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DISSERTATION

Titel der Dissertation

Unraveling functions of the *Arabidopsis* histone deacetylase HDA6 in RNA-mediated silencing, DNA damage response and plant development

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I. Summary

The reversible process of posttranslational histone modifications is an important mechanism of epigenetic regulation in the control of gene expression and chromatin structure. The acetylation of histone tails is catalyzed by the enzymatic activity of histone acetyltransferases (HATs) and is correlated with transcriptionally active chromatin. Histone deacetylases (HDACs) reversibly counteract HATs, thereby balancing the chromatin acetylation state. Deacetylated histones are therefore related with transcriptional gene silencing and maintenance of heterochromatin. A large number of HDACs have been identified in plants and other eukaryotes, and they were found to play crucial roles in plant growth and development.

Genetic forward screens identified the RPD3-like histone deacetylase HDA6 as the sole enzyme responsible for the histone deacetylase step of RNA-directed DNA methylation (RdDM), suggesting that HDA6 might have acquired specific functions for transcriptional silencing processes mediated by small interfering RNAs (siRNAs). RdDM leads to *de novo* methylation of cytosine residues in all sequence contexts within a region of sequence homology to the siRNA trigger, causing transcriptional repression of a varity of transgenes and endogenous loci. A current model proposes that DNA methylation and histone acetylation are acting upstream of one another in a self-reinforcing pathway, thereby serving as control points for switching between the silenced and active states. Given this functional relationship between histone deacetylation and cytosine methylation during RdDM, the role of HDA6 in RNA silencing and epigenetic phenotypes upon HDA6 deficiency were investigated in this study.

To this aim, a series of different allelic mutations in the *Arabidopsis* gene HDA6 (*rts*1-3, *rts*1-4 and *rts*1-5), all encoding enzymatically inactive proteins, were characterized. All alleles, including the previously described *rts*1-1 null allele, exhibit no severe developmental defect but showed a somewhat retarded growth phenotype and delayed flowering. Furthermore, HDA6 deficiency resulted in a drastic suppression of transcriptional gene silencing at investigated transgenic and endogenous loci. This transcriptional reactivation could be correlated with increased euchromatic acetylation marks (H3K9/14ac2) and decreased heterochromatic methylation marks (H3K9me2; H3K27me1). Interestingly, the release of silencing did not correlate with altered DNA methylation levels for all alleles at the soloLTR and, for 2 out of 4 alleles, at the transgenic NOSpro:NPTII. Therefore, it seems likely that DNA methylation is uncoupled from transcriptional silencing as well as from histone deacetylation at some loci. The observed phenotypes (transcriptional reactivation,

increased histone acetylation and decreased histone K9 and K27 di- and monomethylation, respectively) were all complemented in *rts*1-1 mutant lines constitutively expressing a tagged and functional HDA6 allele. These results suggest that all observed effects are due to HDA6 deficiency and that HDA6 is either acting downstream of cytosine methylation or in a parallel silencing pathway. Furthermore, possible additive effects on transcription upon artificially induced DNA demethylation were investigated in this study. A significantly increased transcriptional reactivation compared to mock grown plants could be observed. Interestingly, however, DNA methylation levels were only affected to a minor extent.

Lysine acetylation was shown to preferentially target protein complexes involved in diverse cellular processes, including the DNA damage response. Since HDA6 mutants are sensitive to zebularine, a DNA demethylating drug also known to induce DNA damage, *rts*1-1 sensitivity to different DNA damaging drugs was investigated in this study. An increased hypersensitivity to genotoxic stress was observed, which could be complemented by the expression of a tagged and functional HDA6 allele. This suggests that HDA6 has a dual role as a guardian of both, epigenetic information and genomic stability.

Additionally, the influence of HDA6 on the *Arabidopsis* transcriptome was investigated using Affymetrix ATH1 microarrays. HDA6 could be shown to be both a transcriptional repressor and activator as both up- and downregulated genes were identified upon HDA6 deficiency. It remains to be determined, however, which of these transcriptional changes are direct consequences of the loss of HDA6 function. The analysis of the transcriptome profiling data further suggests diverse roles of HDA6 next to general transcriptional regulation, for example the involvement in responses to abiotic and biotic stresses.

II. Zusammenfassung

Der reversible Prozess der posttranslationalen Histonmodifikationen ist ein wichtiger Mechanismus in der Regulation von Genexpression und Chromatinstruktur. Die Acetylierung der Histon „Tails“ wird durch die enzymatische Aktivität der Histonacetyltransferasen (HATs) katalysiert und korreliert mit transkriptionell aktivem Chromatin. Histondeacetylasen (HDACs) wirken der Acetylierung durch HATs reversibel entgegen und balancieren so den Acetylierungsstatus des Chromatins. Deacetylierte Histone werden mit transkriptionaler Genrepression und der Aufrechterhaltung von Heterochromatin assoziiert. In Pflanzen und anderen Eukaryoten wurde eine Vielzahl von HDACs identfiziert, welche eine wichtige Rolle im Pflanzenwachstum und der Pflanzenentwicklung haben.

Genetische Screens haben die RPD3-verwandte Histondeacetylase HDA6 als einziges Enzym identifiziert, welches für den Deacetylierungschritt während der RNA-dirigierten DNA Methylierung (RdDM) verantwortlich ist. Dies legt den Schluss nahe, das HDA6 spezifische Funktionen für den Prozess der Genrepression mittels „small interfering RNAs“ (siRNAs) erworben hat. RdDM führt zur *de novo* Methylierung von Cytosinen in allen Sequenzkontexten innerhalb der zur siRNA homologen genomischen Sequenz. Diese Methylierung führt zur transkriptionellen Repression von Transgenen und endogenen Zielregionen. In einem aktuellen Modell sind die DNA Methylierung und die Histonacetylierung jeweils dem anderen vorgeschaltet, was zu einen „Feedback Loop“ führt. Hier werden beide epigentischen Modifikationen als mögliche Kontrollpunkte in der Regulation von aktivem und repressivem Chromatin gesehen. Bezugnehmend auf den funktionalen Zusammenhang zwischen DNA Methylierung und Histondeacetylierung wurden in dieser Arbeit die Funktion von HDA6 in der RNA-dirigierten Genrepression und die epigenetisch korrelierenden Phenotypen in der Abwesenheit von HDA6 analysiert.

Zu diesem Zweck wurde eine Serie von HDA6 Mutantenallelen (*rts*1-3, *rts*1-4 und *rts*1-5) charakterisiert, welche jeweils enzymatisch inaktive Proteine kodieren. Alle Allele, inklusive dem bereits beschriebenen *rts*1-1 Nullallel, zeigten keine schwerwiegenden Entwicklungsdefekte, sondern nur einen etwas retardierten Größenphenotyp gepaart mit einer verspäteten Blütezeit. Des Weiteren zeigten alle Mutanten eine drastische Reaktivierung des getesteten Transgenes sowie einiger endogenen RdDM Zielgene. Diese transkriptionelle Reaktivierung korrelierte mit der Zunahme von euchromatischer Histonacetylierung (H3K9/14ac2) sowie mit der Abnahme von repressiven Histonmethylierungen (H3K9me2, H3K27me1). Interessanterweise wurde für keines der getesteten Allele eine Abnahme der DNA Methylierung am soloLTR und, für 2 der 4 getesteten Allele, am transgenen

NOSpro:NPTII festgestellt. Folglich scheint bei einigen Zielgenen DNA Methylierung von transkriptioneller Repression und von Histondeacetylierung unabhängig zu sein. Die beobachteten Phenotypen (transkriptionelle Reaktivierung, induzierte Histonacetylierung und reduzierte Histon K9 und K27 Di- bzw. Monomethylierung) konnten mittels Überexpression eines funktionalen HDA6 Alleles komplementiert werden. Daraus folgt, dass alle beobachteten Effekte auf einer Defizienz von HDA6 beruhen und dass HDA6 entweder nach der DNA Methylierung oder in einem parallelen Mechanismus agiert. Des Weiteren wurde der additive Effekt von artifiziell induzierter DNA Demethylierung auf die Reaktivierung von Zielgenen getestet. Es konnte eine bedeutsame Erhöhung der Trankriptionslevel gezeigt werden. Erstaunlicherweise wurde jedoch keine signifikante Veränderung des DNA Methylierungstatus beobachtet.

In einigen Studien konnte gezeigt werden, dass die Lysinacetylierung von Proteinkomplexen eine wichtige Rolle in verschieden zellulären Prozessen hat, wie z.B. im Reparaturprozess von DNA Defekten. In dieser Arbeit wurde gezeigt, dass alle HDA6 Mutantenallele sensitiv auf Zebularin, einer Substanz die neben DNA Demethylierung auch DNA Defekte auslöst, reagieren. Daher wurde auch die Sensitivität des *rts*1-1 Nullallels auf weitere Substanzen, welche einen DNA Defekt induzieren, getestet. Tatsächlich konnte eine erhöhte Hypersensitivität gegenüber genotoxischem Stress beobachtet werden. Die Überexpression eines funktionalen HDA6 Alleles konnte diese Hypersensitivität allerdings komplementieren. Daraus kann geschlussfolgert werden, dass HDA6 eine Doppelfunktion als „Wächter“ von sowohl epigenetischer Information und der Genomstabilität hat.

Zusätzlich wurde der globale Effekt von HDA6 auf das *Arabidopsis* Transkriptom mittels Affymetrix ATH1 Microarrays getestet. Hier konnte gezeigt werden, dass HDA6 sowohl als transkriptioneller Aktivator wie auch als Repressor fungiert, da sowohl induzierte als auch unterdrückte Gene identifiziert wurden. Auch konnte gezeigt werden, dass HDA6 neben der Rolle als genereller transkriptioneller Regulator möglicherweise in weitere diverse Prozesse involviert ist, wie z.B. in der Antwort auf abiotische und biotische Stresse.

