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Titel der Dissertation

Identification and characterization of HDA6 interaction partners and complexes

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Abstract

The acetylation of histone tails is a reversible post-translational modification, and thus provides a flexible mechanism for transcriptional regulation. The controlled action of histone acetyltransferases (HATs) and histone deacetylases (HDACs) specifies the acetylation levels of histones and is therefore linked to transcriptional activation and repression, respectively. The *Arabidopsis* histone deacetylase HDA6 is involved in RNA-directed DNA methylation (RdDM), a pathway required for silencing of transgenes, transposons and ribosomal RNA (rRNA) genes. Beside its function in RdDM, HDA6 has been implicated in flowering, senescence and jasmonate, ABA and salt stress responses. However, neither interaction partners nor complexes containing HDA6 have been identified so far.

In this work, we investigated the role of HDA6 in the RdDM pathway and the mechanisms of its recruitment to RdDM targets. We have characterized three new mutant alleles of *HDA6* that code for enzymatically inactive proteins. Interestingly, they all show transcriptional reactivation of several known RdDM targets without a decrease of DNA methylation. This result was surprising, since HDA6 has been implicated in the maintenance of DNA methylation, a major hallmark of RdDM silencing. It also indicates that methylation is not sufficient for silencing in RdDM.

In a large yeast two hybrid screen, we identified FIBRILLARIN and an RRM domain protein as possible HDA6 interaction partners. Additionally, we confirmed these interactions *in planta* using bimolecular fluorescence complementation (BiFC). Both proteins have RNA-binding domains, suggesting that HDA6 can be recruited directly via siRNAs in RdDM, which is consistent with a methylation-independent recruitment model. Mass spectrometry on immunoprecipitated epitope-tagged HDA6 identified several plant orthologs of mammalian and yeast components of the SIN3 complex and represents the first purified HDA6 complex from *Arabidopsis*. Additionally, our study indicates that the *Arabidopsis* SIN3-like complex is not involved in RdDM and highlights possibly separate roles of HDA6 in RdDM and non-RdDM silencing.

Zusammenfassung

Die Acetylierung von N-terminalen Histonenden ist eine reversible post-translationale Modifikation und stellt daher einen flexiblen Mechanismus für die Regulation der Transkription dar. Das kontrollierte Zusammenspiel von Histon Acetyltransferasen (HATs) und Histon Deacetylasen (HDAcs) legt Acetylierungslevels von Histonen fest und ist daher eng mit transkriptioneller Aktivierung oder Repression verknüpft. Die *Arabidopsis* Histon Deacetylase HDA6 ist in "RNA-directed DNA methylation" (RdDM) involviert, einem Regulationsweg, der für das Stilllegen von Transgenen, Transposons und ribosomalen Genen eine Rolle spielt. Neben der Funktion in RdDM wurde für HDA6 auch Rollen in der Regulation der Blühzeit, in der Senezenz und in der Antwort auf Jasmonat, ABA und Salzstress festgestellt. Bis jetzt wurden jedoch weder Interaktionspartner noch Proteinkomplexe von HDA6 identifiziert.

In dieser Arbeit untersuchten wir die Rolle von HDA6 in RdDM und die Mechanismen der seiner Rekrutierung zu RdDM Zielgenen. Wir haben drei neue Mutantenallele von HDA6 charakterisiert, welche für enzymatisch inaktive Proteine kodieren. Interessanterweise zeigen alle drei Mutanten die Reaktivierung von einigen bekannten RdDM Zielgenen, ohne zu einer Abnahme der DNA Methylierung zu führen. Dieses Ergebnis war überraschend, da zuvor eine Rolle von HDA6 in der Aufrechterhaltung von DNA Methylierung festgestellt wurde, welche ein wesentliches Kennzeichen von RdDM darstellt. Dieses Ergebnis deutet auch darauf hin, daß DNA Methylierung nicht ausreichend für RdDM-vermittelte Genrepression ist.

Wir identifizierten mittels eines Interaktionsscreens in Hefe FIBRILLARIN und ein Protein mit einer RNA-Interaktionsdomäne (Lorkovic et al.) als mögliche HDA6 Interaktionspartner. Wir bestätigten diese Interaktionen *in planta* mittels "bimolecular fluorescence complementation" (BiFC). Beide Proteine besitzen RNA-Bindungsdomänen, was auf eine Rekrutierung von HDA6 direkt mittels siRNAs in einem Methylierungs-unabhängigen Mechanismus hindeuten könnte. Massenspektrometrie von immun-gereinigtem, Epitop-markiertem HDA6 führte zu der Identifizierung von *Arabidopsis* Orthologen einiger Hefe- und Säugerkomponenten des SIN3 Komplex, was den ersten gereinigten HDA6 Komplex

aus *Arabidopsis* darstellt. Unsere Studien zeigten weiters, dass der *Arabidopsis* SIN3 Komplex wahrscheinlich nicht in RdDM involviert ist und lassen den Schluß auf wahrscheinliche unterschiedlichen Rollen von HDA6 in RdDM und RdDM-unabhängigen Repressionmechanismen zu.

Introduction

1. Histone acetylation: a posttranslational histone modification associated with active transcription

Eukaryotic DNA is organized into chromatin with nucleosomes as the basic building unit. A single nucleosome consists of two copies each of the core histones H2A, H2B, H3 and H4 and is typically enfolded by 147 bp of DNA (Kornberg, 1974; Kornberg and Thomas, 1974; Luger et al., 1997). Each histone has a structured globular domain and an unstructured amino-terminal tail that extends from the core nucleosome (Campos and Reinberg, 2009; Luger et al., 1997). These histone tails provide sites for a variety of posttranslational modifications, which ultimately influence chromatin structure. Extensive literature on these modifications documents different types including acetylation, phosphorylation, methylation, ubiquitination and ADP-ribosylation (reviewed in Grant, 2001). All of these modifications are reversible and maintained by the controlled action of different histone modifying enzymes providing dynamics to chromatin. Considering that DNA in a living cell is never naked but always confined in chromatin, histone modifications play an important role in the regulation of several biological processes involving DNA dynamics like transcription, DNA repair and replication (MacDonald and Howe, 2009). Two generally not exclusive hypotheses regarding the mode of action of histone modifications have been accepted. One hypothesis states that each distinct modification is bound and "read-out" by different non-histone proteins. The combination of different modifications thus represents a histone-code that is underlying the activity state of a given gene (reviewed in Jenuwein and Allis, 2001). The other hypothesis puts more emphasis on direct structural changes of chromatin that are due to changed histone properties (reviewed in Bernstein and Allis, 2005).

The acetylation state of the ε-amino group of conserved lysine residues within all four core histone has been long linked to transcriptional activity (Allfrey et al., 1964). The final molecular link between histone acetylation and transcriptional states was the discovery of the histone acetyltransferase activity of the Gcn5p transcriptional

reactivation factor in yeast (Brownell et al., 1996) and histone deacetylase (HDAC) activity of transcriptional co-repressors (Nagy et al., 1997). Thus, the controlled action of HAT and HDAC enzymes specifies acetylation levels of histones and are linked to transcriptional activation and repression, respectively (Figure 1) (reviewed in Yang and Seto, 2007).

The exact mechanisms by which histone acetylation increases transcription are still not exactly understood. Based on the previously mentioned two hypothesis three models are possible. Two models are based on the fact that the acetylation of lysine residues reduces the net basic charge of histones. One consequence might be that histone affinity to DNA is decreased, resulting in partial decondensation of the DNA from the nucleosome (Figure 2a). Alternatively, the presence of acetyl-groups might alter histone-histone interactions between adjacent nucleosomes, which in turn enhances octamer mobility, leading to the decompaction of nucleosome arrays (Figure 2b) (Ferreira et al., 2007; Toth et al., 2006). The third model considers histone modifications only in the context of the histone code, which is perceived by distinct sets of proteins resulting in specific downstream responses (Figure 2c) (Strahl and Allis, 2000). It has been demonstrated that acetylation influences the interaction of histones with other proteins. Acetylated histone H3 and H4, for example, serve as binding targets for the bromodomain which is found in several remodelling factors and transcriptional regulators (Ladurner et al., 2003; Mujtaba et al., 2007).

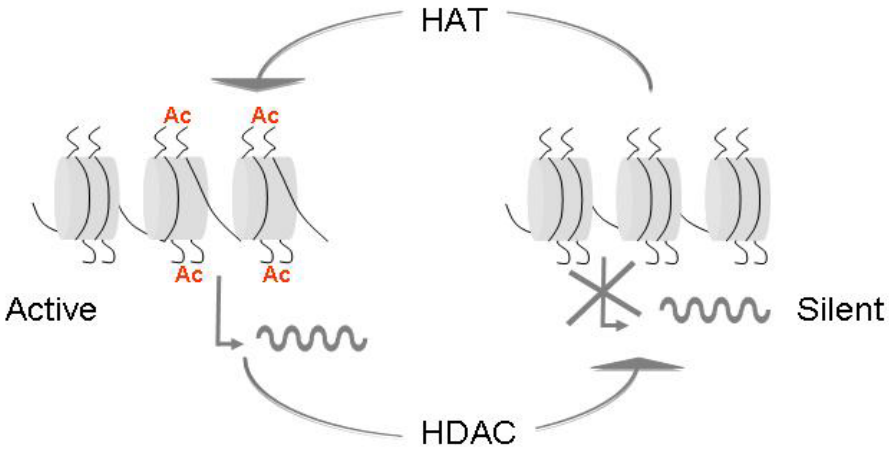


Figure 1. Histone acetylation is a reversible posttranslational modification that is regulated via a dynamic interplay of histone acetyltransferase and histone deacetylase (HDAC) activities. Acetylated histones are associated with transcriptionally active chromatin while histone deacetylation correlates with transcriptionally silent states.

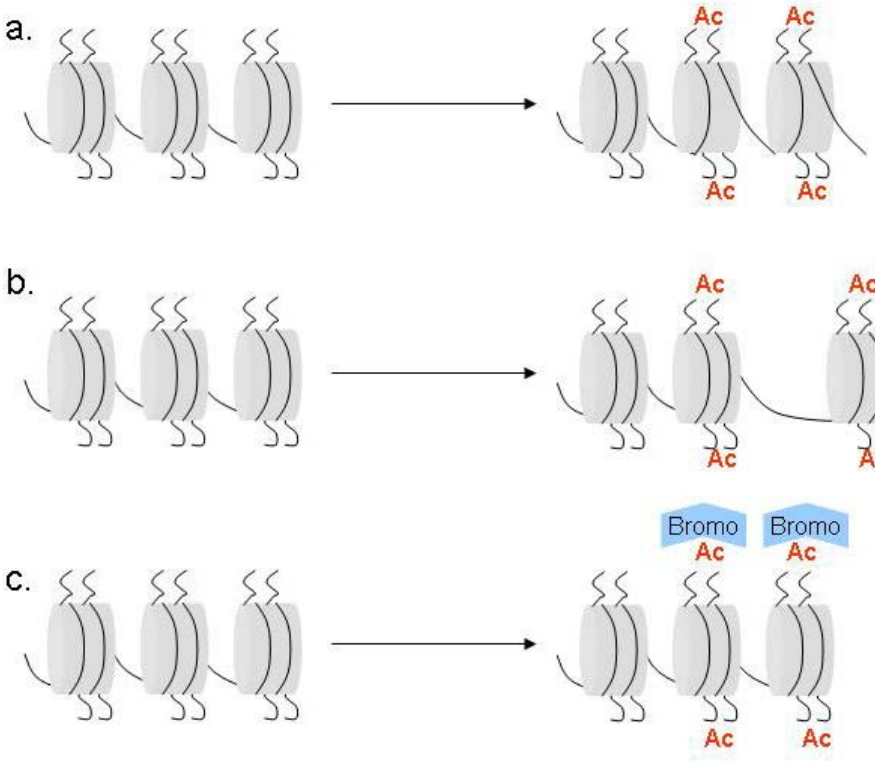


Figure 2. Three models of how acetylation of lysines within histone tails promotes gene activity.

- **a. The presence of acetyl groups changes the charge of histones, leading to a reduced affinity of histones to DNA.**
- **b. Histone acetylation alters histone interactions between adjacent nucleosomes thereby opening the chromatin structure.**
- **c. Acetylated histones form a binding surface for non-histone proteins that have a bromodomain.**

2. The enzymatic players of histone acetylation: histone acetyltransferases (HATs) and histone deacetylases (HDACs)

2.1. HATs

Histone acetyltransferases can be defined as enzymes that transfer the acetyl group from acetyl coenzyme A (acetyl CoA) onto lysine residues of histone tails. HATs are evolutionary conserved from yeast to mammals and they exist as multi-

subunit complexes (reviewed in Lee and Workman, 2007). Each complex generally consists of a catalytic subunit and auxiliary proteins (MacDonald and Howe, 2009).

Based on their catalytic domain, HATs can be divided into two predominant classes: GNAT (Gcn5 N-acetyltransferases) and MYST (Morf, Ybf2, Sas2 and Tip60), named according to their founding members. Members of the GNAT superfamily have been grouped together on the basis of their similarity within several homology regions and acetylation-related motifs of the catalytic subunit. Four sequence motifs whose functions are not yet fully understood (C, D, A, and B, in N-terminal to C-terminal order) define this superfamily (Sterner and Berger, 2000). Motif A is the most highly conserved region and it is shared with MYST proteins. Members of the MYST superfamily are grouped based on their close sequence similarities particularly within the acetyltransferase region that includes part of motif A of the GNAT superfamily (Sterner and Berger, 2000). In addition several proteins, like p300/CBP, Taf1 and a number of nuclear receptor co-activators, possess intrinsic HAT activity, though without a true consensus HAT domain. Consequently, they form a third "orphan class" of HATs (Lee and Workman, 2007; Yang and Seto, 2007).

Besides having the unique catalytic subunit, HAT complexes possess a diverse set of auxiliary proteins. The combination of these contribute to the unique function of each HAT (Lee and Workman, 2007). For example, yeast recombinant Gcn5 was found to acetylate only free histones *in vitro*, however in the context of the multi-subunit complex it is able to acetylate nucleosomal histones (Sterner and Berger, 2000). Additionally, complexes can share a catalytic subunit (the yeast Gcn5 is part of three HAT complexes) or have an overlapping substrate specificity (SAGA modifies H3K9 and to lesser extent H3K14, while NuA3 modifies H3K14) and still have a specific biological function due to auxiliary factors (Lee and Workman, 2007; Yang and Seto, 2007).

Some of these auxiliary proteins possess chromatin-binding domains that recognize modified histone tails, like the bromodomain (acetyl-lysine), chromodomain (methyl-lysine), WD40 repeats (H3K4me2), Tudor domains (methylated lysines and arginines) and plant specific PHD fingers (H3K4me3) and are important for complex targeting. Each complex possesses a mixture of different auxiliary factors with specific set of domains. Interestingly not only the presence of these domains but also their context is crucial for the correct targeting of HATs. For example, the SAGA

complex possesses 15 putative chromatin binding domains among two of them, Gcn5 and Spt7, both have a bromodomain. *In vitro* studies, however, showed that only the Gcn5 is binding to acetylated lysines (Hassan et al., 2002).

The complex structure of HATs is the major reason for their very diverse function. The correct interpretation of intracellular signals and subsequent recruitment to genome targets enables HATs to carry out several different biological functions, including transcriptional regulation, DNA replication and repair, recombination and maintenance of overall genome stability (Yang and Seto, 2007). Although these functions are mainly mediated through histone acetylation, an increasing number of non-histone substrates have been identified. More than 60 transcription factors have been shown to be targets of acetylation by HATs. Additionally, regulatory factors of DNA repair, recombination and replication, some classical metabolic enymes like bacterial and mammalian acetyl-CoA syntheses and several signalling factors (kinases and phosphatases) have been demonstrated to be HAT substrates (Yang and Seto, 2007).

The *Arabidopsis* genome is predicted to encode 12 HAT proteins, which is somewhat more than found in other sequenced eukaryotic genomes (Pandey et al., 2002). These can be grouped into four families: GNAT, MYST (defined by the presence of a conserved activity domain) and two families belonging to “orphan class” of HATs (CBP and TAFII250 related families) (Lee and Workman, 2007; Pandey et al., 2002). The degree of evolutionary conservation varies significantly among the different HAT families. Based on phylogenetic and domain analyses, a partial functional diversification of these proteins during plant development can be predicted (Pandey et al., 2002).

2.2. HDACs

Histone deacetylases are enzymes with an opposing function to HATs; they remove acetyl groups from histone tail lysines. Like HATs, HDACs have several non-histone substrates, including transcription factors, proteins involved in DNA repair and replication, metabolism, cytoskeleton dynamics, apoptosis and cell signalling. Based on sequence similarity and cofactor dependency, HDACs are grouped into two families: Rpd3/Hda1 (reduced potassium dependence 3) and Sir2 (silent

information regulator 2) related protein families (reviewed in Yang and Seto, 2007).

The Rpd3/Hda1 and Sir2 families show no homology on the amino acid sequence level. Members of the Sir2 family (sirtuins) have a catalytic domain that is characterized by the requirement for nicotine adenine dinucleotide (NAD) as a cofactor (reviewed in Haigis and Guarente, 2006). Sirtuins occur in prokaryotes, fungi, plants and animals and can be found in a wide variety of subcellular localizations. The main function of these proteins resides within transcriptional silencing, which is mediated by diverse multi subunit complexes. For example, the yeast Sir2p protein exerts its silencing function on different genomic loci through different complexes: Sir2p is recruited to telomeres and mating-type loci in a sequence specific manner by DNA-binding proteins together with the structurally unrelated proteins Sir3p and Sir4p. Transcription and homologous recombination of rDNA repeats, however, is controlled by a distinct Sir2p complex, regulator of nucleolar silencing and telophase exit (RNT)(Haigis and Guarente, 2006; North and Verdin, 2004).

Members of the Rpd3/Hda1 superfamily share sequence homology especially in the HDAC domain and require Zn^{2+} cofactor for deacetylase activity (reviewed in Yang and Seto, 2008). The availability of crystal structures from two mammalian members of this group and two bacterial HDACs indicates conservation of the deacetylase domain with regard to overall tertiary structure. In humans, proteins of this family can be further subgrouped into three classes. Class I includes proteins homologous to yeast Rpd3, while members of class II are more related to yeast Hda1. One human HDAC, HDAC11, is homologous to both Hda1 and Rpd3 and is thus grouped into the separate class III. Class I HDACs, with an exception of HDAC8, can function as catalytic subunits of multiprotein complexes. HDAC1 and 2 interact with one another and provide the catalytic core of several complexes like SIN3, NuRD (nucleosome remodelling deacetylase) and CoREST (corepressor of RE1-silencing transcription factor). These are silencing complexes, recruited to genomic targets by DNA-binding proteins to repress transcription and modify chromatin (reviewed in Ahringer, 2000; Yang and Seto, 2008).

The *Arabidopsis* genome encodes 16 HDACs. Ten of them belong to the Rpd3/Hda1 superfamily, two are sirtuins and four are members of the plant specific HD2 family (Figure 3). The HD2 family has been first identified and characterized in

maize (Lusser et al., 1997). By sequence homology searches, four members of the HD2 family, HDT1, HDT2, HDT3 and HDT4, were identified in the *Arabidopsis* proteome (Pandey et al., 2002). HDT1 and HDT2 have been further analyzed and connected to gene silencing and seed development (Lusser et al., 1997).

- **HD2 family** (plant-specific HDACs)
  HDT1, HDT2, HDT3, HDT4
- **SIR2 family**
  SRT1, SRT2
- **RPD3/HDA1 superfamily**
  Class I
  Cluster A HDA9
  HDA19
  Cluster B HDA6
  HDA7
  Class II
  HDA5, HDA15, HDA18
  Class III
  HDA2
  Unclassified
  HDA8, HDA14

Figure 3. Classification of Arabidopsis HDACs (Pandey et al., 2002). Arabidopsis harbors 16 predicted HDAC genes. They are classified in three families. HD2 is a plant specific family, while the other two families are defined by homology to yeast RPD3 (reduced potassium dependence 3)/HDA1 and SIR2 (silent mating-type information regulation 2), respectively. The RPD3/HDA1 superfamily can be further subdivided into three classes and an unclassified category.

2.2.1. The Arabidopsis RPD3/HDA1 family of histone deacetylases

Members of the RPD3/HDA1 superfamily, especially those of class I, represent the functionally best characterized HDACs in *Arabidopsis*. Like in mammals, this superfamily can be further divided based on sequence homology within the conserved HDAC domain. They are grouped into three classes and further include two proteins, HDA8 and HDA14, which cannot be assigned to any of these

classes (Figure 3) (Pandey et al., 2002).

Class one has four members with similarity to the yeast RPD3 protein. They can be further grouped into cluster A and B. Cluster A proteins, HDA19 and HDA9, share high sequence homology and may comprise an orthologous group. Cluster B proteins, which include HDA6 and HDA7, are more divergent than those of cluster A (Figure 3). This strong separation into two clusters supports the possibility of functional divergence (Pandey et al., 2002). This is supported by the fact that *hda19* mutants (cluster A) have a strong developmental phenotype, while no developmental abnormalities, except late flowering, have been reported for *hda6* mutants (cluster B) (Probst et al., 2004). Both HDA19 and HDA6 have been reported to be involved in similar processes as well, such as jasmonate response, senescence, flowering and repression of embryonic properties after germination (Devoto et al., 2002; Tanaka et al., 2008; Wu et al., 2008; Zhou et al., 2005). They may, however, differ in the type of target genes they regulate throughout these processes. Like in mammals, the main function of RPD3-type proteins from *Arabidopsis* is transcriptional repression. Orthologs of components of SIN3 and NuRD complexes are present in plants, which points to an evolutionary conservation of mechanisms of class I HDAC function (Ahringer, 2000; Yang and Seto, 2008).

Interestingly, among the four members of *Arabidopsis* RPD3-type HDACs, HDA6 is the only one involved in one type of RNA silencing, called RNA-directed DNA methylation. Several highly saturated screens recovered HDA6 as crucial actor in this pathway. Through this mechanism HDA6 regulates transcriptional silencing of rDNA, several transposons and transgenes inserted in repetitive manner (Earley et al., 2006; Lippman et al., 2003; Murfett et al., 2001).

3. The role of HDACs in small RNA-triggered silencing pathways

Besides regulating the activity of protein-coding genes, RPD3-type HDACs are also involved in the control of permanent silent chromatin, heterochromatin, and in genome-defense against transposable elements. In fission yeast as well as in plants this processes are controlled by silencing pathways involving small RNAs and components of the RNA interference (RNAi) machinery, which are called RNAi-mediated heterochromatin formation and RNA-directed DNA methylation (RdDM),

respectively (Lippman and Martienssen, 2004; Matzke and Birchler, 2005; Wako and Fukui, 2010). Both pathways result in repressive histone modifications and are also linked with DNA methylation in methylation-competent organisms. Histone deacetylation is an important step in both pathways and RPD3-type HDACs have been implicated in both of them.

