RESEARCH PAPER

Expression analysis of Arabidopsis XH/XS-domain proteins indicates overlapping and distinct functions for members of this gene family

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Abstract

RNA-directed DNA methylation (RdDM) is essential for de novo DNA methylation in higher plants, and recent reports established novel elements of this silencing pathway in the model organism Arabidopsis thaliana. INVOLVED IN DE NOVO DNA METHYLATION 2 (IDN2) and the closely related FACTOR OF DNA METHYLATION (FDM) are members of a plant-specific family of dsRNA-binding proteins characterized by conserved XH/XS domains and implicated in the regulation of RdDM at chromatin targets. Genetic analyses have suggested redundant as well as non-overlapping activities for different members of the gene family. However, detailed insights into the function of XH/XS-domain proteins are still elusive. By the generation and analysis of higher-order mutant combinations affected in IDN2 and further members of the gene family, we have provided additional evidence for their redundant activity. Distinct roles for members of the XH/XS-domain gene family were indicated by differences in their expression and subcellular localization. Fluorescent protein-tagged FDM genes were expressed either in nuclei or in the cytoplasm, suggestive of activities of XH/XS-domain proteins in association with chromatin as well as outside the nuclear compartment. In addition, we observed altered location of a functional FDM1–VENUS reporter from the nucleus into the cytoplasm under conditions when availability of further FDM proteins was limited. This is suggestive of a mechanism by which redistribution of XH/XS-domain proteins could compensate for the loss of closely related proteins.

Key words: Arabidopsis, gene family, protein localization, RdDM.

Introduction

Redundant activities exhibited by closely related but distinct genes is a widely observed phenomenon in higher plants (Nowak et al., 1997; Gottlieb, 2003; Gasciolli et al., 2005; Vaucheret, 2008). Duplication of individual genes or even entire genomes results in the establishment of gene families, members of which frequently exhibit overlapping activities. In the model plant Arabidopsis thaliana, more than 80% of its present genome appears to have arisen from such duplication events (Cannon et al., 2004). Once duplicated, loci might sustain redundant activities over an extended period of time. This predicts mechanisms by which homologous loci escape from the fate of pseudogenization by quickly diverging in functionality either by the acquisition of new functions (neofunctionalization; Ohno, 1970) or by subdivision of ancestral functions (subfunctionalization; Lynch and Force, 2000), or a combination of both (Qian et al., 2010).

Ongoing functional diversification is exemplified by XH/XS-domain proteins, which constitute a gene family in the Arabidopsis genome, implicated in exerting redundant and distinct functions in the regulation of RNA-directed DNA methylation (RdDM).
methylation (RdDM) (Ausin et al., 2012; Xie et al., 2012a, b; Zhang et al., 2012). SUPPRESSOR OF GENE SILENCING 3 (SGS3) and the rice gene X1 represent founder members of this plant-specific group of genes, both characterized by the occurrence of a single conserved XS domain (rice gene X and SGS3; Bateman, 2002). In Arabidopsis, nine additional proteins have been predicted, which, apart from the XS domain, encode a closely related XH (rice gene X homology) domain, located in the C-terminal portion (Bateman, 2002). Furthermore, XH/ XS-domain proteins have been predicted to contain a coiled-coil domain, and six of the nine proteins encode a zinc finger (Zf)-XS cysteine/histidine signature in the N-terminal portion of the protein (Bateman, 2002; Qin et al., 2009).

SGS3 was identified as a loss-of-function mutant exhibiting increased susceptibility to viral infections (Mourrain et al., 2000), and was found to act in the generation of trans-activating small interfering RNAs (siRNAs) (Peragine et al., 2004; Elmayan et al., 2009), as well as in post-transcriptional gene silencing-induced viral/bacterial resistance and salt tolerance (Borsani et al., 2005; Katiyar-Agarwal et al., 2006).

More recently, Arabidopsis XH/XS-domain proteins were characterized as regulators of DNA methylation (Ausin et al., 2009; Zheng et al., 2010; Finke et al., 2012). Initially, a loss-of-function mutation in INVOLVED IN DE NOVO DNA METHYLATION2 (IDN2)/RNA-DIRECTED DNA METHYLATION12 (RD29A) was found to interfere with RdDM (Ausin et al., 2009), whereas in another study IDN2/ RD29A was identified by screening for release of silencing of a hypermethylated RD29A::LUC transgene (Zheng et al., 2010). Apart from reduced DNA methylation in the promoter region of transgenic loci, endogenous loci were also affected in idn2/idr1dm2 alleles, supporting the notion that IDN2/RD29A acts in the RdDM pathway (Ausin et al., 2009; Zheng et al., 2010).