1. Introduction

1.1 Epigenetic gene regulation

Epigenetic gene regulation mediates short term (mitotic) and long term (meiotic) heritable, but dynamic control of gene expression without altering the primary DNA sequence (Bird 2007). This epigenetic memory is achieved by covalent modifications which are superimposed on the DNA sequence and chromatin to form a "second code" (Jenuwein and Allis 2001). Such modifications allow not only the inheritance of gene expression patterns (Ringrose and Paro 2004) but also chromosomal properties such as replication, cohesion, condensation and kinetochore function (Karpen and Allshire 1997; Harvey et al. 2002; McNairn and Gilbert 2003).

Chromatin is a DNA-protein complex consisting of repeating units of nucleosomes, which are composed of two copies of each core histone (H2A, H2B, H3 and H4) encircled by approximately 146 base pairs of DNA (Kornberg and Thomas 1974; Luger et al. 1997; Kornberg and Lorch 1999) (Figure I1). Histone H1 binds to non-nucleosomal "linker" DNA and contributes to DNA packaging by stabilizing the 30 nm chromatin fiber (Baldwin et al. 1975; Shaw et al. 1976; Thoma and Koller 1977). The core histones are subjected to different posttranslational modifications such as acetylation, methylation, phosphorylation, ubiquitination, glycosylation, ADP-ribosylation, carbonylation and sumoylation (Kouzarides 2007) (Figure I1). These modifications are introduced by multiple and highly specific enzymes (Allis et al. 2007; Kouzarides 2007) and occur primarily at their N-terminal tails. As nucleosomes are not static entities, but dedicated to sliding, destabilization or disassembly (Aoyagi et al. 2003; Boeger et al. 2003; Ranjith et al. 2007), nucleosome density together with posttranslational modifications affect the structure and packaging of the chromatin and the DNA accessibility (Kornberg and Lorch 1999; Becker and Horz 2002). As a consequence, the combinatorial set of histone modifications at a given genomic location can alter chromatin conformation to dictate the transcriptional activity of a single loci or a whole chromosomal region (Gaszner and Felsenfeld 2006; Talbert and Henikoff 2006).

The "silent" or "closed" chromatin state is called heterochromatin, whereas the "active" or "open" counterpart is called euchromatin (Grunstein et al. 1995). Euchromatin is gene dense, transcripitonally active and contains only few repetitive elements. Constitutively expressed genes in plants and other organsisms, for example, typically reside within euchromatic regions and often have nucleosome free regions within their promoters (Rando and Ahmad 2007; Zhang et al. 2007). Constitutive heterochromatin, in contrast, is rich in repetitive DNA, such as transposons and other duplicated sequences, permanently condensed,

transcriptionally inert and capable of silencing genes within adjacent euchromatin by spreading, a phenomenon that is called position effect variagation (PEV) (Karpen and Allshire 1997; Hennig 1999). In addition to repressive posttranslational modifications of the histone tails, heterochromatic loci are enriched in DNA methylation (Zhang et al. 2006; Weber and Schubeler 2007).

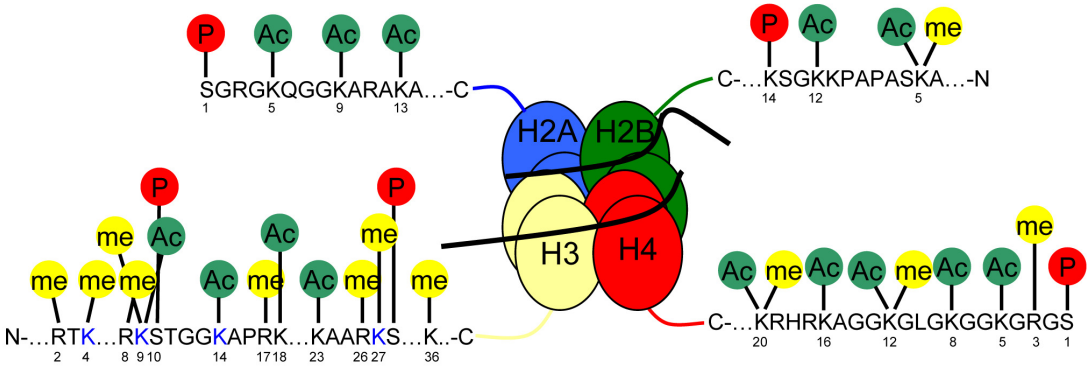

Figure I1. Nucleosome structure and histone tail modifications. Chromatin is a DNA-protein complex consisting of repeating units of nucleosomes, which are composed of two copies of each core histone H2A (blue), H2B (green), H3 (yellow) and H4 (red) encircled by approximately 146 base pairs of DNA (black line). The histone tails can be posttranslationally modified by specific enzymes. These modifications include acetylation (green circles), methylation (yellow circles), and phosphorylation (red circles). Lysine residues investigated in this study for their epigentic marks are highlighted in blue.

1.2 DNA methylation

DNA methylation is an important epigenetic mark involved in diverse biological processes. DNA methylation refers to the addition of a methyl group to either the 5th carbon residue, the 4th nitrogen of the cytosine pyrimidine ring or the 6th nitrogen of the adenine purine ring. DNA methylation is heritable and occurs in both prokaryotes and eukaryotes. In eukaryotes, however, DNA methylation is exclusively found at cytosine residues. (Palmer and Marinus 1994; Martienssen and Richards 1995; Pristas et al. 1998; Bird and Wolffe 1999; Low et al. 2001; Hattman 2005). The DNA methylation landscapes vary between species. Mammalian genomes, for example, exhibit global DNA methylation with the exception of short unmethylated regions called “CpG islands” (Bird 1986; Bird 2002). Fungi and some plants, in contrast, methylate their genome in a mosaic pattern, resulting in domains of heavily methylated DNA that are interspersed with stretches of unmethylated DNA (Bird et al. 1979; Tweedie et al. 1997). Among all eukaryotes, the highest levels of DNA methylation are observed in plants, with up to 50% of cytosines being methylated in some species (Montero et al. 1992). Unlike mammals, which predominantly methylate their genome in CG sequence contexts, plants are able to modify CG, CHG and CHH sites (where H is either T, A or C) (Chan et al. 2005; Henderson and Jacobsen 2007). Deep sequencing of the *Arabidopsis*

genome revealed that approximately 5 % of all cytosines are methylated, with 55% of that methylation in CG, 23% in CHG and 22% in CHH contexts, respectively (Cokus et al. 2008; Lister et al. 2008).

In plants, DNA methylation in all sequence contexts is predominantly established through the RNA-directed DNA methylation pathway (see section 1.6) harnessing the enzymatic activity of *de novo* DNA methyltransferases. The domains rearranged methyltransferase (DRM) proteins DRM1 and DRM2 are orthologs of the mammalian *de novo* methyltransferase DNMT3, although the catalytic domains of the plant DRM proteins are differentially arranged in a linear amino acid sequence (Cao et al. 2000). DRM1 is expressed at a much lower level than DRM2 and *drm*2 mutants recapitulate all tested phenotypes of *drm*1 *drm*2 double mutants. Therefore, DRM2 seems to be the major *de novo* methyltransferases involved in RdDM (Cao and Jacobsen 2002).

In contrast to CHH cytosine methylation, CG and CHG methylation is maintained throughout DNA replication even in the absence of a constant siRNA trigger (Jones et al. 2001; Aufsatz et al. 2002a) by the maintenance DNA methyltransferases MET1 and CMT3. MET1 is the *Arabidopsis* ortholog of the mammalian CG specific maintenance enzyme DNMT1 (Bestor et al. 1988), and like DNMT1 controls CG methylation (Finnegan and Dennis 1993; Finnegan et al. 1996; Ronemus et al. 1996; Kankel et al. 2003; Saze et al. 2003). Mutations in MET1 result in a genome wide loss of CG methylation from 55% in wild-type plants to about 1% in some mutants (Lister et al. 2008) and transcriptional reactivation of many transposons and pseudogenes (Zhang et al. 2006; To et al. 2011). CHG methylation is maintained by CMT3, a member of the chromomethyltransferase class, which is unique to the plant kingdom and characterized by the presence of a chromodomain embedded within the catalytic domain (Henikoff and Comai 1998; Genger et al. 1999). CMT3 loss of function mutants show a reduction of global CHG methylation and more subtle and locus specific effects on asymmetric CHH methylation, indicating that CMT3 also controls CHH methylation at some loci (McCallum et al. 2000; Bartee et al. 2001; Lindroth et al. 2001; Papa et al. 2001; Cao and Jacobsen 2002).

Since epigenetic regulation is potentially reversible, mechanisms must exist to remove cytosine methylation. The removal of DNA methylation can be accomplished either passively or actively. Passive demethylation is achieved by replacing methylated cytosines with unmethylated ones during DNA replication (Saze et al. 2003), whereas active demethylation requires the enzymatic activity of DNA glycosylases followed by base excision repair. The *Arabidopsis* genome encodes a small family of four known DNA glycosylases: ROS1, DME,

DML2 and DML3 (Gong et al. 2002; Penterman et al. 2007; Zhu et al. 2007; Ortega-Galisteo et al. 2008; Zhu 2009). Repressor of silencing (ROS1) is a DNA repair protein shown to repress DNA methylation at numerous endogenous loci including many transposons (Gong et al. 2002; Zhu et al. 2007; Penterman et al. 2007b). The DNA glycosylase Demeter (DME) is preferentially expressed in the central cell of the female reproductive organ and is required for genomic imprinting during female gametophyte development, in the fertilized egg cell and during endosperm formation (Gehring et al. 2006; Morales-Ruiz et al. 2006; Hsieh et al. 2009). The two Demeter-like genes DML2 and DML3 are required for appropriate distribution of DNA methylation marks within the genome (Penterman et al. 2007; Penterman et al. 2007b; Ortega-Galisteo et al. 2008).