3.1. Heterochromatin

Heterochromatin has originally been distinguished from euchromatin by cytology on the basis of differential compaction during interphase. It is highly ordered in nucleosome arrays and is considered to be transcriptionally inactive, in contrast to euchromatin that is lightly packed and transcriptionally active (Grewal and Jia, 2007). Heterochromatic features are maintained epigenetically over cell divisions and across generations. Typically, heterochromatin is composed of DNA sequence with little or no coding potential and is usually found in centromeres, pericentromeric regions and telomers. On the DNA level heterochromatin consists of endogenous repeats of variable size arranged in megabase-long arrays, as well as of multiple copies of defective and intact transposons (reviewed in Henderson and Jacobsen, 2007; Matzke and Birchler, 2005). In *Arabidopsis*, pericentromeric repeats, transposable elements, and silenced rRNA genes are assembled into heterochromatin within nuclear structures that can be seen as chromocenters in interphase nuclei. The defining marks of heterochromatin are posttranslational histone modifications (e.g. H3K9me and histone H3/4 hypoacetylation) and, in some organisms, DNA methylation. Plant heterochromatic histone marks, however, partly differ from the ones in yeast, mammals or *Drosophila* (Fischer et al., 2006). Even though heterochromatin has been long recognized, the pathway of its assembly came into focus only recently with the discovery of RNAi involvement (Volpe et al., 2003).

3.1.1. Heterochromatic marks

As already mentioned, major heterochromatin marks in all organisms are histone methylation and hypoacetylation. However, heterochromatin in yeast, mammals and *Drosophila* is mainly characterized by histone H3 di- and tri- methylation at lysine 9

(H3K9me2 and me3) (Lachner et al., 2001), while in plants heterochromatic marks are mono- and dimethyl H3K9 and H3K27 (Naumann et al., 2005). This indicates that the position of methylated lysine residues of H3 in heterochromatin is conserved among eukaryotes while the degree of methylation varies. In contrast to euchromatin, heterochromatin is typically associated with hypoacetylated histones (Grewal and Jia, 2007), which also holds true for *Arabidopsis* (Soppe et al., 2002). The main acetylation sites of histones that are conserved between species include lysines 9, 14, 18, 23 of histone H3 and lysines 5, 8, 12, 16 of histone H4 (reviewed in Strahl and Allis, 2000).

Another hallmark of heterochromatin in yeast, mammals and flies are homologs of the heterochromatin binding protein HP1, which bind to H3K9me3 or H3K9me2 via a chromodomain (Bannister et al., 2001; Jacobs et al., 2001; Lachner et al., 2001). The unique *Arabidopsis* HP1 homolog LHP1 has the same domain architecture like animal HP1 proteins, it is, however, mainly located outside of chromocenters in gene-rich euchromatic regions (Gaudin et al., 2001; Libault et al., 2005). In contrast to yeast and animal HP1 proteins, LHP1 shows high affinity for H3K27me3 and functions as a suppressor of euchromatic genes (Zhang et al., 2007).

A third hallmark of heterochromatin in plants and animals is DNA methylation. Fission yeast lacks DNA methylation and *Drosophila* shows methylation only in early stages of embryo development (Lyko et al., 2000). In mammals, DNA methylation is restricted to symmetric CG sequence, although cytosines out of the CG context are methylated in mouse embryonic stem cells (Ramsahoye et al., 2000). Plants have by far the most elaborate DNA methylation system: DNA methylation occurs at symmetrical CG, CHG and asymmetrical CHH cytosines, where H is A, C or T (reviewed in Chan et al., 2005).

DNA methylation, histone methylation and deacetylation are interconnected in complex self-reinforcing loops. A decrease in DNA methylation for example causes reduced methylation of H3K9 and chromocenter decondensation in *Arabidopsis* (Jackson et al., 2002; Soppe et al., 2002). In addition, CHG and CHH methylation in *Arabidopsis* depend on both the histone methyltransferase SUVH4 and the DNA methyltransferase CMT3 (Bartee et al., 2001; Malagnac et al., 2002). Thus, DNA methylation appears to be upstream as well as downstream of histone modifications. Evidence for a physical connection between these two epigenetic marks orginates

primarily from research in mammals. There, a direct interaction between the H3K9 methyltransferase SUV39H1 and the DNA methyltransferases Dnmt1 and Dnmt3a has been reported, indicating that these modifying enzymes can recruit each other to target loci (Fuks et al., 2003). Moreover, histone methyltransferase activity has been found associated with MeCP2, an methyl CpG-binding protein, providing another link between DNA methylation and histone methylation (Fuks et al., 2003). Similar inter-dependencies have been observed between histone acetylation and DNA methylation. For example in *Arabidopsis*, mutations of the SWI/SNF remodelling factor *Decrease in DNA methylation* (DDM1) (Vongs et al., 1993) induces global DNA hypomethylation (Jeddeloh et al., 1999), dispersion of chromocenters followed by a reduction in H3K9 methylation and an increase of histone acetylation (Soppe et al., 2002). In animal systems it has been shown, that HDACs can either directly interact with DNA methyltransferases (Fuks et al., 2000) or can be recruited to methylated DNA via methyl-CpG binding proteins (Jones et al., 1998; Nan et al., 1998).

3.1.2. RNAi-mediated heterochromatin formation

RNAi-mediated heterochromatin formation was originally identified and is best understood in fission yeast, which is due to the low genetic complexity of RNAi components in this organism. The fission yeast genome encodes only one copy of the RNAi components Argonaute (Ago1), Dicer (Dcr1) and RNA-dependent RNA polymerase (Rdr1) (Wood et al., 2002). RNAi-mediated heterochromatin formation is the major mechanism for maintaining centromere function in fission yeast, while the establishment of heterochromatin at the silent mating type locus and telomeres involves redundant pathways (Lippman and Martienssen, 2004; Martienssen et al., 2005). Defective heterochromatin formation at centromeres causes failures in chromosome segregation during mitotic cell divisions, which is due to loss of cohesin. At wild-type centromeres cohesin is recruited by the HP1-ortholog Swi6, which binds to di-methylated histone H3 lysine 9 via its chromodomain (Volpe et al., 2003).

Initial results from fission yeast demonstrated that mutants deficient for components of the RNAi silencing machinery are impaired in heterochromatic silencing of transgenes that are integrated within centromeric regions (Volpe et al., 2003). A crucial advance, however, in understanding the mechanism of

transcriptional silencing in the establishment and maintenance of heterochromatin formation originated from the purification of the RITS (RNAi induced transcriptional silencing) complex from fission yeast (Verdel et al., 2004). This effector complex consists of the Ago1, which is able to bind siRNAs via its PAZ domain, the chromodomain protein Chp1 and the adaptor protein Tas3, which links Ago1 to Chp1. From extensive studies in several labs, a general model of heterochromatin formation has emerged (Figure 4). In RNAi mutants, centromeric repeats generate forward and reverse transcripts by PolII transcription, indicating that double-stranded RNA (dsRNA) of centromeric repeats provides the basis for the formation of small interfering RNAs (siRNAs). In fission yeast wild-type cells, however, the transcription of forward repeats is repressed by the action of the fission yeast HP1-homolog, Swi6 (Volpe et al., 2003) and dsRNA formation requires the RNA-dependent RNA polymerase Rdr1, which acts on the reverse centromeric repeats. Thus, in wild-type cells forward transcripts are absent and reverse transcripts accumulate only at low levels due to fast processing by the RNAi machinery. Centromeric dsRNA produced by Rdr1 is then processed by the RNAse III enzyme Dcr1 into siRNAs. Double-stranded siRNAs are initially loaded to the Ago1 complex ARC (Argonaute siRNA chaperone). ARC is distinct from the RITS complex (Buker et al., 2007) and contains Arb1 and Arb2, which negatively control the intrinsic RNAse H activity of the Ago1 PIWI domain (Figure 4). This Ago1 slicer activity is necessary for the removal of complementary passenger strand and formation of mature Ago/siRNA complexes containing only single-stranded siRNAs (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). Only upon dissociation of Arb1 and 2, and incorporation of Ago1 in RITS, the slicer activity of Ago1 is stimulated resulting in the formation of a mature siRNA/Ago1 complex. This complex subsequently can serve as a sequence-specific guide for RITS during heterochromatin formation (Buker et al., 2007).

In subsequent steps, targeting of the RITS complex induces repressive histone modifications. The combined action of the RPD3-type HDAC Clr6 and the class II HDAC Clr3 is required for efficient heterochromatin formation at fission yeast centromeres. *Clr6* and *Clr3* mutants, result in hyperacetylation of centromeres and defective chromosome segregation (Ekwall, 2007; Grewal and Jia, 2007). Furthermore, mutants show an accumulation of centromeric transcripts to similar levels as in RNAi mutants (Hansen et al., 2005), indicating that heterochromatic

silencing is abolished. Clr6 is recruited to centromeres via the transcriptional corepressor SIN3 homolog Pst1 and *pst1* mutants exhibit similar centromere defects as *clr6* mutants (Silverstein et al., 2003). The deacetylation of centromeric histones by Clr3 and Clr6 is essential for the subsequent action of the histone-methyltransferase Clr4, which results in histone H3 lysine 9 methylation (Nakayama et al., 2001).

The RITS complex is stabilized at fission yeast centromers via the binding of Ago1/siRNAs to nascent centromeric transcripts and via binding of the chromodomain protein Chp1 to methylated histone H3 lysine 9 (H3K9me2) (Martienssen et al., 2005; Verdel and Moazed, 2005). Furthermore the RITS complex interacts physically with the Rdr1 complex RDRC in a siRNA-dependent fashion (Motamedi et al., 2004) making it likely that one important RITS function is the recruitment of the RDRC complex to nascent centromeric transcripts. Besides Rdr1, the RDRC complex consists of the putative RNA helicase Hrr1 and the putative polyA polymerase Cid12, suggesting that RDRC interacts directly with nascent transcripts (Motamedi et al., 2004). Thus, in this pathway RDRC and RITS complexes are connected in a reinforcing loop resulting in the amplification of siRNA formation at PolII-transcribed chromatin that is characterized by histone H3 lysine 9 methylation.

While a potential function of the RITS complex in the recruitment of SIN3/HDAC complexes to heterochromatic centromeric repeats is still obscure, RITS play a role in recruiting the Clr4 histone-methyltransferase complex. Clr4 is a component of a multisubunit complex, ClrC, which additionally contains Cul4, Rik1, Raf1 and Raf2 proteins (Hong et al., 2005; Horn et al., 2005; Jia et al., 2005). These factors are components of an E3 ubiquitin ligase, whose activity is essential for heterochromatin assembly in fission yeast, indicating that heterochromatin formation requires the ubiquitination of a yet unidentified substrate (Horn et al., 2005; Jia et al., 2005). The ClrC component Rik1, a WD repeat protein with similarity to nucleic acid binding proteins, interacts directly with the RITS complex and might additionally associate with repeat transcripts, thus promoting ClrC localization to nucleate heterochromatin (Zhang et al., 2008). Recently, it has been shown that association of Ago1 with ClrC depends on the LIM domain protein (Bayne et al., 2010), which therefore acts as an important bridging factor of RNAi components and histone modifiers during heterochromatin formation in fission yeast (Bayne et al., 2010).

One important feature of heterochromatin is the ability to spread from nucleation zones. This spreading might lead to the silencing of genes adjacent to heterochromatin, a phenomenon that is known as position effect variegation (PEV) (Lippman and Martienssen, 2004). An elegant model that explains spreading originates from the domain architecture of the histone-methyltransferase Clr4 (Zhang et al., 2008). Clr4 induces histone H3 lysine 9 methylation via the SET domain but is also able to bind to this modifaction by its chromodomain. Thus, after the initial methylation of H3K9, Clr4 bound to H3K9me can trigger the methylation of adjacent histones which creates additional binding sites for Clr4 itself as well as for HP1-orthologs like Swi6 and Chp2. Oligomerization of Swi6 via its chromoshadow domain and additional recruitment of other histone modifiers by the HP1-orthologs might finally promote higher-order chromatin compaction and long-range spreading (Zhang et al., 2008).

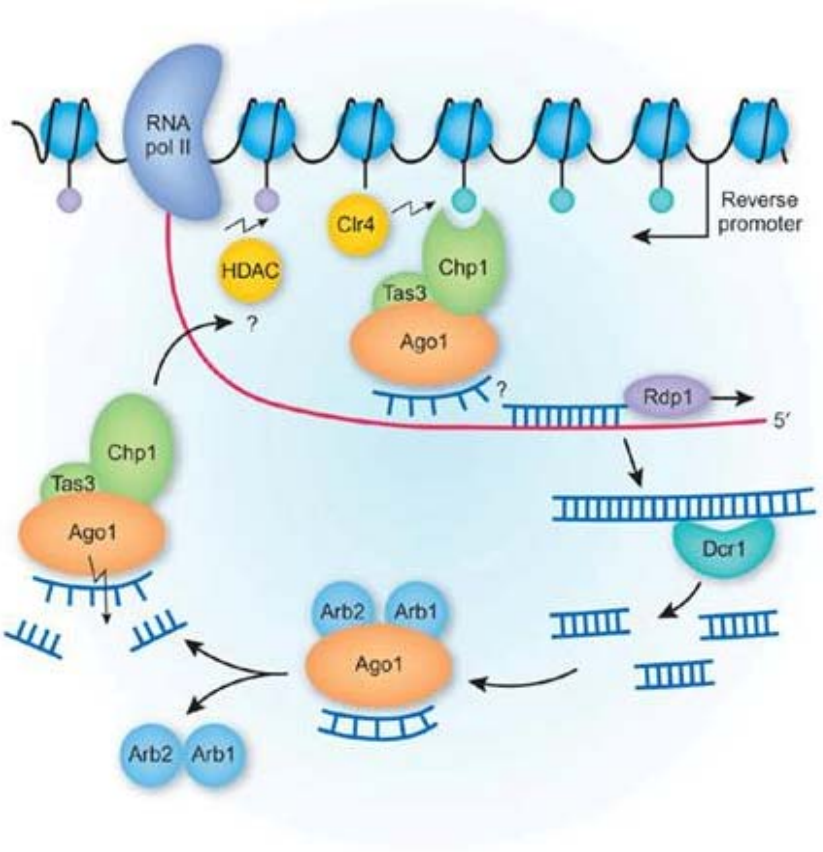


Figure 4. Model of RNAi mediated heterochromatin formation in yeast. Heterochromatic repeats generate dsRNA by the action of the RNA-dependent RNA polymerase Rdr1 that is cleaved by Dicer1 (Dcr1) to produce small interfering RNAs (siRNAs). These small RNAs are loaded onto Ago1, first in the ARC complex and then in the RNA induced transcriptional silencing complex (RITS), where the siRNA duplex is cleaved and the passenger strand is removed. The RITS complex is then recruited in a sequence-specific fashion to nascent repeat transcripts generated by PolII and recruits the RDCR complex resulting in an amplified production of siRNAs. Subsequently, binding of RITS to chromatin induces histone deacetylation by HDACs followed by Clr4 mediated histone methylation by the recruitment of the ClrC complex. Histone methylation creates binding sites for the RITS component further stabilizing the association of RITS with chromatin. From Ekwall, 2007.

3.2. RNA directed DNA methylation

RNA-directed DNA methylation (RdDM) in plants was the first identified mechanism of RNA-mediated epigenetic modifications. RdDM represents another nuclear RNAi pathway mechanistically similar to RNAi-mediated heterochromatin

formation in fission yeast but involves plant-specific components as well (see below). Unlike fission yeast, however, the plant model *Arabidopsis* exhibits an eloborate RNAi machinery, which made a genetic analysis of the pathway more difficult. The *Arabidopsis* genome encodes four Dicer like proteins, DCL1-4 (Gasciolli et al., 2005), ten Argonautes, Ago1-10, (Vaucheret, 2008) and six RNA-dependent RNA polymerases, RdRP1-6 (Zong et al., 2009).

RdDM majorily controls silencing of transposable elements and intergenic repeats in *Arabidopsis,* but also has a role in silencing of centromeric repeats (Lippman and Martienssen, 2004; Matzke and Birchler, 2005). However, in *Arabidopsis,* unlike in fission yeast, centromeric heterochromatin formation is additionally controlled by alternative epigenetic pathways, providing an explanation why RNAi mutants do not exhibit chromocenter decondensation and loss of centromere function (May et al., 2005; Pontes et al., 2009). The first evidence for the involvement of dsRNA in transcriptional gene silencing came from a study of plants infected with viroids, which are pathogens with a highly structured RNA genome (Wassenegger et al., 1994). In that study, viroid cDNA integrated into the host genome as a transgene became methylated and transcriptionally inactivated only in the presence of viroid replication, which leads to the formation of double-stranded viroid RNA. Since then, several forward and reverse genetic screens were designed to identify components of the RdDM silencing pathway. These screens were based on the fact that transgenes and endogenous genes in plant genomes can inactivate homologous genes in *trans* and/or be silenced in *cis* if they are of repetitive nature or harbor inverted repeats (Furner et al., 1998; Murfett et al., 2001).

RdDM is triggered by the presence of double stranded RNA (dsRNA) (Figure 5). This dsRNA can be produced by transcription of inverted DNA repeats, from bidirectional transcription as well as by RNA-dependent RNA polymerase 2 (RDR2) activity on ssRNA templates (Jia et al., 2009; Lu et al., 2006; Xie et al., 2004). Templates for RDR2 activity are provided by the plant specific RNA polymerase PolIV, probably by transcribing methylated DNA (Chan et al., 2006; Daxinger et al., 2009; Herr et al., 2005; Onodera et al., 2005). Subsequently, dsRNA is processed by one of the four *Arabidopsis* Dicer like proteins, DCL3, to 24nt long small RNAs (Xie et al., 2004) and loaded onto Argonaute proteins. AGO4 and AGO6 have partially redundant functions in RdDM (Zheng et al., 2007; Zilberman et al., 2003; Zilberman

et al., 2004). The production of siRNAs and loading into effector complexes takes place in the nucleolus (Li et al., 2006; Pontes et al., 2006). The AGO4/6-siRNA complex is subsequently recruited to target loci in a sequence-dependent manner via interaction with nascent transcripts that are produced by the plant-specific RNA polymerase PolV (Wierzbicki et al., 2008; Wierzbicki et al., 2009). Efficient PolV transcript formation depends on the SWI/SNF remodelling factor, DRD1, the SMC hinge protein, DMS3, and a single-stranded methyl DNA binding protein, RDM1, but is independent of siRNA biogenesis (Kanno et al., 2008; Kanno et al., 2004; Law et al., 2010). Thus, whereas in fission yeast transcripts for siRNA biogenesis and for the assembly of the RITS complex to chromation are both provided by PolII, these functions have separated in plants and are carried out by the specialized polymerases PolIV and PolV, respectively. However, recent evidence suggests that PolII transcription is also necessary to coordinate PolIV and PolV functions (Zheng et al., 2009). While it is thought that AGO4/6 recruitment to chromatin is primarily based on RNA-RNA recognition, AGO4 additionally interacts weakly or transiently with the C-terminal domain of the largest PolV subunit (El-Shami et al., 2007; Li et al., 2006). This interaction is stabilised by the protein KTF1, which binds to PolV generated transcripts as well as Ago4 (He et al., 2009).

The effector complex bound to PolV transcripts subsequently recruits downstream RdDM proteins, such as the *de novo* DNA methyltransferase DRM2 and histone modifying enzymes, such as HDACs and histone methyltransferases in a yet unknown way. DRM2, and at some loci also the chromo-methyltransferase CMT3, direct *de novo* cytosine methylation in all sequence contexts (CG, CHG and CHH) (Cao and Jacobsen, 2002.a; Cao and Jacobsen, 2002.b). Additionally, the CG-specific maintenance DNA methyltransferase MET1 has been reported to have a minor contribution to *de novo* methylation during RdDM (Aufsatz et al., 2004). Once established, the DNA methylation pattern is maintained over cell divisions by different mechanisms depending on the sequence context (reviewed in Chan et al., 2005), CG methylation, for example, is maintained by MET1 and the SWI/SNF remodelling factor DDM1. Loss of DDM1 function additionally causes loss of histone H3 lysine 9 methylation. CHG methylation, on the other hand, is maintained by the plant specific chromomethylase (Espada et al., 2007) and the histone methyltransferase SUVH4, thus highlighting another feedback loop involving DNA and histone methylation.

Asymmetric CHH methylation, in contrast, must be re-established after each DNA replication cycle, because there is no complementary pattern on the daughter strand to serve as a guide for remethylation. Therefore, “maintenance” of CHH methylation depends on DRM2 and the continuous presence of siRNAs.

RdDM target loci are characterized by cytosine methylation and in some, but not all cases, histone H3 lysine 9 methylation (Huettel et al., 2006; Xie et al., 2004). Another feature of RdDM-based silencing is histone deacetylation that is established by the RPD3-type HDAC HDA6. Among the four RPD3-type enzymes present in *Arabidopsis,* HDA6 appears to have developed a specialized function for small RNA-based silencing processes. HDA6 is necessary for transgene RdDM-silencing by hairpin constructs (Aufsatz et al. 2002b). Furthermore, HDA6 controls silencing of rDNA repeats during rRNA gene dosage control in *Arabidopsis*, a process that involves the RdDM machinery (Earley et al., 2010; Probst et al., 2004), and is required for megabase-silencing of rDNA in nucleolar dominance that occurs in genetic hybrids (Earley et al., 2006; Pontes et al., 2007). However, the timing and mechanism of HDA6 recruitment during RdDM is still uncertain. Present models of HDA6 recruitment are based on the discovery that loss of HDA6 function results in a reduction of specifically CG methylation (Aufsatz et al., 2002b; Murfett et al., 2001; Probst et al., 2004). This led to the conclusion that HDA6 controls maintenance methylation and is recruited together with or dependent on MET1 and DDM1 (Lippman et al., 2003; Matzke and Birchler, 2005). Complexes of HDA6 that act in RdDM, however, have not been reported so far.