In vitro binding assays have demonstrated that IDN2/ RD12 and a close relative, termed FACTOR OF DNA METHYLATION1 (FDM1; also described as IDN2-LIKE1 and IDN2 PARALOG1), have the ability to bind dsRNA with a 5′ overhang (Ausin et al., 2009; Xie et al., 2012a). Moreover, a systematic analysis performed with idn2 and mutants deficient in FDM paralogues demonstrated significant alterations in gene expression and DNA methylation that resembled further RdDM mutants (Xie et al., 2012a). It was suggested that IDN2/RD12 might act in an early step of siRNA generation, potentially facilitating their biogenesis (Ausin et al., 2009; Zheng et al., 2010; Xie et al., 2012a). Alternatively, IDN2/RD12 and related proteins were suggested to function in a later step of RdDM. This is supported by findings demonstrating that, whilst DNA methylation at RdDM target loci is affected in idn2 alleles, generation of siRNAs is not, indicating that IDN2 is dispensable for this latter process (Ausin et al., 2012; Finke et al., 2012; Xie et al., 2012b; Zhang et al., 2012).

Analysis of RdDM target loci in idn2/idr dm mutant combinations has indicated partially redundant activities within this gene family (Ausin et al., 2012; Xie et al., 2012a). In addition, studies performed by three laboratories demonstrated the formation of heteromeric protein complexes, consisting of IDN2 and two of its paralogues, with different subunits potentially exerting non-overlapping activities (Ausin et al., 2012; Xie et al., 2012b; Zhang et al., 2012). The individual function of distinct XH/XS-domain proteins within such complexes, however, remains to be determined (Ausin et al., 2012; Xie et al., 2012b; Zhang et al., 2012).

In this report, we have extended the analysis of Arabidopsis XH/XS-domain proteins and provide evidence for redundant activities by analysis of higher-order loss-of-function mutants. In addition, we performed expression and localization studies with FDM genes that indicated functional diversification within the XH/XS-domain family. We showed a differing subcellular distribution of closely related FDM proteins and present indications for intracellular protein relocation under conditions when the availability of additional FDM proteins is limited. This offers an explanation for the partially overlapping activities of XH/XS-domain proteins and highlights mechanisms by which members of this gene family could compensate for the loss of closely related proteins.

**Material and methods**

**Plant growth and lines**

Plants were grown on plant nutrient agar plates (5 mM KNO3, 2 mM MgSO4, 2 mM Ca(NO3)2, 250 mM KPO4, 70 μM H2BO3, 14 μM MnCl2, 500 mM CuSO4, 1 μM ZnSO4, 200 mM Na2MoO4, 10 μM NaCl, 10 mM CoCl2, 50 μM FeSO4; pH adjusted to 5.7; supplemented with 1% (w/v) agar and 1% (w/v) sucrose; Haughn and Somerville, 1986) in a 16/8 h light/dark regime at 21°C.

**TS-GUS**, containing a transcriptionally silenced β-glucuronidase (GUS) transgene, has been described elsewhere (Morel et al., 2000). All transgenic lines were generated in A. thaliana ecotype Col-0 using the floral dip method (Clough and Bent, 1998), unless indicated otherwise. The T-DNA insertion lines are displayed in Supplementary Fig. S1 at JXB online and were obtained from NASC (http://www.arabidopsis.info; McElver et al., 2001; Alonso et al., 2003). Primers used for genotyping mutant lines are summarized in Supplementary Table S1 at JXB online.

For generation of mutant combinations, single mutants were crossed, and confirmed F1 individuals were then used for further crossing into additional mutant lines. Segregation of the TS-GUS transgene was confirmed by GUS staining as described previously (Lang-Mladek et al., 2010). The idn2/idr dm hexatuple and idr dm triple mutants were confirmed by genotyping in at least two independent lines each.