1.3 Histone methylation

Protein methylation is a covalent modification commonly occurring on carboxyl groups of glutamate, leucine, and isoprenylated cysteine, or on the side-chain nitrogen atoms of lysine, arginine and histidine residues (Clarke 1993). Histones, which have long been known as subtrates for methylation, are, however, only methylated on lysines (K) or arginines (R) (Murray 1964) (Figure I1). Histone methylation plays a fundamental role in epigenetic regulation and chromatin formation and is one of the most important and complex epigenetic marks, since it can occur at different degrees on a given residue (Bannister and Kouzarides 2005).

Crucial roles of arginine methylation in transcriptional regulation, RNA processing, nuclear transport, DNA damage response (DDR) and signal transduction are just emerging (Bedford and Richard 2005). Histone arginine methylation can occur in the mono- (me1) or dimethylated (me2) state, with the latter in symmetric or asymmetric configuration and contributes to both active and repressive effects on chromatin function. Arginine methylation is catalyzed by the PRMT (protein arginine methyltransferases) class of histone methyltransferases and is typically found on histone 3 residues 2, 8, 17 and 26 (H3R2, H3R8, H3R17 and H3R26) and residue 3 of histone H4 (H4R3) (Chen et al. 1999; Strahl et al. 2001; Zhang and Reinberg 2001; Wysocka et al. 2006; Yu et al. 2006).

Histone lysine methylation is another important and complex epigenetic mark that can also occur in multiple methylated states (mono-, di- and trimethylation). Depending on which lysine residues are methylated and the degree of methylation, it can contribute to both transcriptionally active and silent chromatin domains. In particular, repressive histone marks such as H3K9, H3K27 and H4K20 are generally found in heterochromatin and silent regions,

whereas permissive marks like H3K4 and H3K36 are associated with active regions of euchromatin (Grasser 2005; Berger 2007; Volkel and Angrand 2007; Zhou 2009). The complexity of lysine methylation contributes to an expanded potential to encode epigenetic information of different "flavor". Defined methylation states, for example, can lead to differing functional consequences, as effector proteins might only recognize specific modification states while having comparatively little affinity to others (Shi et al. 2006; Wysocka et al. 2006b; Shi et al. 2007). This specificity is also mirrored by the fact that histone lysine methyltransferases (HKMTs) are capable of only catalyzing defined and exclusive methylation states (Xiao et al. 2003). In plants, all known HKMTs have an evolutionary conserved protein domain, the SET (Suppressor of variegation (Su(var)), Enhancer of zeste (E(Z)) and Trithorax) domain (Baumbusch et al. 2001). The SET domain constitutes the catalytic activity of the Set domain group (SDG) protein superfamily (Qian and Zhou 2006; Gendler et al. 2008). The *Arabidopsis* genome encodes 49 putative SET domain containing proteins (Baumbusch et al. 2001; Ng et al. 2007; Gendler et al. 2008), which are classified into four categories: (I) SU(VAR)3-9 group, including SU(VAR)3–9 homologs (SUVH) and SU(VAR)3–9 related proteins (SUVR), (II) E(Z) (enhancer of zeste) homologs, (III) TRX (trithorax) group, including TRX homologs and TRX-related proteins, and (IV) ASH1 (absent, small, or homeotic discs 1) group with ASH1 homologs (ASHH) and ASH1-related proteins (ASHR) (Baumbusch et al. 2001; Springer et al. 2003; Zhao and Shen 2004; Ng et al. 2007). HKMTs belonging to the SU(VAR)3-9 group have, additional to the SET domain, a pre-SET, a post-SET and a characteristic SRA domain which serves as a methylcytosine-binding motif in both animals and plants, thus interconnecting DNA methylation and histone methylation (Citterio et al. 2004; Unoki et al. 2004; Woo et al. 2007; Kraft et al. 2008; Woo et al. 2008). Different studies on SUVH1, SUVH5 and SUVH6 indicate that these proteins are H3K9 methyltransferases, raising the possibility that all SUVH proteins methylate H3K9 (Ebbs et al. 2005; Naumann et al. 2005; Ebbs and Bender 2006) and thereby regulate the activity of loci present both in euchromatic and heterochromatic regions and telomere stability (Grafi et al. 2007). *Arabidopsis* HKMTs homologs of Enhancer of Zeste E(Z) are Curly leaf (CLF), Medea (MEA) and Swinger (SWN) (Guitton and Berger 2005). They have H3K27 methyltransferase activity and are components of *Arabidopsis* Polycomb Repressive Complex 2 (PRC2)-like complexes that function as transcriptional regulator during plant development (Kohler et al. 2003; Makarevich et al. 2006; Jiang et al. 2008). The TRX group of HKMTs encodes homologs of Trithorax and are involved in flowering time regulation (Alvarez-Venegas et al. 2003; Pien et al. 2008; Saleh et al. 2008;

Avramova 2009). Studies on ATX1 and ATX2 indicate that this class of HKMTs specifically methylates H3K4, and potentially H3K36 (Pien et al. 2008; Saleh et al. 2008; Berr et al. 2009). HKMTs implicated in H3K36 methylation also belong to the ASH1 group of histone lysine methyltransferases. Proteins of this group were shown to be involved in flowering time regulation, pollen and stamen development as well as fertility (Zhao et al. 2005; Cartagena et al. 2008; Thorstensen et al. 2008).

Even though histone methylation was thought to be a permanent modification, it is now known to be dynamically regulated by writers and erasers. Histone demethylases play vital roles in regulating histone methylation homeostasis and are divided into two classes habouring distinct mechanisms (Liu et al. 2010). The first histone demethylase discovered was the lysine specific demethylase 1 (LSD1) (Shi et al. 2004; Metzger et al. 2005), also known as AOF2 and KDM1. LSD1 belongs to the first group of histone demethylases, the flavin adenine dinucleotide (FAD)-dependent enzyme family which only act on mono- and di-methylated lysines. The second class of histone demethylases encompasses a large protein family of Jumonji C (JmjC) domain containing proteins (Klose et al. 2006). The demethylation reaction is carried out by JmjC domain, which is conserved from bacteria to eukaryotes and belongs to the cupin superfamily of metalloenzymes (Clissold and Ponting 2001). These metalloenzymes allow the removal of mono-, di- and trimethylated lysines in the presence of Fe(II) and α-ketoglutarate as cofactors (Tsukada et al. 2006; Couture et al. 2007; Ng et al. 2007b). JmjC proteins can also demethylate arginine residues (Chang et al. 2007), and, at least in theory, other protein substrates or nucleotides.

1.4 Histone acetylation and deacetylation

The reversible posttranslational acetylation of core histones is a process involving histone acetyltransferases (HATs) and histone deacetylases (HDACs) as co-regulators of transcription (Brownell and Allis 1996; Kuo and Allis 1998; Roth et al. 2001). Histone acetylation occurs through the action of HATs which transfer the acetyl moiety of acetyl-coenzyme A (acetyl-CoA) to the ε-amino group of lysine residues in all core histones, mainly at the tails but also at a few residues within the globular domain (Berger 2007). This reaction can be reversed by HDACs (Figure I2).

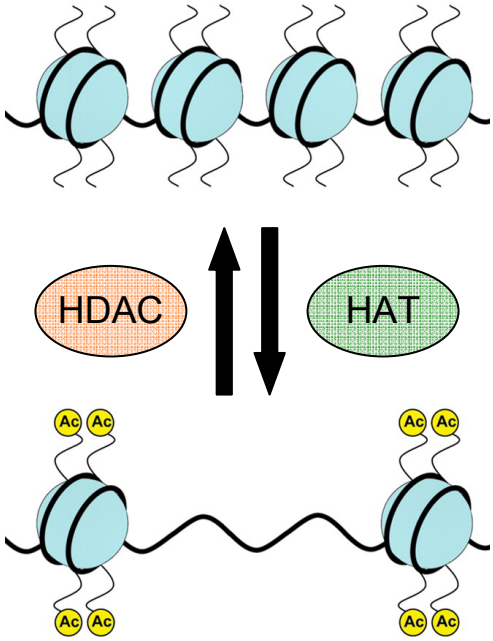

Figure I2. Histone acetylation and deacetylation. Histone acetylation is a reversible posttranslational modification catalyzed by the enzymatic activity of histone acetyltransferases (HATs). HATs transfer an acetyl group to the ε-amino group of lysine residues, predominantly within histone tails. The acetyl group can be removed by the action of histone deacetylases (HDACs), which contribute to balancing the chromatin acetylation state. Acetylated histones are associated with transcriptionally active chromatin whereas deacetylated histones correlate with transcriptionally repressive chromatin.

1.4.1 Histone acetyltransferases (HATs)

HATs are evolutionary conserved from yeast to mammals and generally exist as multisubunit complexes. The functions of the catalytic subunit depend largely on the context of the other complex subunits, the auxiliary proteins, which are required for enzymatic activity and targeting (Lee and Workman 2007; MacDonald and Howe 2009). Based on their cellular distribution and mechanism of catalysis, HATs are classified into two categories, HAT A and HAT B (Brownell and Allis 1996; Roth et al. 2001).

