9 (Jenuwein *et al*, 1998). In *Arabidopsis*, several of these genes were identified by developmental phenotypes of mutations. Like animal E(Z), CLF (Goodrich *et al*, 1997) and MEA (Grossniklaus *et al*, 1998) act as negative regulators and are involved in control of flower and endosperm development, respectively. The homeotic effects of *clf* and the function of MEA in imprinting of paternal genes first indicated an important role of SET domain proteins for chromatin regulation and epigenetic inheritance in plants (Goodrich *et al*, 1997; Vielle-Calcada *et al*, 1999). Similar to TRITHORAX in animals, *Arabidopsis* ATX1 acts as an activator of homeotic genes (Alvarez-Venegas *et al*, 2003).

Epigenetically stable transmission of a condensed and transcriptionally inert chromatin state is characteristic for heterochromatin and heterochromatic gene silencing. With the evolutionary conserved SU(VAR)3–9 SET domain protein (Tschiersch *et al*, 1994; Ivanova *et al*, 1998; Aagaard *et al*, 1999) and demonstration of its function in histone H3K9 methylation (Rea *et al*, 2000), a basic factor of heterochromatin formation has been identified. In plants, in general, a large fraction of the genome is heterochromatic and extensive heterochromatic silencing processes are found. Involvement of histone H3K9 and DNA methylation in heterochromatin formation and heterochromatic gene silencing was documented in studies of the SU(VAR)3–9 homologue SUVH4 (KYP) and DNA methylation defective mutations of the *MET1*, *CMT3*, *DRM* and *DDM1* genes (Cao and Jacobsen, 2002; Gendrel *et al*, 2002; Jackson *et al*, 2002; Tariq *et al*, 2003). In contrast to mammals, plants contain a large number of SU(VAR)3–9 homologues (Baumbusch *et al*, 2001). In *Arabidopsis*, 10 different SU(VAR)3–9 homologous SUVH proteins are found and we studied the function of SUVH1, SUVH2 and SUVH4, representative members of three subgroups of *Arabidopsis* SU(VAR)3–9 homologues.

Here, for the first time, we show the heterochromatin association of the *Arabidopsis* SU(VAR)3–9 homologues SUVH1 and SUVH2 and present evidence that SUVH proteins differentially control heterochromatic histone methylation marks. Our data define SUVH2 as a central function in heterochromatin formation and heterochromatic gene silencing in *Arabidopsis*. In *suvh2* null mutations all heterochromatin-specific histone methylation marks are significantly reduced, which is connected with strong suppression of transcriptional gene silencing (TGS). After overexpression, SUVH2 shows ectopic nuclear distribution and causes extensive heterochromatization, accompanied with an increase of DNA and heterochromatic histone methylation. SUVH2-dependent suppression or enhancement of TGS shows partial epigenetic stability connected with heritable changes in symmetric and nonsymmetric DNA methylation over consecutive generations. Mutational dissection of *SUVH2* revealed a role of its N-terminus in control of nuclear protein distribution and a function of its YDG domain in directing DNA methylation to the target sequences. We also show that this DNA methylation does not cause silencing but rather is a prerequisite for consecutive SUVH2-dependent histone H3 and H4 methylation to establish heterochromatic silencing.

Results

Heterochromatin association of SUVH proteins in Arabidopsis

The heterochromatin-associated SU(VAR)3–9 proteins control repressive chromatin structures by histone H3K9 methylation (Rea *et al*, 2000; Nakayama *et al*, 2001; Schotta *et al*, 2002). In contrast to animals and fungi, which have one or two *Su(var)3–9* homologues, in *Arabidopsis* 10 *SUVH* genes encode SU(VAR)3–9 homologous proteins (Baumbusch *et al*, 2001). Tree reconstruction of 21 SUVH protein sequences from different plant species results in four clearly distinct subgroups of SUVH proteins in angiosperms (Figure 1A). Branching of both moss and fern SUVH fragments at the root of the angiosperm SUVH subgroups argues for a phylogenetic split of *SUVH* genes during early evolution of seed plants. The *SUVH4*-like genes are more distant to all groups of *SUVH* genes. Only *SUVH4*-like genes contain introns and retrotransposition might have been involved in evolution of the different *SUVH* gene families. Branching of gymnosperm *PtaSUV1f* with the *SUVH4* subgroup suggests that functional differentiation of SUVH proteins had already occurred before the angiosperm–gymnosperm split.

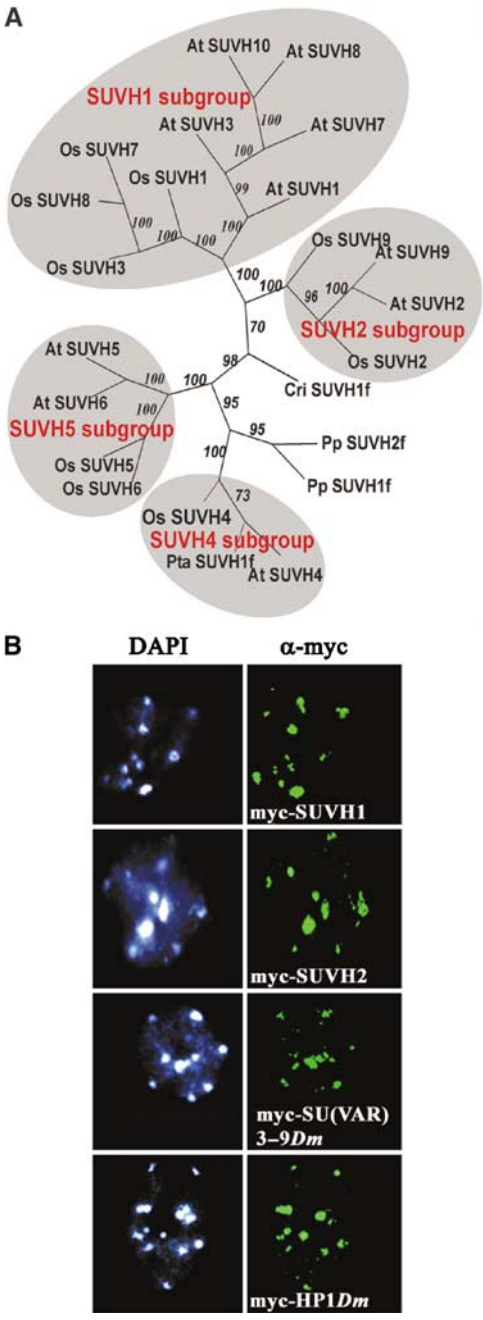

Figure 1 SU(VAR)3–9 homologous proteins in plants. (**A**) Four conserved groups of *SUVH* genes are found in angiosperms. Phylogenetic analysis of 21 SUVH protein sequences from *Arabidopsis* (AtSUVH), rice (OsSUVH), *Pinus taeda* (Pta), *Physcomitrella patens* (Pp) and *Ceratopteris richardii* (Cri). (**B**) Immunostaining of plants expressing myc fusion protein of SUVH1, SUVH2 and *Drosophila* SU(VAR)3–9 and HP1 in *Arabidopsis* interphase nuclei with α-myc. Heterochromatin association is found for the SUVH1 and SUVH2 proteins as well as for *Drosophila* SU(VAR)3–9 and HP1.

To identify chromatin targets of the *Arabidopsis* SU(VAR)3–9 homologues SUVH1 and SUVH2 proteins, we studied their nuclear distribution in transgenic *Arabidopsis* plants expressing myc or EGFP fusion proteins. The SUVH1 and SUVH2 fusion proteins associate with the DAPI bright regions of interphase nuclei (Figure 1B), which represent pericentromeric heterochromatin in *Arabidopsis* (Fransz *et al*, 2000; Soppe *et al*, 2002). We also tested nuclear distribution of the *Drosophila* SU(VAR)3–9 and HP1 heterochromatin proteins in *Arabidopsis*. The *Drosophila* proteins in *Arabidopsis* also associate with heterochromatin (Figure 1B), indicating conserved mechanisms of heterochromatin association of these proteins in plants and animals. SUVH1 and SUVH2 proteins are both heterochromatin associated and could comprise redundant functions. Alternatively, these proteins could control distinct heterochromatic histone methylation marks as well as their combinatorial interplay.