**Generation of constructs**

For generation of FDM1p::GUS, we used primers 5′-GGGTGCA CAATGGGAGGCTGTGCT-3′ and 5′-GGGTGCACTTCCCTCTCTCTCT-3′ to amplify an FDM1 promoter fragment (200 bp), which was subsequently cloned into pRFP-GUS (Diener et al., 2000). As similar strategy was employed for FDM2p::GUS (primers 5′-GGGTGCCGACGTCTCATCAGCTCAGCT-3′ and 5′-GGGTGC GACCATGTCATCCTTCTTACCT3′ to give a fragment of 2061 bp) and for FDM5p::GUS (primers 5′-GGGTGCCAGACGACT CTCCCTTACGTCGG-5′ and 5′-GGGTGCCAGACCATCTATGAGT GCCGCGT-3′ to give a fragment of 1670 bp).

For the generation of FDM translational reporter constructs, we first amplified full-length cDNAs using primers 5′-GGGGCGG GGATGAGCAGTTTCTGATGAAAG-3′ plus 5′-GGGCGGGGG TTAGGCTGTCGCGTTGAGC-3′ for FDM1, and 5′-GGCG CGGGGATGAAACAGTCTGAGT-3′ plus 5′-GGCGGGGG TTAGGCTGTCGCGTTGAGC-3′ for FDM5, and confirmed
them by sequencing. Expression cassettes were cloned into a derivative of pZZP221 modified with the 3SS terminator sequence (Hadjukiewicz et al., 1994; Leitner et al., 2012). For FDM1– and FDM5–VENUS reporters, a VENUS cassette was fused to the 3′ end of the respective coding regions of cDNAs, and the resulting constructs were either expressed under the control of the RP40 promoter (Scheer et al., 1997) or by endogenous FDM promoters (as was used for promoter–GUS constructs). For overexpression constructs, expression cassettes were cloned 3′ of an RP40 promoter fragment, which was generated by PCR with primers 5′-GGGAATTCTCTGAGATATACTGGTAAGATGG-3′ and 5′-GGGAATTCTATCTTTCTTTTCTTGCAGGAAA-3′.

**Expression analysis**

For analysis of transcript levels, poly-A+ enriched RNA was isolated from different tissues as described previously (Sieberer et al., 2003) and used for RT-PCR. GUS staining of FDM reporters and TS-GUS was performed as described previously (Lang-Mladek et al., 2010). For determination of TS-GUS activity, we used established protocols (Morel et al., 2000; Sieberer et al., 2000). Briefly, soluble proteins from 12-d-old seedlings were extracted and incubated in 4-MUG assay buffer [50 mM sodium phosphate, pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA, 0.1% (v/v) SDS, 0.1% (v/v) Triton X-100, 1 mM 4-methylumbelliferyl β-D-glucuronic acid (4-MUG)] at 37 °C. The reaction was stopped by adding 0.2 M Na2CO3, and fluorescence was determined on a fluorescence spectrophotometer (Hitachi F-2000, excitation 365 nm; emission 456 nm). Emission values were normalized to the protein content of the samples.

For visualization of FDM–VENUS reporter lines, we used an SP2 Leica confocal laser-scanning microscope. Seedlings were either viewed alive after brief staining in propidium iodide (100 μg ml⁻¹) to visualize cell boundaries or fixed in 3.7% (v/v) formaldehyde in microtubule stabilization buffer (MTSB: 50 mM Pipes, 5 mM EGTA, 5 mM MgSO4) for 15 min. After three washes in MTSB, seedlings were mounted in MTSB containing 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.5 ng ml⁻¹) and viewed under the confocal laser-scanning microscope. For imaging, we used the following excitation conditions: 514 nm (VENUS), 405 nm (DAPI), and 561 nm (propidium iodide).

**DNA extraction and methylation analysis**

For comparative DNA methylation analysis, genomic DNA was extracted from 10-d-old seedlings (Dellaporta et al., 1983). Digoxigenin-labelled probes were generated by PCR using primers 5′-GGATCCGATCATAACCAAG-3′ and 5′-GGAGGATGAC CACSGA-3′ for the 5S rRNA gene or 5′-GGGGGATCCATGTTG-3′ and 5′-CTTAGCCTTCTTTTCAATCTCA-3′ for AtMu1 (Zheng et al., 2010). Oligonucleotides 5′-AAACCTTTCGTAAGCT ACAGCCACCTTTGTT-3′ and 5′-TCGGATTGGTTCTTCCTACC TCTTACCTT-3′ were used for the generation of a MEA-ISR probe (Cao and Jacobsen, 2002; Ausin et al., 2009).