Members of the HAT A family are found in the nucleus where they are responsible for acetylation of nuclear histones after their incorporation into nucleosomes. Thus, HAT A family members are directly involved in regulating chromatin assembly and gene transcription. The type A HATs can further be divided into three subclasses, depending on their homology with yeast proteins. Based on their catalytic domain, the GNAT (Gcn5 N-acetyltransferase) and the MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60) family was named according to their founding members (Borrow et al. 1996; Reifsnyder et al. 1996; Neuwald and Landsman 1997). The GNAT subclass is the best understood set of HATs, which have been grouped together on the basis of their similarity in several homology regions and acetylation related motifs (Roth et al. 2001). Four sequence motifs (C, D, A and B; in N- to C-terminal order) define this family, even though their functions are not yet fully understood. Of

particular note is motif A, which is the most highly conserved and is shared with the MYST subclass of HATs (Sterner and Berger 2000). It contains an Arg/Gln-X-X-Gly-X-Gly/Ala segment that is important for acetyl-CoA substrate recognition and binding (Dutnall et al. 1998; Wolf et al. 1998). The MYST subfamily members are defined by a distinct conserved acetyltransferase domain. The MYST domain contains a C_2HC zinc finger and includes a part of motif A of the GNAT superfamily (Neuwald and Landsman 1997; Sterner and Berger 2000; Sapountzi and Cote 2011). Furthermore, individual members of the MYST family contain additional structural features, such as chromodomains, PHD (plant homeo domain) and zinc fingers (Sapountzi and Cote 2011). Proteins belonging to the third subclass ("orphan class") possess intrinsic HAT activity, though without a true consensus HAT domain (Lee and Workman 2007; Yang and Seto 2007). The "orphan class" includes proteins with orthologs in many eukaryotes, including plants (e.g p300/CBR, $TAF_{II}250$, Elp3, Hpa2) as well as HATs specific to mammals, that lack orthologs in plants, fungi or other animals (e.g ACTR/SRC1).

The members of the HAT B family are cytoplasmic proteins that catalyze the acetylation of free histones, pior to their deposition into newly replicated chromatin (Parthun et al. 1996; Verreault et al. 1998). HAT1 is, up to date, the sole known example of a type B histone acetyltransferases and is highly active on free histone substrates but has no detectable activity on nucleosomal histones (Parthun et al. 1996). HAT1 has high specificity for histone H4, where it modifies H4K5 and H4K12. This pattern of acetylation is found consistently on newly synthesized histone H4 (Kleff et al. 1995; Parthun et al. 1996).

Since HAT complexes are composed of various subunits, each complex might have exclusive features making it capable to perform specific and unique functions. One example highlighting the dichotomy between overlapping substrates and specialized functions comes from yeast. The SAGA complex preferentially modifies H3K9 and, to lesser extent H3K14, whereas the NuA3 complex preferentially modifies H3K14 (John et al. 2000). On the other hand, SAGA and the Elongator complex have overlapping substrate specificity, but the Elongator complex is thought to function in gene coding regions, rather than at promoters (as the SAGA complex does) to acetylate nucleosomes during transcription (Wittschieben et al. 1999). HAT recruitment to the appropriate locations is also specific for each complex, and is determined through distinct auxiliary proteins. These proteins might possess specific chromatin binding domains that recognize modified histone tails, like bromo- and chromodomains, WD40 repeats, Tudor domains and PHD fingers.

The *Arabidopsis* genome is predicted to encode 12 histone acetyltransferases, of which five belong to the GNAT/MYST family (HAG1 to HAG5), and seven to the "orphan

class" of HATs (HAC1, HAC2, HAC4, HAC5, HAC12 with similarities to CBP and HAF1 and HAF2 similar to the $TAF_{II}250$ gene family) (Pandey et al. 2002). Within the GNAT/MYST family, *Arabidopsis* appears to have the same representation of HATs as animals, suggesting that the plant proteins may form complexes similar to those found in yeast and animals (Ogryzko 2001). The *Arabidopsis* CBP and $TAF_{II}250$ family, however, seems expanded, as it harbors 5 and 2 predicted HATs proteins, respectively. Searches against the complete *C.elegans*, *D.melanogaster*, *S.pombe, S.cerevisiae* and human genome identified only one homolog of the $TAF_{II}250$ family and one to two homologs of the CBP protein family in each organism (Pandey et al. 2002).

1.4.2. Histone deacetylases (HDACs)

Histone deacetylases are the enzymatic counterpart of the HATs described above. They remove acetyl groups from histone tail lysines, thereby contributing to balance the chromatin acetylation state. Histone deacetylases constitute an ancient enzyme family, are conserved from yeast to plants and animals and are also found in eubacteria and archaebacteria (Leipe and Landsman 1997; Gregoretti et al. 2004). HDACs are central players in the area of posttranslational modifications, and they themselves are regulated by covalent modifications after translation. Depending on the type of posttranslational modifications, HDACs aquire different levels of enzymatic activity, shuffle between different complexes or are targeted for degradation (Brandl et al. 2009). Known HDACs are grouped into five classes, according to phylogenetic analyses and sequence homology to the corresponding yeast proteins. Class I, II and III consist of enzymes homologous to yeast RPD3 (*r*educed *p*otassium *d*ependency protein 3), HDA1 (*h*istone *dea*cetylase 1) and SIR2 (silent information regulator 2) proteins, respectively (Wu et al. 2003). A fourth class of HDACs, the HD2-like proteins, are plant specific histone deacetylases (Brosch et al. 1996; Lusser et al. 1997) (Figure I3). HDAC11 is so far the only member of class V HDACs. Even though it shows sequence homology to class I and II HDACs and is conserved from *C.elegans* and *D.melanogaster* to humans, phylogenetic analysis indicated that this deacetylase and its orthologs belong to a separate class (Gao et al. 2002; Gregoretti et al. 2004; Yang and Seto 2008).

Class I (RPD3-like HDACs) are ubiquitously expressed in all tissue types (Witt et al. 2009). They are sensitive to the HDAC inhibitor (HDACi) trichostatin A (TSA) and are predominantly localized in the nucleus (Luo et al. 2001). Class I HDACs are mostly found in large multisubunit complexes that mediate their target specificity (Oberdoerffer et al. 2008)

and are involved in diverse biological processes, e.g. cellular proliferation, cell cycle regulation, apoptosis and DNA damage response (Bhaskara et al. 2008; Eot-Houllier et al. 2008; Kachhap et al. 2010; Miller et al. 2010; Zupkovitz et al. 2010). Sequence analysis revealed that most class I HDACs contain a large conserved domain homologous to the N-terminal region of yeast RPD3 and a short C-terminal region with a more variable sequence (Khochbin and Wolffe 1997). In yeast and mammals, the RPD3-like HDACs mediate transcriptional repression by interacting with specific DNA-binding proteins and association with corepressor complexes (Alland et al. 1997; Hassig et al. 1997; Kadosh and Struhl 1997). In mammals, class I HDACs are known to associate with different multiprotein complexes, including SIN3, NuRD (nucleosome remodelling deacetylase), CoREST (corepressor of RE1-silencing transcription factor) and N-CoR (nuclear receptor corepressor) (Hassig et al. 1997; Laherty et al. 1997; Tong et al. 1998; Zhang et al. 1998; Ayer 1999; Humphrey et al. 2001; You et al. 2001; Zhang et al. 2002). Yeast RPD3 is tightly associated with SIN3 in large multiprotein complexes that repress target genes involved in diverse processes, such as meisosis, cell type specificity, potassium transport, phosphate and phospholipid metabolism, methionine biosynthesis, and responses to stress and developmental changes. There is evidence that *Arabidopsis* RPD3-like HDACs also assemble in SIN3-like multisubunit complexes (Song et al. 2005; Rakic 2010).

The class II includes HDACs that possess catalytic domains similar to the yeast enzyme HDA1 (histone deacetylase 1) and share a common structural organization, with a carboxyl-terminal catalytic domain and an amino-terminal extension (Zhang et al. 2001). Based on sequence homology, they are further subdivided into subclasses class IIa and class IIb (Fischle et al. 1999; Grozinger et al. 1999; Miska et al. 1999; Fischer et al. 2002; Gao et al. 2002; Guardiola and Yao 2002; Kao et al. 2002; Tong et al. 2002). Members of this family are TSA sensitive and localize to both the nucleus and the cytoplasm, where they potentially undergo stimulus dependent shuttling to the nucleus (Kao et al. 2001). HDA1-like HDACs are expressed in a tissue specific manner and are modulated by several signal transduction pathways (Khochbin et al. 2001; Kramer et al. 2001; Grozinger and Schreiber 2002; de Ruijter et al. 2003; Hildmann et al. 2007). Class II HDACs are also part of large multiprotein complexes, like N-CoR and SMRT (silencing mediator for retinoid and thyroid receptors) (Huang et al. 2000). Other interactions occur with CtBP (COOH-terminal binding protein) (Zhang et al. 2001), 14-3-3 proteins (Grozinger and Schreiber 2000; McKinsey et al. 2000; Wang et al. 2000), calmodulin (McKinsey et al. 2000b), heterochromatin protein HP1a (Verdin et al. 2003) and SUMO (David et al. 2002; Kirsh et al. 2002).

Class III enzymes correspond to the Sirtuin family. Their enzymatic activity depends on the cofactor NAD^+, rather then zinc, and they localize to the nucleus, the cytoplasm or the mitochondria (Tamburini and Tyler 2005). The founding member of class III HDACs is the yeast SIR2 protein which has a documented function in transcriptional repression at rDNA loci (Bryk et al. 1997; Smith and Boeke 1997), telomers (Gottschling et al. 1990; Palladino et al. 1993) and the silent mating-type loci (Klar et al. 1981; Nasmyth et al. 1981; Rine and Herskowitz 1987). Furthermore, SIR2 has been implicated in the repair of double strand breaks (DSB), cell cycle progression, chromosomal stability and plays a pivotal role in the molecular mechanism of aging in *S.cerevisiae* and *C.elegans* (Brachmann et al. 1995; Martin et al. 1999; Mills et al. 1999; Lin et al. 2000; Tissenbaum and Guarente 2001). The mammalian SIR2 homolog SIRT1 controls the cellular response to stress by regulating the FOXO family of Forkhead transcription factors, leading to an increase in organismal longevity (Brunet et al. 2004). Unlike that of other HDAC families, Sirtuin deacetylase activity cannot be inhibited by known HDACi. SIR2-like proteins are conserved among species from bacteria to human with apparent homologs also in plants (Brachmann et al. 1995). In *Arabidopsis,* two SIR2 homologs are identified (Figure I3), but so far little is known about their functional role and targets (Pandey et al. 2002).