Differential control of heterochromatic histone methylation by SUVH proteins

We analysed the effect of SUVH1, SUVH2 and SUVH4 on heterochromatic histone H3 and H4 methylation marks using specific histone methylation antibodies (Peters *et al*, 2003). In these studies, we used loss-of-function T-DNA insertion mutations or antisense lines (cf. Supplementary Figures 1 and 2). None of the insertional mutations or the antisense lines show a significant phenotypic defect in homozygous constitution.

Arabidopsis heterochromatin is enriched in methylated DNA and mono- and dimethylated histone H3K9 (Tariq *et al*, 2003; Figure 2A). Other heterochromatic histone methylation marks in *Arabidopsis* are mono- and dimethyl H3K27 and monomethyl H4K20 (Figure 2A). In contrast, trimethyl H3K9, trimethyl H3K27, and di- and trimethyl H4K20 are found together with methylated H3K4 and H3K36 in *Arabidopsis* euchromatin (Figure 2A and B). In our studies loss-of-function mutations of *SUVH1* and *SUVH4* appear to show only weak reduction of mono- and dimethyl H3K9 in pericentromeric heterochromatin (Figure 2A and Supplementary Figure 1). In contrast, *SUVH2* loss-of-function mutations strongly reduce all heterochromatin-specific histone and DNA methylation marks (Figure 2A and C). The most dramatic effect of SUVH2 is on H4K20 monomethylation. Reduction of DNA methylation at heterochromatic sequences in *SUVH2* null plants was further quantified by bisulphite sequence analysis of Athila transposons (Supplementary Figure 3). *In vitro* analysis shows that SUVH2 is a nucleosome-dependent HMTase and methylates histone H3 and H4 in recombinant nucleosomes (Figure 2D).

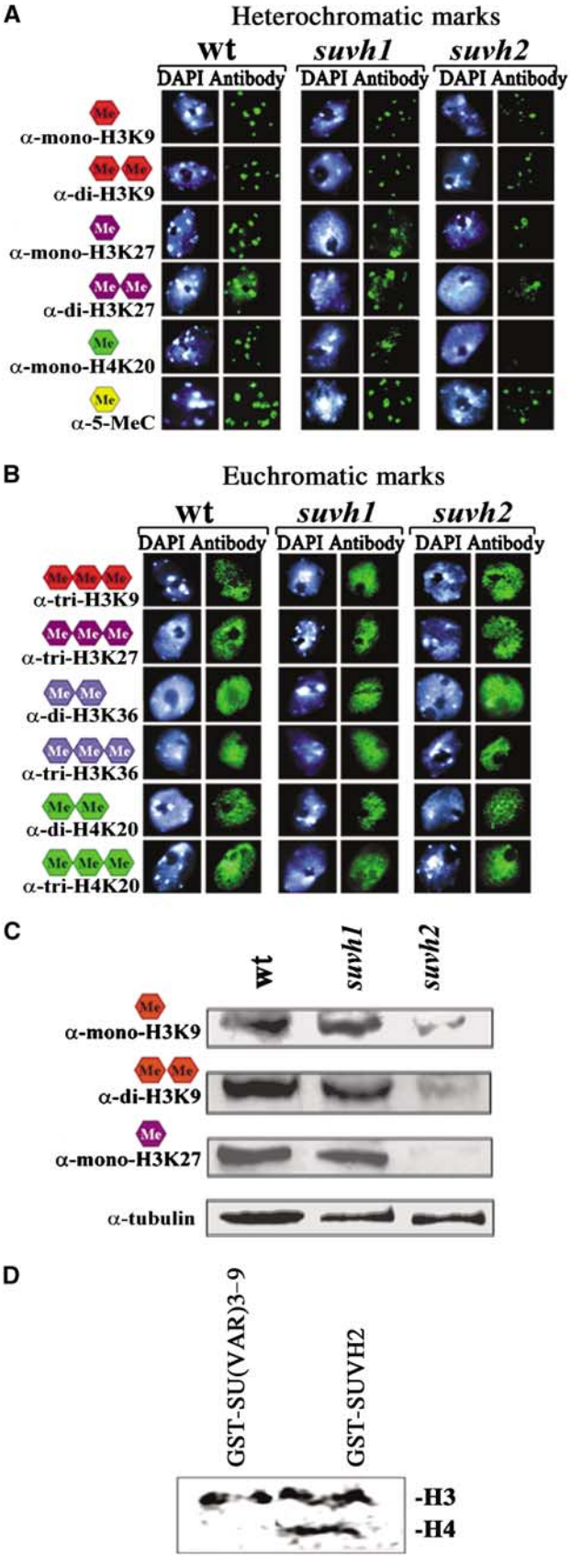

Figure 2 Differential effects of *suvh1* and *suvh2* mutations on histone and DNA methylation. (**A, B**) Immunohistochemical staining of wild-type, *suvh1* and *suvh2* interphase nuclei with antibodies recognizing specific histone and DNA methylation marks. In *suvh2*, but not in *suvh1*, mono- and dimethyl H3K9, mono- and dimethyl H3K27, monomethyl H4K20 and 5-methylcytosine (heterochromatic marks) are significantly reduced (A). No effects are found on the trimethyl H3K9, trimethyl H3K27, di- and trimethyl H3K36 and di- and trimethyl H4K20 euchromatic marks (B). (**C**) Western analysis of nuclear extracts of wild-type, *suvh1* and *suvh2* mutant plants. Only in *suvh2* significant reduction of mono- and dimethyl H3K9 and monomethyl H3K27 is found. (**D**) *In vitro* recombinant SUVH2 shows H3 and H4 HMTase activity in assays with reconstituted nucleosomes.

Drosophila Su(var)3–9 is a haplo- and triplo-dosage-dependent modifier of heterochromatic gene silencing (Schotta *et al*, 2002). In order to study the dosage-dependent effects of *Arabidopsis SUVH1* and *SUVH2*, we constructed transgenes with the 35S* promotor (Mindrinos *et al*, 1994) or used a glucocorticoid-mediated transcriptional system, which allows controlled induction of gene expression by dexamethasone treatment (Aoyama and Chua, 1997). Overexpression of *SUVH1* has no significant effect on pericentromeric heterochromatin and the protein remains heterochromatin associated (Figure 3A). In contrast, after overexpression, SUVH2 shows dispersed nuclear distribution, resulting in ectopic heterochromatization. By electron microscopic analysis, additional blocks of heterochromatic material can be detected (Figure 3A). Immunocytological analysis of *SUVH2* overexpression plants reveals a significant increase in mono- and dimethyl H3K9, mono- and dimethyl H3K27, and monomethyl H4K20, as well as cytosine methylation (Figure 3B). We have quantified the dosage-dependent effects of SUVH2 on five histone methylation marks by Western analysis of bulk histones. In *suvh2* mutant plants, heterochromatic H3K9 mono- and dimethylation, H3K27 monomethylation and H4K20 monomethylation are significantly reduced (Figures 2C and 3C), whereas *SUVH2* overexpression causes enhancement of H3K9 dimethylation and H4K20 monomethylation (Figure 3B and C). In contrast, trimethylation of H3K9, which is a euchromatic histone methylation mark in *Arabidopsis*, is significantly reduced after *SUVH2* overexpression (Figure 3C and D). Significant reduction in immunostaining for other euchromatic histone modification marks like dimethyl H3K4, acetyl H3K9 and trimethyl H3K27 is also found in *SUVH2* overexpression plants (Figure 3D).