**Results**

**Identification of XH/XS-domain protein loss-of-function alleles**

For analysis of XH/XS-domain proteins, we first obtained potential loss-of-function mutants for all nine predicted members of the family, characterized by an XH and a closely related XS domain (Bateman, 2002). We adopted the nomenclature introduced recently (Xie et al., 2012a; Zhang et al., 2012), and characterized T-DNA insertion mutants in FDM1–FDMS5 and identified a potentially leaky T-DNA insertion line for IDN2/RDM12 with an insertion in the 5′ untranslated region (Supplementary Fig. S1; Xie et al., 2012a). Moreover, we obtained insertion mutants for all three additional loci predicted to encode proteins with an XH and an XS domain but to lack a zf-XS domain. Phylogenetic analyses performed with the entire predicted coding region of these loci highlighted an overall similarity to XH/XS-domain genes (Supplementary Fig. S1A). We therefore termed these loci IDR1–IDR3 (for IDN2 RELATED; Supplementary Fig. S1). RT-PCR analysis of the insertion lines demonstrated defects in expression of full-length mRNAs for all lines except for IDR2, for which no transcript could be detected under our experimental conditions (Supplementary Fig. S1B). With respect to idn2-3, we found defects in expression of its 5′ untranslated region (Supplementary Fig. S1B). Nevertheless, earlier analysis of this IDN2 allele suggested that it has retained some of its functionality (Xie et al., 2012a).

Taken together, analysis of T-DNA insertion lines suggested identification of loss-of-function mutants for the XH/ XS-domain loci, although we cannot rule out the possibility that the idn2-3 and idr2-1 alleles do not represent null alleles but still exhibit some remaining activity.

**Combinatorial loss of IDN2/FDM/IDR genes interferes with DNA methylation at selected RdDM target loci and antagonizes silencing of a GUS transgene**

Analysis of idn2/rdm12 mutant lines revealed defects in RdDM that appeared weaker than those of mutants deficient in additional, characterized components of the RdDM pathway (Aisin et al., 2009; Zheng et al., 2010). This led to suggestions of partially redundant activities among XH/XS-domain proteins, corroborated further by analysis of fdm mutant combinations (Xie et al., 2012a). However, recent reports indicated only limited functional redundancy within the gene family, substantiated by the finding that IDN2 and at least two of its paralogues form complexes in which they could exhibit discernible functions in the control of RdDM (Aisin et al., 2012; Xie et al., 2012b; Zhang et al., 2012).

Having identified potential loss-of-function lines for the *Arabidopsis* XH/XS-domain proteins, we determined their roles in DNA methylation at selected RdDM loci. Moreover, we obtained mutant combinations, which culminated in the generation of the idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 hextuple and the idr1-1 idr2-1 idr3-1 triple mutant.

Loss-of-function mutants affected in IDN2 and FDM loci have been described to impact on DNA methylation of RdDM targets, and we therefore tested some of these loci (Zheng et al., 2010). When probing methylation of the 5S rRNA gene by using restriction digests performed with HpaII (for methylation analysis in the CG and CHG context) and with HaeIII (for methylation analysis in the CHH context), a difference to that of the wild type was apparent in idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 DNA, indicative of weakly reduced CHG and CG methylation and a reduction in CHH methylation in the hextuple mutant (Fig. 1A).
Single mutants as well as the idr1-1 idr2-1 idr3-1 triple mutant combination exhibited no strong differences to wild-type DNA in comparison (Fig. 1A and Supplementary Fig. S2A). A similar observation was made when analysing the AtMU1 locus in HaeIII-cut DNA samples, with a reduction in DNA methylation in the CHH context apparent specifically for idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 DNA, whereas less pronounced or no differences were observed for the other mutant lines under our experimental conditions (Fig. 1B and Supplementary Fig. S2B). To further test the effects of XH/XS-domain proteins on methylation in the CG and CHG context, we analysed HpaII- and MspI-cut genomic DNA that was probed for MEA-ISR (Cao and Jacobsen, 2002). This suggested reduced symmetric DNA methylation most apparent in the idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 hextuple mutant (Fig. 1C). In contrast, we did not observe
clear differences when analysing RdDM loci in idr single and idrl-1 idr2-1 idr3-1 triple mutant lines, indicating that these loci are not essential for DNA methylation of analysed targets (Supplementary Fig. S2C).