The plant specific HD2-like HDAC family (Figure I3) does not share sequence similarities with other known HDAC proteins (Pandey et al. 2002), is not found in animals and fungi (Lusser et al. 1997) and is distantly related to the FKBP family of peptidyl-prolyl *cis-trans* isomerases found in insects, *S.cerevisiae* and parasitic apicomplexans (Aravind and Koonin 1998). HD2-like proteins are comprised of a conserved N-terminal domain, a central acidic domain and a variant C-terminal domain (Pandey et al. 2002). Four HD2 homologs have been identified in *Arabidopsis* (Figure I3), where their similar mRNA expression profiles suggest potential functional redundancy (Zhou et al. 2004; Hollender and Liu 2008). With the exception of HDT4, the other three HD2-like HDACs have been characterized. Reporter gene assays demonstrated that HDT1, HDT2 and HDT3 mediate transcriptional gene repression through interactions with transcription factors (Wu et al. 2003). HDT1, like the RPD3-like HDAC HDA6, plays a role in rRNA silencing and nucleolar dominance (Earley et al. 2006). Furthermore, HDT3 mediates abscisic acid (ABA) responses and is likely to be indirectly involved in stress-induced gene expression (Sridha and Wu 2006). Therefore, HD2-like HDACs may function in the same biological processes as RPD3-like HDACs, for example by modulating gene expression in complexes containing both HD2-like and RPD3-like HDACs.

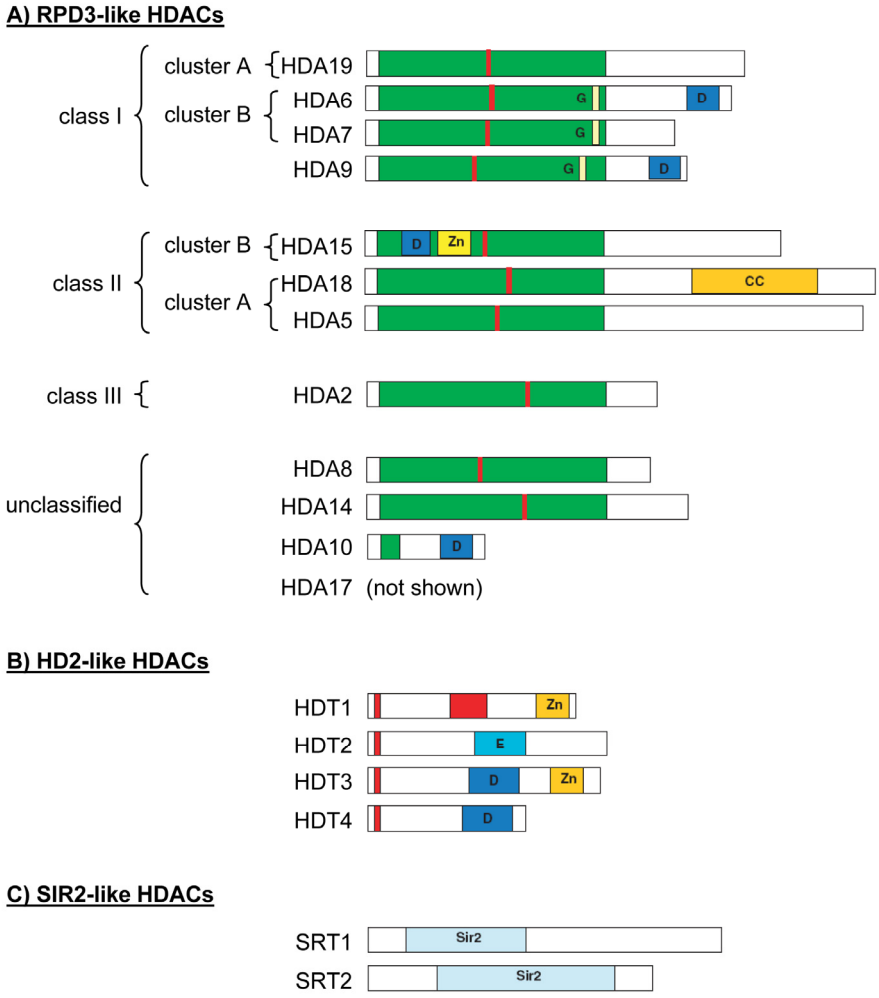

Figure I3. Classification and domain organization of *Arabidopsis thaliana* histone deacetylases. HDACs are classified into 3 superfamilies, the RPD3-like HDACs, the HD2-like HDACs and the SIR2-like HDACs. (A) The RPD3-like HDACs are further subdivided into three classes and unclassified HDACs. The green boxes depict the conserved HDAC domain, red regions represent the active sites necessary for the enzymatic activity. The domain structure of HDA17, which is similar to that of HDA9, is not shown. (B) HD2-like HDACs are plant specific HDACs and do not share sequence similarities with other known HDAC proteins. The red bar at the amino terminus represents the conserved EFWG region required for repression. (C) The SIR2-like HDACs family is represended by two homologs in *Arabidopsis*. The enzymatic activity of this family depends on the cofactor NAD^+. The blue box represents the conserved SIR2 domain. In all cases, G, D and E represent high glycine-, aspartate- and glutamate-rich regions, respectively. CC represents a coiled-coil domain, and Zn represents a zinc finger domain. (adapted from Hollender and Liu 2008)

1.4.2.1 *Arabidopsis* RPD3-like HDACs

The RPD3-like superfamily of HDACs in *Arabidopsis* comprises 12 putative members, which all have a characteristic histone deacetylase domain. Based on sequence similarity, they are further subdivided into three classes and some unclassified HDACs (Figure I3) (Pandey et al. 2002). The *Arabidopsis* genome encodes three class II proteins (HDA5, HDA15 and HDA18), which form two clusters. HDA15 is the sole HDAC forming cluster B. Cluster A comprises of HDA5 and HDA18, which appear to be closely related to the two tandem HDAC domain proteins in animals, suggesting that they may act on proteins

other than histones (Pandey et al. 2002). The four HDACs of class I, together with the yeast RPD3 protein and several animal class I proteins, also fall into two clusters. HDA19 is the only RPD3-like *Arabidopsis* HDAC belonging to cluster A, together with *Mc*Hdeac1, *Zm*RPD3, *Os*HDA702 and *Gm*HDA1201. These proteins share high sequence homology and may comprise an orthologous group. The proteins in cluster B (HDA6, HDA7 and *Zm*HD1bII) are more divergent than the proteins in cluster A, supporting functional diversification among the clusters. HDA9, not falling into any cluster, shows similarity at the nucleotide level to HDA10 and HDA17, which belong to the unclassified RPD3-like HDACs in *Arabidopsis*. Among the RPD3-like HDACs of class I, HDA6 and HDA19 are the best characterized and exhibit divergent as well as overlapping functions.

Among all *Arabidopsis* HDACS, HDA19 is the best studied. HDA19 is constitutively expressed in all tissues and throughout the plant life cycle (Wu et al. 2000; Zhou et al. 2005), is localized to the nucleus, presumably to euchromatic regions, and is excluded from the nucleolus (Fong et al. 2006). HDA19 is a global regulator of gene expression, since over 7 % of the genome is either up- or downregulated in *hda*19 mutants (Tian et al. 2005). A range of developmental abnormalities, including early senescence, serrated leaves, aerial rosettes, defects in floral organ identity and late flowering were observed in loss of function mutants, highlighting a role of HDA19 in plant development (Wu et al. 2000; Tian and Chen 2001; Tian et al. 2003; Tian et al. 2005). Further studies indicate that HDA19 regulates plant stress and basal defense responses (Kim et al. 2008; Chen and Wu 2010) and, together with HDA6, redundantly controls the repression of embryonic properties after germination (Tanaka et al. 2008).

HDA6 encodes another RPD3-like histone deacetylase (Wu et al. 2000; Murfett et al. 2001) and is expressed in various tissues, including leaf, flower, siliques and young seedlings at a lower level, however, than HDA19 (Gendler et al. 2008) (www.chromdb.org). HDA6 localizes to the nucleoplasm and to the nucleolus (Earley et al. 2006; Rakic 2010). Given the highly similar gene expression profiles and sequence similarity between HDA6 and HDA19 (Hollender and Liu 2008), it is likely that HDA6 and HDA19 regulate, perhaps redundantly, many of the same processes, including suppression of embryonic programs after germination, mediation of jasmonic acid (JA) and ethylene signalling pathways, and promotion of flowering and senescence (Zhou et al. 2005; Tanaka et al. 2008; Wu et al. 2008). Mutations in HDA6 affect the expression of transgenes, transposable elements and endogenous genes, DNA methylation, and regulation of rRNA genes (Murfett et al. 2001; Aufsatz et al. 2002b; Probst et al. 2004; Earley et al. 2006; To et al. 2011; Yu et al. 2011; this study). Interestingly,

HDA6 is so far the only HDAC known to be involved in one type of RNA silencing, called RNA-directed DNA methylation (RdDM) (see section 1.6). Several highly saturated screens recovered HDA6 as a crucial factor in this pathway (Murfett et al. 2001; Aufsatz et al. 2002b), suggesting that HDA6 might have acquired specific functions for RNA-directed transcriptional silencing processes.

Other members of the RPD3-like HDAC family, HDA2 in class III, and the unclassified enzymes HDA8, HDA10, HDA14 and HDA17 are not well characterized.