SUVH2-dependent growth defects and the mutational dissection of its molecular function

None of the 24 *35S*::*mycSUVH1* overexpression lines nor the *GVG*::*SUVH1-EGFP* lines after dexamethasone treatment show any phenotypic defects (not shown). In contrast, four of 24 independent *35S*::*mycSUVH2* transgenic lines show significant growth reduction (mini-plant phenotype) and a curled cotyledon phenotype (Figure 4A and B). Western blot analysis shows direct correlation between the extent of *SUVH2* overexpression and the strength of mini-plant growth defects (Figure 4C). Line *35S*::*mycSUVH2#4* with lower

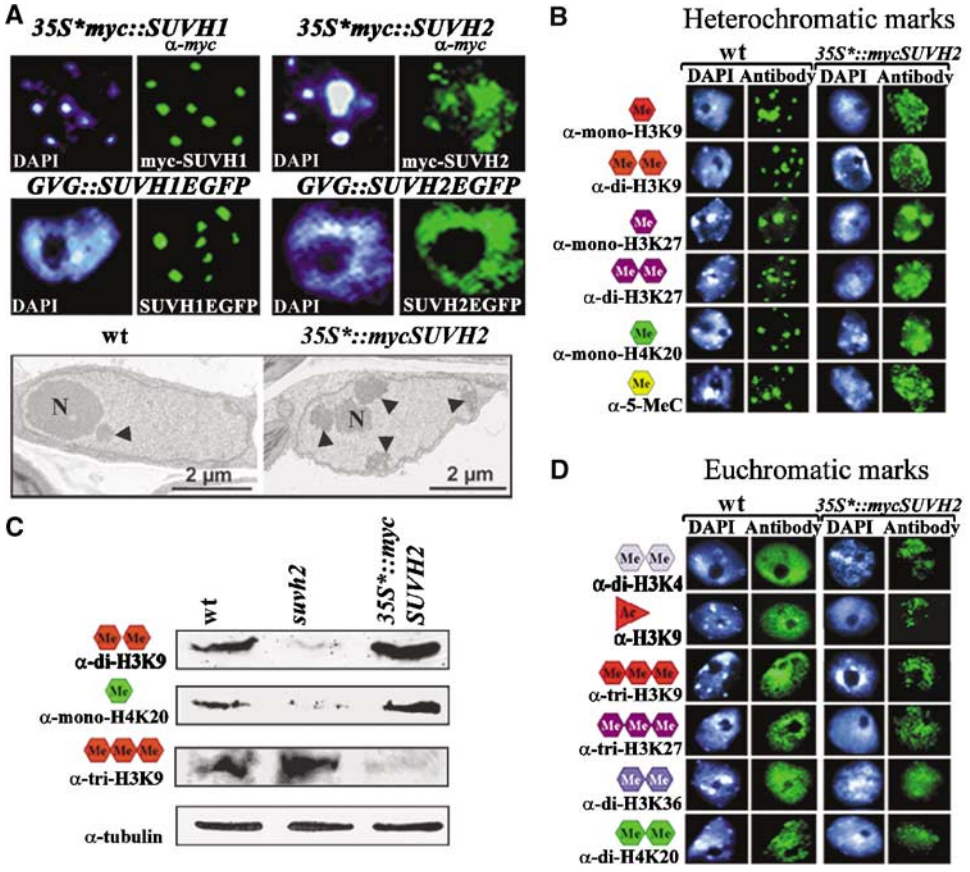

Figure 3 Ectopic heterochromatization in *SUVH2* overexpression lines. (**A**) Immunostaining of nuclei from *35S*::*mycSUVH1*, *35S*::*mycSUVH2* with α-myc and GFP fluorescence analysis in dexamethasone-treated *GVG*::*SUVH1EGFP* and *GVG*::*SUVH2EGFP* plants. Only SUVH2 shows ectopic distribution. Electron microscopic analysis of nuclei from *35S*::*mycSUVH2* plants shows ectopic heterochromatin (arrowheads). (**B**) Immunocytological analysis of heterochromatic histone and 5-methylcytosine methylation in *SUVH2* overexpression plants. Enhanced staining for all heterochromatic marks (mono- and dimethyl H3K9, mono- and dimethyl H3K27, monomethyl H4K20 and 5-methylcytosine) is found. (**C**) Western analysis of *suvh2* mutant and *35S*::*mycSUVH2* overexpression plants. In *suvh2*, dimethyl H3K9 and monomethyl H4K20 are reduced. In *35S*::*mycSUVH2* overexpression plants, heterochromatic H3K9 dimethyl and H4K20 monomethyl are enriched, whereas euchromatic H3K9 trimethyl is reduced. (**D**) Immunostaining for euchromatic histone modification marks in *SUVH2* overexpression plants. Staining for dimethyl H3K4, acetyl H3K9, trimethyl H3K27, dimethyl H3K36 and dimethyl H4K20 is significantly reduced.

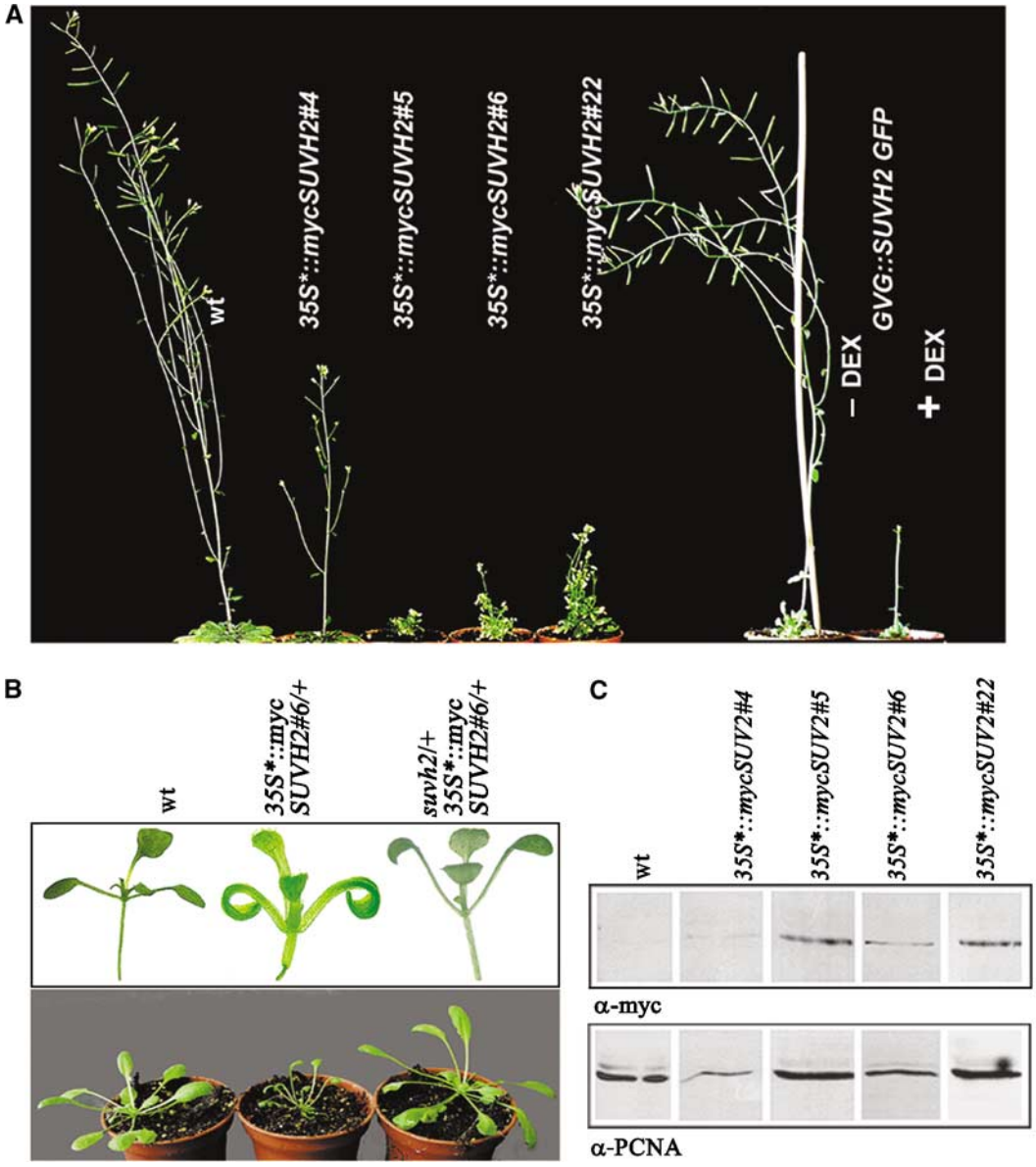

Figure 4 Growth and developmental defects in *SUVH2* overexpression plants. (**A**) *SUVH2* overexpression causes mini-plant phenotype in *35S**::*mycSUVH2#5, #6, #22* and dexamethasone-treated *GVG*::*SUVH2EGFP* lines. (**B**) *SUVH2* overexpression seedlings show a curled cotyledon phenotype (upper panel). By introducing a *suvh2* null allele, the mini-plant and curled cotyledon phenotypes are significantly rescued in *suvh2*/+; *35S**::*mycSUVH2#6*/+ plants (lower panel). (**C**) Western analysis of extracts from *35S**::*mycSUVH2* lines with α-myc. The amount of mycSUVH2 protein correlates with the strength of growth defects. *35S**::*mycSUVH2#4* plants with a weak growth reduction expresses a lower amount of additional SUVH2 as compared to lines with a mini-plant phenotype.

