AtMBD6, a methyl CpG binding domain protein, maintains gene silencing in *Arabidopsis* by interacting with RNA binding proteins

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DNA methylation, mediated by double-stranded RNA, is a conserved epigenetic phenomenon that protects a genome from transposons, silences unwanted genes and has a paramount function in plant or animal development. Methyl CpG binding domain proteins are members of a class of proteins that bind to methylated DNA. The *Arabidopsis thaliana* genome encodes 13 methyl CpG binding domain (MBD) proteins, but the molecular/biological functions of most of these proteins are still not clear. In the present study, we identified four proteins that interact with AtMBD6. Interestingly, three of them contain RNA binding domains and are co-localized with AtMBD6 in the nucleus. The interacting partners includes AtRPS2C (a 40S ribosomal protein), AtNTF2 (nuclear transport factor 2) and AtAGO4 (Argonoute 4). The fourth protein that physically interacts with AtMBD6 is a histone-modifying enzyme, histone deacetylase 6 (AtHDA6), which is a known component of the RNA-mediated gene silencing system. Analysis of genomic DNA methylation in the *atmbd6*, *atrps2c* and *atntf2* mutants, using methylation-sensitive PCR detected decreased DNA methylation at miRNA/siRNA producing loci, pseudogenes and other targets of RNA-directed DNA methylation. Our results indicate that AtMBD6 is involved in RNA-mediated gene silencing and it binds to RNA binding proteins like AtRPS2C, AtAGO4 and AtNTF2. AtMBD6 also interacts with histone deacetylase AtHDA6 that might have a role in chromatin condensation at the targets of RdDM.

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1. Introduction

Epigenetic changes in higher plants involve a complex regulation of small RNAs, DNA methylation and chromatin remodelling. There is a special class of proteins called methyl CpG binding domain (MBD) proteins. Some of the members of this class bind to only methylated DNA. The MBD proteins are present in both plants and animals. The model plant *Arabidopsis thaliana* genome encodes 13 MBD proteins. Two of them, AtMBD6 and AtMBD10, have a role in nucleolar dominance in hybrid plants. The proteins involved in nucleolar dominance like RDR2, DCL3 and DRM2 also participate in RNA-directed DNA methylation (RdDM) (Preuss *et al.* 2008). AtMBD6 is localized in the nucleolus, which is a major site for siRNA metabolism (Li *et al.* 2006; Costa-Nunes *et al.* 2010). Loss of DRM2 activity disturbs the condensation of nucleolar organizing region (NOR) and association of MBD6 with NORs (Preuss *et al.* 2008; Costa-Nunes *et al.* 2010). The MBD domains of AtMBD5, AtMBD6 and AtMBD7 are more similar to those present in human MBDs, suggesting similar molecular functions. AtMBD4, AtMBD6 and AtMBD7 bind to both methylated and unmethylated DNA (Ito *et al.* 2003; Zemach and Grafi 2003). AtMBD6 gene of *Arabidopsis* shows more homology to genes encoding mammalian MBD proteins and the protein is localized in the NOR and has a role in nucleolar dominance in allotetraploid hybrid plant, *Arabidopsis suecica* (Preuss *et al.*

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2008). This protein is localized in the nucleolus and regulates rRNA gene expressions. The localization of the protein is disrupted in *ddm1* and *met1* mutants and DRM2-RNAi lines (Zemach *et al.* 2005; Preuss *et al.* 2008). *AtMBD6*-RNAi lines of *Arabidopsis suecica* show disruption of nucleolar dominance. This protein is able to immunoprecipitate histone deacetylase activity associated with histone H3 deacetylation (Zemach and Grafi 2003).

Two histone modifying enzymes, acting in the RNA-directed DNA methylation pathway, have been identified. The first one is histone deacetylase 6 (HDA6), which is involved in CG methylation induced by RNA (Aufsatz *et al.* 2002) and plays an additional role in gene silencing that is not mediated by RNA (Probst *et al.* 2004). The other one is SUVH4/KRYPTONITE (KYP) that helps to maintain cytosine methylation, particularly at CHG sites (Jackson *et al.* 2002; Malagnac *et al.* 2002).

In plants, small interfering RNAs (siRNAs) are known to guide DNA methyltransferases to the siRNA-generating genomic loci for *de novo* DNA methylation, commonly called RdDM (Wassenegger *et al.* 1994). This process requires proteins involved in small RNA metabolism, specialized RNA binding proteins, DNA methyltransferases and histone-modifying enzymes. The sRNAs are first loaded on to RNA binding proteins to form RNA protein complexes. Some specialized RNA binding proteins play a major role in recognition of target loci in the genomic DNA. AGO4 and AGO6, which are important components of RNA-induced silencing complex (RISC), are predicted to guide DNA methylation at the target loci in the genomic DNA (Pontes *et al*. 2006; Qi *et al*. 2006; Peters and Meister 2007; Zheng *et al*. 2007; Pontes *et al*. 2009; Gao *et al*. 2010; He *et al.* 2009: Wierzbicki *et al*. 2009; Zheng *et al*. 2013). The RNA binding proteins, IDN1 and IDN2, maintain siRNA-mediated *de novo* methylation (Ausin *et al.* 2009). IDN2 is a known component of RdDM and it act downstream to siRNA biogenesis (Ausin *et al.* 2012; Finke *et al.* 2012). It encodes a double-strand RNA binding protein that has homology with SGS3 class of protein. IDN2 along with three SGS3 like proteins (FDM3, FDM4 and FDM5) has overlapping function in RdDM (Zheng *et al*. 2010; Xie *et al*. 2012). The RDM1 protein of DDR complex is also involved in RdDM. It has been reported that RDM1, on the one hand, binds with RNA and, on the other, it can also bind with RNA Polymerase II, AGO4, DMS3 and DRM2 (Gao *et al.* 2010; Law *et al.* 2010; Sasaki *et al.* 2014). These findings suggest a role for RNA binding proteins in gene silencing.