1.4.3 Histone de/acetylation and gene regulation

The acetylation of histone lysine residues is correlatively associated with transcriptional gene activation (Brownell and Allis 1996; Wolffe and Pruss 1996; Grunstein 1997), and genome wide studies showed that the bulk of acetylated histones H3 and H4 are enriched in euchromatin, whereas hypoacetylated histones associate with repressed genes and heterochromatic loci (Vogelauer et al. 2000; Kurdistani et al. 2002; Robyr et al. 2002; Kurdistani et al. 2004; Roh et al. 2005). The exact mechanism by which histone acetylation renders chromatin transcriptionally active is still not exactly understood, however, several alternative explanations are possible. First, the introduction of an acetyl group might neutralize the positive charge of lysine residues, thereby increasing hydrophobicity and weakening the interaction of the histone octamer with the negatively charged DNA (Hong et al. 1993). Thus, the nucleosomes would become destabilized and allow transcriptional regulators to gain access to the DNA (Norton et al. 1989; Kuo and Allis 1998). Indeed, studies using nucleosomal templates reconstituted either with bulk, relatively underacetylated histones, or with hyperacetylated histones, demonstrated that histone acetylation does facilitate the access of transcription factors to the nucleosomal DNA templates and hence enhances transcription (Lee et al. 1993; Vettese-Dadey et al. 1996; Ura et al. 1997). A second hypothesis suggests that the acetyl group might interfere with the higher order packing of chromatin. This might alter the interaction between neighbouring nucleosomes, thereby rendering larger chromatin areas accessible to regulatory proteins (Bauer et al. 1994; Garcia-Ramirez et al. 1995; Toth et al. 2006; Ferreira et al. 2007). The third hypothesis considers histone acetylation in the context of the histone code. Here acetylation, next to histone methylation, could act as a specific signal that alters histone-protein interactions, resulting in recruitment of specific chromatin associated proteins (Tse et al. 1998; Strahl and Allis 2000; Jenuwein and Allis 2001). This possibility is supported by the fact that acetylated histones serve as binding sites for bromodomain containing proteins (Sterner and Berger 2000;

Mujtaba et al. 2007) and that non-histone proteins are also substrates of HATs and HDACs (Sterner and Berger 2000).

1.4.4 Histone de/acetylation in plant responses to environmental signals

The interplay between HATs and HDACs and their role in transcriptional regulation is associated with developmental processes and environmental conditions, including day length, flowering regulation (He et al. 2003; Ausin et al. 2004; Kim et al. 2004), osmotic and oxidative stress (Brunet et al. 2004; De Nadal et al. 2004) and cell aging (Imai et al. 2000). As plants are sessile organisms that cannot choose their living environments, they essentially have to develop rapid responses to changes in environmental conditions for their adaptation and survival.

Light signals, for example, are amongst the most important environmental factors regulating plant growth and development throughout the entire life cycle (Neff et al. 2000; Franklin and Whitelam 2004). Studies from tobacco green shoots could show that light induction of the pea plastocyanin gene (*PetE*) is associated with hyperacetylation of histones H3 and H4 (Chua et al. 2001; Chua et al. 2003), suggesting a role for histone acetylation in a regulatory switch that integrates light signals into the control of gene transcription. Next to histone acetylation, histone deacetylation was demonstrated to be equally important in processing light signals. The *Arabidopsis* HDA6, together with the photoreceptor phytochrome B (PHYB), were shown to control light-dependent chromatin organization (Tessadori et al. 2009). The expression of phytochrome A (PHYA), which is the major photoreceptor of deetiolation, is reversibly repressed by light. Transcriptional repression upon light was proven to be accompanied by HDA19 mediated deacetylation of H3K9/14 (Jang et al. 2011). Other genetic studies of *Arabidopsis* mutants deficient in histone acetyltransferases (TAF1/HAF2 and GCN5) or histone deacetylases (HDA19) provide further evidence for the importance of histone acetylation and deacetylation in the expression of a number of photosynthetic genes (Bertrand et al. 2005; Benhamed et al. 2006).

Additionally, histone deacetylation activity plays a vital role in flower time regulation. In *Arabidopsis*, HDA6 is involved in flowering by epigentically controling FLC expression, since *hda*6 mutants have increased FLC transcription levels (Wu et al. 2008; Yu et al. 2011; this study). The regulatory mechanism involves proteins of the autonomous flowering control pathway such as FLD and FVE. Both proteins participate in chromatin deacetylation as component of an HDAC complex that regulates FLC expression (He et al. 2003; Ausin et al. 2004; Kim et al. 2004; Yu et al. 2011).

The responses of plants to abiotic and biotic stresses further involves plant hormones, such as ethylene, abscisic acid (ABA) and jasmonic acid (JA) (Finkelstein et al. 2002; Wang et al. 2002). Several histone deacetylases and proteins interacting with them have been identified as factors that contribute to those respective responses, suggesting their involvement in integrating hormone signals to modulate stress responsive gene expression (Devoto et al. 2002; Zhou et al. 2005; Sridha and Wu 2006). Upon treatment with ethylene and JA, the expression of both HDA6 and HDA19 is induced. Furthermore, HDA19 expression is increased by wounding and pathogens. Recently, it was demonstrated that HDA6 interacts with COI1, an F-box protein involved in JA-mediated plant defence responses (Devoto et al. 2002). Although the interaction of HDA19 with COI1 has not yet been shown, overexpression of HDA19 induces ethylene and JA-regulated pathogenesis-related (PR) gene expression, resulting in an increased resistance to the pathogen *Alternaria brassicicola* (Zhou et al. 2005). These results suggest a possible role of HDA6 and HDA19 in ethylene and JA signaling and plant-pathogen interactions. Further evidence that HDACs are involved in ABA and stress responses originates from a study demonstrating that ERF7 interacts with the *Arabidopsis thaliana* homolog of a human global corepressor of transcription, SIN3. ERF7 is an apetala2/erebp-type transcription factor that plays an important role in ABA responses (Song et al. 2005). Interestingly, *Arabidopsis* SIN3 orthologs interact with HDA19 (Song et al. 2005) and with HDA6 (Rakic 2010).

1.4.5 Histone de/acetylation in DNA repair

DNA damage is the consequence of errors, which occur during DNA replication or is triggered by exogenous agents, which directly damage the DNA. DNA repair pathways occur in all eukaryotes on nucleosomal DNA, and it has long been suspected that chromatin modifications such as histone acetylation might play a role in this process. Repair synthesis is significantly enhanced by hyperacetylated nucleosomes (Ramanathan and Smerdon 1989), and both HATs and HDACs influence the DNA damage response (DDR) through the acetylation of key DNA repair and checkpoint proteins (Choudhary et al. 2009). First evidence that histone acetyltransferases are involved in DDR and DNA repair resulted from transfection experiments in HeLa cells. There, the ectopic expression of mutated TIP60 lacking histone acetylase activity resulted in defective double stand break (DSB) repair (Ikura et al. 2000). Further studies demonstrated that TIP60, an ubiquitously expressed mammalian acetyltransferase, plays a key role in DSB repair, and is required for the maintenance of genomic integrity and the regulation of DNA damage repair (Bird et al. 2002b; Sun et al.

2005). The human histone deacetylases HDAC1 and HDAC2 respond to DNA damage by mediating changes in histone acetylation. HDAC1 and HDAC2 depleted cells are hypersensitive to DNA damaging agents and show sustained DNA damage signalling, suggesting that these enzymes serve as important components of the DDR by promoting DNA damage signalling and repair (Miller et al. 2010). Additionally, the inhibition of mammalian histone deacetylation by HDACi was shown to induce cell cycle arrest, to activate the apoptotic extrinsic pathway and to result in the downregulation of numerous DDR and repair pathway genes (Kachhap et al.; Martin-Sanchez et al. 2011). In yeast, HDAC inhibition showed similar effects in counteracting DSB processing, single strand DNA-RFA (replication factor A) nucleofilament formation and the induction of autophagy of SAE2 (Robert et al. 2011), which is a cyclin dependent kinase 1 (CDK1) target involved in DSB processing (Huertas et al. 2008). Furthermore, homologous recombination in yeast, a DSB repair pathway, triggers the acetylation of N-terminal lysines on histones H3 and H4 flanking a DSB, followed by their deacetylation. This acetylation/deacetylation cycle seems to be controlled by the histone acetyltransferases GCN5 and ESA1 and the histone deacetylases RPD3, SIR2, and HST1, since all of these factors are recruited to the HO lesion during repair by homologous recombination (Downs et al. 2004; Tamburini and Tyler 2005). In summary, these findings suggest that dynamic changes in histone acetylation accompany DDR and DNA repair pathways and that the ability to modulate histone acetylation is essential for the viability of organisms upon DNA damage.

1.5 RNA silencing in plants

RNA silencing is a widespread and fundamental component of gene expression. This process is found in a large variety of eukaryotes such as plants, fungi and animals. Although respective pathways may differ in detail, they result from the same highly conserved mechanism, indicating an ancient origin (Baulcombe 1996; Sanchez Alvarado and Newmark 1999; Cogoni and Macino 2000; Plasterk and Ketting 2000; Carthew 2001; Sharp 2001; Tuschl 2001; Vance and Vaucheret 2001). The term RNA silencing collectively refers to diverse RNA-based processes that main characteristic is the use of small RNA (sRNA) molecules. These typically are 20-26 nucleotide (nt) in length and confer high specificity to a target sequence by exploiting sequence homology. RNA silencing results in sequence specific inhibition of gene expression, either at the levels of transcription, mRNA stability or translation (Baulcombe 2004; Grewal and Rice 2004; Lippman and Martienssen 2004; Almeida and Allshire 2005; Brodersen and Voinnet 2006; Buhler et al. 2006). All of those

processes share three mechanistic features: (I) the formation of double stranded RNA (dsRNA), (II) the processing of the dsRNA by a dsRNA-directed endonuclease activity, termed Dicer, that processes dsRNA into sense and antisense sRNAs, and (III) the targeting of a sRNA loaded inhibitory effector complex, RISC (RNA-induced silencing complex), which contains a member of the Argonaute (AGO) protein family, to complementary DNA or RNA to induce silencing.