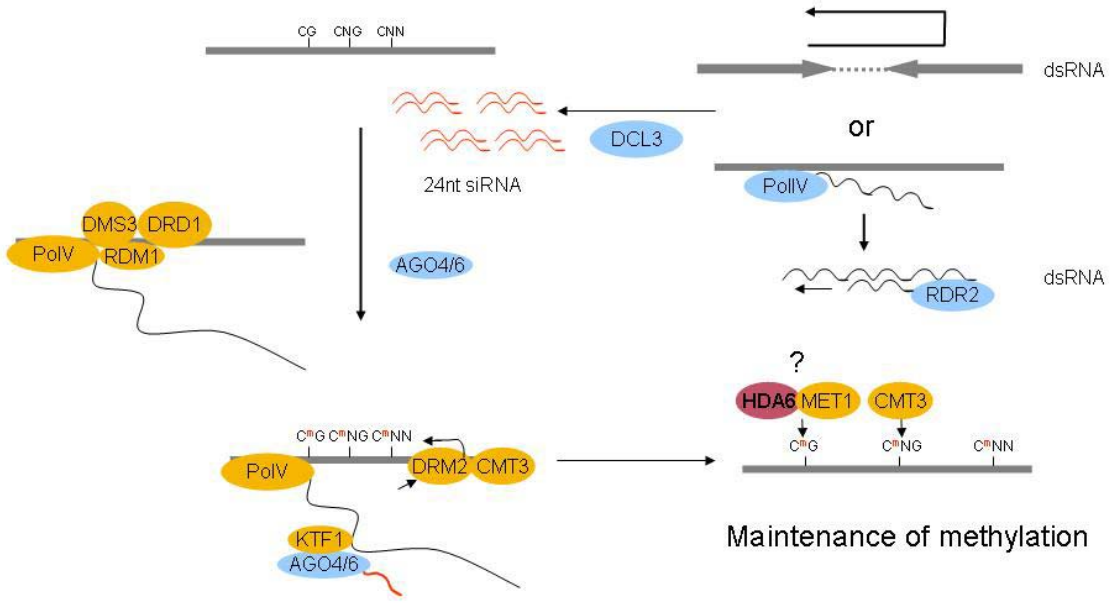


Figure 5. Model of RNA directed DNA methylation in Arabidopsis. In the nucleus, dsRNA can be produced by RNA-dependent RNA polymerase 2 (RDR2) acting on the ssRNA template produced by PolIV or by transcription of inverted repeats by PolII. The dsRNA is then further processed in the nucleolus by Dicer like 3 (DCL3) into 24 nt long small RNAs which are loaded onto the Argonaute proteins AGO4 and/or AGO6. Indicated in blue are all of the components that are required for siRNA biogenesis or stability. The SWI/SNF remodelling factor DRD1, the SMC hinge protein DMS3 and the methyl- ssDNA binding protein RDM1 facilitate noncoding PolV transcription at target loci independently of siRNAs. The programmed AGO4/6 RITS complex is targeted by siRNAs to nascent PolV transcripts and is stabilized by interactions with the largest PolV subunit and the RNA-binding protein KTF1. Assembly of RITS at chromatin results in the recuitment of the de novo methyltransferase DRM2 (and sometimes CMT3) as well as histone modifying factors by yet poorly defined mechanisms. Once established, DNA methylation is maintained by MET1 in the CG context and by CMT3 in the CNG context. CNN can only be maintained by DRM2 in the continuous presence of siRNA. The recruitment mechnisms of HDA6 are still uncertain, though HDA6 is proposed to have a role together with or dependent of MET1 in the maintenance of CG methylation.

4. Aims of this work

The *hda6* alleles used in this work were isolated from a forward genetic screen designed to identify RdDM components in *Arabidopsis* (Figure 6) (Aufsatz et al., 2002a; Aufsatz et al., 2002b). This screen was based on a two-component transgene system that was originally established in tobacco (Mette et al., 2000). One transgene represents the target (T) for silencing by RdDM and consists of a kanamycin-resistance (KanR) gene under the control of the NOS (nopaline synthase) promoter (NOSpro). A homozygous line stably expressing the target transgene (ST- single transformed plants) was transformed with a Silencer (S) transgene that harbors a transcribed inverted repeat of the NOSpro under control of the strong, constitutive 35S promoter. Plants harbouring both transgenes are named double transformed (DT) plants. The S transgene produces dsRNA that is processed into 21-24 nt long siRNAs by the action of Dicers, including DCL3. The T transgene is transcriptionally silenced in the presence of the S locus, which results in kanamycin resistance. Transcriptional silencing is accompanied by cytosine methylation of the NOSpro in all sequence contexts and by histone deacetylation as the major histone mark.

DT plants were subjected to T-DNA or EMS mutagenesis and the second generation of plants was screened for reactivation of the KanR reporter gene, allowing for the identification of recessive mutants that are impaired in RdDM. Despite the screen being saturated, as judged by the number of identified alleles, HDA6 was the only histone modifier that was isolated from that screen (Aufsatz et al., 2002b). From T-DNA mutagenesis, the *rts1-1* (RNA-mediated transcriptional silencing) allele was recovered, which represents an *hda6* null allele with a premature stop codon just downstream of the start codon. EMS mutagenesis additionally resulted in the isolation of several point mutations (Aufsatz et al., 2007) from which three were used in this work. All of the point mutations reside within the conserved HDAC domain and affect amino acids that are highly conserved among RPD3-type HDACs from yeast to mammals (Figure 7).

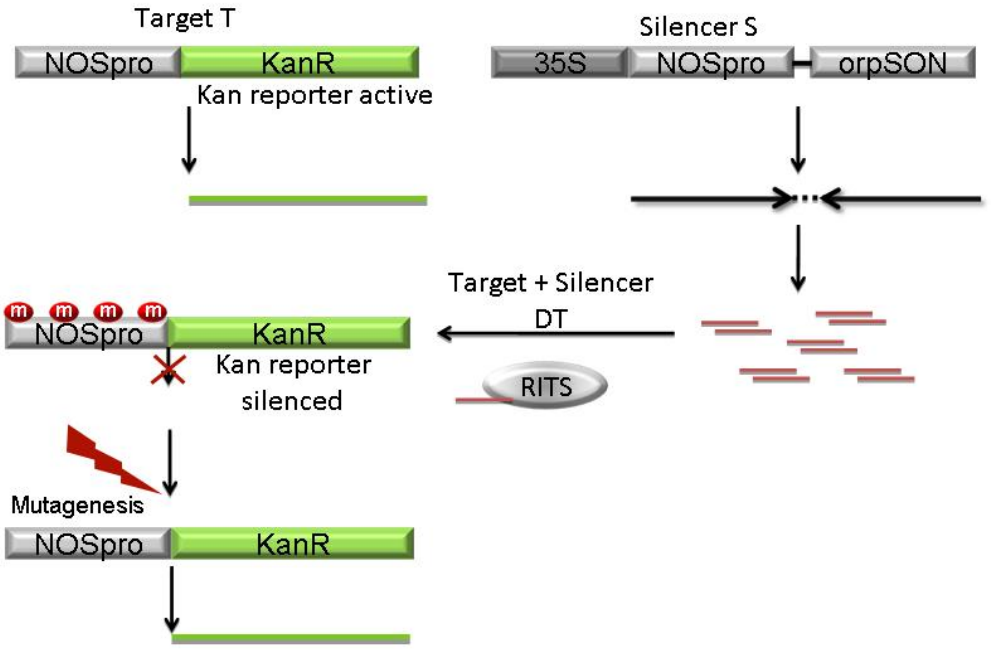


Figure 6. A transgene-based two component RdDM model in Arabidopsis. The target for RdDM silencing (T) consists of the NOSpro driving the expression of a kanamycin-resistance (KanR) gene. The Silencer (S) transgene harbors a NOSpro inverted repeat under control of the 35S promoter. S is a constant source of siRNAs corresponding to the NOSpro. In plants having both transgenes (DT for double transformants), T is transcriptionally silenced by the RdDM pathway. The DT plant population was mutated by T-DNA insertion mutagenesis and by EMS and screened for RdDM pathway mutants by scoring for the reactivation of T (Kan resistance phenotype).

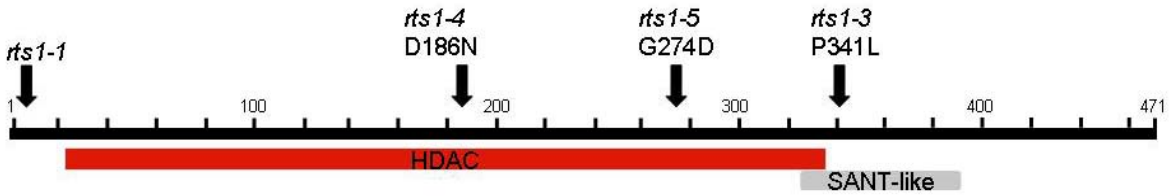


Figure 7. HDA6 mutations and their position with regard to the protein sequence. The *rts1-1* mutation resides at the beginning of the coding region resulting in a premature stop codon. *rts1-3*, *rts1-4* and *rts1-5* are point mutations within or close to the conserved HDAC domain (shown in red) and affect invariable amino acids that are conserved within RPD3-type enzymes from yeast to mammals.

All *rts* mutants show transcriptional reactivation of several known endogenous targets confirming the role of HDA6 in RdDM (Figure 8a) (Stille, unpublished). Levels of histone acetylation were increased as expected from transcriptional reactivation (Figure 8c). Previously it has been reported that transcriptional reactivation of some targets in *hda6* mutants is accompanied by major decreases in DNA methylation (Aufsatz et al., 2002b; Earley et al., 2006). Interestingly, however, reactivation of some targets, including IG5 (intergenic locus 5) and soloLTR (Huettel et al., 2006), occurs despite nearly wild-type levels of DNA methylation (Figure 8b) (Stille, unpublished).

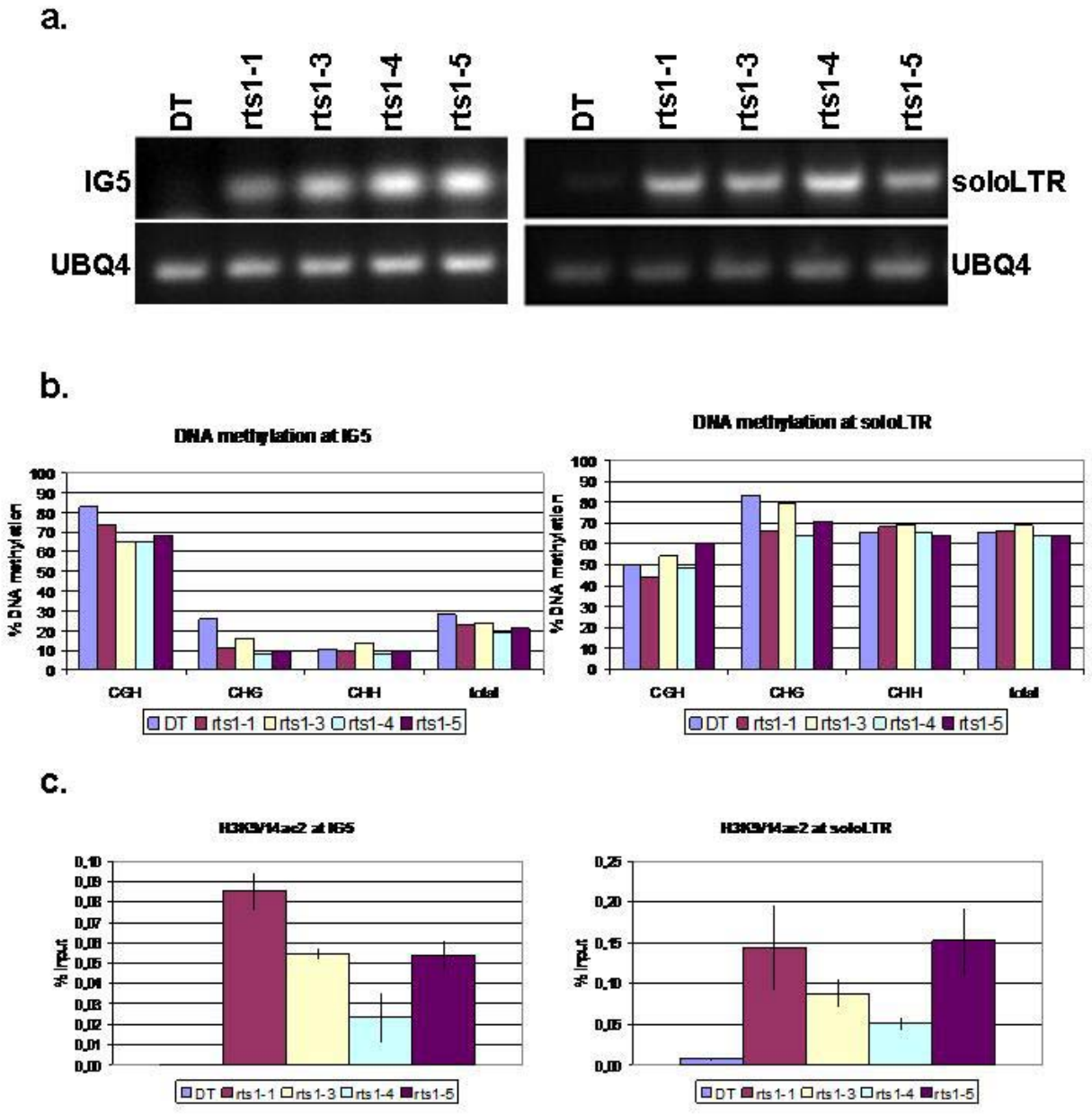


Figure 8. Transcriptional status, DNA methylation and histone acetylation of IG5 and soloLTR in rts mutants.

- **a. RT-PCR data demonstrate transcriptional reactivation of IG5 and soloLTR in all rts mutants. UBQ4 was used as a loading control.**
- **b. Bisulfite sequencing analysis reveals no or only minor reduction of DNA methylation at IG5 and soloLTR in rts mutants.**
- **c. ChIP data show increased levels of histone H3K97K14 acetylation of IG5 and soloLTR in rts mutants.**

Although HDA6 has an evident and important role in RdDM, details on mechanisms of HDA6-dependent silencing are still unclear. It is neither known how HDA6 is recruited to RdDM target loci nor whether enzymatic function or just the full protein is needed, e.g. for structural reasons. Therefore, a first objective of this work was an in depth analysis of *hda6* missense mutants, including the assessment of wild-type and mutant protein localization and enzymatic activity. Fusions of the wild-type and defective HDA6 proteins with fluorescent proteins were expressed transiently in tobacco cells to assess whether the point mutations affect localization of HDA6. Furthermore, epitope-tagged wild-type and defective HDA6 proteins were stably expressed in *Arabidopsis rts1-1* mutants and transgenic plants were analyzed for complementation. The various HDA6 versions were then immunoprecipitated with tag-specific antibodies and subjected to *in vitro* activity measurements.

The second and third objectives of this work were the characterization and further identification of HDA6 interaction partners, respectively, to shed light on recruitment mechanisms of HDA6 in RdDM. Previous yeast two hybrid screens identified two RNA-associated proteins, fibrillarin and a novel RRM domain protein, as well as an RNA helicase (Stoiber, unpublish). This led to the conclusion that HDA6 is part of a protein complex that might be targeted by (small) RNAs (Aufsatz et al., 2007). Analysis of potential co-localization as well as *in planta* interaction studies using bimolecular fluorescent complementation were used to further characterize these potential interactors. Finally immunoprecipitation of epitope-tagged HDA6 and mass spectrometry was used to identify hitherto unknown HDA6 complex partners.

Results

1. Characterization of *hda6* missense mutants

All of the *hda6* missense mutants have a slight late flowering phenotype as was previously reported for the null mutants *axe1-5,* (Wu et al., 2008) and *rts1-1* (Aufsatz et al., 2002b). Otherwise phenotypically normal, the missense mutants show aberrant transcriptional reactivation of silent RdDM targets. The *rts1-4* mutation affects a conserved aspartic acid residue important for cofactor binding within the active site of HDACs, indicating that the encoded protein might be catalytically impaired (Finnin et al., 1999). Alternatively to the loss of enzymatic activity, however, proteins encoded by the missense *rts* alleles might have an altered structure resulting in the inability to interact with complex partners and/or mislocalization within the cell. To analyze the mutants more closely, we examined the localization of the respective defective HDA6 proteins within the cell (see Figure 9) along with their ability to complement the *rts1-1* null mutation (see Figure 11) and their *in vitro* activity (see Figure 12).

1.1. Localization of HDA6 wild-type and mutant proteins by confocal laser scanning and fluorescence microscopy.

HDA6 is a nuclear protein distributed throughout the nucleus with a distinct focus within the nucleolus (Earley et al., 2006). To investigate potential mislocalisation of the HDA6 missense mutant (RTS) proteins, we engineered a C-terminal GFP fusion of HDA6 wild-type and RTS proteins, all under the transcriptional control of the strong, constitutive 35S promoter (Sanders et al., 1987). We transiently expressed those constructs in tobacco (*Nicotiana benthamiana*) leaf cells by *Agrobacterium*-mediated transformation (agro-infiltration) and analyzed the localization of the fusion proteins by confocal laser scanning microscopy (CLSM) and fluorescence microscopy.

First, we tested whether overexpression of HDA6-GFP results in normal subcellular localization. When analyzing whole cells, HDA6-GFP was localized

exclusively in the nucleus as previously reported (Figure 9A). To analyze the sub-nuclear distribution of HDA6-GFP more closely, we isolated tobacco leaf nuclei and observed a localization pattern that closely resembles that of the endogenous HDA6 protein (Earley et al., 2006). Overexpressed HDA6 is distributed in the nucleoplasm and in addition shows strong localization within the nucleolus (Figure 9E). This result indicates that overexpression of HDA6 does not alter its normal localization pattern.

A similar analysis was done for the RTS-GFP fusion proteins. All of the RTS proteins showed the same subcellular (Figure 9B-D) and subnuclear (Figure 9F-H) localization pattern as the wild-type HDA6 protein. We conclude that for all studied missense alleles the mutations do not interfere with the localization of the encoded proteins and that the silencing defects are not due to protein mislocalization.

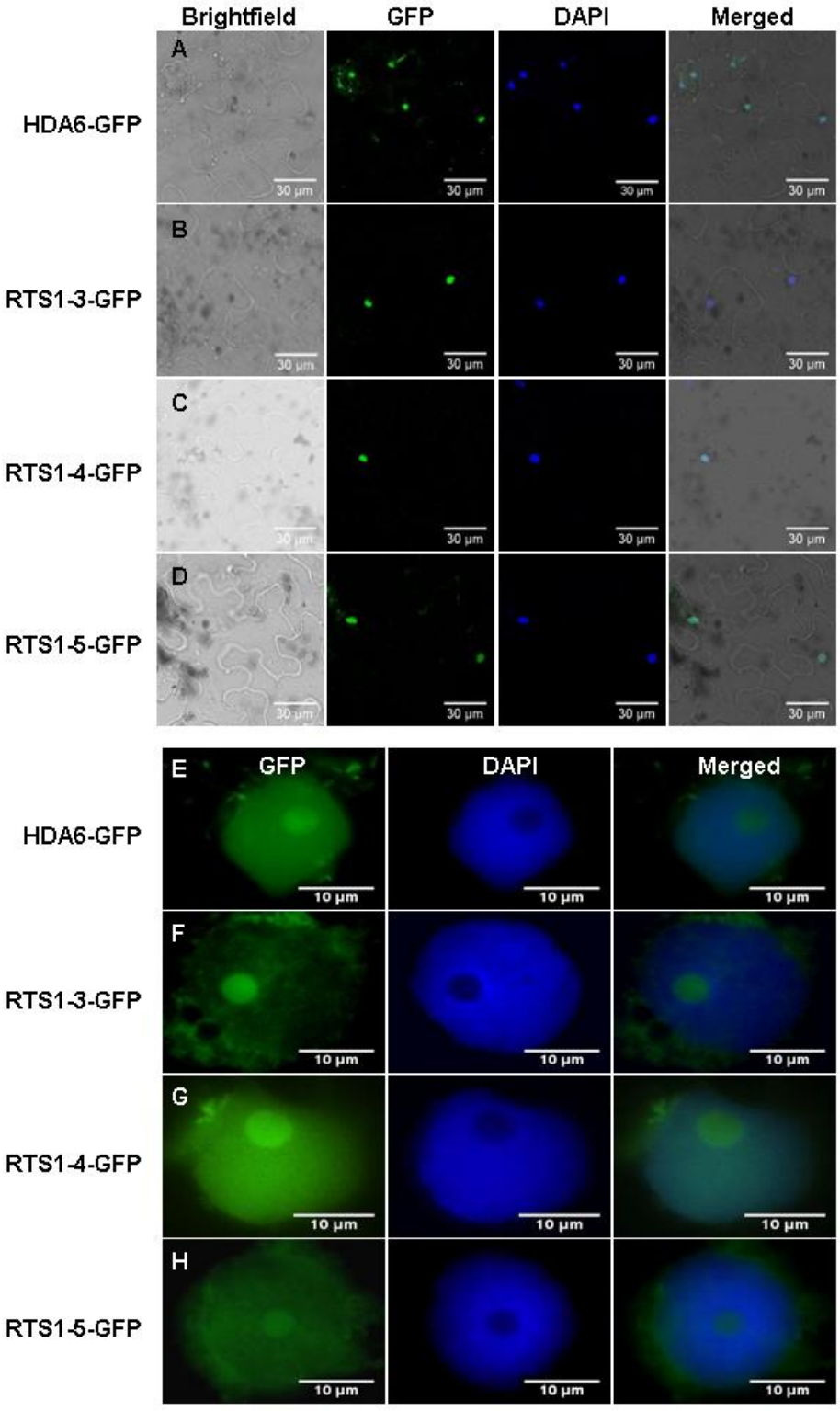


Figure 9. For localization studies, HDA6-GFP and RTS-GFP fusion proteins were transiently expressed in tobacco (*N. benthamiana*) leaves. Localization was either monitored in epidermal cells of tobacco leaves by CLSM (A-D) or in isolated leaves nuclei by fluorescence microscopy (E-H).

(A-D) HDA6-GFP and all of the RTS-GFP fusion proteins localize exclusively in the nucleus.

(E-H) For subnuclear localization, isolated nuclei were analyzed. All of the tested proteins are localized in the nucleoplasm as well as show show a strong signal within the nucleolus.

1.2. Complementation studies of the null *rts1-1* mutant with tagged wild-type and defective HDA6 proteins.

In order to perform biochemical analysis of wild-type and mutant HDA6 proteins, we created transgenic plants that overexpress tagged versions of both in the *rts1-1* background. We chose an overexpression strategy because first the cellular localization of HDA6 does not seem to be altered by overexpression (Figure 9) and second endogenous HDA6 is poorly expressed (Figure 10b), which would make biochemical analysis more difficult. The wild-type HDA6 (tag-HDA6) and all three RTS proteins (tag-RTS) were N-terminally tagged with a poly-histidine (6xHIS), an XPRESS and an hemagglutinin (HA) tag and expressed under control of the strong, constitutive 35S promoter (Figure 10a). After transformation plants were propagated to the third generation to obtain homozygous lines. All transgenic plant lines examined show high levels of transgene-specific HDA6 mRNA in contrast to non-transformed wild-type plants, where (endogenous) HDA6 RNA is undetectable (Figure 10b.). The fusion proteins were readily detectable by Western blot with both anti-HA and anti-express antibody (Figure 10c.) even from crude whole cell protein extracts without any enrichment of the nuclear fraction.