In the present study, using yeast two-hybrid screening and *in planta* FRET analysis we have tried to determine the way AtMBD6 controls post-transcriptional gene silencing. Here we report interaction of AtMBD6 protein with four proteins, three of which are RNA binding proteins and the other one is a histone deacetylase. Our results will help in understanding of the molecular mechanism of the action of AtMBD6 in post-transcriptional gene silencing.

2. Methods

2.1 *Yeast two-hybrid screening*

Gene encoding AtMBD6 was cloned in fusion with the DNA encoding GAL4-DNA binding domain in pGBK-T7 vector. The yeast strain Y187, transformed with pGBK-T7-*AtMBD6*, produced the bait. A yeast two-hybrid library was prepared in yeast strain AH109, according to the manufacturer's instructions (Clontech), using cDNA from ten days old *Arabidopsis* seedlings. One aliquot of library and 5 mL culture of overnight grown bait were inoculated in 50 mL 2X YPDA medium, containing 50 μg/mL of kanamycin, followed by incubation for 24 h at 30°C with 35 rpm shaking. After 24 h, the cells were harvested and grown on –AHLT medium (-Ade, -His, -Leu, -Trp). The plates were incubated at 30°C until the colonies appeared. The yeast colonies that grew were further patched on –AHLT media containing 5 mM 3-AT (3-amino-1,2,4-triazole). The selected colonies were further analysed by α and β galactosidase activities.

β-Galactosidase was assayed by chloroform overlay method as described by Golemis *et al*. (2009). Briefly, 5 mL of chloroform was poured on to the colonies and incubated for 5 min at room temperature. The chloroform was discarded, and the plates were air-dried. The colonies were overlaid with 1% agarose, prepared in 100 mM phosphate buffer (pH 7) containing 0.25 mg/mL of X-gal. The plates were then incubated at 30°C and the colour change was monitored. For α-galactosidase activity, the colonies were patched on –AHLW medium containing the substrate α-gal and incubated overnight at 30°C.

2.2 *Subcellular localization and FRET analysis*

For subcellular localization and Fluorescence Resonance Energy Transfer (FRET) analysis, the ORFs of baits and preys were cloned in-frame with the 3′ end of gene encoding Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP), using pSITE1CA and pSITE3CA vectors respectively (Chakrabarty *et al.* 2007). For this, ORFs were first cloned in pENTR/D-TOPO entry vector and then mobilized into pSITE1CA and/or pSITE3CA destination vector using Gateway cloning (Invirtogen). The resultant binary plasmids were coated on gold particles and used for transient expression in onion epidermal cells by biolistic method and placed on MS medium (Murashige and Skoog 1962).

For studying the interaction of AtMBD6 and AtRPS2C/AtNTF2/AtHDA6/AtAGO4, pSITE vectors carrying DNA encoding one partner as a CFP fusion protein and another as YFP fusion protein were used to transform onion epidermal cells. A single cell that showed expression of both CFP and YFP was targeted for FRET analysis by acceptor bleaching protocol. While CFP was excited by argon laser at a

wavelength of 458 nm and emission was measured between 465 nm and 505 nm, YFP was excited at a wavelength of 512 nm and emission was measured between 525 nm and 600 nm. The fluorescence detected from CFP or YFP proteins was recorded. FRET analysis was performed following FRET wizard available in Leica Application Suite software (LAS-AF 2.2, Germany). For each FRET analysis, we used a minimum of three independent cells. The nucleus where both the fluorophores were localized were selected as regions of interest for the FRET analysis.

2.3 *DNA methylation analysis*

The genomic DNA was isolated by GenElute Plant Genomic DNA Miniprep Kit (Sigma). The quality was verified by electrophoresis on 0.8% agarose gel and the quantitation was done using ND-1000 spectrophotometer (Nanodrop Technologies, USA). One microgram of genomic DNA was digested with McrBC endonuclease, which uses methylated DNA as substrate. Further, 10–50 ng of digested genomic DNA was used to perform PCR. All primers used in this study are listed in supplementary table 3.

3. Results

3.1 *Yeast two-hybrid library screening to identify proteins that interact with AtMBD6*

To find out the interacting partners of AtMBD6, yeast two-hybrid assay was carried out using AtMBD6 as bait. We prepared a yeast two-hybrid library using the cDNA from 10 day old seedlings of *Arabidopsis*. Initially we got 149 colonies that grew on -AHLW medium. Another layer of screening was performed to determine the activation of α-galactosidase reporter gene. After the α-galactosidase assay, we found 74 positive colonies. Out of the 74 colonies we sequenced DNA of the gene encoding the interacting protein from 33 colonies. Finally, we identified two putative interacting partners. These partners were AtNTF2 (nuclear transport factor 2, AT5G60980) and AtRPS2C (40S ribosomal protein, AT2G41840). Both of these proteins possessed RNA binding domains, which indicates that AtMBD6 might be involved in RNA-mediated gene silencing. To determine whether the full-length AtRPS2C and AtNTF2 can interact with AtMBD6, we co-transformed yeast with vectors carrying genes encoding these proteins, pAD-AtRPS2C or pAD-AtNTF2 along with pBD-AtMBD6 in two independent transformations. Only the yeast cells expressing both bait and prey were able to grow on –AHLW (-Ade, -His, -Leu, -Trp) plates, containing 5 mM of 3-AT (3-amino-1,2,4-triazole) and showed α-galactosidase activity (figure 1A). The yeast cells transformed with pBD-MBD6 along with pAD vector did not grow in –AHLW medium, suggesting there is no transactivation activity associated with AtMBD6. We also transformed yeast with pAD-NTF2/pAD-RPS2C along with pBD vector. In this case the colonies did not grow on –AHLW medium. These results confirm that AtMBD6 interacts with AtRPS2C and AtNTF2 (figure 1B).