Plants encode multiple RNA silencing components, including four Dicer-like proteins (DCL1 to DCL4), ten Argonautes (AGO1 to AGO10) and six RNA-dependent RNA polymerases (RDR1 to RDR6), implying specialized functions and a diversified tool kit for conducting RNA silencing. Although there are some overlaps and shared components, three major silencing systems with dedicated functions, including regulation of endogenous gene expression, transposon taming, viral defense and heterochromatin formation, can be distinguished in plants (Meins et al. 2005) (Figure I4).

The transgene-related posttranscriptional and virus-induced gene silencing (PTGS/VIGS) pathway primarily functions as a host defense to foreign or invasive nucleic acids, including viruses, transposons and transgenes (Figure I4A). The dsRNA can therefore derive from a variety of sources and by a variety of routes, either entering the pathway directly (e.g RNA viruses) or being produced by the cellular RDR6 as well as several other factors. In a subsequent step, the dsRNA is processed into short (21-22 nt) and long (24-26 nt) sRNAs by DCL2/4 or DCL3, respectively (Xie et al. 2004; Gasciolli et al. 2005; Xie et al. 2005; Moissiard et al. 2007). The antisense strand of the 21-22 nt long sRNAs is loaded into the RISC complex to guide mRNA cleavage, whereas the 24-26 nt sRNAs are believed to exclusively mediate chromatin modifications (Hamilton et al. 2002; Tang et al. 2003; Zilberman et al. 2003).

Another form of RNA-mediated gene regulation is promoted by endogenous 21-24 nt long microRNAs (miRNAs) and is very similar to that described for animals (Bartel 2004; Kidner and Martienssen 2005). Most miRNAs arise from smRNA/miRNA genes that do not encode proteins. The large precursors (pre-miRNA) are cleaved by DCL activities, in most cases by DCL1, single stranded miRNAs are released from the miRNA duplex and subsequently transported to the cytoplasm by the exportin5 homolog hasty (HST) (Bollman et al. 2003; Park et al. 2005). After the export, the miRNAs are assumed to enter the AGO1/RISC complex, which guides the cleavage of the mRNA target by a slicer endocnuclease intrinsic to AGO1 (Liu et al. 2004). MiRNAs may also function in the nucleus

to direct epigenetic modifications, suggesting that dual mechanisms for miRNA targets may exist in plants (Bao et al. 2004; Chen 2004) (Figure 4B).

In addition to acting on RNA, siRNAs can also guide the formation of transcriptionally silent heterochromatin, which is characterized by DNA methylation and histone modifications. In plants, this heterochromatin formation involves a pathway known as RNA-directed DNA methylation (Figure I4C).

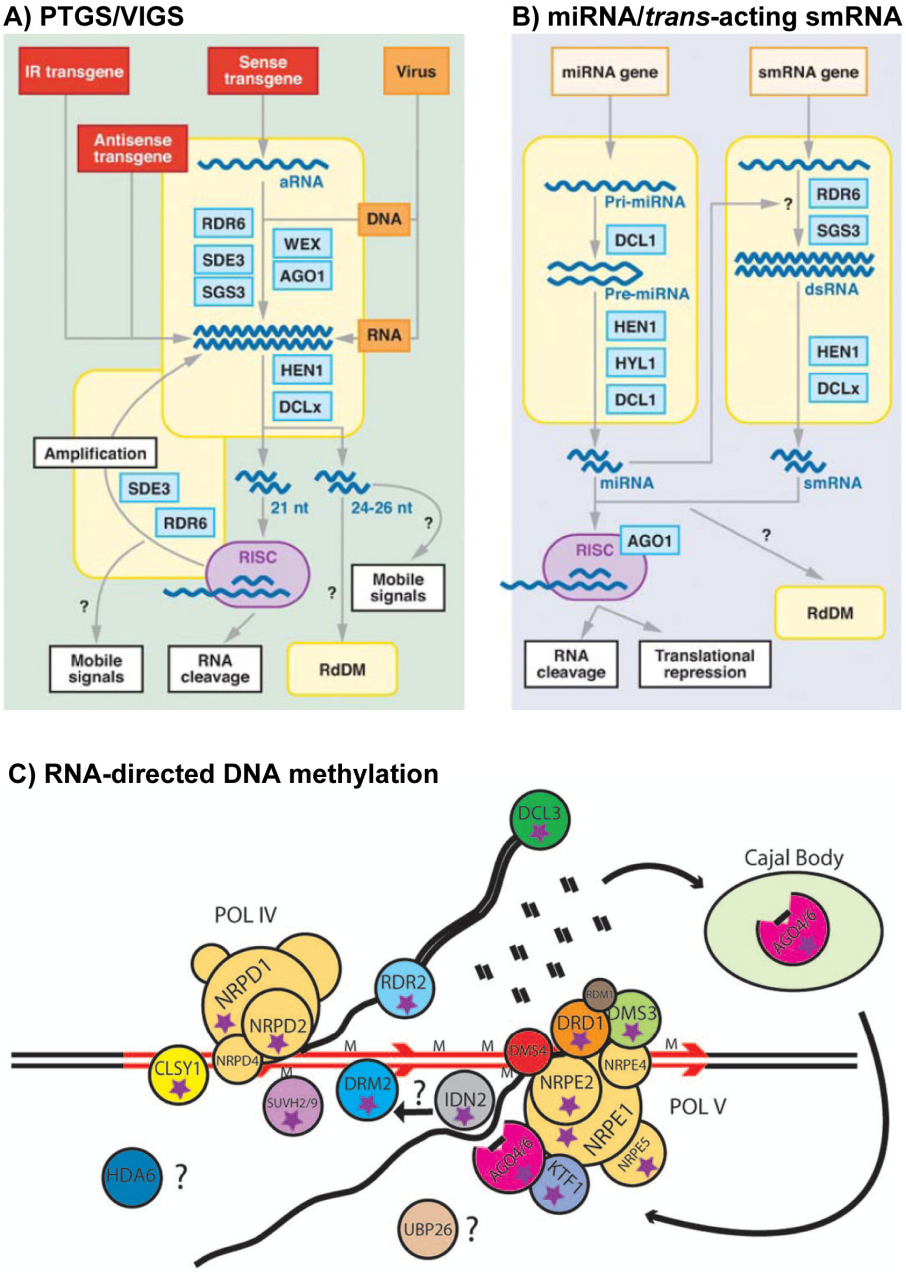

Figure I4. RNA silencing pathways in plants. Even though the three major RNA silencing pathways share some overlapping components, they can be distinguished by the source of dsRNA, by the class of sRNAs, by the nature of the target sequence and by the level of gene silencing. (A) Posttranscriptional (PTGS) and virus-induced gene silencing (VIGS), (B) micro RNA (miRNA) and *trans*-acting siRNA pathways and (C) RNA-directed DNA methylation. (A and B are adopted from Meins et al. 2005, C is taken from Greenberg et al. 2011).

1.6 RNA-directed DNA methylation

In plants, the majority of short interfering RNAs (siRNAs) are 24 nt in size and correspond to transposons and other repetitive elements (Zhang et al. 2007b; Mosher et al. 2008). The 24 nt siRNAs cause silencing by directing epigenetic modifications like *de novo* methylation, which in plants is generally considered to occur as the result of RNA-directed DNA methylation (RdDM) (Chan et al. 2005; Matzke and Birchler 2005). RdDM is mechanistically related and interconnected with a different mechanism of gene silencing called RNA interference (RNAi). RdDM primarily serves as a defense system against the transcriptional activity and mobility of transposable elements and the recombination between such elements, but also has a role in the silencing of intergenic regions and centromeric repeats (Colot and Rossignol 1999; Lippman and Martienssen 2004). The first evidence of the involvement of dsRNA in transcriptional gene silencing came from a study of viroid-infected tobacco plants (Wassenegger et al. 1994). Since then, several forward and reverse genetic screens were carried out to identify components of the RdDM pathway (Furner et al. 1998; Murfett et al. 2001; Aufsatz et al. 2002a; He et al. 2009b; Gao et al. 2010; Zheng et al. 2010; Greenberg et al. 2011).

A hallmark of RdDM is the methylation of cytosines in all sequence contexts (CG, CHG and CHH), which leads to the question of how these complex methylation pattern are established. RdDM is a stepwise process that is initiated by RNA signals (Figure I4C). First, single stranded RNAs (ssRNA) are produced from target loci by the action of the plant-specific RNA polymerase IV (PolIV) complex, probably by transcribing methylated DNA (Herr et al. 2005; Onodera et al. 2005; Chan et al. 2006; Daxinger et al. 2009). This ssRNA serves as template for RDR2, which converts it into dsRNA (Xie et al. 2004; Lu et al. 2006; Jia et al. 2009). Furthermore, dsRNA can also be produced by the transcription of inverted repeats or by bidirectional transcription. The proper localization of both PolIV and RDR2 might depend on CLSY1, a member of the SNF2-like domain containing class of proteins (Smith et al. 2007). The dsRNA is processed into 24 nt long siRNAs by DCL3 in the Cajal bodies (Xie et al. 2004; Henderson et al. 2006), which are subsequently loaded onto an effector complex. This complex harbours the PAZ- and PIWI-domain containing proteins AGO4 or AGO6, which have partially redundant functions in RdDM (Zilberman et al. 2003; Zilberman et al. 2004; Zheng et al. 2007). This AGO/siRNA complex subsequently associates with nascent transcripts produced by the plant specific RNA polymerase V (PolV), resulting in its recruitment to homologous genomic sequences (Wierzbicki et al. 2008; Wierzbicki et al. 2009). Efficient PolV transcription depends on the SWI/SNF remodeling factor DRD1, the

SMC hinge protein DMS3, and the single stranded methyl DNA-binding protein RDM1 (Kanno et al. 2004; Kanno et al. 2008; Law et al. 2010). Even though AGO4/6 recruitment is mainly based on RNA-RNA interaction, it was recently shown that AGO4 directly interacts with the hydrophilic, C-terminal domain of the largest PolV subunit (Li et al. 2006; El-Shami et al. 2007). This interaction is stabilized by the protein KTF1, which has RNA-binding capability and interacts with both AGO4 and PolV (Bies-Etheve et al. 2009; He et al. 2009; Huang et al. 2009). After binding to PolV transcripts, the AGO/siRNA effector recruits downstream RdDM proteins, such as the *de novo* methyltransferase DRM2, histone deactetylases like HDA6 and histone methyltransferases such as SUVH2/9. The recruitment of chromatin modifiers by the effector complex most likely involves IDN2, a previously unknown dsRNA-binding protein with homology to SGS3 (Ausin et al. 2009) (Figure I4C). After *de novo* establishment, cytosine methylation needs to be maintained, otherwise it will be lost by passive or active processes. Major players involved in the maintenance of DNA methylation are described in section 1.2.