To ensure that the three different tags on the N-terminus do not interfere with the function of the protein, we tested whether the tagged HDA6 is able to complement the *rts1-1* null mutation. To assess complementation we made use of the kanamycin (Kan) sensitivity of the *rts1-1* mutant. The *rts1-1* mutation has a double transgene (DT) background, consisting of a kanamycin resistance gene (KanR) as one, and a silencer of KanR as the second transgene. Wildtype plants having both transgenes do not express the KanR gene due to RdDM silencing, whereas *rts1-1* mutants show reactivation (Aufsatz et al., 2002) (see Figure 6 and introduction). For the complementation analysis, lines overexpressing tagged HDA6 constructs as well as *rts1-1* and DT plants as controls were grown on MS medium supplemented with kanamycin. The control plants behaved as expected: DT plants showed kanamycin sensitivity (Figure 11A), while *rts1-1* plants were resistant (see Figure 11B). *rts1-1* mutants expressing tag-HDA6 showed Kan sensitivity and died early in development at cotyledon stage (Figure 11C), whereas plants expressing tag-RTS did not show complementation and exhibited Kan resistance (Figure 11D-F).

Since tag-HDA6 can complement the *rts1-1* mutation, we conclude that the

presence of multiple tags does not interfere with the function of the protein. Overexpression of tag-RTS proteins, however, did not resilence the KanR gene, although the respective proteins are readily detectable on Western blots. This indicates that the observed phenotypes are not due to protein instability.

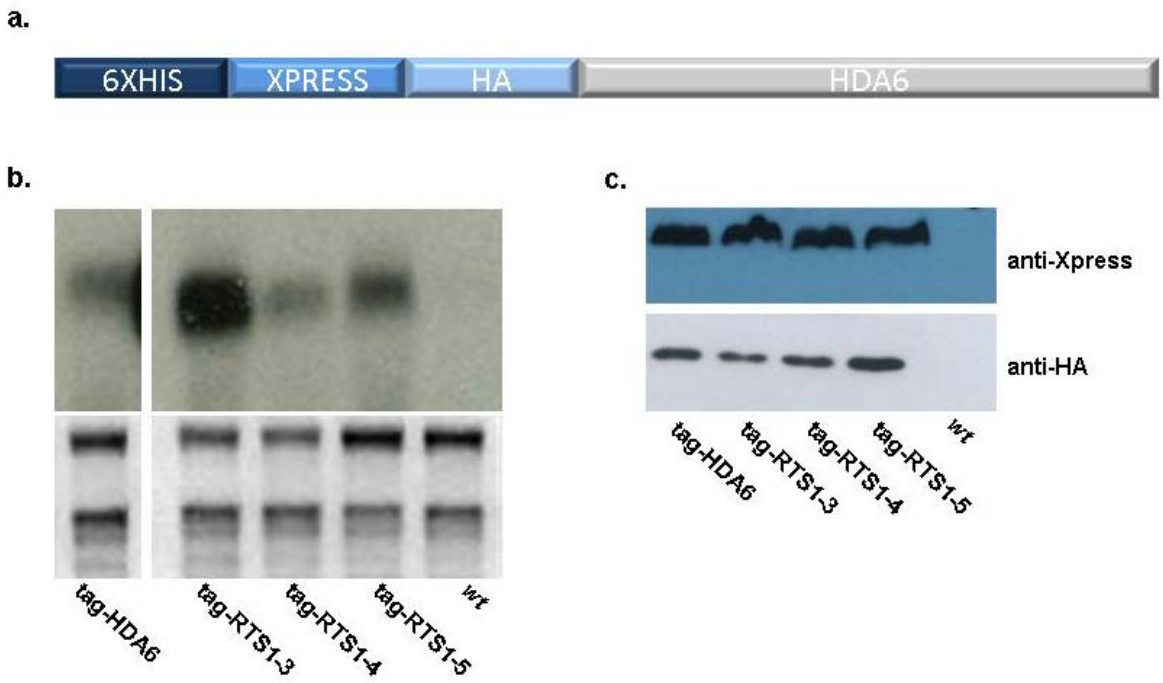


Figure 10. Characterization of Tag-HDA6 and Tag-RTS constructs.

- **a. HDA6 wt and *RTS* alleles were N terminally tagged. The tag consisted of 6xHIS, HA and XPRESS epitopes, respectively. These constructs were expressed in the *rts1-1* mutant background under control of the 35S promoter.**
- **b. Northern blot analysis with a HDA6-specific probe showing that all of the constructs are highly expressed in transgenic plants. *Arabidopsis* Col-0 wild-type (wt) plants were used as a non-transformed control. The absence of a signal in wild-type plants indicates low-level of HDA6 expression.**
- **c. Western blot analysis of crude protein extracts with anti-HA and anti-XPRESS antibodies showing that the tagged proteins can be easily detected without nuclear enrichment.**

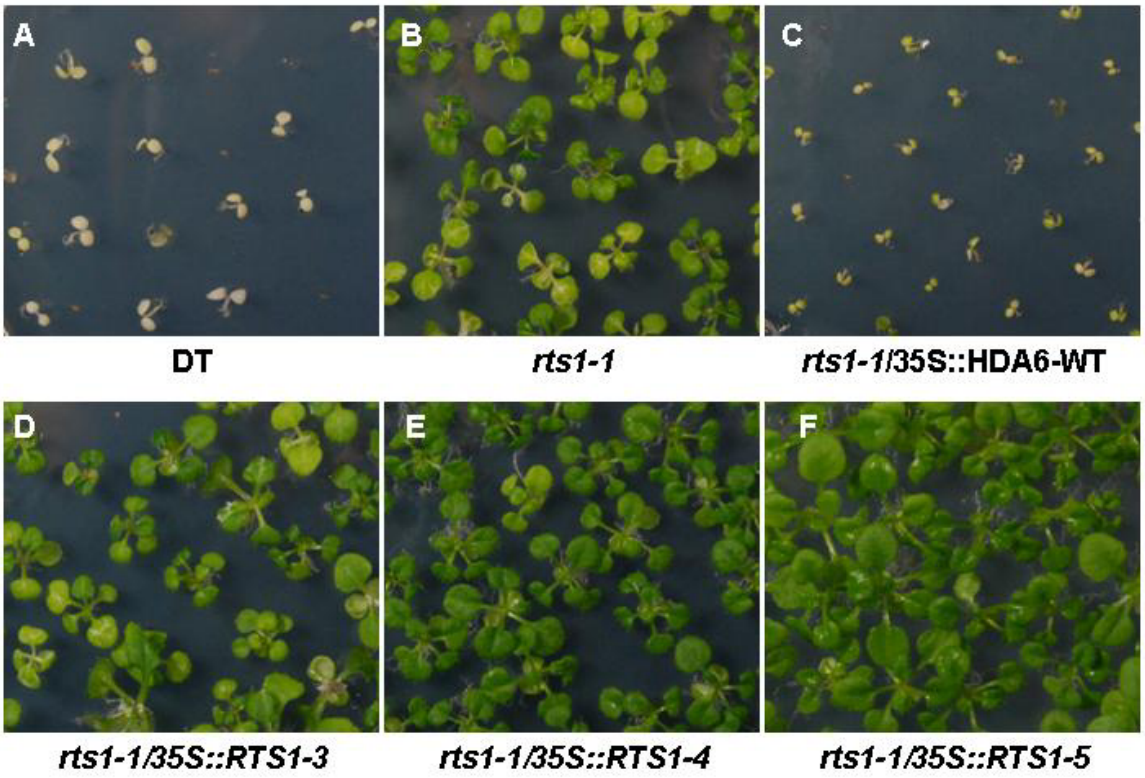


Figure 11. Complementation tests of *rts1-1* null mutants expressing tagged HDA6 wild-type and mutant proteins. Seedlings grown for four weeks on kanamycin medium are displayed. While DT (A) plants are kanamycin sensitive, *rts1-1* (B) plants are resistant due to the absence of functional HDA6 and abolished silencing of the KanR reporter gene. Plants overexpressing tagged wild-type HDA6 (C) can complement the *rts1-1* mutation resulting in kanamycin sensitivity again, while overexpression of mutant HDA6 proteins (RTS) does not result in complementation (D-F).

1.3. HDA6 mutant proteins are enzymatically inactive *in vitro*

HDA6 is a broad-range histone deacetylase that removes acetyl groups from multiple lysine residues of core histones, thereby facilitating gene silencing (Earley et al., 2006). As described above, the *rts1-4* mutation affects the catalytic centre of HDA6, which suggests that RTS1-4 might be an inactive protein. The other mutations (*rts1-3* and *rts1-5)*, however, reside further downstream, outside of the predicted catalytic domain, thus leaving it unclear whether the encoded proteins still have enzymatic activity.

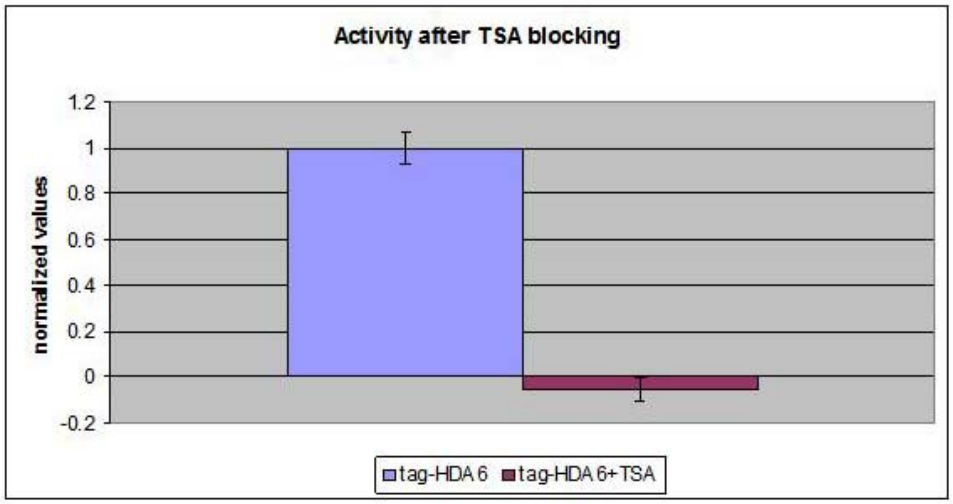
To determine whether the loss of HDAC activity of RTS proteins is responsible for their ineffectiveness in RdDM, we performed an *in vitro* activity assay. This assay

uses a synthetic peptide with an acetylated lysine residue and a covalently attached fluorescence group as a substrate. Upon deacetylation, the fluorescence group can be removed from the peptide by a developer, which results in an increase of fluorescence. The measured fluorescence is directly proportional to the deacetylation activity of tested extracts added to the substrate.

Wild-type and mutant HDA6 proteins were HA-affinity purified from *Arabidopsis* seedlings expressing the tagged proteins and input concentration was determined by Western blot analysis. The measurement of histone deacetylase activity was done in three biological replicates with three technical replicates each. Data were normalized to background levels obtained from immunoprecipitates of non-transformed *rts1-1* plants. To confirm that measured fluorescence corresponds specifically to HDAC activity, we measured the activity of tag-HDA6 sample after blocking with the HDAC inhibitor trichostatin A (TSA) (Yoshida et al., 1995). Upon TSA blocking activity was at background levels, confirming that the measured fluorescence indeed corresponds to HDAC activity of the sample (Figure 12a).

As expected, the RTS1-4 protein with the mutation in the predicted catalytic domain was inactive *in vitro* when compared to tag-HDA6. Surprisingly, also RTS1-3 and RTS1-5 showed no enzymatic activity (Figure 12b). This suggests that the whole HDAC domain is needed for normal HDAC activity. As a summary, we can conclude that enzymatic inactivity is the reason for the dysfunction of RTS proteins in RdDM.

a.



b.

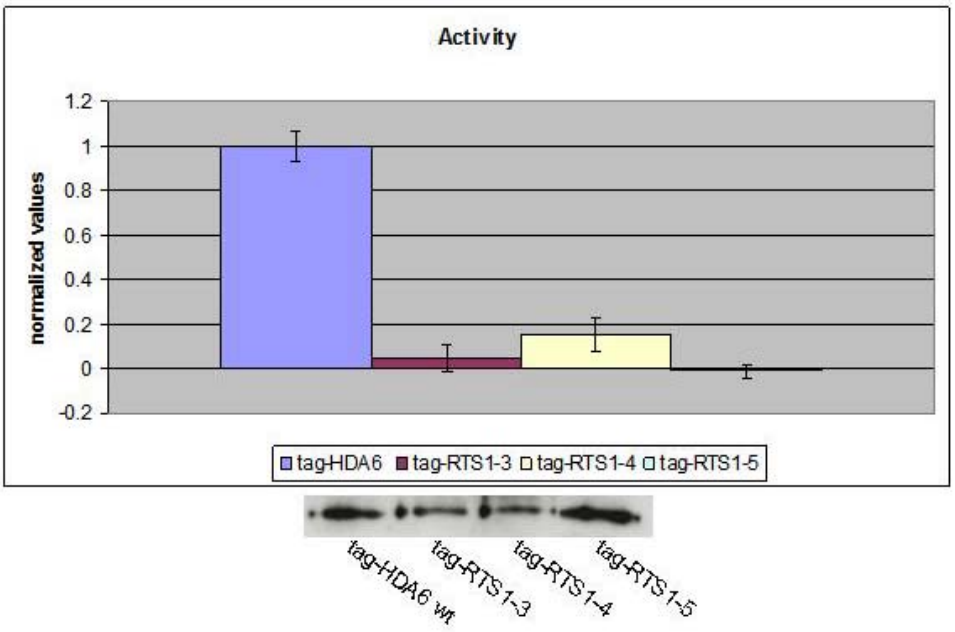


Figure 12. ***In vitro*** **activity assay of HDA6 wild-type (tag-HDA6) and mutant proteins (tag-RTS). Proteins were purified by HA affinity purification and input concentration of proteins was adjusted according to Western blot analysis. The measured fluorescence of tag-HDA6 was arbitrarily set to 1.**

- **a. Tag-HDA6 protein activity was efficiently blocked after treatment with the HDAC inhibitor TSA.**
- **b. Mutant proteins (Tag-RTS) were not active in the** ***in vitro*** **assay. Western blot analysis below the graph shows that similar quantities of tagged proteins proteins were used in the assay.**

1.4. Analysis of global histone acetylation in *hda6* mutants

Several HDA6 targets have been identified as being transcriptionaly reactivated and hypoacetylated in *hda6* mutants (Aufsatz et al., 2002a; Earley et al., 2006; Lippman et al., 2003; Murfett et al., 2001). Since HDA6 has a broad specificity towards histone residues and RTS alleles are enzymatically inactive (Figure 12b) we asked whether these mutants have global changes in histone acetylation levels.

To answer this question we performed histone extraction from *rts* mutants and wild-type DT plants, followed by western blot analysis with anti-H3K9ac, anti-H3K9/K27ac and anti-H4ac (ac stands for acetylation) antibodies. Although purified mutant proteins where enzymatically inactive *in vitro* (Figure 12b) and had an affect on specific targets (Figure 8a), we observed no obvious changes in global H3 and H4 acetylation levels (Figure 13). The fact that global acetylation levels were unchanged indicates that HDA6 has high target specificity.

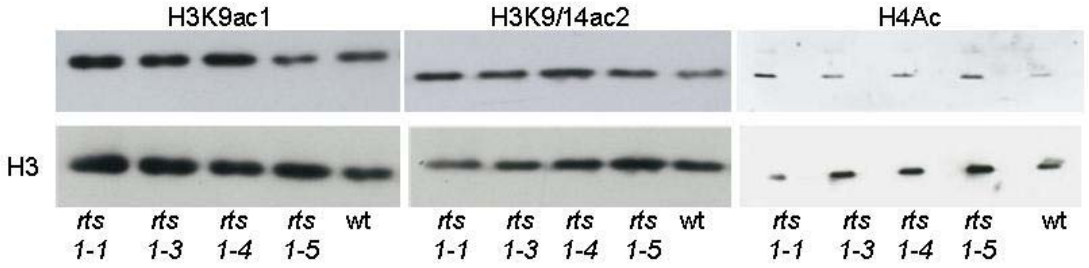


Figure 13. Western blot analysis of changes in global histone acetylation in *rts* mutants. Histone acetylation levels were tested with anti-H3K9ac, anti-H3K9/27ac and anti-H4ac antibodies. The anti-H3 antibody served as a loading control. All *hda6* mutants show no significant change for all tested acetylation marks.

2. Characterization of HDA6 interactors

Although HDA6 was identified in several independent mutant screens as a component of the RdDM pathway (Aufsatz et al., 2002b; Furner et al., 1998; Murfett et al., 2001; Probst et al., 2004), mechanistic details of its function in RdDM with regard to HDA6 protein complexes and their recruitment to RdDM targets are still unclear. One model of HDA6 recruitment is based on the concept that DNA methylation is an essential and sufficient mark for gene silencing and that HDA6 has been shown to be required for DNA methylation maintenance (Lippman et al., 2003; Matzke and Birchler, 2005). Hence, HDA6 could be recruited dependent on or concomitant to DNA methylation, e.g. by interaction with methyl-binding domain proteins (MBD) or DNA methyltransferases (DMT), respectively (Figure 14a). Findings, however, that in the absence of HDA6 activity silencing is lost without changes in DNA methylation (see Figure 8 and Figure 12) are in agreement with DNA methylation and histone deacetylation acting independently during RdDM. The latter hypothesis would require methylation-independent, sequence-specific recruitment of HDA6. In RNA silencing pathways, sequence-specific recruitment could for example be provided by (short) RNAs and association of HDA6 with RNA-binding proteins (RBP) (Figure 14b).

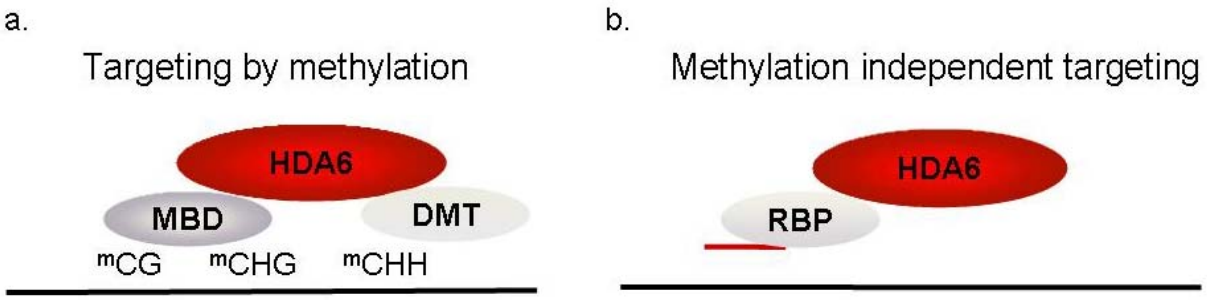


Figure 14 Models of HDA6 recruitment in RdDM

a. HDA6 recruitment involving DNA methylation via association with DNA methyltransferases (DMT) or methyl-binding domain proteins (MBD)

b. HDA6 recruitment independent of DNA methylation, e.g by RNA binding proteins (RBP)

Previously, we tested for interactions of HDA6 with six MBP proteins (MBD4-7, MBD10 and MBD11) in a targeted yeast two hybrid (Y2H) (Schuller, 2007). None of the tested MBD proteins, however, interacted with HDA6. Thus, the identification of HDA6 interacting proteins by unbiased approaches was necessary to shed light on potential HDA6 complexes and discriminate between different recruitment mechanisms.

2.1. HDA6 interactors identified by Y2H assay

In order to identify new HDA6 interaction partners, we previously performed a large Y2H screen against different *Arabidopsis* cDNA libraries. We recovered Fibrillarin1 (FIB1), a DEAD-box helicase (PRH75) and a novel RRM-domain protein (Lorkovic et al.) from this screen (Figure 15) (Stoiber, unpublished). Interestingly, all of these proteins can associate with RNA and thus provide candidates for sequence-specific, RNA-dependent recruitment of HDA6 (see Figure 14).

Fibrillarins are nucleolar proteins involved in the processing of rRNA precursors (Aris and Blobel, 1991; Lischwe et al., 1985; Maxwell and Fournier, 1995). *Arabidopsis* has two genes, *AtFib1* and *AtFib2,* that encode almost identical proteins, presumably with the same function (Barneche et al., 2000; Pih et al., 2000). Supposedly, this is a result of a large ancestral duplication of the *Arabidopsis* genome (Barneche et al., 2000). FIB2 was identified by mass spectrometry analysis to be associated with the PolV subunit NRPE1 (Huang et al., 2009) and might therefore have a role with PolV in RdDM.

The DEAD-box helicase PRH75 is a nuclear protein with very weak ATPase activity and, at least transiently, interacts with RNA in an ATP-independent manner (Lorkovic et al., 1997).

The RRM-domain protein is a novel *Arabidopsis* protein that is comprised of two previously separately annotated ORFs, At2g33435 and At2g33440. The protein has two RRM domains (Marchler-Bauer et al., 2009) and so far no predicted function or localization has been reported. The RRM domain (RNA recognition motif), also

known as RBD (RNA-binding domain) or RNP (ribonucleoprotein domain), is a highly abundant domain in eukaryotes, which usually interacts with single stranded RNA (ssRNA) but also with single stranded DNA (ssDNA), as well as, proteins (Auweter et al., 2006).

Fibrillarin1 (FIB1)	At5g52470
DEAD-box helicase-(PRH75)	At5g62190
RRM-domain protein	At2g33435/ At2g33440

Figure 15. Table of names and accession numbers of HDA6 interaction partners obtained by large yeast two hybrid screen.

2.2. The potential HDA6 interactors localize to the nucleus

Previous studies have already shown that Fibrillarin1 has a nucleolar localization (Pih et al., 2000). PRH75 was also shown to be nuclear although the precise localization within the nucleus is not clear (Lorkovic et al., 1997). Nothing is known about the localization of the RRM protein. To test for a potential co-localization with HDA6 in plant cells, we analyzed the cellular localization of the three potential interactors.

To this aim, C-terminal YFP fusion proteins were transiently expressed in tobacco leaf cells under control of 35S promoter. The localization was analysed first in whole epidermal cells by CLSM (Figure 16A-C) and subsequently in isolated nuclei by fluorescence microscopy (Figure 16D-F).

All three proteins showed an exclusive nuclear localization (Figure 16A-C). Fluorescence microscopy of isolated nuclei demonstrates strong signals for all three potential interactors in the nucleolus (Figure 16D-F). RRM-YFP and PRH75-YFP are additionally located in the nucleoplasm, while FIB1-YFP is exclusively nucleolar as expected from the work of others (Pih et al., 2000). In summary, these results

indicate that HDA6 and its potential interactors have partially overlapping localization patterns and could thus form complexes *in planta*.