3.2 *In planta interaction between AtMBD6 and AtRPS2C*

The putative interacting partner AtRPS2C, identified in a yeast two-hybrid assay, is a 40S ribosomal protein. The protein is part of the protein synthesis machinery and is localized in the cytoplasm (Barakat *et al.* 2001; Carroll *et al.* 2008). It contains the N-terminal ribosomal S5 domain, the C-terminal ribosomal S5 domain and the double-stranded RNA binding domain. This protein was also reported to be present in the nucleolus (Pendle *et al.* 2005).

To verify the yeast two-hybrid result *in vivo*, we performed co-localization and Florescence Resonance Energy Transfer (FRET) analysis. The genes encoding AtMBD6 and AtRPS2C were fused with genes encoding CFP and YFP respectively and the chimeric genes were transiently expressed in onion epidermal cells. The strong signal in some sub-nuclear regions was detected for AtMBD6 (figure 2A, B). Previous reports suggested that AtMBD6 is localized in the nucleolus and is co-localized with centromeric repeats, which are highly methylated (Preuss *et al.* 2008; Costa-Nunes *et al.* 2010). The strong localization of AtMBD6 in the sub-nuclear regions of onion epidermal cell suggests that the densely fluorescing areas may be highly methylated DNA stretches. The YFP-tagged AtRPS2C was found to be localized in the cytoplasm as well as nucleus. The presence of AtRPS2C in cytoplasm supports the previous report that the protein is a part of the protein synthesis machinery (Barakat *et al.* 2001). The localization of the protein in the nucleus of onion peels showed a distinct pattern, which was similar to that of AtMBD6 (figure 2A, B). Both the proteins co-localized in the nucleus. The common niche suggests their close association. The interaction between AtMBD6 and AtRPS2C was again verified by FRET analysis. This was carried out using CFP-AtMBD6 as a donor and YFP-AtRPS2C as an acceptor. The efficiency of the energy transfer between CFP and YFP was found to be 13.69%, which confirmed the association of these two proteins (figure 2C). The CFP fused AtMBD6 and YFP were used as a negative control for FRET analysis (supplementary figure 1A). The FRET efficiency was found to be 1.5% when CFP-MBD6 and YFP were used for FRET analysis (supplementary figure 1B).

3.3 *AtNTF2, a nuclear transport factor, interacts with AtMBD6*

Another protein that was identified using yeast two-hybrid assay was AtNTF2, a nuclear transport factor, containing a

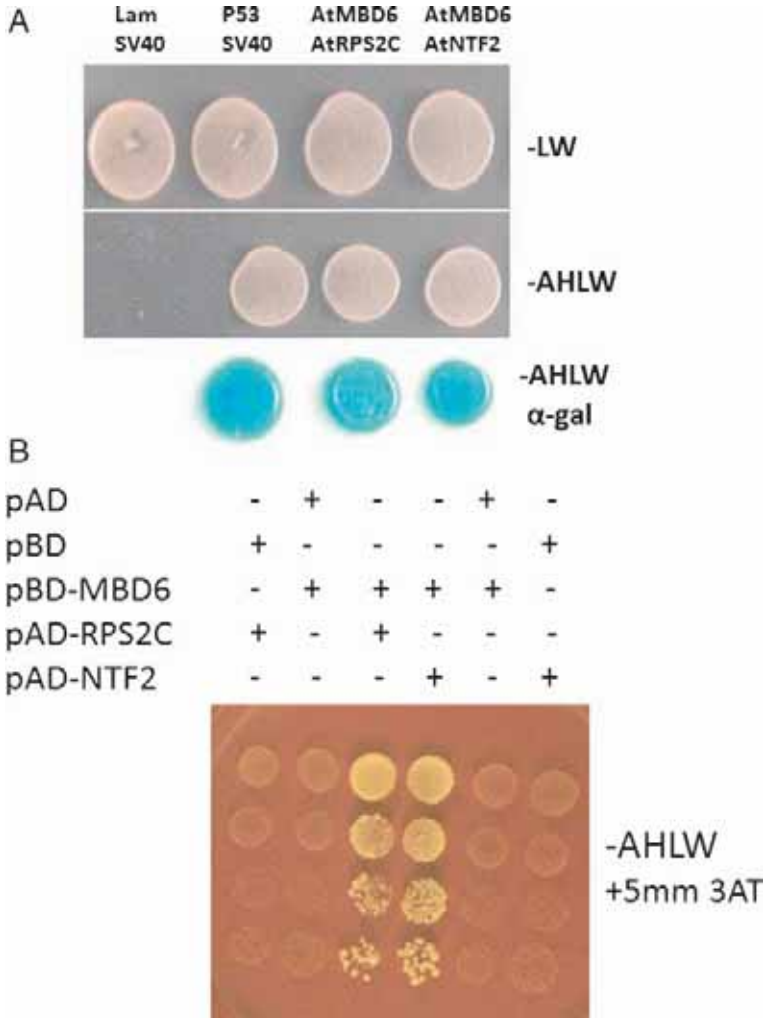

Figure 1. Screening for AtMBD6-interacting proteins by yeast two-hybrid assay: (**A**) The yeast two-hybrid screening identified that AtNTF2 and AtRPS2C interact with AtMBD6. The yeast cells transformed with genes encoding the bait (AtMBD6) and prey (RPS2C/NTF2) grew on –AHLW medium and were positive for α-galactosidase activity. Yeast cells transformed with pGBKT7-p53 and pGADT7rec-SV40 were used as a positive control. Yeast cells transformed with pGBKT7Lam and pGADT7rec-SV40 were used as a negative control. (**B**) Yeast cells co-transformed with pBD-MBD6 + pAD vector or pBD vector + pAD-NTF2/pAD-RPS2C did not grow on –AHLW medium containing 5 mM 3 (3-amino-1,2,4-triazole), whereas yeast cell co-transformed with pBD-AtMBD6 and pAD-AtRPS2C/pAD-AtNTF2 were able to grow in same medium. Dilution analysis was performed using the initial OD 1 at 600 nm and subsequently 1/10th dilution was used for the analysis.