amount of additional SUVH2 is only weakly affected, whereas the three lines *35S**::*mycSUVH2#5*, *#6* and *#22* with higher amount of SUVH2 manifest a strong mini-plant phenotype. Similarly, the *GVG*::*SUVH2-EGFP* lines manifest a mini-plant phenotype only after dexamethasone treatment (Figure 4A). Significant rescue of *SUVH2* overexpression phenotypes is found in *suvh2*/+; *35S**::*mycSUVH2#6*/+ plants by introducing a *suvh2* null allele (Figure 4B).

For functional dissection of *SUVH2*, we isolated mutations within the *35S**::*mycSUVH2#5* transgene after EMS mutagenesis. These mutations were identified as dominant suppressors of the curled cotyledon phenotype in M1 seedlings. All confirmed dominant suppressors rescued the mini-plant growth defect. In M3 two classes of mutant lines could be established. In 224 lines the suppressor mutation and the *SUVH2* transgene segregate independently, whereas in 96 lines complete linkage is found. The latter class represents *35S**::*mycSUVH2#5* transgene mutations and we studied the functional consequence of seven such mutations (Figure 5). All are missense mutations located either in the N-terminus (*5–1*), the YDG (*5–2*) or the SET domain (*5–3* to *5–7*) of SUVH2 (Figure 5A). Only the N-terminus mutation *5–1* interferes with ectopic nuclear distribution of SUVH2 (Figure 5B). Mutations *5–2* and *5–3* located in the YDG domain and the region between the YDG domain and the preSET region, respectively, cause loss of ectopic 5-methylcytosine, H3K9 dimethylation and H4K20 monomethylation, although ectopic distribution of SUVH2 is not affected (Figure

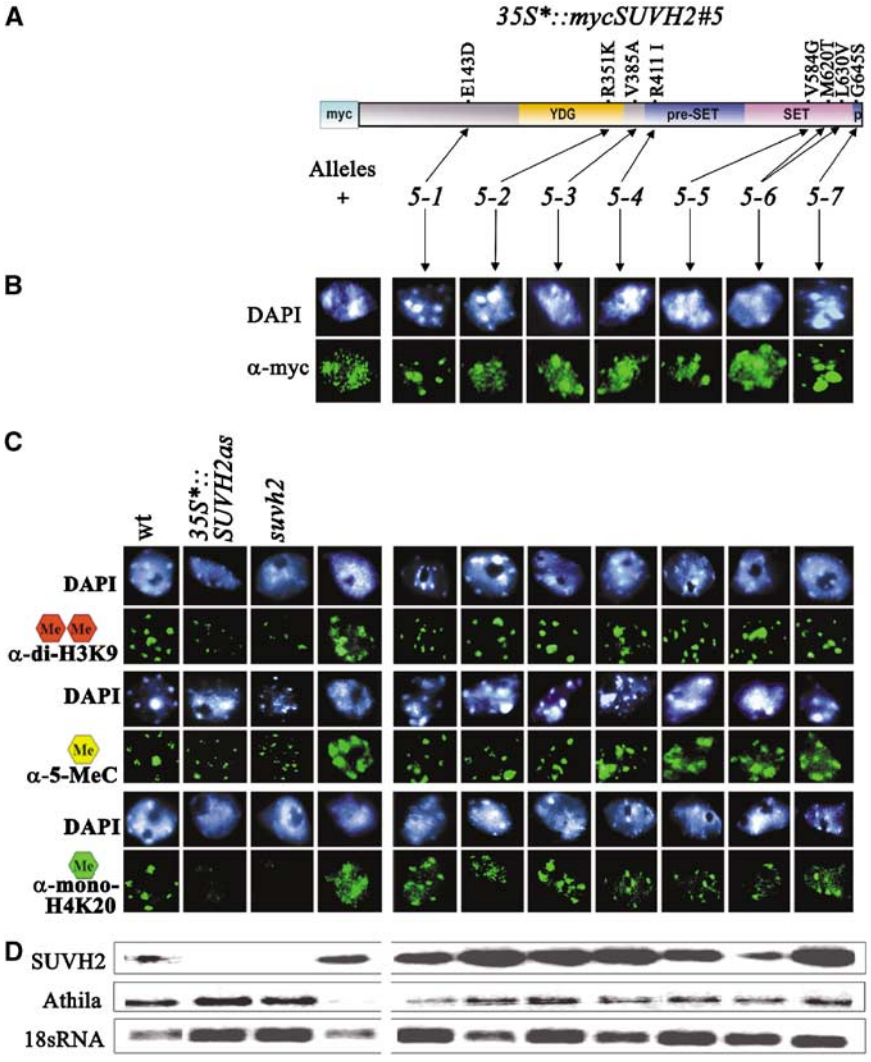

Figure 5 Functional dissection of *SUVH2* by transgene mutations. (**A**) Molecular nature of *35S**::*mycSUVH2#5* transgene mutations. Structure of SUVH2 with the conserved YDG, preSET, SET and postSET (p) domains. (**B**) Immunostaining with α-myc shows that only the N-terminus mutation *5-1* eliminates ectopic distribution of SUVH2. (**C**) All mutations eliminate ectopic H3K9 and H4K20 methylation. The N-terminus mutation *5-1*, the YDG mutation *5-2* and mutation *5-3* eliminate ectopic DNA methylation, whereas in plants with the SET domain mutations *5-4*, *5-5*, *5-6* and *5-7* ectopic DNA methylation is observed. (**D**) Silencing of Athila transposons by *SUVH2* overexpression is rescued by all transgene mutations independent of DNA hypermethylation (RT–PCR) (cf. Supplementary Figure 3 for bisulphite data).

5B and C). Mutations in the SET domain (*5–3* to *5–7*) eliminate ectopic H3K9 and H4K20 methylation, but leave ectopic DNA methylation unaffected.

In order to study *SUVH2* overexpression effects on heterochromatic silencing of endogenous sequences, we analysed expression of Athila transposons. Overexpression of *SUVH2* causes strong repression of Athila, whereas all *SUVH2* transgene mutations rescue this silencing effect (Figure 5D). Immunocytological and bisulphite sequence analysis shows that none of the SET domain mutations reduce DNA hypermethylation at Athila although its silencing is released (Figure 5D and Supplementary Figure 3).

Taken together, our mutant analysis resolves a sequence of molecular events connected with SUVH2-induced heterochromatic gene silencing. Mutation *5–1* indicates a possible role of the SUVH2 N-terminus in target sequences recognition, whereas the YDG domain region appears to be involved in directing DNA methylation to these sequences. DNA methylation alone is not sufficient for silencing, but rather functions as a mark directing SUVH2-dependent histone methylation to sequences subjected to silencing. Moreover, our data suggest that SUVH2-mediated DNA methylation precedes histone methylation.