Apart from well-established RdDM targets, we also analysed expression of the TS-GUS reporter line, containing a transcriptionally silenced GUS transgene that exhibits reactivation in a range of Arabidopsis gene-silencing mutants (Morel et al., 2000; Elmayan et al., 2005). Within this context, limited TS-GUS reactivation has been described for Arabidopsis mutants affected in loci functionally connected to RdDM (Elmayan et al., 2005; Lang-Mladek et al., 2010), and we asked whether or not XH/XS-domain proteins could be involved in the control of TS-GUS expression as well. idn2-3 fdm1-1 idp2-1 fdn3-2 fdm4-2 fdm5-2 TS-GUS exhibited a reproducible, statistically significant increase in TS-GUS activity when compared with TS-GUS controls (Fig. 1D, E). However, no prominent alterations in GUS activity were observed in any of the single mutants or in idrl-1 idr2-1 idr3-1. This indicated combinatorial activities of a subset of XH/XS-domain proteins in the regulation of TS-GUS.

Taken together, analysis of single mutant lines suggested only a limited impact on DNA methylation at endogenous RdDM target sites and activation of TS-GUS. In the case of IDN2, this might be a consequence of using a leaky allele, which would explain discrepancies between our results and findings made with potentially more severe idn2 alleles (Ausin et al., 2009; Finke et al., 2012; Zhang et al., 2012). Nevertheless, the more pronounced alterations in DNA methylation that were observed in the idn2/fdm hextuple mutant indicated that these loci exhibit overlapping, redundant activities in the control of DNA methylation. This corroborates findings by Xie et al. (2012a), who described additive effects on DNA methylation in fdm mutant combinations.

**FDM expression analysis**

To determine expression of XH/XS-domain genes, we generated cDNAs from different plant tissues and analysed transcript levels by RT-PCR after normalization to UBQ5 (Fig. 2A). These experiments revealed expression of all loci except for IDR2, transcripts of which were not detectable under our conditions. No prominent differences in tissue specificity in gene expression were observed when analysing IDN2/RDM12 and FDM transcript levels in mRNA extracted from seedlings, roots, leaves, or flowers, whereas expression of IDR1 and IDR3 appeared most pronounced in floral organs (Fig. 2A).

To address gene expression in planta, we generated promoter–GUS reporter constructs of selected FDM genes. Specifically, we analysed FDM1, FDM2, and FDM5, which represent a distinct subclade in the phylogenetic tree of the protein family (Supplementary Fig. S1A), in order to determine overlaps and differences in gene expression of these closely related loci. In seedlings, FDM1p::GUS staining was detectable in distal portions of cotyledons, and weaker expression was observed in root tips, a pattern that remained essentially unaltered throughout vegetative development (Fig. 2B, E, H). Additional FDM1p::GUS activity was observed in flowers, where it appeared to be restricted to the male gametophyte (Fig. 2L). Only weak FDM1p::GUS activity was observed in root meristems, which differed from RT-PCR analyses and root expression of a VENUS-tagged FDM1 translational reporter line (Figs 2A, B, and 3), suggesting that cis-acting elements required for FDM1 expression in root tissue are not fully active in FDM1p::GUS. FDM2p::GUS and FDM5p::GUS expression was stronger in comparison, exhibiting signals in roots, hypocotyls, and cotyledons of seedlings (Fig. 2C, D, F, G), whereas true leaves showed strong expression only of FDM2p::GUS (Fig. 2J). In flowers, FDM2p::GUS staining produced signals in the vasculature of sepals and petals as well as in anther filaments and parts of the style (Fig. 2M), whereas FDM5p::GUS staining was visible in the anthers, including pollen as well as in the stigma (Fig. 2N). In addition, FDM5p::GUS showed activity in the entire root, both at the seedling stage and in older plants (Fig. 2D, G), whereas FDM2::GUS staining was essentially restricted to the root vasculature and emerging lateral roots (Fig. 2C).

Taken together, the results obtained from gene expression and reporter analysis indicated distinct gene expression patterns, reflecting tissue specificity in activity of these closely related Arabidopsis XH/XS-domain genes.