RNA recognition motif (RRM). The RRM domain is predicted to bind to ssRNA (Allain *et al.* 2000; Maris *et al.* 2005). The proteins of this class mediate nuclear transport of small proteins like RanGTPase (Ribbeck *et al.* 1998; Quimby *et al.* 2000). The YFP-fused AtNTF2 was found to be localized in the nucleus and cytoplasm. The protein was also found to be present in some sub-nuclear regions where AtMBD6 was abundant (figure 3A, B). The florescence resonance energy transfer between CFP-fused AtMBD6 and YFP-fused NTF2 in the nucleus was found to be 21.47%, suggesting their close association *in vivo* (figure 3C).

3.4 *Interaction of AtMBD6 with AtHDA6 and AtAGO4*

The histone deacetylase enzyme is also an integral component of RNA-mediated gene silencing pathway and is involved in the process of nucleolar dominance (Aufsatz *et al.* 2002; Probst *et al.* 2004; Earley *et al*. 2006). These results substantiate the earlier finding of association of AtMBD6 with histone deacetylase activity (Zemach and Grafi 2003). Yeast two-hybrid assay was used to examine if there is any direct physical interaction between AtMBD6 and AtHDA6. The ORF-encoding AtMBD6 and AtHDA6 were cloned in

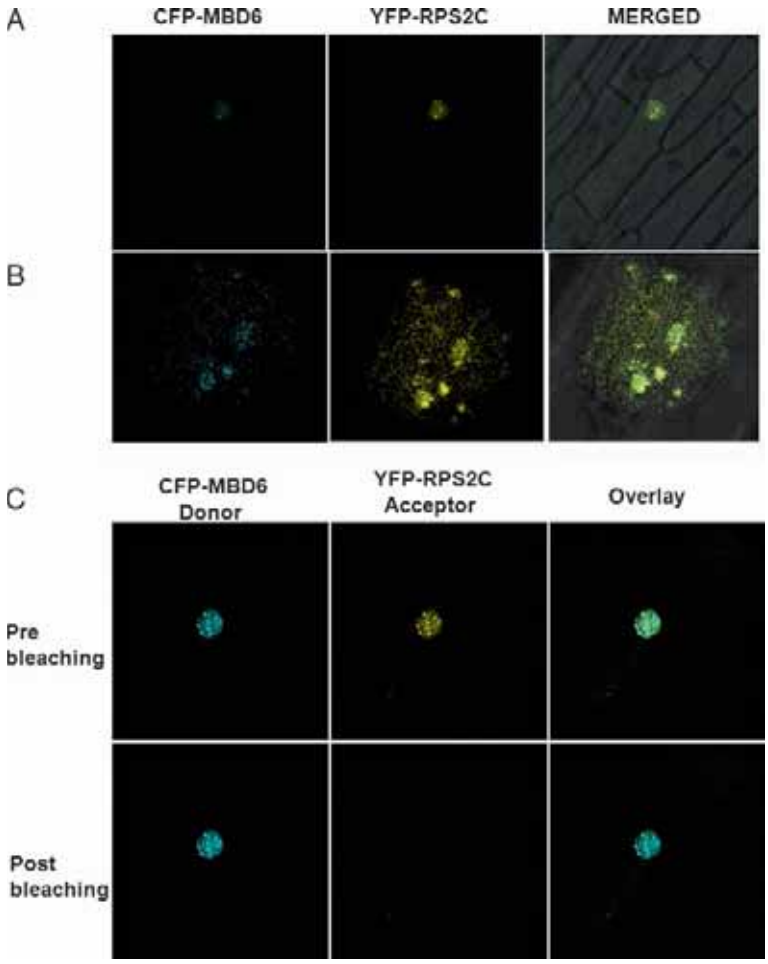

Figure 2. Co-localization and FRET analysis for AtMBD6 and AtRPS2C in onion cell using confocal microscope: (**A**) Subcellular localization of CFP-AtMBD6 and YFP-AtRPS2C fusion proteins was performed in onion epidermal cells. AtMBD6 was localized in the nucleus, whereas AtRPS2C was found to be present in the nucleus as well as in the cytoplasm. Cyan and yellow are pseudo-colours representing fluorescence of CFP and YFP respectively. (**B**) Close-up of the nucleus. (**C**) The physical interaction of AtMBD6 and AtRPS2C was studied by FRET-acceptor photo-bleaching protocol. The CFP fused with AtMBD6 was used as donor and YFP fused with AtRPS2C was the acceptor. The FRET efficiency was calculated based on the intensity of CFP before and after photo-bleaching of the acceptor.

fusion with GAL4-DNA binding domain and GAL4-activation domain respectively. The yeast cells expressing both the chimeric genes were able to grow on –AHLW medium. The positive interaction was indicated by α-galactosidase activity (figure 4A). We have also tested interaction of AtMBD6 with AtAGO4, another known component of RdDM. We used AtAGO4, as it is a RNA binding protein and known to be localized in the nucleolus. The past observations suggested that it was involved in miRNA-mediated gene silencing. These two proteins AtMBD6 and AtAGO4 may be part of a single complex. To find out the interaction between AtMBD6 and AtAGO4, yeast-two-hybrid assay was carried out using pBD-AtMBD6 and pAD-AtAGO4 vectors. Yeast transformation was carried out using these vectors. Only yeast cells expressing both bait and prey were able to grow on –AHLW plates and showed α-galactosidase activity. These results confirmed that AtMBD6 interacted with AtAGO4 (figure 4B).

Further, the *in vivo* association of AtMBD6 and AtHDA6 was analysed using FRET. The FRET analysis was carried out by co-expressing genes encoding CFP, fused to AtMBD6 and YFP, fused AtHDA6, in onion epidermal cells. Both the proteins were localized in the nucleus and showed more florescence at some sub-nuclear regions (figure 5A, B). The efficiency of energy transfer from CFP to YFP was found to be 15.92%, suggesting a positive interaction (figure 5C).