Dosage dependence and epigenetic maintenance of SUVH2-induced transgene silencing

To study dosage-dependent effects of SUVH proteins on repeat-induced TGS, we used a new type of transgene constructs that contain four tandem copies of the luciferase gene (cf. Materials and methods). The *LUC2* transgene shows moderate luciferase silencing, which is significantly enhanced by *SUVH2* (Figure 6) but not *SUVH1* overexpression (data not shown). Significant enhancement of TGS is already found in crosses with the *35S**::*mycSUVH2#4* line showing only weak growth defects (Figure 5), indicating that TGS is already efficiently enhanced by low levels of *SUVH2* overexpression. The loss-of-function effect of *SUVH2* on TGS was studied using a specific antisense line. Out of 25 independent

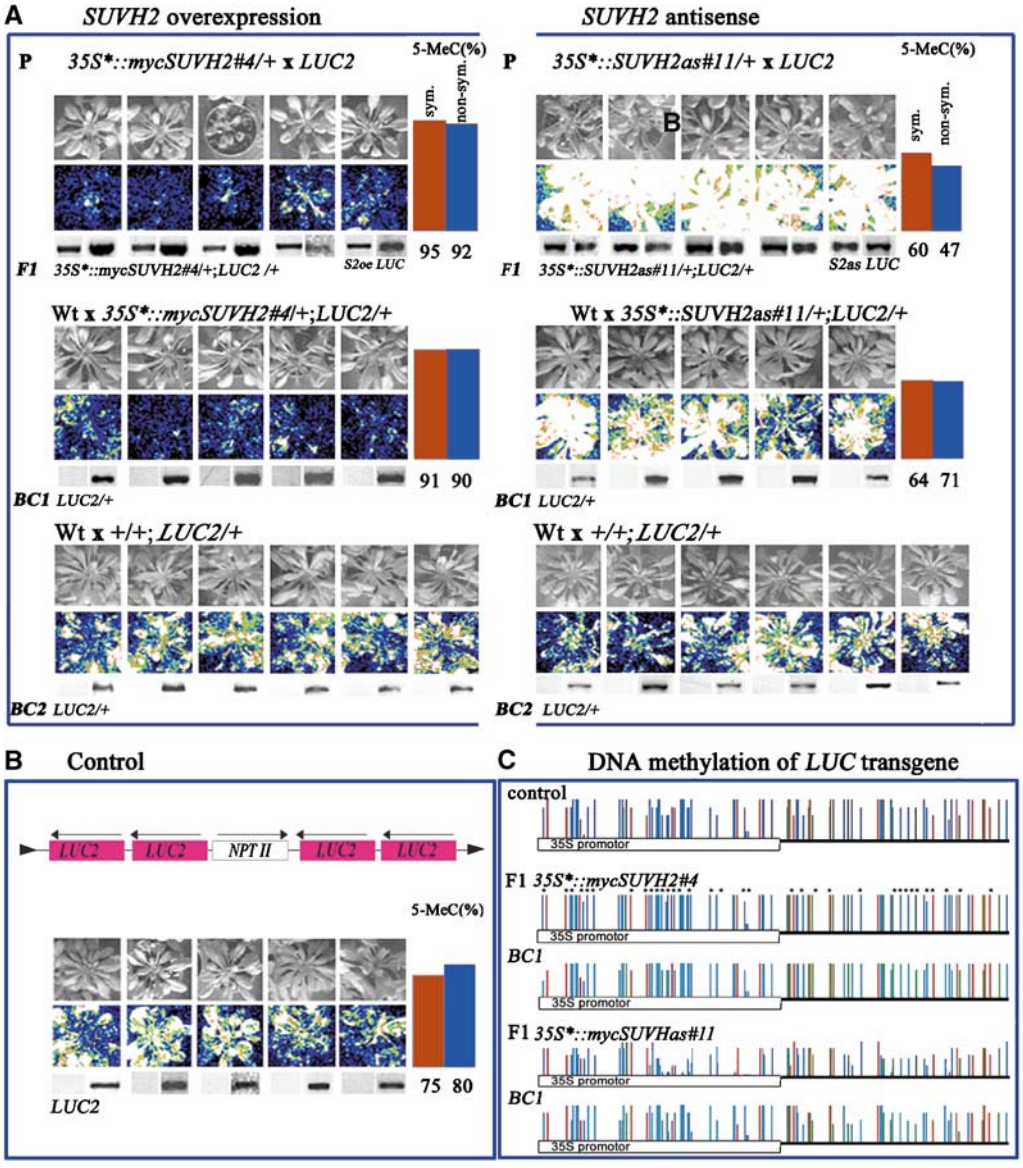

Figure 6 Dosage-dependent modifier effects of SUVH2 on *LUC2* transgene silencing. (**A–C**) Crosses of *35S**::*mycSUVH2#4/+* and *35S**::*SUVH2as#11/+* with *LUC2* homozygous plants and backcrosses (BC) of F1 and F2 *LUC2/+* plants to wild type (A). Structure and activity of the *LUC2* repeated transgene in control plants (B). In *35S**::*mycSUVH2#4/+*; *LUC2/+* plants with *SUVH2* overexpression, *LUC2* silencing is enhanced, whereas in *35S**::*SUVH2as#11/+*; *LUC2/+* plants without SUVH2 *LUC2* silencing is strongly released. The repressed or activated state of the *LUC2* is maintained after a backcross of *35S**::*mycSUVH2#4/+*;*LUC2/+* and *35S**::*SUVH2as#11/+*;*LUC2/+* with wild-type plants, respectively. In *LUC2/+* offspring from the second backcross generation, reversion of transgene silencing to the control level is found. Symmetric (red bars) and nonsymmetric (blue bars) DNA methylation at *LUC2* transgenes was studied by bisulphite sequencing (A and B). Bisulphite sequence analysis of control *LUC2/+*, F1 *35S**::*mycSUVH2#4/+*; *LUC2/+* and *35S**::*SUVH2as#11/+*; *LUC2/+* as well as *LUC2/+* BC1 progeny plants. (C) Stars denote significantly changed symmetric CpG (red) and CpNpG (green) and nonsymmetric CpNpN (blue) cytosine residues (N, no G).

*35S**::*SUVH2as* antisense lines, we selected line *SUVH2as#11* causing complete elimination of SUVH2 transcript, whereas other tested *SUVH* genes are not affected (cf. Supplementary Figure 1). After a cross of *SUVH2as#11/SUVH2as#11* with *LUC2* homozygous plants, all *SUVH2as#11/+*; *LUC2/+* offspring show strong suppression of TGS (Figure 6). Significant suppressor effects are also seen with antisense lines only partially eliminating the SUVH2 transcript (not shown). Our experiments reveal the dosage-dependent modifier effect of SUVH2 on TGS. Overexpression enhances whereas loss-of-function suppresses TGS. We further studied the epigenetic stability of SUVH2 modified *LUC2* transgene silencing in progeny plants, lacking *SUVH2* overexpression or antisense constructs which were generated by a backcross of the F1 progeny with wild-type plants. Partial epigenetic stability of modified *LUC2* transgene silencing is found in the offspring plants for the enhanced as well as suppressed state of *LUC2*. Reciprocal crosses produced identical results (data not shown). Reversion to the level typical for control plants is found in offspring of the second backcross generation (Figure 6A and B). To study whether epigenetic maintenance of SUVH2-modified TGS is based on stable transmission of changes in DNA methylation, we performed bisulphite sequence analysis of the *LUC2* transgene. In plants produced by

the first backcross (BC1) to wild type, the *LUC2* transgene inherited either from *SUVH2* overexpression or *SUVH2* antisense plants still shows increased and decreased DNA methylation, respectively (Figure 6A and C). The data suggest that DNA methylation is involved in maintenance of the SUVH2-induced epigenetic effects.

Modification of TGS by SUVH2 is connected with complex DNA methylation pattern

We analysed the effect of SUVH2 on DNA methylation at the *LUC2* transgene, *GUS* transgene repeats and Athila transposon sequences (Figure 6 and Supplementary Figure 3). All these sequences show already in wild-type a consistent amount of DNA methylation. In our studies, we compared CpNpN (N, no G) nonsymmetric with CpG and CpNpG symmetric DNA methylation. Loss or overexpression of *SUVH2* affects both symmetrical and nonsymmetrical DNA methylation at all studied sequences. In *suvh2* mutant as well as *SUVH2as#11* antisense plants, strongest reduction of nonsymmetrical methylation at CpC is observed (Supplementary Figure 3).