To determine the subcellular localization of FDM proteins, we generated FDM1::VENUS and FDM5::VENUS expression cassettes, in which the entire FDM-coding regions were fused to the VENUS fluorescent protein (Nagai et al., 2002), and expressed under control of their endogenous promoters. Confocal laser-scanning microscopy analysis revealed that both FDM1p::FDM1::VENUS and FDM5p::FDM5::VENUS expression could be observed in root meristems, but the signals differed in their subcellular localization. When expressed in Col-0, FMD1p::FDM1::VENUS lines accumulated signals in the nucleus, whereas FDM5p::FDM5::VENUS exhibited signals predominantly in the cytoplasm (Fig. 3A, B).

We then asked whether or not differences in FDM reporter localization could result from differences in expression levels, and fused both FDM–VENUS expression cassettes to the strong ribosomal protein RP40 promoter (Scheer et al., 1997) and analysed expression of the resultant reporter constructs in wild-type Col-0. Ectopic overexpression of both transgenes confirmed the results obtained with endogenous reporters, with RP40p::FDM1::VENUS root meristems exhibiting signals in nuclei, whereas RP40p::FDM5::VENUS signals were most pronounced in the cytoplasm (Fig. 3C, D). In additional experiments, we performed promoter swaps and expressed FDM1::VENUS under the control of the FDM5 promoter and vice versa, expressing FDM5::VENUS by the FDM1 promoter. These experiments demonstrated nuclear signals in FDM5p::FDM1::VENUS lines and cytoplasmic signals in FDM5p::FDM5::VENUS lines (Fig. 3E, F). Collectively, our analyses indicated that neither variations in expression domains (promoter swaps) nor expression levels (overexpression) significantly affected the distinct subcellular distribution of the FDM1 and FDM5 reporter proteins. This is in
agreement with roles for FDM proteins in both the nucleus and the cytoplasm.

**Relocation of FDM1–VENUS coincides with defects in XH/XS-domain proteins**

Differences in the subcellular distribution of closely related FDM proteins are suggestive of differing functions. We further addressed this issue, and first determined FDM reporter protein functionality in the \textit{idn2/fdm} mutant background. To this end, we introduced \textit{RP40p::FDM1:VENUS} and \textit{RP40p::FDM5:VENUS} into \textit{idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2} expressing the \textit{TS-GUS} transgene. TS-GUS activity was increased in \textit{idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2}, reflecting defects in controlling the silenced state of the transgene (Figs 1D, E, and 4A, B, E). This was different in \textit{idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 TS-GUS RP40p::FDM1:VENUS} and \textit{idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 TS-GUS RP40p::FDM5:VENUS}, which both exhibited reduced TS-GUS activity, a response that appeared more pronounced upon expression of the \textit{RP40p::FDM1:VENUS} reporter construct (Fig. 4C, D, E).
These findings suggested that both FDM–VENUS reporter proteins are capable of rescuing defects in TS-GUS silencing (Fig. 4C, D).

Analysis of FDM–VENUS reporters in Col-0 revealed distinct FDM1–VENUS and FDM5–VENUS signals in root meristem cells (Fig. 3). On the other hand, both reporters rescued defects in TS-GUS expression control in idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 at least to some extent (Fig. 4), raising questions about the mechanisms involved. To address this issue, we assayed the FDM–VENUS reporter localization in the idn2/fdm mutant background. We first analysed RP40p::FDM1:VENUS and RP40p::FDM5:VENUS expression in the corresponding fdm1-1 and fdm5-2 single mutants. Similar to Col-0, we found FDM1:VENUS signals in the nucleus of fdm1-1 root meristem cells, whereas FDM5:VENUS was visible in the cytoplasm of fdm5-2 root meristem cells (Fig. 3A). Identical results were obtained when analysing FDM1:VENUS distribution in fdm5-2 and FDM5:VENUS distribution in fdm1-1 (Fig. 3B), suggesting that defects in endogenous FDM1 and FDM5 do not influence FDM–VENUS reporter distribution.

We then expressed RP40p::FDM1:VENUS and RP40p::FDM5:VENUS in the idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 hextuplet mutant and determined reporter localization in root meristem cells. Subcellular distribution of FDM5:VENUS remained detectable predominantly in the cytoplasm (Fig. 3C), but alterations were observed for RP40p::FDM1:VENUS. This reporter line exhibited signals in the nuclei and cytoplasm when expressed in idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 (Fig. 3C). Thus, unlike in Col-0, fdm1-1, or fdm5-2, in which FDM1:VENUS signals are restricted to the nucleus, a combinatorial loss of XH/XS-domain proteins coincided with a visibly altered subcellular localization of FDM1, no longer confined to the nuclear compartment.