To validate the yeast two-hybrid result of AtMBD6 and AtAGO4 interaction, co-localization and FRET analysis was

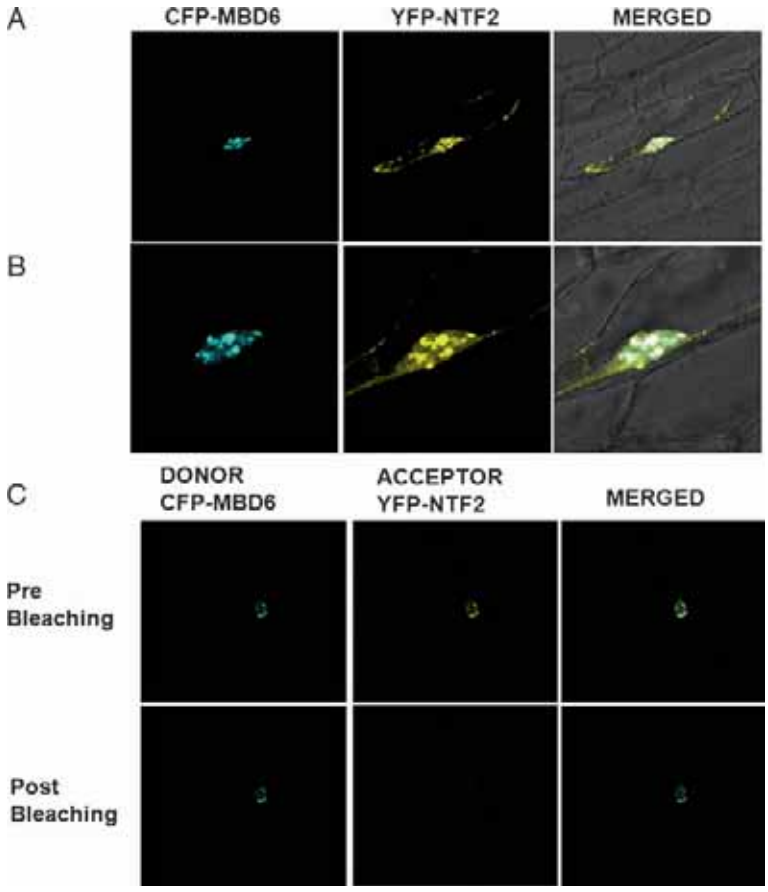

Figure 3. Co-localization and FRET analysis for interaction of AtMBD6 and AtNTF2 in onion cell by confocal microscopy: (**A**) Subcellular localization of CFP-AtMBD6 and YFP-AtNTF2 fusion proteins was performed in onion peels. AtNTF2 was found to be present in the nucleus as well as in the cytoplasm, whereas AtMBD6 was found only in the nucleus. Cyan and yellow are pseudo-colours representing fluorescence of CFP and YFP respectively (**B**) Close-up of the nucleus. (**C**) The energy transfer between CFP-AtMBD6 and YFP-AtNTF2 was studied by FRET-acceptor photo-bleaching protocol. The CFP fused with AtMBD6 was used as donor and YFP fused with AtNTF2 as the acceptor. The FRET efficiency was calculated based on the intensity of CFP before and after photo-bleaching of acceptor.

performed using CFP-tagged AtMBD6 and YFP-tagged AtAGO4. Genes encoding both the YFP-AtAGO4 and CFP-AtMBD6 proteins were transiently expressed in onion epidermal cells and analysed by confocal microscope. AtMBD6 and AtAGO4 were found to be localized in the nucleus showing strong signal in some sub nuclear regions (figure 6A, B). The interaction between AtMBD6 and AtAGO4 was verified by FRET. This was carried out using CFP-AtMBD6 as a donor and YFP fused to AtAGO4 as acceptor. The efficiency of energy transfer between CFP-AtMBD6 and YFP-AtAGO4 was found to be 18.09%, which confirms the association of these two proteins and supports the yeast two-hybrid result (figure 6C). The association of AtMBD6 with AtAGO4, which is known to have an important role in RNA-directed DNA methylation, suggests that AtMBD6 is also involved in the RdDM pathway.

3.5 *Mutants of AtMBD6 and its interacting partners show altered DNA methylation*

Mutant plants impaired in RdDM are associated with decreased DNA methylation (Aufsatz *et al.* 2002; Ausin *et al.* 2009; Lorkovic *et al.* 2012). The involvement of AtMBD6 in RNA-mediated gene silencing and its interaction with AtRPS2C and AtNTF2 prompted us to check the methylation pattern of the genomic DNA of their respective mutants. DNA methylation was analysed by MSP (methylation-sensitive PCR). The PCR amplification of several loci, using McrBC-digested genomic DNA from wild-type and mutant plants, revealed loss of DNA methylation at many loci. The reduced DNA methylation was found in DNA of 18S rRNA gene in *atmbd6* and *atntf2* mutants, whereas 180 bp centromeric repeats showed loss of DNA methylation in *atmbd6*