Since DNA methylase MET1 is suggested to function in maintenance of cytosine methylation (Finnegan and Kovac, 2000), we studied at the completely silenced *LUC7* transgene the effects of a newly isolated strong *met1-h1* mutation on SUVH2-induced transgene silencing (Figure 7A and B). Furthermore, we also studied interaction between *SUVH2* and a newly isolated strong *cmt3-h1 CHROMOMETHYLASE* mutation (Figure 7C). In *LUC7 met1-h1* and *LUC7 cmt3-h1* plants, silencing of the *LUC7* transgene is significantly released (Figure 7B and C). *LUC7* transgene silencing is differentially affected in *35S*::mycSUVH2#5/+*; *LUC7/LUC7*; *met1-h1/met1-h1* and *35S*::mycSUVH2#5/+*; *LUC7/LUC7*; *cmt3-h1/cmt3-h1* plants. The suppressor effect of *met1-h1* dominates the enhancer effect of *SUVH2* overexpression, whereas the enhancer effect of SUVH2 dominates the suppressor effect of *cmt3-h1*. These results demonstrate that transgene silencing by SUVH2 depends on MET1, but not significantly on CMT3.

In addition to DNA methylases, the DDM1 protein, which shows homology to the SWI2/SNF2 family of chromatin remodelling factors, controls DNA methylation independent of their sequence context (Mittelsten Scheid *et al*, 1998; Jeddeloh *et al*, 1999). In *35S*::mycSUVH2#6/+*; *ddm1–2/ddm1–2* plants, the mini-plant phenotype caused by *SUVH2* overexpression as well as ectopic dimethyl H3K9, monomethyl H4K20 and 5-methylcytosine are all significantly suppressed (Figure 7E). Our results suggest that the function of SUVH2 in heterochromatin formation and gene silencing depends on both MET1 and DDM1. Genetic interaction of SUVH2 with different silencing factors also demonstrate that the clear-cut *SUVH2* overexpression phenotypes allow systematic genetic dissection of functions involved in the control of heterochromatic gene silencing in *Arabidopsis*.

Discussion

SUVH proteins function differentially in control of heterochromatin formation

SU(VAR)3–9 proteins are evolutionary conserved and their H3K9 HMTase activity controls heterochromatin formation in eukaryotic organisms (Nakayama *et al*, 2001; Peters *et al*, 2001; Tamaru and Selker, 2001; Jackson *et al*, 2002; Schotta *et al*, 2002). In *Arabidopsis* 10 *SUVH* genes encode *SU(VAR)3–9* homologues and sequence analysis suggests that these genes evolved via gene duplications (Baumbusch *et al*, 2001). It is likely that a duplicated gene, which does not adopt a distinct function, tends to become lost by mutation. Functional difference could be reached by either having different expression patterns or by acquiring distinct functions. As *SUVH* genes are expressed in all tissues, it is likely that they have adopted at least partially nonoverlapping functions. The first SUVH protein analysed is SUVH4 (KYP), which shows *in vitro* H3K9 HMTase activity and *in vivo* is required for CMT3-dependent CpNpG DNA methylation of several transposon and repeat sequences (Jackson *et al*, 2002; Malagnac *et al*, 2002; Lindroth *et al*, 2004). Our analysis could not confirm the report that dimethyl H3K9 is lost from the chromocentre of *kyp-2* plants (Supplementary Figure 2; Jasencakova *et al*, 2003). Analysis of *suvh1* and *suvh4* null mutations shows only weak reduction of total histone H3K9 dimethylation (Figure 2 and Supplementary Figure 2), whereas all other histone methylation marks or DNA methylation in chromocentres are not significantly changed. This suggests that both SUVH1 and SUVH4 have no primary role in overall heterochromatin organization. Loss-of-function and overexpression of SUVH1 do not affect silencing of repeated *LUC* or *GUS* transgenes and therefore these proteins might only affect a restricted number of heterochromatic sequences.

In contrast, the SUVH2 protein has a strong impact on heterochromatin formation and gene silencing. The protein shows dosage-dependent nuclear distribution and affects all heterochromatin-specific histone methylation marks. *In vitro* SUVH2 methylates H3 and H4, and our data demonstrate that *in vivo* SUVH2 is one of the major mono- and dimethyl H3K9 and monomethyl H4K20 HMTase. SUVH2 also significantly affects mono- and dimethylation of H3K27. As none of the heterochromatic histone methylation marks are completely lost in the *suvh2* null mutant line, other SUVH proteins also contribute to the establishment of these marks. Whether this is mainly connected with target site specificity of different SUVH protein complexes remains to be studied. Finally, differences in HMTase activities, target sequence recognition specificity, differential recruitment of DNA methylases as well as functional interdependence could all contribute to the functional complexity of SUVH protein complexes. In mammals and *Drosophila*, functional interdependence between the H3K9 HMTase SU(VAR)3–9 and the H4K20 trimethyl specific HMTase SUV4–20 was recently shown (Schotta *et al*, 2004). In these organisms, binding of HP1 to heterochromatin depends on H3K9 di- and trimethylation by SU(VAR)3–9. HP1 not only binds SU(VAR)3–9 but also recruits the SUV4–20 HMTase to heterochromatin, and therefore a loss of SU(VAR)3–9 activity excludes HP1 heterochromatin association and successive recruitment of the SUV4–20 HMTase.

Proteins binding specific heterochromatic histone methylation marks *in vivo* are still unknown in plants. *In vitro*, a high affinity of a CMT3 chromodomain homodimer to lysine 9 and lysine 27 double methylated histone H3 tails was recently shown (Lindroth *et al*, 2004), indicating that in plants also proteins with differential binding activities to histone methylation marks exist. Complex interactions between such proteins, various SUVH proteins, other SET