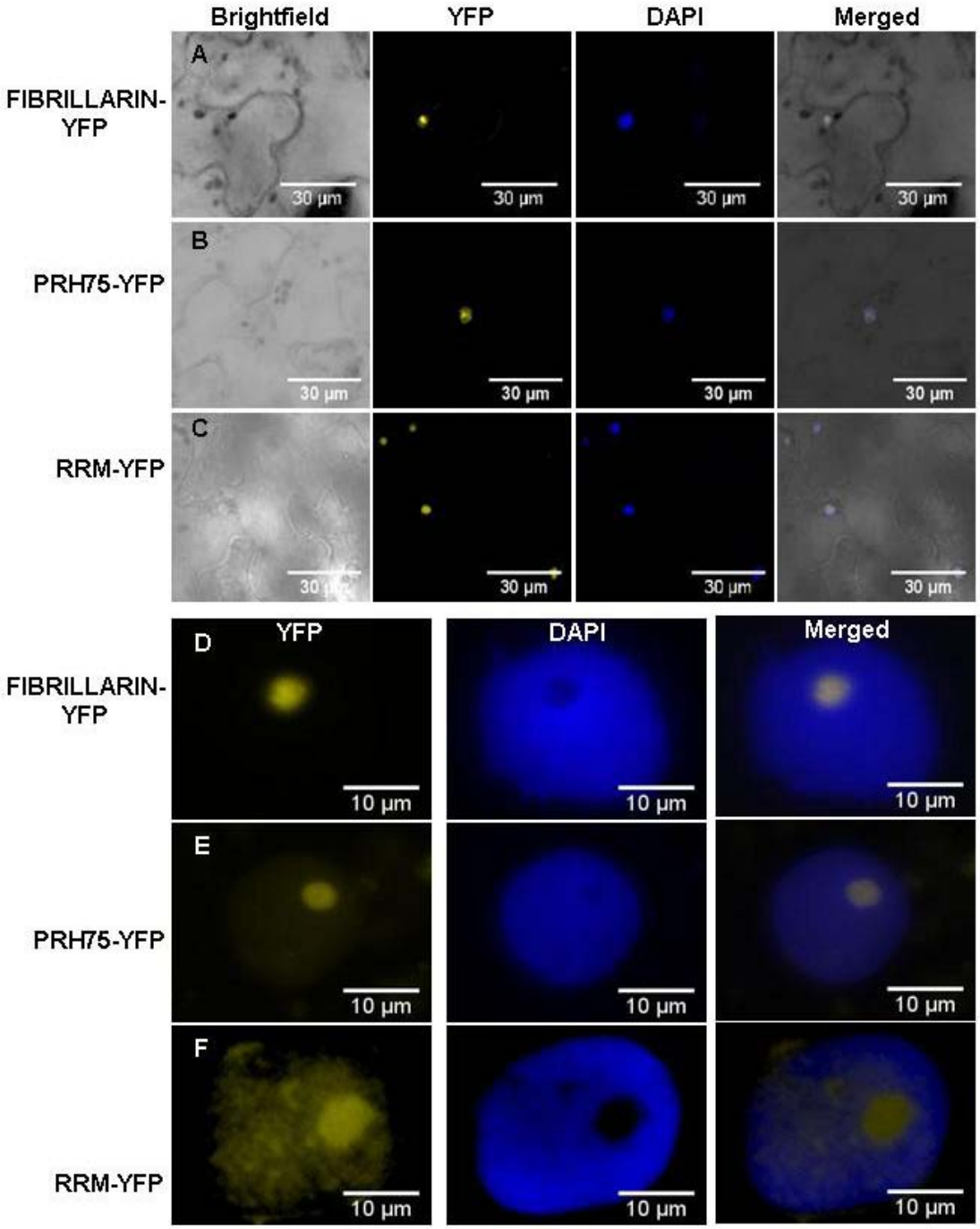


Figure 16. Localization of potential HDA6 interacting partners. YFP fusion proteins were transiently expressed in tobacco (*N. benthamiana*) leaf cells. Localization was analysed in leaf epidermal cells by CLSM (A-C) and in isolated leaf nuclei by fluorescence microscopy (D-F).

(A-C) All of the tested interactors are exclusively localized in the nucleus of epidermal cells.

(D-F) UV florescence microscopy analysis of isolated tobacco leaf nuclei revealed the fine nuclear localization.

2.3. Proof of interaction by Bimolecular Florescence Complementation (BiFC)

We further tested whether these proteins can indeed interact with HDA6 *in planta* by bimolecular fluorescence complementation (BiFC). BiFC is a well established and convenient method for the visualization of protein interactions in living cells. The visualization of interactions is based on the restoration of a fluorescent YFP protein from two YFP fragments fused to associated partner proteins (Kerppola, 2006). We constructed fusion proteins of HDA6 and the interactors with the N-terminal and C-terminal half of YFP, respectively (Walter et al., 2004). These constructs were transiently expressed in tobacco leaf cells and the interaction was monitored in isolated nuclei by fluorescence microscopy.

As a positive control for BiFC, we used fusions of the *Arabidopsis* bZIP63 protein with both YFP fragments. bZIP63 is a transcription factor that has a nuclear localization and forms homodimers (Walter et al., 2004). Co-expression of the two YFP fusion variants of bZIP63 results in homodimerization and reconstitution of YFP fluorescence (Figure 17A). As a test for specificity of the assay we co-expressed each of the studied YFP fusion proteins (HDA6, FIB1, PHR75 and RRM) with bZIP63 fused to the complementary YFP part. Although bZIP63 has overlapping localization with the studied proteins, it does not interact with them, resulting in no or very low reconstructed YFP signal (Figure 17B-E).

Fibrillarin1 and RRM showed a clear interaction with HDA6 (Figure 18A-B). The localization of the signal corresponds to the localization of these proteins as described before (see Figure 16). In contrast, PRH75 showed no interaction with HDA6 (Figure 18.C).

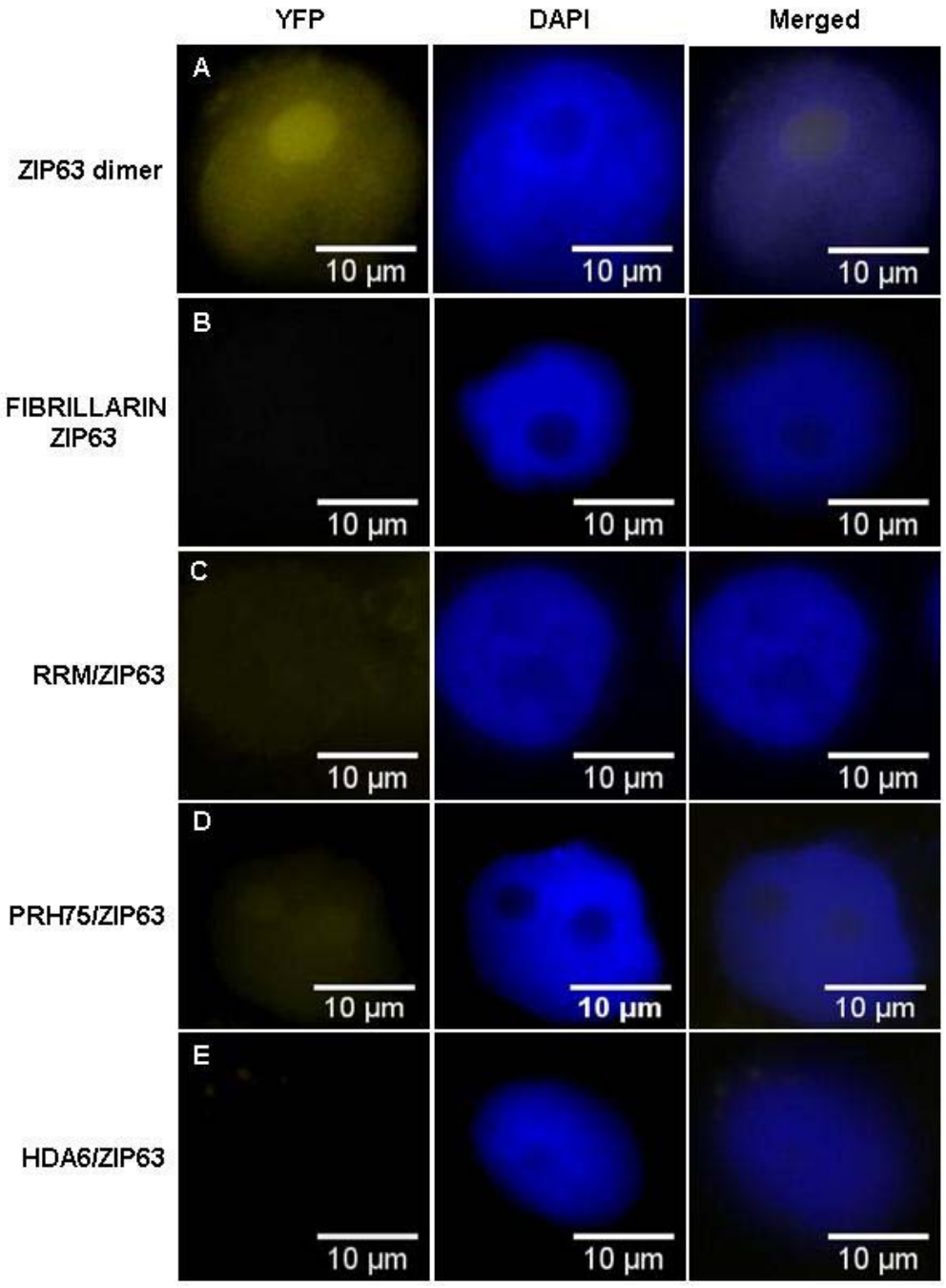


Figure 17. BiFC study of protein interactions. Protein fusions of HDA6 and interactors (FIB1, RRM and PRH75) to N- and C-terminal halves of YFP (YFP^N/YFP^C) were transiently co-expressed in tobacco cells. Interaction analysis was done in isolated leaf nuclei by fluorescence microscopy.

A. Positive control for the BiFC assay: bZIP6-YFP^N and bZIP6-YFP^C was co-expressed, resulting in heterodimerization and YFP signal reconstitution.

B.-E. Negative controls for interaction: HDA6, FIB1, PRH75 or RRM fusions with YFP were co-expressed with bZIP63-YFP fusions and show no or very low level of YFP fluorescence.

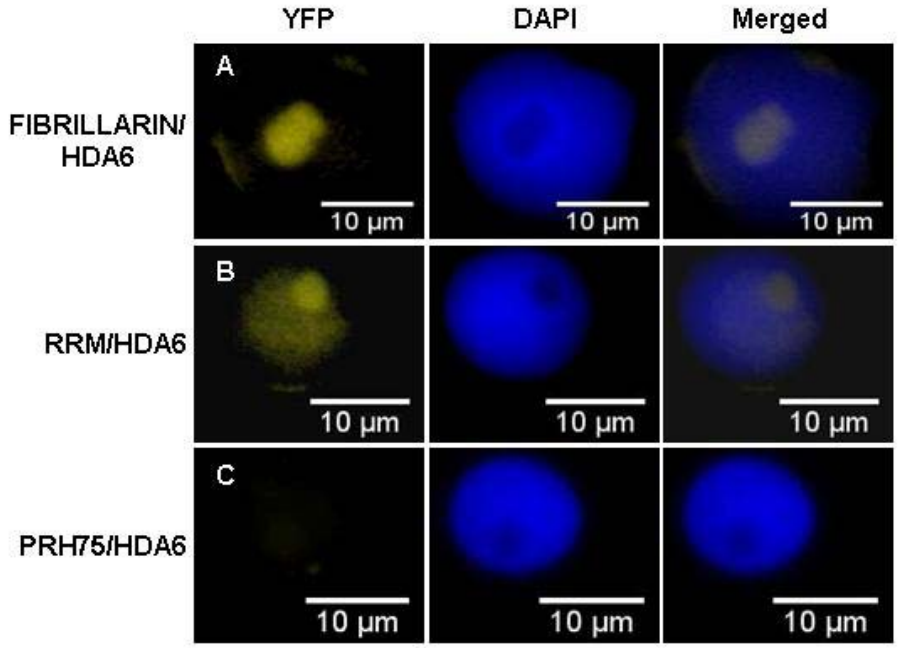


Figure 18. BiFC study of protein interactions. Protein fusions of HDA6 and interactors (FIB1, RRM and PRH75) to N- and C-terminal halves of YFP (YFP^N/YFP^C) were transiently co-expressed in tobacco cells. Interaction analysis was done in isolated leaf nuclei by fluorescence microscopy.

A.-B. Co-expression of FIB1/HDA6 or RRM/HDA6 reconstitutes a strong YFP signal indicative for interaction.

C. Co-expression of PRH75/HDA6 does not result in YFP signal reconstitution above background levels (compare to D).

2.4. Interactor mutants are not impaired in RdDM

We further wanted to test whether *fib1* and *rrm* mutant plants are impaired in RdDM. For that analysis, we used homozygous mutants harbouring a T-DNA insertion in respected genomic sequence. The T-DNA insertions were located in the 5' UTR of *Fib1* (SALK_031583) and in the first intron of the *Rrm* gene (SAIL_48_B11), respectively (Figure 19). As assayed by RT-PCR, the expression of both *Fib1* and *Rrm* was drastically reduced in homozygous mutant plants (Figure 20). We were unable to test the expression of these genes by Northern Blot analysis, possibly due to low expression levels (data not shown).

Homozygous mutant plants were used to check the expression of soloLTR (retroelement) and IG5 (intergenic sequence 5). These are well described RdDM

targets, which are upregulated in several RdDM mutants, including *hda6* (see Figure 8a) (Huettel et al., 2006). RT-PCR analysis, however, showed no reactivation of these targets (Figure 21). This means that we genetically could not prove an involvement of FIB1 and RRM in RdDM.

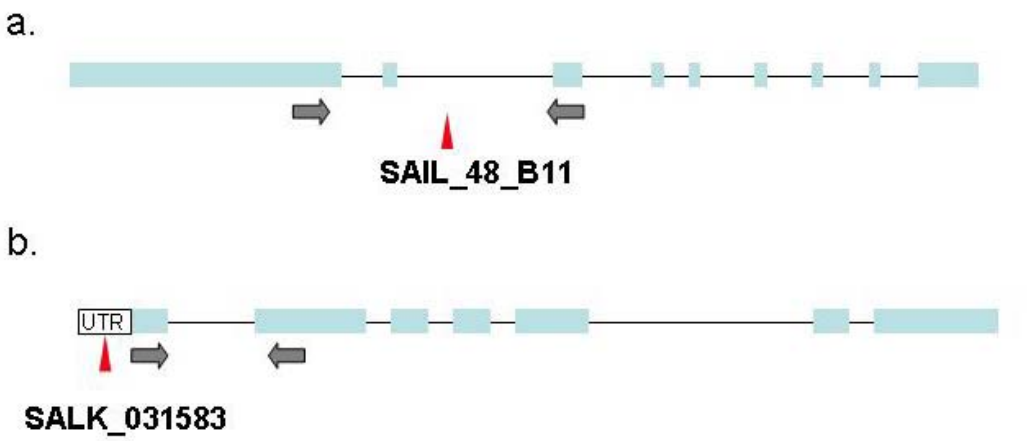


Figure 19. Location of rrm mutation, SAIL_48_B11 (a) and fibrillarin1, SALK_031583 (b).

- **a. The T-DNA insertion in the SAIL_48_B11 line is located in the second intron of the *rrm* gene. Primers used for transcript analysis are positioned in the first and third exon.**
- **b. The T-DNA insertion in the SALK_031583 line resides within the 5'UTR of the *fibrillarin1* gene. Primers are located in the first and third exon.**

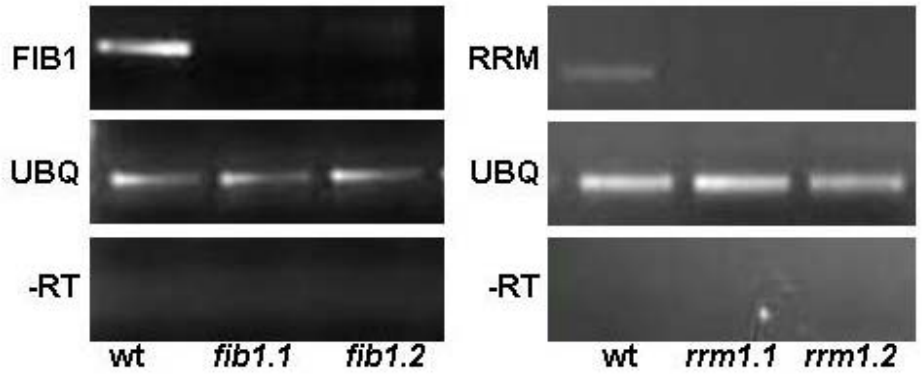


Figure 20. RT-PCR analysis of the T-DNA insertion mutants. Both, expression of FIB1 and RRM is detectable in wild-type plants but not in mutants (upper panels). UBQ4 was used as a loading control (middle panels). The lower panel shows a –RT control for UBQ4.

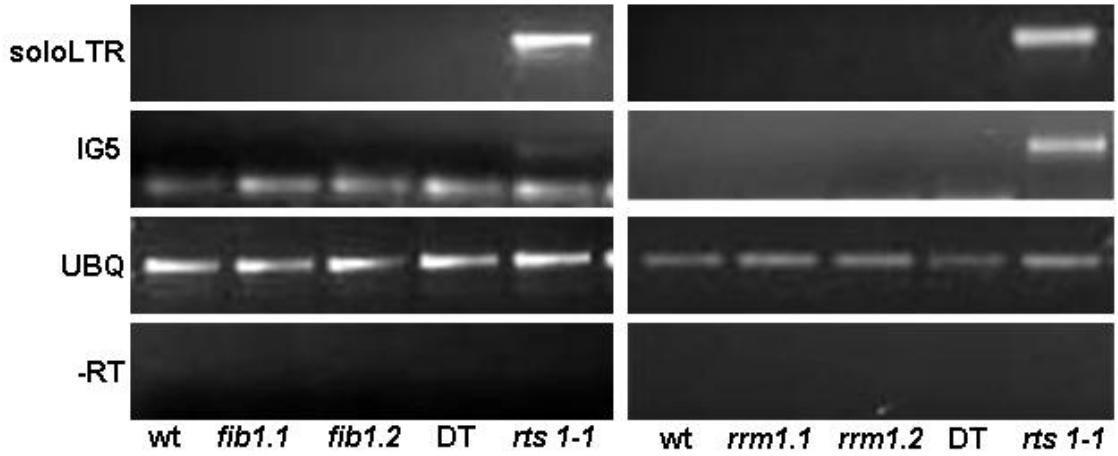


Figure 21. RT-PCR analysis of RdDM targets (soloLTR and IG5) in *fib1* and *rrm* mutants. Both targets (panel 1 and 2) are upregulated in *rts1-1* mutants as expected. No reactivation is observed in *rrm* and *fib1* mutants. UBQ4 was used as a loading control (panel 3). The lower panel shows a –RT control for UBQ4.

3. Identification of a novel HDA6 complex by mass spectrometry

Up to date, numerous HDA6 targets are known. Some of these, a number of transposons, transgenes and rDNA repeats, are silenced by the RdDM pathway (Aufsatz et al., 2002b; Aufsatz et al., 2007; Earley et al., 2006; Earley et al., 2010; Lippman et al., 2003; Probst et al., 2004). Beside these, several genes involved in embryo development, biotic and abiotic stress response and flowering are also silenced by HDA6 (Chen et al., 2010; Tanaka et al., 2008; Wu et al., 2008). The mechanism of silencing, however, is not clear for these protein coding genes. In theory, silencing could depend on RdDM or on some other epigenetic pathways. This opens the question of whether distinct HDA6 complexes are involved in RdDM and other silencing pathways, respectively. We aimed to identify novel HDA6 interactors and HDA6 complexes by mass spectrometry analyses of purified tag-HDA6.

3.1. Identification of novel HDA6 interactors

To identify HDA6 complexes, we purified tag-HDA6 from transgenic *rts1-1* seedlings and performed mass spectrometry (MS) analysis of four different immunoprecipitated (IP) samples. As a negative control, IP was done from non-transformed *rts1-1* seedlings and subjected to MS analysis in parallel. Three samples were prepared from non-crosslinked seedlings with different IP-washing stringency (see Material and Methods), and one sample was from *in vivo* crosslinked seedlings (Figure 22). Crosslinking is supposed to stabilize transient interactions, potentially resulting in the identification of less prominent HDA6 interactors (Vasilescu et al., 2004). We tested three different commonly used crosslinking agents: Formaldehyde (FA), Dimethyl adipimidate (DMA) and Ethylene glycol bis[succinimidylsuccinate] (EGS) (Browning and Ribolini, 1989; Dihazi and Sinz, 2003). All of these crosslinkers are potentially reversible and the main difference between them is the length of the spacer arm: FA has approximately 2Å, DMA 8.6Å and EGS 12Å long spacers, respectively. From these three crosslinking agents, EGS was the most compatible with our IP procedure since this sample had the highest concentration of purified HDA6 (even visible on silver staining) with the least background (Figure 22). MS data generated from the three independent IPs identified eight proteins with high scoring peptides that were present in at least three out of the four preparations with at least two individual peptides (Figure 23). In the second and third samples, washing stringency was reduced compared to the first one, so the identified proteins were covered by more unique peptides. Surprisingly, no additional proteins were identified after EGS crosslinking, and the coverage of identified proteins was about the same in crosslinked and non-crosslinked samples.

Four of the identified proteins, SIN3 like 3 (SNL3), SIN3 like 1 (SNL1) and MSI1, are plant homologues of components of a SIN3 co-repressor complex. All of these proteins together with HDA6 as an RPD3-like histone deacetylase can be considered as the core of the SIN3 complex that is conserved between species (Grzenda et al., 2009).

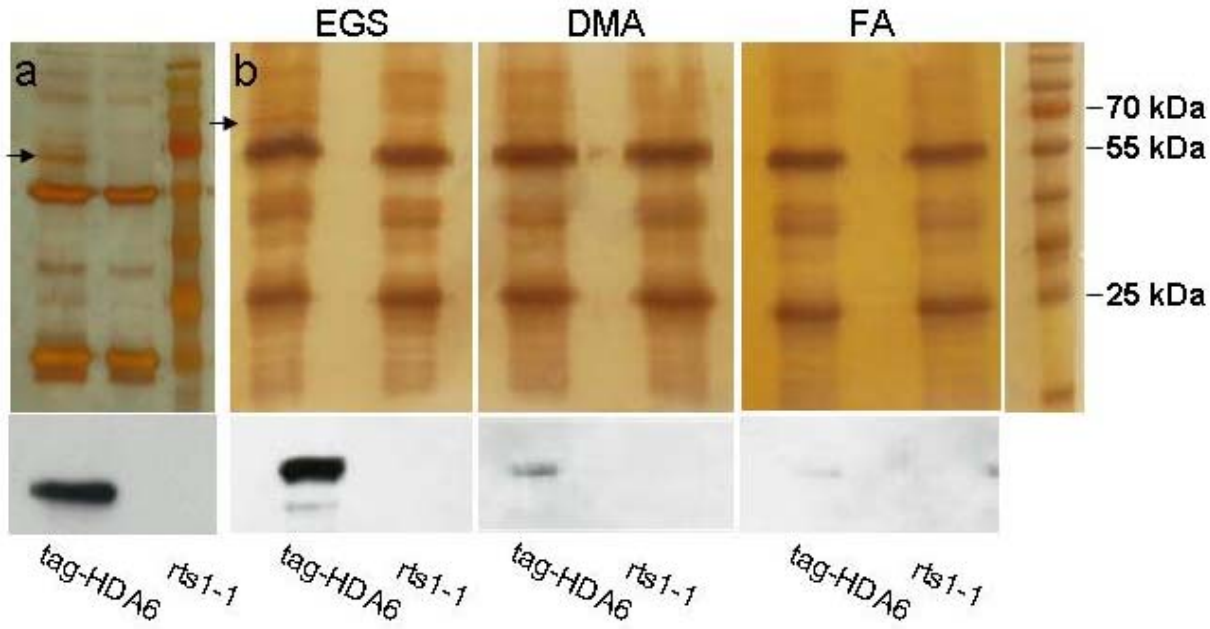


Figure 22. Silver staining of different tag-HDA6 IPs (upper panel) and detection of purified tag-HDA6 by Western blot (lower panel), compared to IPs from non-transformed *rts1-1* mutant plants. The arrow indicates the position of the tagged HDA6 proteins.