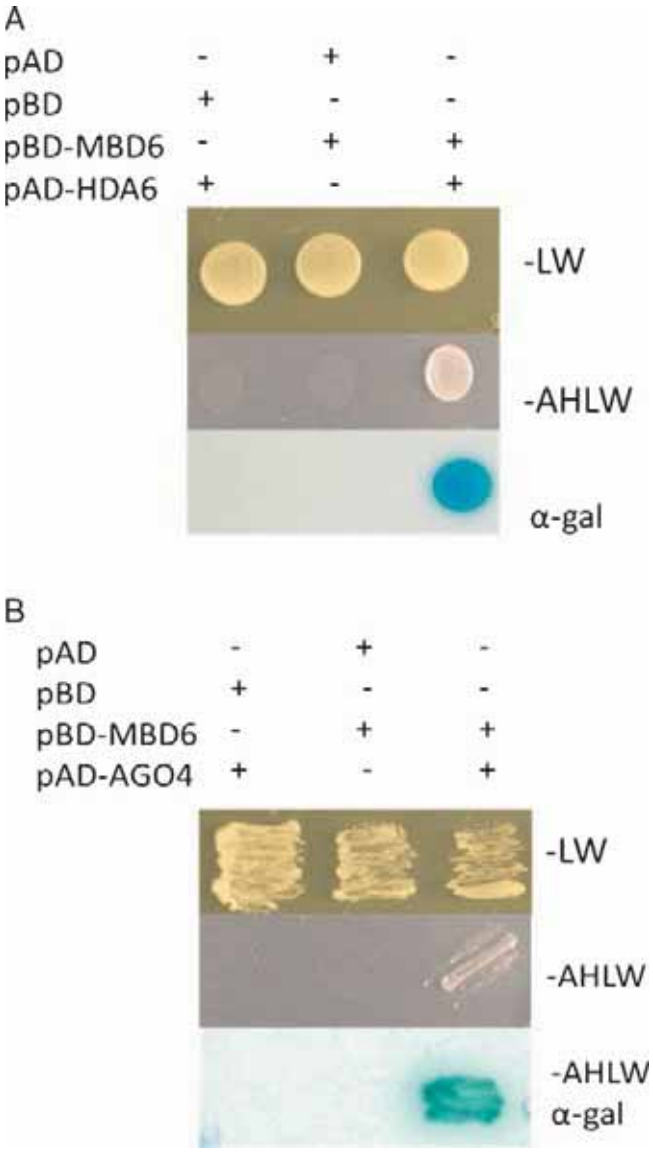

Figure 4. Study of physical interaction between AtMBD6 and AtHDA6/AGO4 by yeast two-hybrid assay: (**A**) Yeast two-hybrid assay was performed to detect the interaction between AtMBD6 and AtHDA6. The yeast cell transformed with the pGBKT7-AtMBD6 and pGAD-AtHDA6 were able to grow on –AHLW medium. Yeast cells co-transformed with pBD-MBD6 + pAD vector or pAD-HDA6 + pBD vector did not grow on –AHLW medium. The transformation cells were also verified by α-galactosidase activity. (**B**) Yeast two-hybrid assay was performed to detect the interaction between AtMBD6 and AtAGO4. The yeast cell transformed with the pGBKT7-AtMBD6 and pGAD-AtAGO4 were able to grow on –AHLW medium. Yeast cells co-transformed with pBD-MBD6 + pAD vector or pAD-AGO4 + pBD vector did not grow in –AHLW medium. The transformed was also verified by α-galactosidase activity.

and *atrps2c* (SAIL_569_G11) mutants (figure 7A). Some of the targets of RdDM, including pseudogenes, showed hypomethylation in all the mutants as compared with the wild-type (figure 7A). The loss of methylation was more in *atntf2* (SAIL_87_G09) mutant. When DNA methylation status of some miRNA loci was analysed, decreased DNA methylation was observed in all the mutants tested (figure 7B). The methylation of the constitutively expressed gene *ACTIN12* was unchanged (figure 7B).

4. Discussion

4.1 *AtMBD6 interacts with RNA binding proteins*

AtRPS2C is a component of 40S ribosome, localized in the cytoplasm and involved in protein synthesis. It is also found in the nucleolus (Pendle *et al.* 2005). Its interaction with AtMBD6 suggests its novel function. The S5 domain of AtRPS2C binds to dsRNA molecules and this motif is essential for the trafficking of the protein from cytoplasm to nucleus in mice (Bycroft *et al.* 1995; Matragkou *et al.* 2009). The localization of AtRPS2C and its RNA binding property suggests its connection with RNA-mediated gene regulation. Its interaction with AtMBD6 suggests that AtRPS2C may be involved in the process of RNA-directed DNA methylation.

Sequence analysis of NTF2 protein revealed several important features. The protein is made of 460 amino acids with a predicted molecular mass of 49.55 kDa. AtNTF2 shares similarity with other members of the *Arabidopsis* and human NTF2 domain containing proteins. Based on homology the protein can be divided into two domains, a nuclear transport factor 2 (NTF2) domain and an RNA recognition motif (RRM). The *Arabidopsis* genome encodes eight proteins that contain NTF2 and RRM domains (supplementary figure 2; supplementary table 1). There are homologs of genes encoding proteins containing NTF2 and RRM domains. These domains are conserved in other flowering plant species (supplementary figure 3; supplementary table 2).

AtNTF2 is essential for the transport of NLS-containing proteins from cytoplasm to nucleus (Moore and Blobel 1994). Proteins containing RRM domains bind to RNA and are involved in post-transcriptional gene regulation (Allain *et al.* 2000; Maris *et al.* 2005). The two NTF2 domain containing proteins, AtNTF2a and AtNTF2b, are involved in nuclear transport and interact with AtRAN1 (Zhao *et al.* 2006). Ran-GTPase cycle is also required for the nuclear transport of ribosomal subunits (Moy and Silver 1999). AtRAN3 has been reported to interact with AtMBD5, another MBD protein known to interact with AtMBD6 (Zemach *et al.* 2005; Yano *et al.* 2006). It can be concluded from these reports that Ran-GTPase cycle may be involved in the transport of AtMBD6 into the nucleus.

The involvement of AGO4 in RdDM is well characterized in *Arabidopsis*. The newly generated siRNAs have been reported to be loaded on to AGO4, which is a signal for *de*