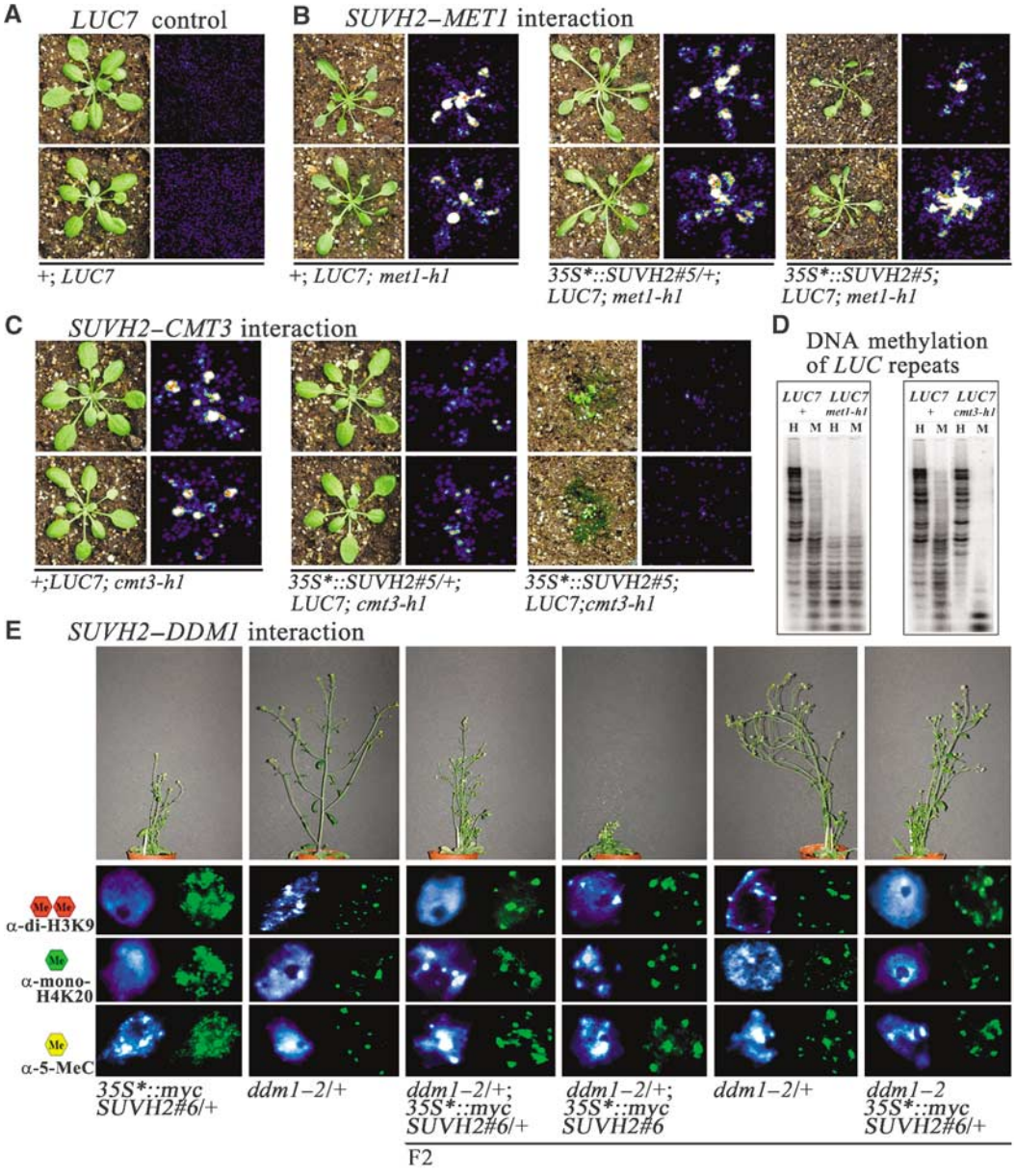

Figure 7 Genetic interaction of *SUVH2* with *MET1*, *CMT3* and *DDM1*. (**A**) The *LUC7* transgene shows complete repression of *LUC* activity (no fluorescence). (**B**) Relaxation of gene silencing by *met1-h1* dominates the enhancer effect of *SUVH2* overexpression and significantly rescues the *SUVH2* overexpression mini-plant phenotype. (**C**) The silencing enhancer effect of *SUVH2* overexpression dominates the suppressor effect of *cmt3-h1* on *LUC7* silencing. (**D**) The *met1-h1* and *cmt3-h1* mutations confer reduced CpG and CpNpG methylation, respectively. *Hpa*II (H) and *Msp*I (M) restriction map of *LUC7* with *LUC* as a probe. (**E**) Suppression of SUVH2-dependent mini-plant phenotype in *35S*::SUVH2#6/+*; *ddm1–2* plants and rescue of ectopic dimethyl H3K9, monomethyl H4K20 and 5-methylcytosine methylation.

domain proteins and DNA methylases could finally result in a highly complex network of regulatory interactions.

The interplay between DNA and histone methylation in heterochromatic silencing

Histone H3K9 and DNA methylation represent interrelated marks of repressed chromatin (Martienssen and Colot, 2001; Selker, 2002). Recently, two alternative models are discussed. Either DNA methylation is triggered by histone methylation (Tamaru and Selker, 2001; Jackson *et al*, 2002; Malagnac *et al*, 2002; Lindroth *et al*, 2004) or *vice versa* histone methylation is initiated after DNA methylation (Johnson *et al*, 2002; Soppe *et al*, 2002; Tariq *et al*, 2003). Our analysis with *SUVH2* transgene mutations clearly favours the latter hypothesis. Overexpression of *SUVH2* results in silencing of Athila transposons. This effect is completely released with an HMTase inactive SUVH2 protein although Athila sequences remain hypermethylated. Mutant analysis of a *SUVH2* transgene revealed an interesting hierarchic sequence of molecular events. N-terminal regions of SUVH2 appear to be involved in target sequence recognition. Specificity of these processes might also depend on a variety of unknown interactors. The YDG domain and possibly a region N-terminal to the preSET region of SUVH2 appear to be involved in recruitment of DNA methylation to target sequences, which is a prerequisite for histone methylation by SUVH2. Preference for CpNpG symmetric methylation is shown for SUVH4-dependent silencing (Jackson *et al*, 2002; Lindroth *et al*, 2004), whereas in

silencing processes induced by SUVH2 both symmetric and nonsymmetric DNA methylation is involved. This difference is also reflected by dependence of SUVH4-induced silencing on CMT3, whereas SUVH2 is largely independent of CMT3. The epistatic effect of *met1-h1* mutation on SUVH2-induced silencing and suppression of ectopic SUVH2 distribution in *ddm1–2*, *35S*::mycSUVH2#5* mutant plants shows that SUVH2-induced gene silencing requires both MET1 and DDM1 but not CMT3. Furthermore, in plants a large number of genes encoding MBD proteins is found and these proteins could also be necessary to convey differentially cytosine methylation to histone methylation. Interaction with HDAC complexes was demonstrated for AtMBD5, AtMBD6 and AtMBD7 (Zemach and Grafi, 2003), and MBD proteins might be involved in control of euchromatic as well as heterochromatic chromosomal subdomains. Genetic dissection of gene-silencing processes which depend either on SUVH2 or other SUVH proteins with suppressor and enhancer mutations should contribute substantially to understanding of complex regulatory networks involved in chromatin regulation, heterochromatin formation and gene silencing in plants.

Materials and methods

Plant material and growth conditions

The *35S*::mycSUVH1*, *35S*::mycSUVH2*, *35S*::mycDmSU(-VAR)3–9* and *35S*::mycDmHP1 Arabidopsis* transgenes were introduced with the pBI1-4tr vector. *SUVH1* and *SUVH2* ORFs were PCR-amplified from genomic DNA using primers Su1start-Su1stop and Su2start-Su2stop. All primers are listed in Supplementary Table 1. *Sal*I fragments (*SUVH1* and *SUVH2*) and the *Drosophila Bam*HI (*Dm*SU(VAR)3–9) and *Eco*RI–*Bam*HI (*Dm*HP1) fragments (Schotta *et al*, 2002) were cloned with 3xmyc in frame in sense orientation and in antisense orientation without 3xmyc (*35S*::SUVH1as* and *35S*::SUVH2as* transgenes) into pBI1-4tr, which was derived from pBI121 (Clontech) after insertion of a 1.4 kb *Xba*I–*Sac*I fragment of pKEx4tr (Baumbusch *et al*, 2001) containing the 35*S promotor (Mindrinos *et al*, 1994).

For controlled ectopic expression, *GVG::SUVH1EGFP*, *GVG::SUVH2EGFP* transgenic plants were generated. *Sal*I–*Nhe*I fragments of *SUVH1*, *SUVH2* and *GFP* were cloned in frame into the *Xho*I–*Spe*I site of pTA7002 (Aoyama and Chua, 1997). The *Sal*I–*Nhe*I *EGFP* fragment was PCR-amplified from CD3-327 (ABRC Stock Center) with primers GFP-Nhestop and GFPstart. Expression of the SUVH1–GFP and SUVH2–GFP fusion proteins was induced by 0.01 mM dexamethasone (Sigma) treatment. All constructs were confirmed by sequencing using an ABI 377 sequencer.

For studies of repeat-dependent TGS, the pGPTV 2xLUC Kan 2xLUC (*LUC2* and *LUC7* lines) vector was constructed. The *Nco*I–*Xba*I LUC fragment from pSP-LUC (Promega) was cloned into pRT100 (Töpfer *et al*, 1987) and, subsequently, the LUC *Pst*I fragment from pRT100LUC cloned into the *Pst*I site of partially *Pst*I digested pRT100, resulting in pRT100-2xLUC. After *Hin*dIII digestion, the 2xLUC repeat was cloned into pGPTV-Kan, the resulting construct was digested with *Pst*I–*Hin*dIII and the *Pst*I–*Hin*dIII LUC fragment cloned into the *Pst*I–*Hin*dIII site of pGEM-3zf+ (Promega), resulting in pGEM-2xLUC. *Kpn*I–*Hin*dIII digestion of pGEM-2xLUC produces a 2xLUC fragment, which was cloned into pBC-pAnos, resulting in pBC-pAnos-2xLUC. After *Eco*RI–*Ecl*136II digestion of pBC-pAnos-2xLUC, the 2LUC-nos-ter fragment was inserted into the *Eco*RI–*Sma*I site of pBluescript KS (Stratagene). The *Bam*HI fragment of pBluescript 2xLUC-nos-ter was cloned into the *Bam*HI site of pGPTV-Kan 2xLUC producing the pGPTV 2xLUC Kan 2xLUC vector.