When taken together, analysis of FDM reporter lines indicated localization in the nucleus and cytoplasm and highlighted flexible subcellular FDM protein distribution under conditions when cytoplasmic FDM protein abundance appeared restricted.

**Discussion**

Gene amplification by duplication is a common phenomenon observed in virtually all classes of organisms. Plants, in particular, have evolved numerous gene families of variable sizes, which allows evolution of modified gene functions but also represents a resource for redundant gene activities, resulting in an increased genetic robustness. In the case of Arabidopsis IDN2/RDM12 and its closely related paralogues, both mechanisms seem to be in effect, supported by experimental evidence for neofunctionalization but also for redundant activities within this gene family (Ausin et al., 2009; Zheng et al., 2010; Ausin et al., 2012; Xie et al., 2012a, b; Zhang et al., 2012).

Characterization of IDN2/RDM12 demonstrated rather mild defects in siRNA biogenesis and DNA methylation control, which led to the suggestion of redundant gene function (Ausin et al., 2009; Zheng et al., 2010). This is supported by the work by Xie et al. (2012a), demonstrating synergetic effects in some idn2/fdm alleles, in which RNA double and triple mutant combinations. Currently, it cannot be excluded that some of the idn2/fdm alleles in use have retained some of their functional role. For example, idn2-3 has been described to represent a leaky allele (Xie et al., 2012a), which makes it difficult to determine the contribution of individual loci in mutant combinations. Nevertheless, the phenotype of the idn2-3 fdm1-1
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The idp2-1 fdm2-1 fdm3-2 fdm4-2 fdm5-2 hextuple mutant is clearly consistent with a model suggesting overlapping functions for at least some of the XH/XS-domain genes, as it exhibited defects in DNA methylation and transgene silencing that were more pronounced than in any of the corresponding single mutants.

Analysis of the idr mutants did not reveal alterations in DNA methylation at selected RdDM target sites. This suggests roles for IDR genes, either different from, or only partially overlapping with, the activities of the IDN2 and FDM loci. Homology-based searches revealed that IDN2/FDM and IDR proteins differ in their N-terminal portion, as there is no zf-XS domain predicted for IDR proteins. Differing DNA methylation phenotypes observed in idn2/fdm and idr mutant combinations thus could indicate a prominent function for the zf-XS domain in the regulation of RdDM, but further experiments are required to characterize this domain.

Recent reports provided evidence for divergent functions of IDN2 and some of its paralogues (Ausin et al., 2012; Xie et al., 2012b; Zhang et al., 2012). Specifically, analysis of idn2 fdm1 fdm2 mutant combinations have suggested non-redundant roles for IDN2 and FDM1/FDM2 (Ausin et al., 2012). Strong experimental support for a functional diversification of XH/XS-domain proteins comes from observations demonstrating that IDN2 as well as FDM1 have the ability to form homo- and heteromeric protein complexes, in which monomers might carry out different functions (Ausin et al.,

Fig. 4. Functional analysis of FDM–VENUS reporter proteins. (A–D) TS-GUS staining in 10-d-old TS-GUS (A), idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 TS-GUS (B), idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 TS-GUS RP40::FDM1::VENUS (C), and idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 TS-GUS RP40::FDM5::VENUS (D). (E) TS-GUS activity in 14-d-old plantlets as determined by a 4-MUG assay. Activities are plotted as fold induction of activity found in the TS-GUS controls (given a value of 1). Two independent experiments are shown and standard deviation is indicated. Asterisks and brackets highlight significant differences in TS-GUS activity between samples (t-test; P<0.05).
Another report demonstrated a physical interaction between IDN2/RDM12 and a subunit of a SWI/SNF ATP-dependent nucleosome-remodelling complex, possibly involved in mediating the effects of long non-coding RNAs on the epigenetic status of chromatin (Zhu et al., 2013). It thus appears that variations in configuration and composition of XH/XS-domain protein complexes might reflect distinct roles for these proteins, depending on the type of complex being formed. Our analysis of FDM reporter proteins provides additional evidence for distinct roles of FDM proteins, indicated by the prominent differences in their subcellular localization.