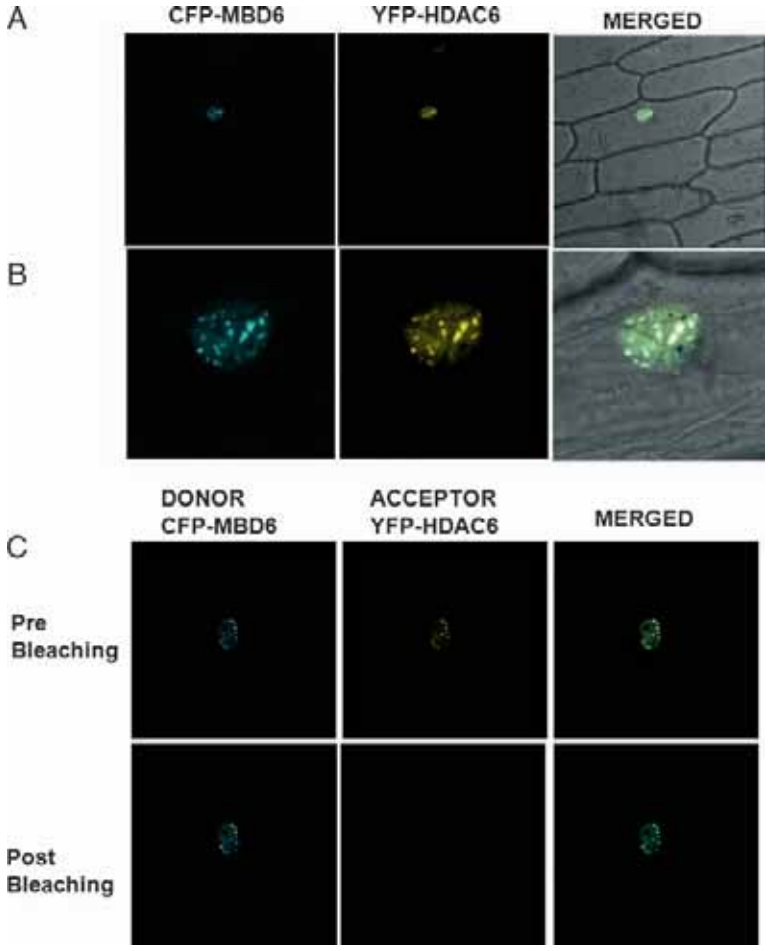

Figure 5. Subcellular localization and FRET analysis for interaction of AtMBD6 and AtHDA6: (**A**) Subcellular localization of CFP-AtMBD6 and YFP-AtHDA6 fusion proteins was performed in onion epidermal cells. Both the proteins were localized in the nucleus. Cyan and Yellow are pseudo-colours representing fluorescence of CFP and YFP respectively. (**B**) Close-up of the nucleus. (**C**) The energy transfer between CFP-AtMBD6 and YFP-AtHDA6 was studied by FRET-acceptor photo-bleaching protocol. The decreased pre-bleaching and post-bleaching intensity of YFP indicates the energy transfer from CFP to YFP.

novo DNA methylation, catalysed by DRM2 (Li *et al*. 2006; Pontes *et al*. 2006; Law and Jacobsen 2010). The siRNA production and loading onto AGO4 takes place in nuclear processing centres called Cajal bodies (Li *et al*. 2006; Pontes *et al*. 2006: Ye *et al*. 2012). The interaction between AtMBD6 and AtAGO4 suggests that AGO4, loaded with siRNA targets *de novo* methylation with the help of DRM2 and binding of AtMBD6 to the methylated DNA might lead to formation of heterochromatin.

4.2 *AtMBD6 interact with AtHDA6*

Earlier, histone deacetylase activity was found to be associated with AtMBD6 (Zemach and Grafi 2003). However, which histone deacetylase out of several is associated with AtMBD6 was not clear. In *Arabidopsis* 18 histone deacetylases are present, and of them we chose AtHDA6 because of its association with RdDM and nucleolar dominance (Aufsatz *et al.* 2002; Earley *et al.* 2006). When *MET1* is mutated, it results in loss of methylation. Mutation in *AtHDA6* negated this loss of DNA methylation in *met1* mutant (Aufsatz *et al.* 2002; To *et al.* 2011; Earley *et al.* 2010). The *de novo* DNA methylation activity of MET1 and its interaction with AtHDA6 suggests that although DRM2 and CMT3 are well characterized methyltransferases involved in RdDM, MET1 may also be a possible methyltransferase involved in RdDM (Cao *et al.* 2003; Cao and Jacobsen 2002; Zubko *et al.* 2012). Since AtHDA6 is known to be involved in RdDM, in the present study, we attempted to study the interaction of AtMBD6 with AtHDA6. We could find positive interaction between AtMBD6 and

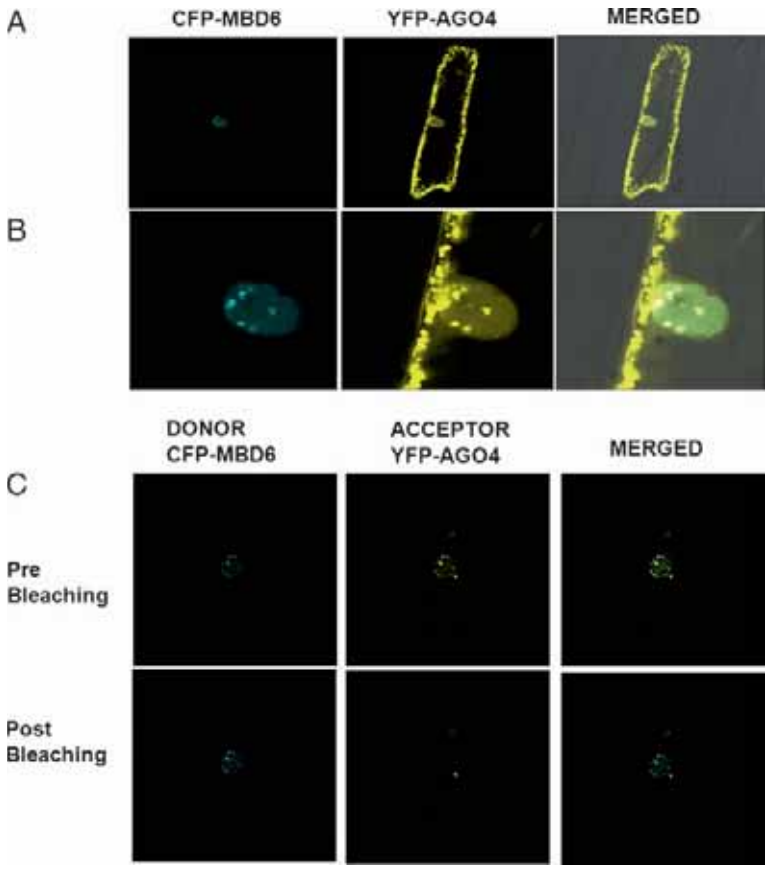

Figure 6. Subcellular localization and FRET analysis for interaction of AtMBD6 and AtAGO4: (**A**) Subcellular localization of CFP-AtMBD6 and YFP-AtAGO4 fusion proteins was performed in onion epidermal cells. Both the proteins were localized in the nucleus. Cyan and Yellow are pseudo-colours representing fluorescence of CFP and YFP respectively. (**B**) Close-up of the nucleus. (**C**) The energy transfer between CFP-AtMBD6 and YFP-AtAGO4 was studied by FRET-acceptor photo-bleaching protocol. The decreased pre-bleaching and post-bleaching intensity of YFP indicates the energy transfer from CFP to YFP.