- **a. Anti-HA IPs from *rts1-1* plants expressing tag-HDA6 and non-transformed *rts1-1* mutant plants were electrophorized on a 12% polyacrylamide/SDS gel and subsequently silver stained upper panel). The ~ 70 kd protein band marked with an arrow corresponds to tag-HDA6, which was verified by Western blot analysis with an anti-HA antibody (lower panel).**
- **b. *In vivo* crosslinking of tag-HDA6 and *rts1-1* plant tissue was done with the three different crosslinking agents EGS, DMA and FA. Upon crosslinking, anti-HA IPs were separated on a 12% polyacrylamide/SDS gel. In tag-HDA6 samples crosslinked with EGS, a protein band corresponding to tag-HDA6 is visible (marked by an arrow), which is absent in EGS-crosslinked IPs from *rts1-1* samples. The Tag-HDA6 band is hardly visible after DMA and FA crosslinking (upper panel). From the Western blot analysis of these samples it is evident that HDA6 can be purified upon all of the crosslinking procedures, but is present at highest amount after EGS crosslinking (lower panel).**

Identified proteins	Arabidopsis accession	Molecular Weight	Sample preparation	Number of unique peptides	
				sample	control
HDA6	AT5gG63110	53 kDa	PBS Tween I	18	0
			PBS II	23	2
			PBS III	22	0
			EGS	21	0
SNL1 SIN3-like1	AT3G01320.1	156 kDa	PBS Tween I	1	0
			PBS II	4	0
			PBS III	3	0
			EGS	3	0
SNL3 SIN3-like3	AT1G24190	154 kDa	PBS Tween I	1	0
			PBS II	10	0
			PBS III	7	0
			EGS	7	0
MSI1	AT5G58230	48 kDa	PBS Tween I	1	0
			PBS II	4	0
			PBS III	7	0
			EGS	6	0
Putative protein	AT5G08450	104 kDa	PBS Tween I	0	0
			PBS II	16	0
			PBS III	6	0
			EGS	10	0
Unknown protein	AT2G47820	91 kDa	PBS Tween I	2	0
			PBS II	2	0
			PBS III	6	0
			EGS	5	0
Unknown protein	AT2G47820	91 kDa	PBS Tween I	2	0
			PBS II	2	0
			PBS III	6	0
			EGS	5	0
Unknown protein	AT1G09050	102 kDa	PBSII	5	0
			PBS III	3	0
			EGS	4	0
MEE58 (maternal effect embryo arrest 58) HOG1	AT4G13940	53 kDa	PBSII	2	0
			PBS III	2	0
			EGS	3	0
LOS1	AT1G56070	94 kDa	PBS Tween I	4	0
			PBS II	9	2
			PBS III	6	0
			EGS	6	0

Figure 23. Table of proteins identified by mass spectrometry. The numbers of identified unique peptides for each protein and sample preparation are presented. The sample preparation with PBS Tween washing generally shows less identified peptides than the other preparations.

	Arabidopsis gene accession	Frequency	Number of peptides
HDA6 53 kDa	At5g63110	4/4	84
SNL3 SIN3-like3 153 kDa	At1g24190	4/4	25
SNL1 SIN3-like1 156 kDa	At3g01320	4/4	11
MSI1 48 kDa	At5g58230	4/4	18
Putative protein 104 kDa	At5g08450	3/4	32
Unknown protein 91 kDa	At2g47820	4/4	15
Unknown protein 102 kDa	At1g09050	3/4	12
LOS1 94 kDa	At1g56070	4/4	25
MEE58,HOG1 53 kDa	At4g13940	3/4	7

Figure 24. Summary table of mass spectrometry results. Proteins present in a minimum of 3 out of 4 sample preparations and covered by at least two unique peptides are shown. The last column presents the total number of identified peptides from all four preparations.

3.2. Other HDA6-associated proteins identified by mass spectrometry

In addition to components of the plant SIN3 complex we identified the three unknown proteins At5g08450, At2g47820 and At1g09050, the translational elongation factor LOS1 and the adenosyl-L-homocysteine (SAH) hydrolase, HOG1 (Figure 23 and Figure 24).

We performed blast search for domains in the identified unknown proteins, using the Conserved Domain Database (CDD) and clusters of euKaryotic Orthologous

Groups (KOG) database (Marchler-Bauer et al., 2009; Marchler-Bauer and Bryant, 2004). Searches against the CDD database revealed that one of these proteins, At5g08450, has an RXT3 domain (Figure 25). Interestingly, yeast Rxt3 was recently identified by MS as a component of the yeast SIN3 complex (Carrozza et al., 2005). Blast searches of At5g08450 against the KOG database substantiated the initially found homology and identified a much larger domain, KOG4843, that is present as well in the yeast Rxt3 protein and has been reported to be involved in transcriptional regulation (Wood et al., 2002).

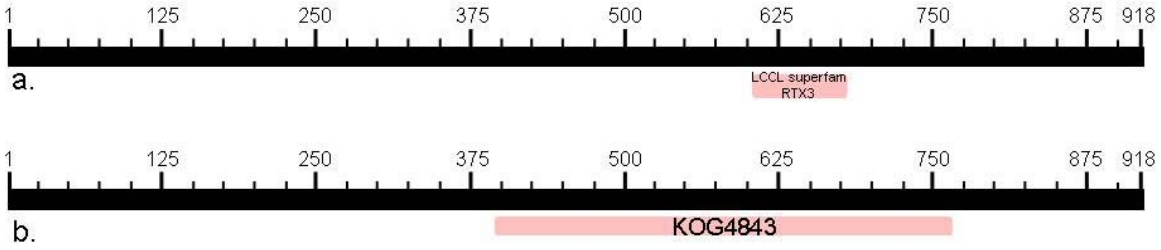


Figure 25. NCBI blast searches for conserved domain present in the putative protein At5g08450.

a. Blast search against the CDD data base identified a LCCL superfamily domain present in RXT3 protein from yeast.

b. Searches against the KOG data base identified an even larger domain also present in the yeast RTX3 protein.

An NCBI blast search against the CDD data base revealed that At2g47820 harbors a domain belonging to the RST superfamily (Figure 26a). This domain is found in many plant proteins (Ahlfors et al., 2004; Belles-Boix et al., 2000) and is required for interaction with multiple plant transcription factors. A search for the At1g09050 protein against the KOG data base, showed a presence of one KOG4329 domain in this protein (Figure 26b). The predicted function for this protein domain is DNA-binding.

LOS1 is a translation elongation factor 2-like and it is involved in cold induced protein synthesis (Guo et al., 2002). It is predicted to localize in the plasma membrane, vacuoles, cytosol and in the nucleus.

HOG1 is an S-adenosyl-L-homocysteine hydrolase (Furner et al., 1998; Rocha et al., 2005), an enzyme involved in recycling of SAM, the substrate for trans-methylation reactions. The predicted localization of HOG1 is within the plasma membrane, cytosol and vacuoles, but not the nucleus.

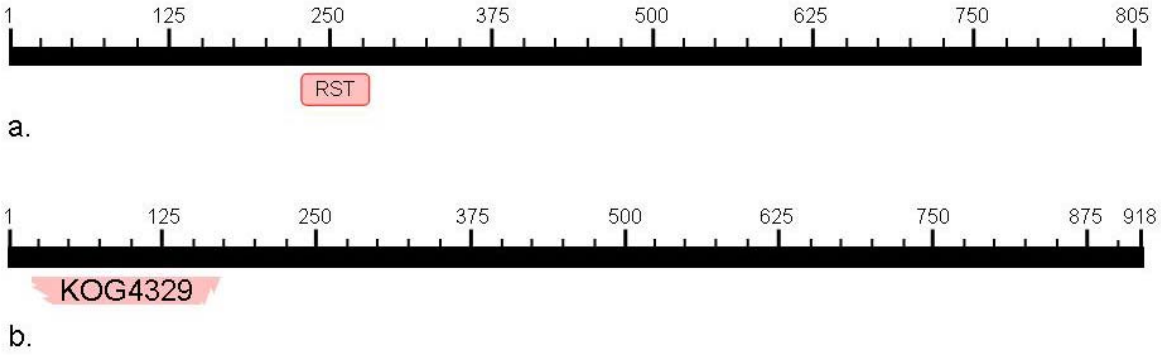


Figure 26. NCBI blast searches for conserved domains present in unknown proteins At2g47820 and At1g09050

- **a. Blast searches against the CDD data base identified an RST superfamily domain present in At2g47820. This domain is present in many plant proteins and is required for interaction with multiple plant transcription factors.**
- **b. Blast searches against the KOG data base identified the KOG4329 domain present in At1g09050. The predicted function for this protein domain is DNA-binding.**

3.3. Role of SNL3 in RdDM

Given that HDA6 is part of the SIN3 co-repressor complex, RdDM silencing might be mediated by this HDAC complex. To test this assumption, we analysed the expression levels of known RdDM targets in *snl3* mutant plants and compared them to *hda6* mutants.

In our analysis, we used homozygous *snl3* mutant plants (SALK_020633). These mutants have a T-DNA insertion within the first exon of the *Snl3* gene (Figure 27). No *Snl3* transcript was detected by RT-PCR, indicating that the T-DNA insertion results in a complete knock-out (Figure 28a)

To test whether RdDM is alleviated in *snl3* mutants, we chose two RdDM targets that have a prominent expression phenotype in *hda6* mutants, the retrotransposon-derived soloLTR and the intergenic sequence IG5 (Huettel et al., 2006). In contrast to *hda6* mutant plants, however, the expression of both RdDM targets is unchanged in the *snl3* mutant background (Figure 28b). Although functional redundancy of SNL3 and SNL1 cannot be ruled out at the moment, this result indicates that SNL3 does not have a major function in RdDM silencing.

Figure 27. Location of *snl3* mutation. The T-DNA insertion in the SALK_020633 line resides within the first exon of the snl3 gene. Primers used for transcript analysis are positioned in the second and third exon.

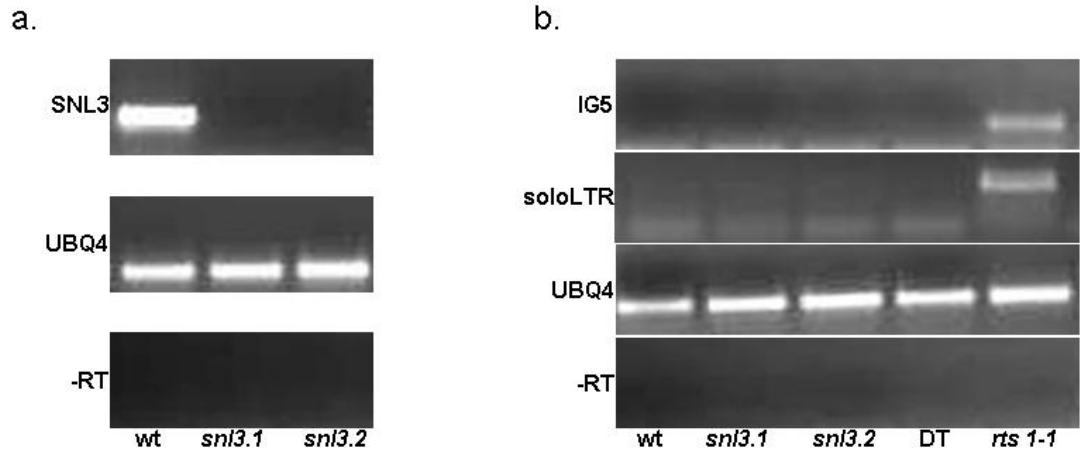


Figure 28. Expression analysis in *snl3* mutants by RT-PCR. Expression of UBQ4 was used as loading control.

a. SNL3 is expressed in wild-type (wt) plants, but not in two homozygous snl3 mutant plants (*snl3.1* and *snl3.2*).

b. IG5 and soloLTR are reactivated in *rts1-1* mutants, but not in *snl3* mutants. Col-0 wild-type (wt) plants were used as a control for the snl3 mutants, whereas DT plants served as control for the *rts1-1* mutants.

3.4. Analysis of global histone acetylation levels in *snl3* mutants

SIN3 complexes have important roles in epigenetic regulation of gene expression and in maintenance of heterochromatin and genome stability (Silverstein and Ekwall, 2005). In mouse as well as *Drosophila*, loss of SIN3 results in lethality during early stages of development (Dannenberg et al., 2005), while *snl3* mutants in *Arabidopsis* have no obvious phenotypes. However, another *Arabidopsis* RPD3-type HDAC, HDA19, has been shown to interact with SIN3 components, and loss HDA19 results in a global increase of histone acetylation levels (Song et al., 2005; Zhou et al., 2005). Therefore we tested whether the same is true for *snl3* mutants.

Upon histone extraction from *snl3* mutants and from wild-type control plants, we performed western blot analysis with anti-H3K9ac, anti-H3K9/K27ac and anti-H4ac antibodies. We observed no obvious changes in global H3 and H4 acetylation levels (Figure 29). The absence of global effects on histone acetylation can be due to functional redundancy between different *Arabidopsis* SIN3 proteins. Alternatively, the SNL3-specific complex might regulate only a limited number of target genes.

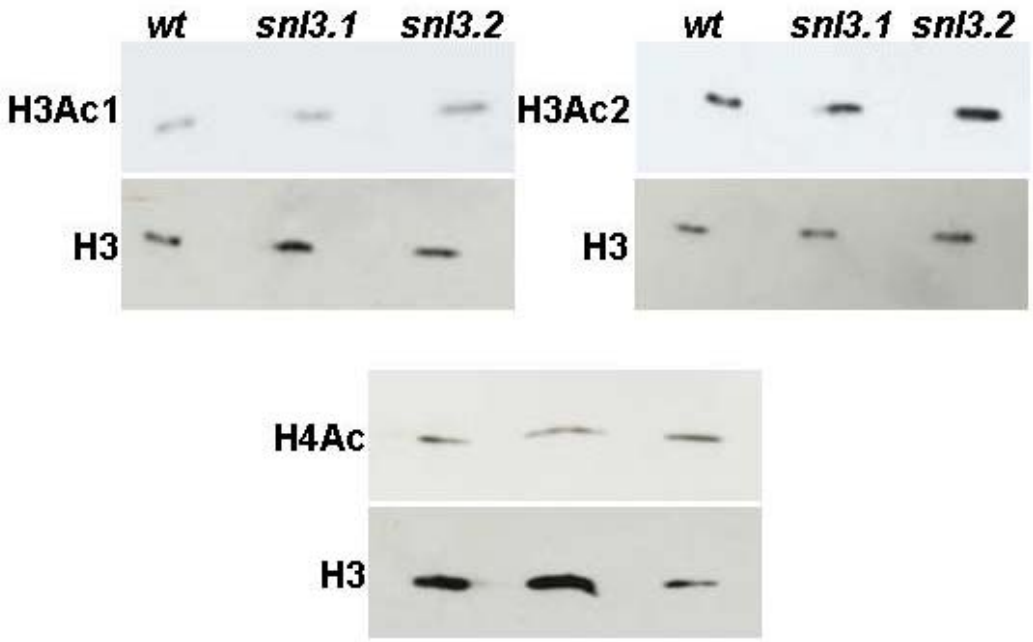


Figure 29. Western blot analysis of changes in global histone acetylation in *snl3* mutants. Histone acetylation was tested with anti-H3K9ac, anti-H3K9/27ac (H3Ac2) and anti-H4ac antibodies. The anti-H3 antibody served as a loading control. There were no significant changes in acetylation levels in *snl3* mutants compared to wild-type (wt) plants.

Discussion

The *Arabidopsis* histone deacetylase HDA6 belongs to the Class I of RPD3 family HDACs. Like its mammalian paralogs (HDAC1 and HDAC2) it is involved in transcriptional gene silencing. On several reported targets, inluding transgenes, transposons and rDNA genes. HDA6 mediates silencing via the RdDM pathway. Some recently discovered targets, however, are silenced by yet unknown mechanisms (Chen et al., 2010; Tanaka et al., 2008; Wu et al., 2008). It is still an open question how histone deacetylation catalyzed by HDA6 interplays with other repressive chromatin modifications, e.g. DNA methylation, during RdDM. Information on HDA6 recruitment to RdDM-silenced loci is scarce, mostly due to the fact that no interacting proteins or complexes of HDA6 have been identified so far. One model of HDA6 function in RdDM states that HDA6 is recruited by and is required for DNA methylation (Figure 31a). It is based on the finding that HDA6 has a role, together with MET1, in the maintenance of DNA methylation in RdDM silencing. In this work we aimed to shed light on the function of HDA6 in RdDM by characterizing mis-sense alleles of HDA6 that are defective in RdDM with regard to localization and activity of the encoded proteins, and by the identification and characterization of HDA6 interaction partners.

1. RdDM-defective *hda6* mutants are compromised in enzymatic activity but not intracellular localization of HDA6

In this study we used HDA6 mutants that were identified in a genetic forward screen for RdDM components in *Arabidopsis* based on a transgene reporter (Aufsatz et al., 2002b). These mutants include one null (*rts1-1* for RNA-mediated transcriptional silencing) and three mis-sense alleles (*rts1-3*, *rts1-4* and *rts1-5*). We demonstrated that all of these HDA6 mutants release the transcriptional silencing of known endogenous RdDM targets (soloLTR, IG5), which is associated with elevated

histone acetylation (see Figure 8c). However, transcriptional activation was not followed by a decrease in DNA methylation. This finding is opposing the concept that HDA6 acts primarily by the reinforcement of DNA methylation, but suggests a more direct and possibly methylation-independent role of histone deacetylation in RdDM silencing.

All of the mis-sense alleles encode full-length proteins that have an intracellular localization indistinguishable from wild-type HDA6. Thus, RdDM-defects of these mutants cannot be explained by the formation of truncated HDA6 or by apparent mislocalization. To further analyse these proteins we overexpressed HDA6 proteins tagged with HA, 6xHIS and anti-XPRESS tags (tag-HDA6 and tag-RTS, respectively) in the genetic background of the *rts1-1* null mutation. Tag-HDA6 could complement the *rts1-1* mutation showing that overexpression or the presence of the tag does not influence protein function. Since mutant proteins could not complement the *rts1-1* mutation despite being fully translated and showing normal localization we suspected that they are enzymatically inactive. Indeed, an *in vitro* HDAC activity assay of HA-purified proteins proved that all of the analyzed mutant proteins are enzymatically inactive. Interestingly, another lab recently reported an enzymatically inactive HDA6 point mutant that has the mutation within the same amino acid residue as the *rts1-4* allele, at D186 (Earley et al., 2010). They identified a conserved part of the HDAC domain by the alignment of *Arabidopsis* HDA6, *Arabidopsis* HDA19, mammalian HDAC1 and *Aquifex aeolicus* HDLP (Earley et al., 2010). From the crystal structure of HDLP bound to the inhibitor TSA (Finnin et al., 1999) it was clear that the catalytic centre of HDLP resides exactly within the identified conserved part. Thus, Earley and coworkers created four different mutations within conserved residues of this domain. Beside amino acid 186, residues 190, 191, and 193 were all required for enzymatic activity, indicating that they contribute to the active site of HDA6. Our work, however, demonstrates that two additional alleles (*rts1-3* and *rts1-5*) affecting amino acids further C-terminal of the predicted catalytic domain (G274 and P341, respectively) also encode enzymatically inactive protein, indicating that a much larger region of the HDAC domain is important for activity.

Considering that HDA6 proteins encoded by the mutant alleles are translated to full length, localize normally within the cell but are enzymatically inactive, we can conclude that reactivation of RdDM target loci in these mutants is due to the loss of

HDA6 enzymatic activity. This result, together with the previous conclusion that DNA methylation is not sufficient for silencing, provides a more direct function for HDA6-catalyzed histone acetylation in RdDM than solely for the reinforcement DNA methylation. Based on those results histone deacetylation either acts downstream or parallel to DNA methylation in RdDM.

2. HDA6 associates *in planta* with RNA-binding proteins

To elucidate the question of HDA6 recruitment mechanisms during RdDM, we set out to identify and further investigate potential interaction partners of HDA6. Considering that one of the models proposes HDA6 recruitment via DNA methylation, methyl-cytosine binding domain (MBD) proteins and DNA methyltransferases (DMTs) are potential complex partners. Direct proofs for these interactions have been obtained from research in mammals: Mammalian MBD proteins and HDAC1/2 are components of two histone deacetylase complexes NuRD and MeCP1 (Feng and Zhang, 2001). Additionally, the DNA methyltransferases DNMT1 and DNMT3B have been shown to interact with HDAC2 and HDAC1/2, respectively (Costello and Plass, 2001; Geiman et al., 2004). In plants, biochemical evidence for these interactions is still missing. For example, we previously failed to identify interaction of HDA6 with several MBD proteins by a targeted yeast two-hybrid assay (Schuller, 2007). Even though a direct proof for methylation-dependent recruitment of HDACs is still missing in plants, pharmacological approaches reveal a close inter-dependence of histone deacetylation and DNA methylation: Blocking cytosine methylation with aza-dC or blocking histone deacetylation with TSA prevents both cytosine methylation and H3K9 deacetylation at some loci, indicating the connection between those two repressive modifications (Lawrence et al., 2004).

In order to identify interaction partners of HDA6, we initially performed a large-scale yeast two-hybrid screen against random- and oligo dT-primed *Arabidopsis* cDNA libraries. Neither DMTs nor MBDs were identified in these screens. We, however, identified several proteins from which three (fibrillarin1, the DEAD-box helicase PRH75 and a novel RRM protein) potentially associate with RNA and are thus good candidates for a model of HDA6 recruitment via RNA (Figure 31b).