XH/XS-domain family genes have been implicated to act in a late step of the RdDM pathway, with XH/XS-domain protein complexes potentially required for stabilization of AGO4-delivered siRNAs when paired to PolIV transcripts at chromatin sites (Ausin et al., 2012; Finke et al., 2012). Furthermore, IDN2 has been suggested to guide an Arabidopsis SWI/SNF remodelling complex to genomic loci in the process of gene silencing (Zhu et al., 2013). All these functions would be in agreement with a nuclear localization of XH/XS-domain proteins as we observed for FDM1–VENUS reporters. The cytoplasmic localization of FDM5–VENUS reporters highlights additional functions for XH/XS-domain proteins apparently detached from their activities at chromatin. Apart from that, FDM1, which otherwise exhibits strictly nuclear localization, appears to relocalize to the cytoplasm under conditions when availability of further FDMs is limited, further suggestive of an essential role for cytoplasmic FDM localization.

The Arabidopsis XS-domain protein SGS3 appears to function in the cytoplasm, and is suggested to be involved in the generation or maturation of viral siRNAs (Mourrain et al., 2000; Peragine et al., 2004; Borsani et al., 2005; Höffer et al., 2011). In addition, Ye et al. (2012) demonstrated that loading of siRNA onto AGO4 occurred in the cytoplasm, and provided evidence suggesting that such complex assembly triggers its subsequent relocation to the nucleus. This finding indicated that mechanisms controlling RdDM are not entirely restricted to the nucleus. A similar scenario could be envisioned for XH/XS-domain proteins, in which cytoplasmic localization of FDM5 and possibly further members of the protein family might reflect involvement in early steps of the RdDM pathway. This could, for example, concern stabilization of AGO/siRNA complex formation in the cytoplasm, as well as potential roles in guidance of such complexes to destination sites in the nucleus. Clearly, further experiments are required to characterize these elusive activities.

Our experiments demonstrated that FDM1-reporter relocation coincided with defects in additional members of the gene family. This observation suggests that FDM1, whilst otherwise exhibiting nuclear localization, could adopt activities in the cytoplasm. XH/XS-domain proteins have been demonstrated to form multimeric complexes (Ausin et al., 2012; Xie et al., 2012b; Zhang et al., 2012), and there is a possibility that some of these complexes might function in

![Fig. 5. Subcellular localization of FDM–VENUS reporter proteins (yellow) in fixed root meristem cells of idn2/fdm mutants. (A) RP40p::FDM1:VENUS (left panels) and RP40p::FDM5:VENUS (right panels) in fdm1-1. Note the accumulation of FDM1–VENUS reporter signals in the nucleolar cavity, whilst weak signals only are found in the surrounding dense fibrillar zone. (B) RP40p::FDM1:VENUS (left panels) and RP40p::FDM5:VENUS (right panels) in fdm5-2. (C) RP40p::FDM1:VENUS (left panels) and RP40p::FDM5:VENUS (right panels) in idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2. Seedlings were fixed in formaldehyde (3.7%, v/v) and counterstained with DAPI (0.5 ng ml⁻¹, blue) to visualize nuclei. Bars, 10 μm.](https://academic.oup.com/jxb/article-abstract/65/4/1217/498888)
the cytoplasm. It is possible that increased cytoplasmic accumulation of FDM1 results from its relocation as part of a protein complex, in which it would take over the function of cytoplasmic FDM proteins like FDM5. This might explain FDM1–VENUS relocation occurring only in the severe idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 hextuple mutant. In an alternate scenario, loss of additional XH/XX-domain proteins in the idn2-3 fdm1-1 idp2-1 fdm3-2 fdm4-2 fdm5-2 mutant might interfere with correct nuclear relocation of otherwise cytoplasmic FDM1. A role for XH/XX proteins in the cytoplasm is supported by the distribution of functional FDM5–VENUS, which accumulates predominantly outside the nucleus, underlining a scenario in which, apart from their activity in association with chromatin domains, cytoplasmic localization of XH/XX-domain proteins contributes to their function in the control of DNA methylation.

Overall, our findings suggest activities of XH/XX proteins in both the nucleus and cytoplasm, and it seems possible that relocation of FDM proteins between these compartments could account for partially redundant activities within this gene family. It will be interesting to study the principles and mechanisms of this potentially highly versatile strategy to compensate for activities within functionally diverging gene families.

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