The *GUS27* line was produced with a pGPTV-3xuidA-Kan vector containing three tandem copies of the *uidA* gene, provided by Dr Bettina Tschiersch (Institute of Plant Biochemistry, Halle).

All binary vectors were transferred to the *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) and used to transform *Arabidopsis* ecotype Columbia (Col) plants by vacuum infiltration. T1 seeds were plated on MS medium supplemented with kanamycin or hygromycin. The plates were left at 4°C in the dark for 1 day and then transferred to a growth chamber and incubated at 23°C under continuous light. Plants were transferred to soil after 2 weeks and grown in a chamber with 16-h-light/8-h-dark cycle at 23°C.

The sequence-indexed *Arabidopsis* T-DNA insertion mutants of *SUVH1*, *SUVH2* and *SUVH4* (SALK 003675, 079574, 105816, 130630) were kindly provided by the Salk Institute Genomic Analysis Laboratory. The new *cmt3-h1* (P715L) and *met1-h1* (E1272K) mutant alleles were selected as suppressors of *LUC7* transgene silencing after EMS treatment of *LUC7* homozygous plants (I Hofmann, unpublished). The alleles strongly reduce CpNpG and CpG DNA methylation, respectively. The *ddm1–2* mutant allele is described in Vongs *et al* (1993). All mutant material is available upon request.

Genetic analysis

35S::mycSUVH2#6* and antisense *35S*::SUVH2as#11* plants were used as female parent in all crosses with *LUC2*, *LUC7* and *GUS27* homozygous lines. Genotypes in offspring were determined with the specific primers 35S-1*Hin*dIII, 2RNAi3*Bam*H1, Lu/1626B and Lu8F, Gus-start and Gus-stop or S2-1631B and myc*Sal*1 (Supplementary Table 1). Luciferase activity was monitored with a CCD camera system employing Argus 50 Software (Hamamatsu Photonic Deutschland) after spraying of plants with 1.3mM Luciferin (Molecular Probes). GUS histochemical staining was performed as previously described (Baumbusch *et al*, 2001).

Functional interaction of SUVH2 with MET1 and CMT3 in transcriptional silencing of the *LUC7* transgene was studied in crosses of the *35S*::mycSUVH2#5* overexpression line with homozygous *LUC7*; *met1-h1* and *LUC7*; *cmt3-h1* mutant plants. F1 *35S*::mycSUVH2#5/+*; *LUC7/+*; *met1h-1/+* and *35S*::mycSUVH2#5/+*; *LUC7/+*; *cmt3-h1/+* plants were selfed over two generations and genotypes in F3 plants originated from *LUC7* homozygous F2 plants by using the derived cleaved amplified polymorphic sequence primers Met1-6f and Met1-6r or CMT3-eF and CMT3-eR (Supplementary Table 1). Amplified fragments were digested with *Eco*57I (*met1-h1*) or *Bsp*I (*cmt3-h1*). In crosses with *ddm1–2*, the F2 offspring originated from *35S*::mycSUVH2#6/+*; *ddm1–2/+* F1 plants were analysed using primers DDM1f and ddm1–2dRsaI (Neff *et al*, 1998). The *35S*::mycSUVH2#6* transgene was identified with primers 35S-1*Hin*dIII and S2RNAi3X/Bstop.

To screen for *SUVH2* transgene mutations, seeds of the *35S*::mycSUVH2#5* overexpression line were mutagenized with ethylmethane sulphonate (EMS, 90 mM). M2 progeny seedlings were grown on agar medium with kanamycin and scored after 10 days for a non-curled cotyledon phenotype. Putative mutants were transferred to soil and genomic DNA PCR-amplified with *SUVH2* transgene-specific primers 35S-1*Hin*dIII and S2RNAi3X/Bstop. The *SUVH2* transgenes were sequenced using primers S2RNAi3X/Bstop, 2RNAi5-*Bam*H1F, 2RNAi5X B and 2RNAi3*Bam*H1.

Immunocytology and EM analysis

For all immunocytological analyses, young rosette leaves from *Arabidopsis* ecotype Columbia plants were used. Leaf pieces were fixed in 4% formaldehyde in PBS on a glass slide, chopped, covered with coverslips and squashed. Slides were freezed in liquid nitrogen and transferred immediately into PBS after removing the coverslips. The slides were pre-incubated for 30 min at 37°C in 1% BSA in PBS and incubated with the following antibodies (1:100): α-mono-, α-di- and α-trimethyl H3K9, α-mono-, α-di- and α-trimethyl H3K27, α-di- and α-trimethyl H3K36, α-di- and α-trimethyl H4K20, α-dimethyl H3K4, α-acetyl H3K9, α-monomethyl H4K20 (Upstate). Detection of antibodies was performed with Alexa-488 conjugated secondary antibodies (1:200; Molecular Probes).

For detection of myc-tagged fusion proteins and 5-methylcytosine, leaves were fixed for 1 h in cold methanol:acetic acid (3:1), washed in citrate buffer (0.01 M citric acid monohydrate, 0.01 M trisodium citrate-2-hydrate, pH 4.8) and digested with 20% pectinase (Onozuka R-10) and 2% cellulase (Merk) in citrate buffer at 37°C for 30 min. The preparations were washed for 30 min in citrate buffer, squashed and coverslips were removed after freezing on dry ice.

For detection of myc-tagged fusion proteins, slides were washed in PBS, preincubated for 30 min at 37°C in 5% dry milk/PBS and incubated with mouse monoclonal α-myc (1:30; Labvision).

For detection of 5-methylcytosine, slides were baked after enzyme treatment at 60°C for 30 min, denatured in a 70% formamide, 2 × SSC, 50 mM sodium phosphate buffer (pH 7.0), washed in ice-cold PBS and preincubated in 1% BSA in PBS for 30 min at 37°C, followed by incubation with mouse monoclonal α-5-methylcytosine (1:400; Eurogenetech). For antibody detection Alexa-488 conjugated secondary antibodies (1:200; Molecular Probes) were used. All preparations were washed in PBS, stained with DAPI in mounting solution and examined with Zeiss Axioscop fluorescence microscope.

For electron microscopic analysis, plant material was processed as described previously (Hause and Hahn, 1998). Ultrathin sections were observed with an EM900 transmission electron microscope (Carl Zeiss NTS).

In vitro histone methyltransferase assay

GST constructs used for the assays were *Arabidopsis* SUVH2 amino acids 291–651 and *Drosophila* SU(VAR)3–9 amino acids 305–635 (Schotta *et al*, 2002). Generation and purification of the GST fusion proteins and histone methyltransferase assay were performed as described previously (Rea *et al*, 2000), with about 2 μg recombinant protein and 1 μg reconstituted recombinant nucleosomes.

Bisulphite sequencing

Cytosine methylation was assayed by the bisulphite genomic sequencing (Grunau *et al*, 2001) with 2 μg of *Eco*RI digested and alkali denatured genomic DNA. Denatured DNA was incubated in 1.2 ml freshly prepared 3.1 M sodium bisulphite/0.5 mM hydoquinone (Sigma), pH 5.0 at 95°C for 1 h, followed by desalting (QIAaexII, Quiagen), desulphonation and neutralization. DNA was precipitated, resuspended in 100 μl of 1 mM Tris, pH 8.0 and stored at −20°C. Gene fragments were PCR amplified and cloned into pGEM (Promega) for sequencing. For each genotype, 10–15 independent clones were analysed.

Protein isolation and Western blot analysis

Proteins from approximately 1 g leaf tissue were isolated as described (Tariq *et al*, 2003), separated on SDS–15% polyacrylamide gels and visualized by Coomassie Brilliant Blue staining or transferred to nitrocellulose membranes.

Membranes were used for immunodetection with α-dimethyl H3K9, and α-monomethyl-H4K20 (Upstate). Detection of primary antibodies was performed with peroxidase-conjugated secondary antibodies using the ECL kit (Amersham).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Dr G Schotta for helpful comments on the manuscript. We are grateful to Dr M Lachner for nucleosome preparations, C Fiedler and A Thuemmler for their help in genotyping of plants, K Kittlaus and R Franke for excellent technical assistance. This work was supported by grants from SFB363 of the Deutsche Forschungsgemeinschaft.

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