Fibrillarins are nucleolar proteins involved in the processing of rRNA precursors

(Aris and Blobel, 1991; Lischwe et al., 1985; Maxwell and Fournier, 1995). The *Arabidopsis* genome harbors two genes, AtFib1 and AtFib2, that encode for almost identical proteins, presumably with the same function (Barneche et al., 2000; Pih et al., 2000). This is supposedly the result of a large ancestral duplication of the *Arabidopsis* genome (Barneche et al., 2000; Grant et al., 2000). Interestingly, fibrillarins have already been connected to RdDM before: fibrillarin2 was identified in a mass spec analysis to be associated with the PolV subunit NRPE1 (Huang et al., 2009) and might therefore have a role together with PolV in RdDM.

The DEAD-box helicase PRH75 is a nuclear protein with very weak ATPase activity that, at least temporarily, interacts with RNA in an ATP- independent manner (Lorkovic, Herrmann et al. 1997). PRH75 belongs to the DEAD- box helixase subfamily IV which is closest to human RNA helicase II/Gu protein involved in rRNA processing (Aubourg et al., 1999).

The RRM-domain protein is a novel *Arabidopsis* protein that is comprised of two previously separately annotated ORFs, At2g33435 and At2g33440. It has two RRM domains that usually interact with single stranded RNA (ssRNA), but are also known to mediate binding to single stranded DNA (ssDNA) as well as to proteins (Auweter et al., 2006).

We demonstrated that these potential interaction partners have an overlapping localization with HDA6. Fibrillarin and PRH75 are localized in nucleolus while the RRM protein and HDA6 are localized in both, nucleolus and nucleoplasm. Additionally, we confirmed by a bimolecular fluorescence complementation (BiFC) assay that HDA6 interacts *in planta* with fibrillarin and the RRM protein. We were, however, not able to demonstrate *in planta* interaction of PRH75 and HDA6. The interaction of PRH75 and HDA6 in the yeast system was weaker compared to the other interactions. Therefore, we cannot exclude the possibility that HDA6 interacts with PRH75 in a transient or weak manner that is undetectable by the BiFC approach.

To genetically prove the interaction of HDA6 with fibrillarin and the RRM protein, we tested for the potential missexpresion of known HDA6 targets, like the soloLTR and IG5, in respective mutant plants. Both mutants showed no reactivation of HDA6 targets in contrast to the *hda6* null mutant *rts1-1*. However, this negative result does not completely exclude the involvement of fibrillarin1 and the RRM protein in RdDM:

In the case of fibrillarin1 the absence of reactivation can be explained by genetic redundancy between the two almost identical *Arabidopsis* fibrillarins. Additionally, recruitment of HDA6 in a sequence-specific manner by RNA-associated complexes could be redundant to methylation-dependent recruitment of HDA6 (Figure 31a and b). SoloLTR and IG5 both represent targets, for which DNA methylation is already maintained over generations of plants and for which methylation-dependent HDA6 recruitment might therefore dominate. The contribution of RNA-based HDA6 recruitment to RdDM could only be unmasked for targets, that are subject to *de novo* silencing by RdDM and that have not yet established DNA methylation patterns.

3. HDA6 is a component of the *Arabidopsis* SIN3 complex

We identified novel interaction partners of HDA6 by mass spectronomy analysis of affinity-purified HDA6. Four of these proteins, SIN3 like 3 (SNL3), SIN3 like 1 (SNL1), MSI1 and RXT3 are plant orthologs of yeast and mammalian components of the conserved SIN3 complex. The SIN3 complex is a well described silencing complex in yeast, mammals and Drosophila (Ahringer, 2000; Grzenda et al., 2009; Pennetta and Pauli, 1998). The core of the complex includes SIN3, RPD-type HDACs, the Sin3 associated proteins SAP18 and SAP30, the retinoblastoma binding protein RBBP4/7 and SDS3 (Figure 30).

SIN3 is a scaffold protein that is thought to mediate multiple protein-protein interactions between core components of the complex and other more peripheral proteins (reviewed in Grzenda et al., 2009). SIN3 contains no demonstrated intrinsic DNA binding activity or any known enzymatic activity (reviewed in Bowen et al., 2009; Grzenda et al., 2009). The SIN3 complex is recruited via different transcriptional regulators (Bowen et al., 2009; Carrozza et al., 2005; Hill et al., 2008; Silverstein et al., 2003; Song et al., 2005) and complex activity is mediated through HDACs (reviewed in Ahringer, 2000; Yang and Seto, 2008). SIN3 has been identified in most eukaryotes (reviewed in Silverstein and Ekwall, 2005), however with a variable number of paralogs. For example, there is only one SIN3 protein in budding yeast, mammals have two isoforms, SIN3A and SIN3B, while plants have six SIN3 like proteins SNL1-6 (Bowen et al., 2009).

The functional redundancy or specificity of these isoforms is an important

question that remains largely unaddressed to date. The two mammalian Sin3 paralogs are both widely expressed and highly homologous (reviewed in Grzenda et al., 2009). Nevertheless, the fact that *sin3a* embryos are not rescued by the presence of sin3b provides a genetic support for distinct functions of these proteins (Dannenberg et al., 2005).

Arabidopsis SNL proteins were all shown to be expressed, suggesting that each of them plays a functional role (Bowen et al., 2009). However, not much is known with regard to their function. The best studied SNL proteins are SNL1 and SNL3 (Bowen et al., 2009) both of which we identified as HDA6 interaction partners. SNL1 has been shown to possess an inherent transcription repression capability that is dependent on functional HDAC activity (Bowen et al., 2009). Additionally number of interaction partners have been identified like DNA binding transcriptional factors and telomere-binding proteins (Bowen et al., 2009). SNL3 has been reported to directly interact with SAP18, HDA19 and several interaction partners (Bowen et al., 2009; Hill et al., 2008; Song et al., 2005; Song and Galbraith, 2006).

The identification of SNL1 and SNL3 as HDA6 interaction partners opens new questions about the structure of *Arabidopsis* SIN3/HDA6 complex. One of the possibilities is that SNL1 and SNL3 are part of the same complex. Considering that plants have no ortholog of SDS3 (reviewed in Grzenda et al., 2009) which is an integral subunit of the SIN3 complex in other species, the presence of two SNL proteins in one complex might compensate for the absence of SDS3. Another possibility is that we have identified two different SIN3/HDA6 complexes having SNL1 or SNL3 as a central scaffold protein. To test this we have to further analyse phenotypes of *snl1*, *snl3* and *snl1/snl3* mutant plants and identify overlapping or specific targets of these paralogs.

RPD3-type HDACs are responsible for the enzymatic activity of the SIN3 complex. Besides HDA6 three other *Arabidopsis* HDACs, HDA7, 9 and 19 belong to this group, which makes them potential components of the complex. Until now most of the research on *Arabidopsis* SIN3 complex focused on the interaction of HDA19 with SAP18 and SNL3 and the identification of associated transcription factors. Yeast two hybrid analysis revealed several different transcription factors as partners of SNL3 and, to lesser extent, of SNL1 (Bowen et al., 2009; Hill et al., 2008; Song et al., 2005; Song and Galbraith, 2006). HDA19 has been shown to interact with SAP18 by

yeast two hybrid analysis and GST pull downs (Hill et al., 2008; Song and Galbraith, 2006). Furthermore HDA19 interacts with SNL3 in yeast two hybrid assays (Song et al., 2005). Although HDA6 has been reported to interact with SAP18 as well (Hill et al., 2008), HDA19 was considered to be the major enzymatic subunit of SIN3 complex. However, in the mass spec analysis we did not identify any other HDAC except HDA6. Considering that both *hda6* and *snl3* have no global changes in histone acetylation levels (see Figure 13 and Figure 29) while *hda19* mutation results in global increase in histone acetylation (Zhou et al., 2005) it is possible that HDA19 is not part of the SNL3 subcomplex. Alternatively, HDA19 could be part of several distinct complexes defined by different SNL proteins. Therefore, the loss of HDA19 would result in a more severe phenotype than loss of SNL3.

The third identified component of SIN3/HDA6 complex is MSI1, which is a plant ortholog of RBBP4/7. RBBP4/7 like proteins are characterized by WD40 repeats that are part of several protein complexes acting on chromatin (Bouveret et al., 2006; Yang and Seto, 2008). They were originally isolated for their ability to bind immobilized retinoblastoma protein (Rb) (reviewed in Hennig et al., 2003; Silverstein and Ekwall, 2005). Additionally RBBP4/7 and MSI proteins can interact directly or indirectly with histones (Grzenda et al., 2009; Hennig et al., 2005). *Arabidopsis* has five MSI1-like genes, MSI1-5, with similar expression patterns but only limited functional redundancy (Ach et al., 1997; Hennig et al., 2003; Kenzior and Folk, 1998). The loss of MSI1 function, but not of MSI2, MSI3, MSI4 or MSI5, causes seed abortion (reviewed in Hennig et al., 2003). MSI1 is a component of *Arabidopsis* Polycomb repressive complexes (PRC2), such the Fertilisation independent seed (FIS) complex and the CURLY-LEAF (CLF) complex (Kohler et al., 2003). MSI1 is involved in imprinting by interaction with the retinoblastoma related protein RBR1 and the transcriptional repression of the DNA methyltransferase MET1 during female gametogenesis (Jullien et al., 2008). Finally, MSI1 is involved in regulating *Arabidopsis* flowering time (Bouveret et al., 2006). The maize RBBp4/7ortholog ZmRbAp1 has been shown to associate with the histone deacetylase ZmRpd3 (HD1B) and the Rb ortholog ZmRBR1 (Lechner et al., 2000). However *Arabidopsis* MSI1-like proteins have so far not been demonstrated to interact with HDACs and/or SIN3 complex component.

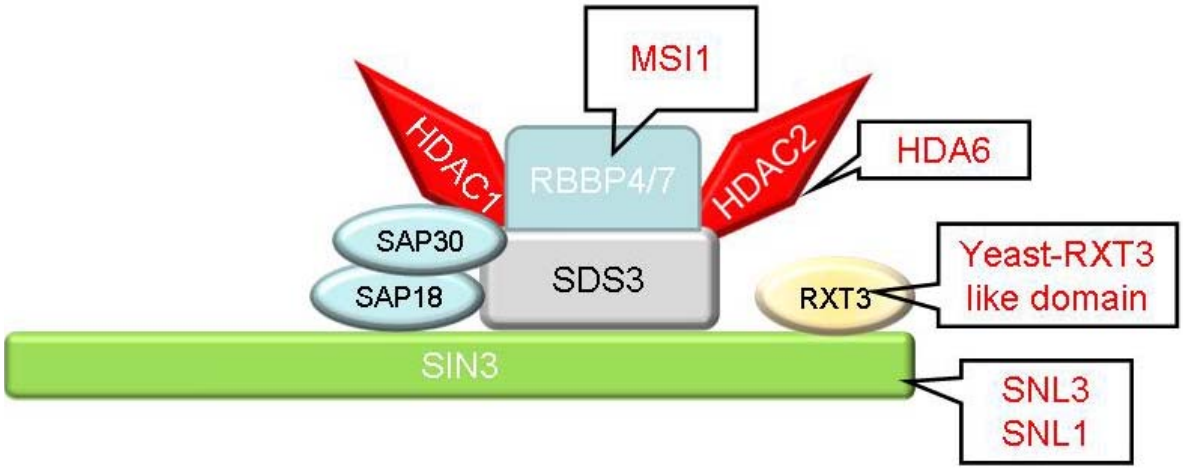


Figure 30. Scheme of the core mammalian SIN3 complex. Additionally shown is RXT3 that was identified as a component of the yeast SIN3 complex. Plant orthologs of SIN3 complex components that were identified in this work are indicated by boxed red type color.

Additional components of the core SIN3 complex that were not identified in this work include the SIN3 associated proteins SAP18 and SAP30. In mammals, SAP18 and SAP30 can directly interact with Sin3 and HDAC1, probably stabilising the SIN3 complex (reviewed in Silverstein and Ekwall, 2005). SAP18 orthologs have been identified in plants and yeast two hybrid results indicate that they are part of plant SIN3 complexes (Hill et al., 2008; Song and Galbraith, 2006). As already mentioned above, plant orthologs of SDS3, which is essential for the integrity and catalytic activity of SIN3 complexes in yeast and mammals, have not been identified.

It is important to note that although these proteins are considered core components of the SIN3 complex, not all of them are always detected in complex purifications. In the literature, there are examples of SIN3 complexes lacking one of the HDACs or SAP proteins (reviewed in Silverstein and Ekwall, 2005). It is not clear how much of this variation is due to experimental limitations. It is likely, however, that some of these variations reflect the true biological plasticity of this complex. The SIN3/HDAC complex can expand its function or alter its specificity by distinct complex components (reviewed in Silverstein and Ekwall, 2005).

Besides the clear orthologs of SIN3 complex constituents, we identified three unknown proteins, which – based on their domain composition – could have a potential function within the SIN3/HDA6 complex. At5g08450 encodes a protein with

an RXT3 domain. This domain is present in yeast RXT3 which was purified as component of the RPD3L-SIN3 complex (Carrozza et al., 2005). The function of RXT3 is unknown, however, it appears to interact indirectly with Rpd3 (Carrozza et al., 2005). The protein encoded by At1g09050 has a domain (KOG4329) with predicted function in DNA-binding, while the At2g47820 protein has the RST superfamily domain required for the interaction with a number of plant transcription factors (Ahlfors et al., 2004; Belles-Boix et al., 2000; Marchler-Bauer et al., 2009; Marchler-Bauer and Bryant, 2004). It is known that transcription factors are peripheral components of the SIN3 complex, which are important for sequence specific targeting (Silverstein and Ekwall, 2005). They normally interact directly with SIN3 through one of several PAH domains present in SIN3 protein. Consequently, At1g09050 and At2g47820 might be part of the SIN3/HDA6 complex and could be important for its proper targeting.

4. HDA6 co-purifies with the S-adenosylhomocysteine hydrolase HOG1

In addition to SIN3 complex components, we identified HOG1 and LOS1 as interaction partners of HDA6. HOG1 (MEE58) is an S-adenosylhomocysteine hydrolase (SAHH) (reviewed in Moffat and Weretilnyk, 2001). SAHH activity is vital for the recycling of S-adenosyl-L-methionine (Sambrook and Russell), the major source of methyl groups for all biological transmethylation reactions. Each methyltransferase reaction involves the transfer of one methyl group from SAM to a specific substrate with the release of one molecule of S-adenosyl-L-homocysteine (SAH) (Espada et al., 2007). SAH is a strong competitive inhibitor of methyltransferases by occupying their active site. Thus, SAH has to be removed by SAHH activity in order to allow methyltransferase reactions to continue. In *Arabidopsis*, for example, the CMT3 pathway, which involves cytosine methylation in non CG contexts as well as histone H3 lysine 9 methylation, has been shown to be particularly sensitive to SAH inhibition. This is probably due to the requirement for two transmethylation reactions (Mull et al., 2006). Because of its role in SAH turnover, it is not surprising that mutations in HOG1 result in decrease in DNA and histone methylation and in the release of gene silencing (Baubec et al., 2010; Furner

et al., 1998; Rocha et al., 2005). HOG1 was predicted to be localized in the cytosol since it lacks a detectable nuclear localization sequence. A recent study employing a GFP-HOG1 fusion protein, however, demonstrated that it is located in the nucleus as well. A closer inspection revealed that HOG1 harbors a 40 amino acid sequence, that is conserved in plants and photosynthetic bacteria and that is essential for targeting to the nucleus (Sanhyun Doxey and Moffatt, 2008). The shared subcellular localization and functions in gene silencing make it likely that HOG1 and HDA6 form a complex. In support of this conclusion, HOG1 and HDA6 have some overlapping targets. CACTA transposons and three unknown expressed genes are reactivated in the *hda6* mutants *rts1-1* and *rts1-5* (Stille, unpublished), as well as in *hog1* mutants (Jordan et al., 2007). The purpose of this interaction could be the recycling of SAH on the spot of DNA methylation. This is in agreement with the fact that mutations in HDA6 can influence DNA and histone methylation levels at some target sequences. However, as already mentioned above, some loci, like soloLTR and IG5 have unchanged levels of methylation in *hda6* mutant plants, which would argue for a specialization of the HOG1/HDA6 complex with regard to target selection.

LOS1 was originally identified in a mutant screen for genes involved in low temperature response (Guo et al., 2002). In *los1-1* mutants, several cold responsive genes are reactivated, among which COR15A is also upregulated in *rts1-1* mutants (Stille, unpublished, Guo et al., 2002). LOS1 is a translational elongation factor similar to eEF-2, which is important for cold temperature protein synthesis and is located in the cytoplasm (Guo et al., 2002). Considering that LOS1 and HDA6 have different localization and function, there is no obvious explanation for LOS1 to co-purify together with HDA6.

5. The role of the SIN3/HDA6 complex in *Arabidopsis*

In yeast and mammals, SIN3 complexes have many different functions, including DNA methylation, gene expression regulation, chromosome segregation, rDNA silencing, DNA damage repair and replication timing (reviewed in Grzenda et al., 2009). In *Arabidopsis*, a number of transcription factors and DNA binding proteins interacting with SAP18 or SNL3 have been identified. They reveal an insight into the function of the SIN3 complex in plants. Interestingly, some of these functions seem to

overlap with those of HDA6.

For instance, SAP18 has been reported to interact with Suppressor of overexpression of constans (SOC1) which is a MADS box transcription factor and one of the key floral activators (Liu et al., 2009). In *soc1* mutants, cold response genes are overexpressed, indicating that SOC1 negatively regulates their expression. The overexpression of cold response genes in turn results in elevated expression of the flowering repressor FLC (flowering locus C), resulting in a late flowering phenotype (Seo et al., 2009). Interestingly, *hda6* mutants also show upregulation of both FLC and cold-regulated genes (Stille, unpublished). Thus, SOC1 might act partially together with the SIN3/HDA6 complex in regulating flowering time in *Arabidopsis.*

Another reported function of HDA6 is the regulation of ABA and salt stress responses in *Arabidopsis* (Chen et al., 2010). *Snl3* mutants, like *hda6* mutants, have increased ABA sensitivity. Similarly, *sap18* and *hda6* mutants are more sensitive to salt stress (Chen et al., 2010; Song et al., 2005; Song and Galbraith, 2006). These findings suggest that HDA6 mediates ABA and salt stress tolerance via the SIN3/HDA6 complex.

So far, there is no experimental evidence for a function of SIN3 complexes in RNA-mediated silencing processes in *Arabidopsis*, which would be analogous to the role of SIN3/Clr6 in RNAi-mediated heterochromatin formation in fission yeast (Silverstein et al., 2003). We demonstrated in this work that *snl3* mutants are not impaired in the silencing of several RdDM targets. Even though SNL3 does not seem to be redundant with any of the other *Arabidopsis* SNL proteins in the ABA response (Song et al., 2005), redundant SIN3/HDA6 complexes could control RdDM, e.g. SNL1/HDA6. The analysis of RdDM target reactivation in *snl1/snl3* double mutants and/or *msi1* mutants will be required to conclude on SIN3 functions in RdDM.

Several studies have suggested that SNL proteins, SAP18 and HDA19 constitute the evolutionary conserved SIN3 complex in *Arabidopsis.* In this study, for the first time, HDA6 was co-purified with *Arabidopsis* SIN3 components. It remains to be clarified whether HDA6 exists in a single complex containing both SNL3 and SNL1, or whether these are separate SIN3 complexes. The analysis of single and double mutants of complex components is now required to address genetic redundancy, to identify target genes and to reveal functions of SIN3 in RdDM.

In summary, this work reveals two possible sequence-specific recruitment mechanisms of HDA6, one by RNA via interactions of HDA6 with an RRM protein and fibrillarin1 and one by DNA binding proteins via a SIN3/HDA6 complex (Figure 31b-c). Whether any of these sequence-specific recruitment mechanisms has a role in RdDM, however, still needs closer examination in future.

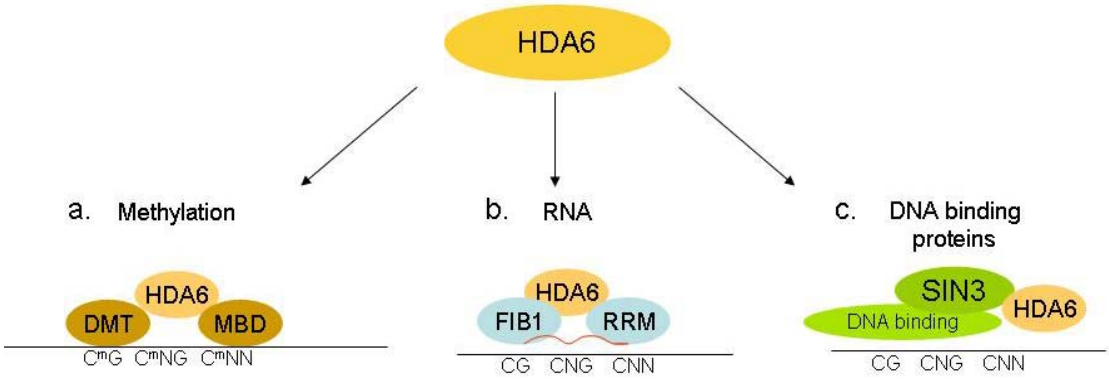


Figure 31. Possible mechanisms of HDA6 recruitment to target genes.

- **a. HDA6 can be recruited to specific DNA sequence in a methylation-dependent way, e.g. by the interaction with methyl-binding domain proteins (MBP) or DNA methyltransferases (DMT)**
- **b. Data in this work suggest the possibility RNA-dependent recruitment of HDA6 by the interaction with an RNA recognition motif (Lorkovic et al.) protein and Fibrillarin1 (FIB1)**
- **c. Data in this work suggest the recruitment of HDA6 by the SIN3 complex and DNA binding proteins.**

Materials and Methods

1. Plant genotypes and growth conditions

In this work, exclusively *Arabidopsis* plants of the ecotype Columbia (Col) were used: DT (double transformed) plants were homozygous for both, the silencer and the target transgenes of the NOSpro-*NPTII* silencing system (Aufsatz et al., 2002a). All *rts* mutants analyzed in this study were in the DT background. The constructs for the expression of tagged wild-type and mutant HDA6 proteins were introduced into the *rts1-1* background (Aufsatz et al., 2002b) by *Agrobacterium* mediated transformation (Clough and Bent, 1998). The *snl3* (SALK_020633), *fib1*

(SALK_031583) and *rrm* (SAIL_48_B11) mutants have been obtained from the Nottingham Arabidopsis Stock center (NASC).

Seeds were surface sterilized by adding 1ml of 70% EtOH/0,05% Triton X-100 and incubation for 20 min with constant shaking, followed by an additional 20 min incubation in 1 ml of 100% EtOH. Seeds were air dried and plated on Murashige Skoog-Basal agar medium (0,8% agar supplemented with 1% sucrose) (Murashige and Skoog, 1962). After stratification for 48h at 4°C, plants were grown at 21°C in 16h light conditions. Depending on the genotypes, plants were selected with 50mg/l Kanamycin (complementation tests of plants expressing tagged HDA6 versions; *snl3* mutants), 10mg/l phosphinotricin (*rrm* mutants) or 100mg/l gentamicin (transgenic plants expressing tagged HDA6 versions). For seed production, plants were transferred to soil and grown at 21°C in a 16h/8h light/dark cycle.