AtHDA6, using yeast two-hybrid assays and FRET. Another protein which has been reported earlier to interact with AtMBD6 is a SNF2 factor DDM1. It also interacts with AtMBD5 and is involved in transcriptional as well as post-transcriptional gene silencing in cooperation with MET1 (Zemach *et al.* 2005; Zubko *et al.* 2012). Human MBDs are known to be a link between DNA methylation and histone deacetylation (Nan *et al.* 1998; Burgers *et al.* 2002; Bogdanovic and Veenstra 2009). Our results suggest that a similar link is likely to be present between AtMBD6 and AtHDA6.

4.3 *AtMBD6 is involved in RNA-mediated gene silencing*

Analysis with methylation-sensitive PCR revealed a reduction in methylation in *atmbd6*, *atrps2c* and *atntf2* mutants. This indicates that AtMBD6 is required to maintain methylation and contributes to silencing. Loss of methylation in both 18S rRNA gene and 180 bp repeats suggest its contribution for both the targets. The results suggest that AtMBD6 interacts with AtNTF2 and AtRPS2C, and these interactions, in addition to interactions with other proteins involved in gene silencing, contribute to regulation DNA methylation and gene silencing.

5. Concluding remarks

The interaction of AtMBD6 with RNA binding proteins like AGO4, NTF2 and RPS2C suggests the involvement of this protein in reorganization of target loci in the genomic DNA for *de novo* methylation. We propose that miRNA or siRNA load onto these RNA binding proteins and then the complexes formed interact with AtMBD6, followed by recognition of RdDM targets in the genomic DNA. Further, *de novo* methylation takes place on the target sites by *de novo* methyl transferase DRM2, allowing binding of AtMBD6 to the

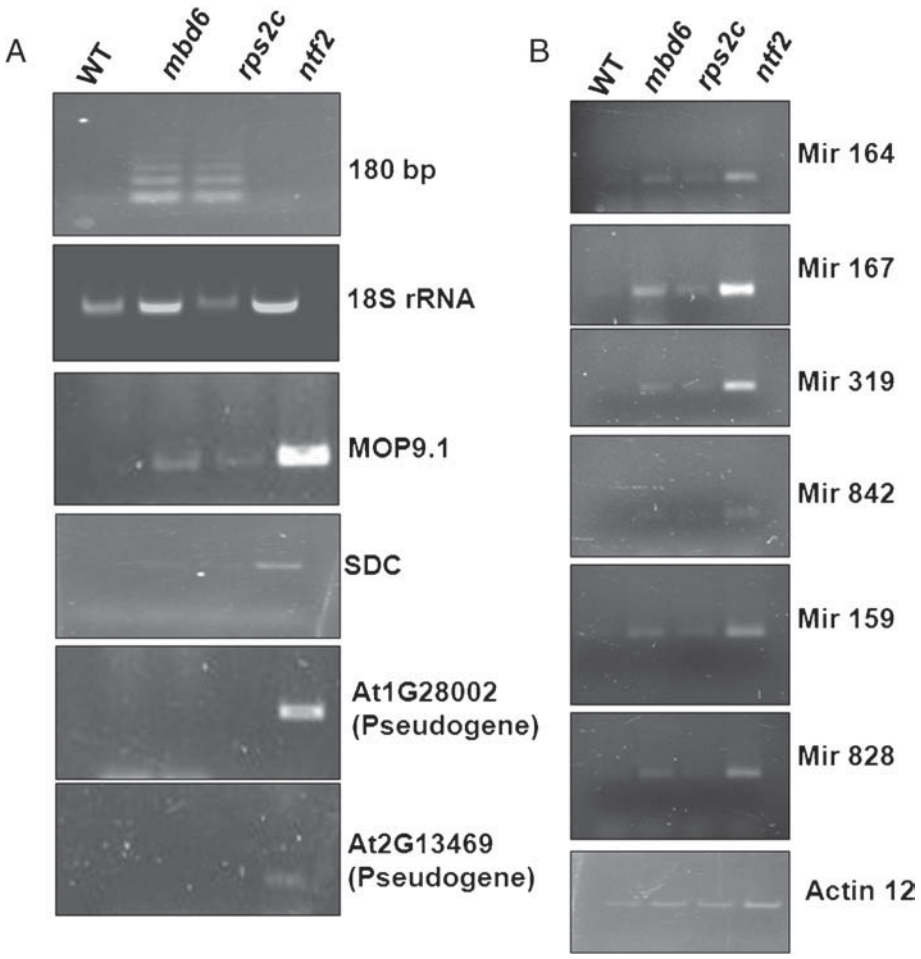

Figure 7. DNA methylation analysis in *atmbd6*, *atrps2c* and *atntf2* mutants: DNA methylation analysis was carried out for repeat elements (180 bp repeat and 18S rRNA), pseudogenes (At1G28002 and At2G13469), targets of RdDM (SDC and MOP9.1) and microRNA producing loci (mir164, mir167, mir319, mir842, mir159 and mir828). Genomic DNA samples isolated from wild type and mutants were digested with methylation requiring restriction enzyme McrBC and used for PCR amplification. *ACTIN12* gene was used as control.

methylated sites, which recruits AtHDA6, resulting in histone deacetylation, leading to formation of heterochromatin.

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