2. Constructs of wild-type and mutant tagged HDA6 proteins

Full length HDA6 was amplified from the cDNA clone U18957 (ABRC, Ohio) with primers HDA6-F1 and HDA6-R3. HDA6-F1 has a HindIII site, followed by a NdeI site that contains the start codon of HDA6. HDA6-R3 contains a Cfr9I site. The PCR product was cloned into the HindIII/Cfr9I sites of pBluescript II SK- (Stratagene). After sequence confirmation, the HDA6 insert was released by digestion with NdeI/Cfr9I and cloned into the respective sites of the yeast two hybrid vector pAS1 (Clontech). In pAS1, HDA6 is N-terminally tagged with a hemagglutinin (HA) tag (pAS1/HA-HDA6). In order to make a multi-epitope tagged HDA6 construct, the HIS/XPRESS-tag from pcDNA3.1 (Invitrogen) was amplified with primers Xpress-F and Xpress-R and cloned as a ClaI/EcoRI fragment into the respective sites of pBluescript II SK- (Stratagene). Into this plasmid, an EcoRI/Cfr9I HDA6 fragment from pAS1/HA-HDA6 was inserted into the respective sites to give rise to pSK-HIS/XPRESS/HA-HDA6. To introduce the point mutations of the *rts1-3*, *rts1-4* and *rts1-5* alleles, a PCR approach was used: The regions of HDA6 encompassing the respective point mutations were amplified from cDNA of mutant plants with primers HDA6-F3/HDA6-R3 (*rts1-3*), HDA6-F1/HDA6-R1 (*rts1-4*) and HDA6-F2/HDA6-R2 (*rts1-5*). The PCR fragments were then exchanged with the corresponding fragments in pSK-HIS/XPRESS/HA-HDA6 (EcoRI/Cfr9I for *rts1-3*, NdeI/NcoI for *rts1-4* and NcoI/PstI for *rts1-5*) to give

rise to tagged RTS1-3, RTS1-4 and RTS1-5 proteins, respectively. For plant transformation, the constructs were cloned as SacI/Cfr9I fragments between the 35S promoter and the pea *rbcS* terminator in the binary vector pCHF1 (a gift from Christian Fankhauser, University of Geneva; based on pPZP212 (Hajdukiewicz et al., 1994).

3. Constructs for localization and interaction studies

Full length cDNAs of wild-type and mutant HDA6 (At5g63110), the RRM domain protein (At2g33435), PRH75 (At5g62190) and Fibrillarin1 (FIB) (At5g52470) were amplified with primers containing AscI and KspAI restriction sites. The PCR products were first cloned into pJET using the CloneJET™ PCR Cloning Kit (Fermentas, acc. to manufacturer's protocol) and inserts were verified by sequencing. The inserts were then cloned as AscI/KspAI fragments into pGPTVII-GFP (HDA6) or pGPTVII-YFP (RRM, PRH75, FIB) binary vectors for localization studies and 35S-pSPYNE (HDA6) or 35S-pSPYCE (RRM, PRH75, FIB) for interaction studies (Walter et al., 2004). Constructs of bZIP63 (At5g28770) in 35S-pSPYNE and 35S-pSPYCE were a gift from Klaus Harter, University of Münster, Germany.

4. Transient expression in tobacco leaf cells

Agrobacterium tumefaciens strain AGL-1 was transformed with the appropriate constructs (see above) by electroporation. The protocol for transient expression was adopted from Kapila and coworkers (Kapila et al., 1997). An *Agrobacterium* pre-culture (grown overnight in LB supplemented with the appropriate antibiotics) was inoculated into LB (pH 5.6) medium supplemented with antibiotics and Acetosyringone (20µM) for virulence gene induction. The culture was grown overnight at 29°C to an OD600 of 0.8. Cells were collected by centrifugation at 3000 rpm for 15 min at 4°C. The pellet was resuspended in MMA infiltration medium (1/2MS supplemented with 10mM MES, 20% sucrose, 200 µM acetosyringone, 5.6 pH adjusted by KOH) to a final OD600 of 2.4. For interaction analysis, where co-

expression of two constructs was performed, equal amounts of bacterial suspensions (both OD600 = 2.4) were combined. The bacterial suspensions were incubated for 1h at room temperature and subsequently used for vacuum infiltration. Leaves of 4 week old *N. benthamiana* plants were placed into the suspension and a continuous vacuum was applied for 20 min. After rapid release of the vacuum, leaves were briefly rinsed with sterile water and kept on wet Whatman paper with their adaxial sides facing up. The material was incubated in 16h light conditions for 48 h and then analyzed by confocal laser scanning microscopy or subjected to nuclei isolation.

5. Leaf nuclei isolation

Nuclei isolation protocol was done according to Baubec and co-workers (Baubec et al., 2010). Transformed *N. benthamiana* leaves were rinsed in 10mM Tris buffer, pH 7.5, fixed by incubation in 4% paraformaldehyde, 1M Tris buffer pH 7.5 under a constant vacuum for 15-20 min. After rapid vacuum release, the leaves were washed 2x10 min with 1M Tris buffer, pH 7.5. The leaves were then cut with a razor blade in the presence of 250 μl of chromosomal isolation buffer (15 mM Tris, 2 mM Na_2EDTA, 0.5 mM spermin, 80 mM KCl, 20 mM NaCl, 15 mM β -mercaptoethanol, and 0.1% Triton X-100, pH 7.5) until the solution was almost homogenous. Then, again 250 μl of chromosomal isolation buffer were added. The suspension was filtered through a 50 μm nylon mesh filter (CellTrics, PARTEC) and the filtrate was kept on ice until centrifugation. 100 μl of filtrate plus 250 μl were transferred onto microscope slides, and nuclei were attached to the slide using a cytospin centrifuge (MPW, Med-Instruments) at 2500 rpm for 10 min. Slides were rinsed shortly in ice cold 1xPBS and immediately analysed by fluorescent microscopy or stored in 50% glycerol at -20°C until further use.

6. RNA extraction for reverse transcription (RT) and for Northern blot analysis

RNA was extracted employing phase separation extraction using the TriFast reagent (Peqlab, according to manufacturer's protocol). For Northern blot analysis,

the RNA was used without further cleaning. For reverse transcription, the RNA was subjected to DNase I digest (Qiagen, according to manufacturer's protocol) after which RNA was further cleaned and concentrated using the RNeasy MiniElute Cleanup Kit (Qiagen), according to the kit protocol. After cleanup, another DNAse I digest was performed using 1u DNaseI (Fermentas) for 1 µg of RNA incubated 30 min on 37°C. For the RT reaction, we used 0.1 µg of RNA, random hexamer primers and M-MulV reverse transcriptase (Fermentas, according to manufacturer's protocol).

7. Northern blot analysis

For Northern blot analysis, 10 µg of RNA were denatured in denaturating buffer (according to Sambrook and Russell, 2001) for 15 min at 65°C and separated using a 1.5% formaldehyde gel in 1xMOPS buffer at 70 V for approximately 1.5 h (according to Sambrook and Russell, 2001). The RNA was then transferred to Hybond-N membranes (Amersham) by capillary downward transfer (Turboblotter, Whatman), according to the manufacturer´s protocol. After transfer, the membrane was briefly washed in the transfer buffer (6xSSC) and UV-crosslinked using a Stratalinker (Stratagene). The membrane was prehybridized for 1h at 65°C in 250 mM Na_2HPO_4/H_3PO_4 (pH 7.2); 4% SDS; 1mM EDTA. For sequence-specific probes, 25mg of DNA were labelled with ^{32}P dCTP (Amersham) using the Rediprime labelling kit (Amersham) and purified via G50 ProbeQuant columns (Amersham). The labeled probe was added to the prehybridization reaction and incubated over night at 65°C. Membranes were washed 2x30min in 2xSSC/0.1% SDS and 2x30min in 0.1xSSC/0.1% SDS at 65°C. Signals were detected by exposure to X ray Hyperfilm (Amersham) at -80°C for 1 to 7 days.

8. Genomic DNA extraction and genotyping

Genomic DNA was extracted from 100 mg of *Arabidopsis* leaf tissue using the DNeasy Plant Mini Kit (Qiagen). For genotyping segregating mutant T-DNA lines (SALK and SAIL collections), we used primer pairs flanking the T-DNA insertion to identify the wild-type fragment and a flanking primer together with a T-DNA specific

primer (recommended e.g. on http://signal.salk.edu/tdnaprimers.2.html) to identify the fragment specific for the insertion.

9. Immunoprecipitation of HA-tagged HDA6

3-4 week old *Arabidopsis* seedlings expressing Tag-HDA6 grown on MS plates were harvested and ground to a powder in liquid nitrogen. The powder was resuspended in 3 vol (w/v) of Tandem lysis buffer (Hafren and Makinen, 2008); 50 mM NaH_2PO_4, 150 mM NaCl, 2% PVP, 0.1% Triton X-100, 0.1 % Tween 20, 13 % sucrose, pH 8 containing Complete Mini protease inhibitor cocktail (Roche). Homogenates were filtered through Miracloth (Calbiochem) and sonicated with a Bioruptor (Diagenode) at setting "High" using 5 times a cycle of 5 sec pulsing and 30 sec resting time. Afterward samples were centrifuged at 13000 rpm for 15 min at 4°C. The supernatant was incubated with 50 µl anti-HA-conjugated agarose (Sigma-Aldrich) for 2 h at 4°C. The resin was then washed three times for 10min with 10 vol of 1xPBS, 0.1% Tween buffer. The washed beads were directly used in HDAC activity assays or for Mass Spec analysis.

10. *In vitro* HDAC activity assay

The HDAC *in vitro* activity assay was performed with purified tag-HDA6 and tag-RTS fusion proteins bound to anti-HA-conjugated agarose beads (Sigma-Aldrich). Immunoprecipitation was done from seedlings expressing tag-HDA6, tag-RTS proteins and from non-transformed *rts1-1* plants. Before incubation with resin, the protein concentration of the samples was measured by Bradford (Roti Quant, Roth) and equal amounts of proteins were loaded on 50 µl of anti-HA-conjugated agarose (Sigma-Aldrich). 5 µl of the beads were then tested by Western blot analysis with HA antibody and normalized to histone H3. The amount of beads used in the assay was adjusted to the amount of normalized HA signal on the Western blot. The HDAC activity assay was performed using the Fluorometric Histone Deacetylase Assay Kit (CS1010, Sigma-Aldrich). Fluorescence was recorded in the plate reader fluorometer (Spectrafluor Plus,Tecan). All the measurements were done on minimum three independent IPs each in three technical replicates. Measured activity was directly

proportional to arbitrary florescent units (FU) and mean value was calculated for each biological sample. The activity of IP-sample from non-transformed *rts1-1* plants was treated as background activity and the mean value of this sample was subtracted from each of the IPs from plants expressing recombinant proteins. After subtraction, tag-HDA6 activity was arbitrarily set to 1 and activities of the tag-RTS proteins adjusted accordingly.

11. Western blot analysis

Protein samples were separated on 12% polyacrylamide/SDS gels and electroblotted to Hybond P membranes (Amersham) using a semi-dry blotting cell (Trans-Blot SD, Bio-Rad). After blotting, membranes was washed for 5 min in 1xPBS and subsequently blocked in blocking buffer (1xPBS, 0.05% Tween 20, 1% non-fat dried milk) for 1h at room temperature. The incubation with primary antibody was done in blocking buffer over night at 4°C. Used antibodies and dilutions: mouse anti-HA (Covance 16B12) 1:2000, rabbit anti-acetyl-H3K9 (Abcam ab10812) 1:1000, rabbit anti-acetyl H3K9/K27 (Millipore 06599) 1:500, rabbit anti-acetyl H4 (Millipore 06866) 1:1000, rabbit anti-H3 (Abcam ab1791) 1:2000. After incubation with primary antibodies, membranes were washed in 1XPBS, 0.05% Tween 20 5x for 2 min at room temperature and incubated for 1h at room temperature in blocking buffer and an appropriate peroxidase-coupled secondary antibody. Used secondary antibodies and dilutions: anti-mouse (Pierce 31444), 1:20000, anti-rabbit (Jackson Immuno Research, 111-035-008) 1:15000. Signals were detected using the Lumi-Light protein gel blotting substrate (Roche).

12. Stripping of PVDF membranes for Western blot reprobing

Membranes were submerged in the stripping buffer (100 mM -β-mercaptoethanol, 2% sodium dodecyl sulphate (SDS), 62.5 mM Tris-HCl pH 6.7) in a sealed bag and incubated at 65°C for 30 min with occasional agitation. Subsequently, membranes were washed 2x 10 minutes in 1XPBS, 0.05% Tween 20 at room temperature using

large volumes of wash buffer. Stripped membranes could be again used for incubation with antibodies according to the protocol above.

13. Silver staining

We used a modified Blum Silver Staining Protocol. Protein gel was fixed in 40% EtOH, 10% AcOH for 1 hour at room temperature. Afterwards, the gel was washed twice in 30% EtOH for 20 min and once in H_2O for 20 min. The gel was sensitized by incubation in 0.02% Na2S2O3 for 1 min followed by brief washing in H_2O 3 x for 20 sec. Next, the gel was incubated in cold 0.1% AgNO3 for 20 min at 4ºC, followed by washing with H_2O for 1 min. The gel was developed in 3% Na_2CO_3, 0.05% formalin at room temperature until bands became visible. Immediately after that, the gel was rinsed in H_2O and staining was terminated in 5% HAc. Gel could be stored in 1% HAc at 4°C.

14. Trypsin cleavage from beads

For trypsin cleavage, beads were subjected to two additional final washing steps with 50 bv (bead volumes) of 150 mM NaCl to remove any detergent. After these washes, the beads are resuspended in 2 bv of 50 mM Triethylammonium bicarbonate (TEAB) buffer. To the bead slurry, Trypsin Gold (Promega) was added at a final concentration of 500 ng per 100 µl bead slurry (i.e. 50% slurry) and incubated at 37°C for 30 min on a rotation wheel at 900 rpm. The beads had to be completely resuspended during incubation, if necessary beads were mixed regularly by flicking the tube. Beads were centrifuged at 500g for 2 min at room temperature and the supernatant was collected, frozen in liquid nitrogen and stored at -80°C. The efficiency of trypsin digests was assessed by Western Blot analysis of samples before and after the digest.

15. *In vivo* cross-linking

For IPs followed by mass spec analysis we used three crosslinking agents: EGS (Sigma-Aldrich, E3257-1G), DMA (Sigma-Aldrich 285625) and formaldehyde (Applichem, A3592,1). Protocols for EGS and DMA crosslinking were the same: 10 mM DMA or 2mM EGS was prepared freshly in 1xPBS and stored at 4°C. 2g of 4 week old *Arabidopsis* seedlings were washed twice with 1xPBS and then submerged in the DMA/EGS crosslinking solutions. Constant vacuum was applied for 15 min. After rapid vacuum release, seedlings were washed twice with 1xPBS.

Formaldehyde (FA) crosslinking had one additional step: After vacuum infiltration with crosslinking buffer (1% FA, 1xPBS), crosslinking was quenched by adding glycine to a final concentration of 125 mM to the crosslinking buffer. Subsequently, vacuum was applied for additional 5 min. After vacuum release, the seedlings were washed once with 1xPBS and once with bidistilled H_2O.

16. NanoLC-Mass Spec Analysis.

The nano HPLC system used was an UltiMate 3000 HPLC system (Dionex, Amsterdam, The Netherlands) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a Proxeon nanospray source (Proxeon, Odense, Denmark). Peptides were loaded onto a trap column (Dionex PepMap C18, 5 mm × 300 µm ID, 5 µm particles, 100 Å pore size) at a flow rate of 25 µL min^{-1} using 0.1% TFA as mobile phase. After 15 min, the trap column was switched in line with the analytical column (Dionex PepMap C18, 250 mm × 75 µm ID, 3 µm, 100 Å). Peptides were eluted using a flow rate of 275 nl min^{-1} and a ternary 106 min gradient, with the following mobile phases: A (water/acetonitrile/formic acid, 95/5/0.1, v/v/v), B (water/acetonitrile/formic acid, 70/30/0.08, v/v/v), and C (water/acetonitrile/trifluoroethanol/formic acid, 10/80/10/0.08, v/v/v/v) at 30°C. The LTQ Orbitrap XL was operated in data-dependent mode, using a full scan in the Orbitrap (m/z range 400-1800, nominal resolution of 60 000 at m/z 400, target value 1E6) followed by MS/ MS scans of the five most abundant ions in the linear ion trap. MS/MS spectra (normalized collision energy, 35%; activation value q, 0.25; activation time, 30 ms; isolation width, 3 m/z

units, target value 5E4) were acquired and subsequent activation was performed on fragment ions through multistage activation. Precursor ions selected for fragmentation (charge state 2 and higher) were put on a dynamic exclusion list for 180s. Additionally, singly-charged parent ions were excluded from selection for MS/MS experiments and the monoisotopic precursor selection feature was enabled.

17. Mass spec Data Analysis

For peptide identification, all MS/MS spectra were searched using Mascot 2.2.04 (Matrix Science, London, UK) against the NCBI non redundant protein sequence database, using the taxonomy arabidopsis thaliana. The generation of dta-files for Mascot was performed using the Extract MSn program (version 4.0, Thermo Scientific). The following search parameters were used: carbamidomethylation on cysteine was set as a fixed modification, oxidation on methionine, pyro-carbamidomethylation on N-terminal cysteine, substitution of Glutamine against pyro-Glutamic Acid were set as variable modifications. Monoisotopic masses were searched within unrestricted protein masses for tryptic peptides. The peptide mass tolerance was set to ±3 ppm and the fragment mass tolerance to ±1.2 Da. The maximal number of missed cleavages was set to 2.

18. Primer list

Gene	Sequence (5'-3')	Details
T-DNA Border Primer (BP) SAIL	TAGCATCTGAATTTCATAACCAATCTCGATACAC	detection SAIL T-DNA
T-DNA Border Primer (BP) SALK	TGGTTCACGTAGTGGGCCATCG	detection SALK T-DNA
Fibrillarin1 SALK	TACGGCCTCTGTCACCACTAC	Genotyping
Fibrillarin1 SALK	AGACATAACGCCATCGTTTTG	Genotyping
Fibrillarin1	GCCCCGCGG**GTTAAC**ATGAGACCCCCAGTTACA	Cloning KspAI restriction site
Fibrillarin1	GCCGGTACC**GGCGCGCC**ATGAGGCTGGGGTCTTTTG	Cloning SgsI restriction site
RRM protein SAIL	AACGGTATGACATGTGGCTTG	Genotyping
RRM protein SAIL	GCTAGTGGAGTTCCTTACGCC	Genotyping
RRM protein SAIL	GGCATGTTTTCGGACTCAGTA	Transcript detection
RRM protein SAIL	AAGCATTTCAGATGAAATAGC	Transcript detection
SNL3 SAIL	GTTGATACAGCTGGTGTGATAACTAG	Transcript detection
SNL3 SAIL	TATACACGATCATCTCCCTGAAACCT	Transcript detection
SoloLTR	CATATAACCGAAGCCGAAGGATGTGAAA	soloLTR RT-PCR
	CAGAAACCTAAGGAACCATTACACGCTAAACC	soloLTR RT-PCR
Intergenic region IG5	TCGCTTGAATCTAATACTTGTGTGC	IG5 RT-PCR
	CGTAAGTGCTTTTCGGACATTACAA	IG5 RT-PCR
Ubiquitin 4	TTACGAAGGCGGTGTTTTTC	RT-PCR-Loading control
	GCTCAGGATGAGCCATCAAT	RT-PCR-Loading control
PRH75-DEAD-box helixase	GCCCCGCGG**GTTAAC**ATGCCTTCCCTAATGTTA	Cloning KspAI restriction site
	GGCGTCGAC**GGCGCG**CCAATATCTCTGGCCTCTACC	Cloning SgsI restriction site
Target nos::NPTII	ATGCCATCTCCATCAACGTC	Genotyping for target transgene
	TTTCTGACGTATGTGCTTAG	
Target wt	ATGGACATCCCCGGCAAATG	Genotyping for target transgene
	CATTGTACTGCTCTGCTTGATACTGCTTGA	
Silencer	GTCCTGCGGGTAAATAGCTGG	Genotyping for silencer transgene
	CGTCTGCTGCTCCATACAAGC	
Silencer wt	GAGATAGTGGAGCAATCTCTGAGATG	Genotyping for silencer transgene
	TTCATACGAGACCCTCTGTTTTGGC	
HDA6 and *rts1-1*	GATTCTGAGTGAGAGACGGAG	Sequencing the mutation *rts1-1*
HDA6 and *rts1-1*	AGCCATACGGATCCGGTGAGG	Sequencing the mutation *rts1-1*
rts1-3 allele	AAGAGACACCAAACCATC	Sequencing the mutation *rts1-3*
rts1-3 allele	CTGCAGTTGCTGTTGGAG	Sequencing the mutation *rts1-3/1-4*
rts1-3/*rts1-4* allele	CTTATCCTCTCCATATCTTTG	Sequencing the mutation *rts1-3/1-4*
rts1-4 allele	ATCGTGCTAGGGATTCTGGAG	Sequencing the mutation *rts1-4*
rts1-5 allele	CCATGGAGATGGAGTGGA	Sequencing the mutation *rts1-5*

Abbreviations

Ago	Argonaute
At	*Arabidopsis thaliana*
BiFC	Bimolecular fluorescence complementation
CLSM	Confocal laser scanning microscopy
DCL	Dicer like proteins
DMA	Dimethyl adipimidate
DMT	DNA methyl transferase
dsRNA	double stranded RNA
DT	double transformed plants (silencer and target transgene)
EGS	Ethylene glycol bis[succinimidylsuccinate]
FA	Formaldehyde
FIB	Fibrillarin
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
Kan	Kanamycin antibiotic
KanR	Kanamycin resistance
MPB	Methyl binding proteins
MS	Mass spectrometry
ORF	Open reading frame
PolIV	DNA Polymerase IV
PolV	DNA Polymerase V
RBD	RNA-binding domain
RBP	RNA binding proteins
RdDM	RNA-directed DNA methylation
RDR	RNA dependent RNA polymerase
RdRP	RNA dependent RNA polymerase
RNAi	RNA interference
RPD3	reduced potassium dependence 3
RRM	RNA recognition motif
rRNA	ribosomal RNA
RTS	RNA-mediated transcriptional silencing
siRNA	small interfering RNA
SNL	SIN3 like
ssRNA	single stranded RNA
ST	single transformed plants (only target)
TSA	Trichostatin A

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