1.7 HDA6 mutant alleles used in this study

Several HDA6 alleles were identified in three independent screens for factors that affect transcriptional transgene silencing (Furner et al. 1998; Murfett et al. 2001; Aufsatz et al. 2002b). The *rts*1 alleles (RNA-mediated transcriptional silencing) are derived from a forward screen that was based on the reactivation of a transgene silenced by promoter dsRNA and RdDM (Aufsatz et al. 2002b). This *Arabidopsis* RdDM *trans*-silencing model consists of a target locus (T), which harbors the NOSpro:NPTII (nopaline synthase promoter: neomycinphosphotransferase II) gene conferring kanamycine resistance, and an unlinked silencing complex (S), which constitutively expresses an inverted NOSpro repeat (Figure I5). The S transgene produces NOSpro dsRNA by transcription through the NOSpro inverted repeat (IR) which is subsequently processed by DCL3 activity into siRNAs of 24 nts in length. The T transgene becomes silenced by those siRNAs via the RdDM pathway, resulting in cytosine methylation of the NOSpro and kanamycine sensitivity (Figure I5). This silencing of the target NOSpro is initiated or substantially reversed within one generation after introducing or removing the silencing locus, respectively. In a T-DNA mutagenesis screen of plants harbouring both the S and the T transgene (DT, double transformed), the HDA6 mutant allele *rts*1-1 was identified (Aufsatz et al. 2002b). A second EMS mutagenesis screen revealed further HDA6 mutant alleles, namely *rts*1-3, *rts*1-4 and *rts*1-5. Despite the screen

being saturated, as judged by the number of identified alleles, HDA6 was the sole histone modifier recovered.

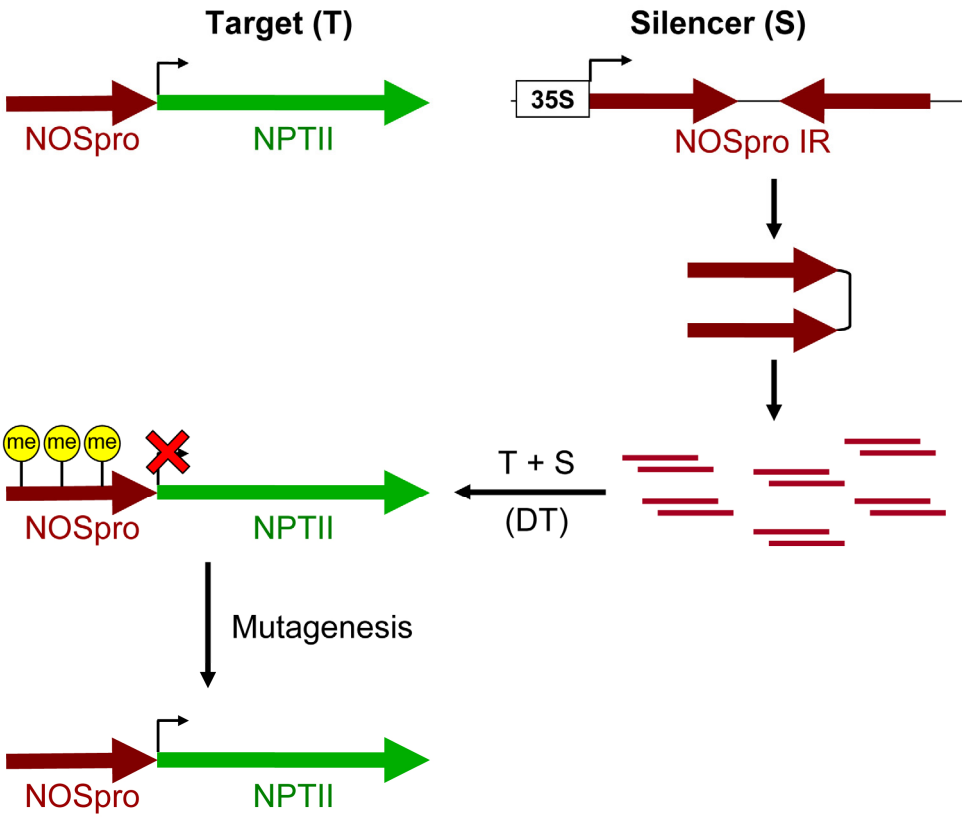

Figure I5: The two component RdDM model system used in *Arabidopsis*. The target transgene (T) consists of a NOPSpro driven NPTII gene, the expression of which confers kanamycin resistance. The physically unlinked silencer transgene (S) provides NOSpro siRNAs by constitutive expression of a NOSpro inverted repeat (IR). In plants with both transgenes (DT), the siRNAs direct promoter methylation at T via the RdDM pathway, thereby silencing the NPTII gene and establishing sensitivity to kanamycin. The DT plant population was mutagenized by T-DNA insertion mutagenesis or by EMS and screened for RdDM mutants by scoring for kanamycin resistant plants due to the reactivation of T.

The *rts*1-1 allele likely is a null allele caused by a 37 bp deletion just downstream of the start codon, which results in frame-shifting and a premature stop codon. The *rts*1-3, *rts*1-4 and *rts*1-5 alleles are missense alleles, all affecting highly conserved amino acid residues invariant in RDP3-type enzymes from yeast to human. The *rts*1-3 allele harbours a C1022 to T1022 point mutation that changes Pro341 to Leu341. Pro341 resides within a SANT-like domain, which is predicted to be able to mediated DNA binding and protein-protein interactions (Aasland et al. 1996). In *rts*1-4 a G556 to A556 mutation causes a change from Asp186 to Asn186, affecting a residue potentially involved in binding of the cofactor zinc (Finnin et al. 1999). The G821 to A821 point mutation in *rts*1-5 is located within the HDAC domain just N-terminal to the SANT-like domain, resulting in transition from Gly274 to Asp274 (Figure I6).

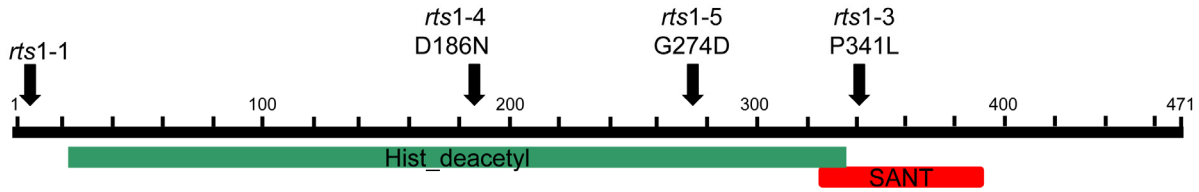

Figure I6. HDA6 mutant alleles used in this stuy. The mutant alleles of HDA6 were identified in a forward genetic screen and are named *rts*1 (for RNA-mediated transcriptional gene silencing). The locations of the mutations are shown with respect to the amino acid sequence of the protein. The green box indicates the conserved HDAC domain, the red box represents the predicted SANT-like domain.

1.8 Aim of this work

Although HDA6 has an evident and important role in RdDM, details on the mechanistic interplay between DNA methylation and histone deacteylation are still unclear. The fact that the inhibition of cytosine methylation (e.g. with 5-aza-2-deoxycytidine) or histone deacetylation activity (e.g. with TSA) both cause promoter demethylation and loss of histone acetylation as well as repressive histone methylation in plants, suggests a model whereby DNA methylation and histone acetylation are acting upstream of one another in a self-reinforcing pathway. In this model, histone acetylation/deacetylation and DNA methylation/demethylation are hypothesized to be the likely control points for switching between silenced and active states (Lawrence et al. 2004) (Figure I7).

Since HDA6 is, so far, the only histone deacetylase known to be involved in RdDM and since there was the given opportunity to use different mutant alleles, the first objective of this work was to investigate to what extend HDA6 deficiency effects the epigenetic state of the above describe target transgene (T) as well as endogenous RdDM targets. For this, the DNA methylation status, transcriptional reactivation and histone modifications (acetylation and methylation) were monitored. Furthermore, possible additive effects on transcription upon artificially induced DNA demethylation in wild-type and mutant plants were investigated in this study. After demonstrating that HDA6 mutants were hypersensitive to zebularine, a DNA demethylating drug also known to induce DNA damage, *rts*1-1 sensitivity to different DNA damaging drugs was investigated. This second part of the study leads to the conclusion that HDA6 might play a crucial role in DNA damage responsive pathways in *Arabidopsis*. A third objective of this study was to identify new HDA6 targets. For this, *rts*1-1 and *rts*1-5 mutant alleles were transcriptionally analysed using Affymetrix ATH1 microarrays, and genes found to be up- or downregulated were compared to publicly available datasets of other *hda*6 mutants as well as different silencing mutants.

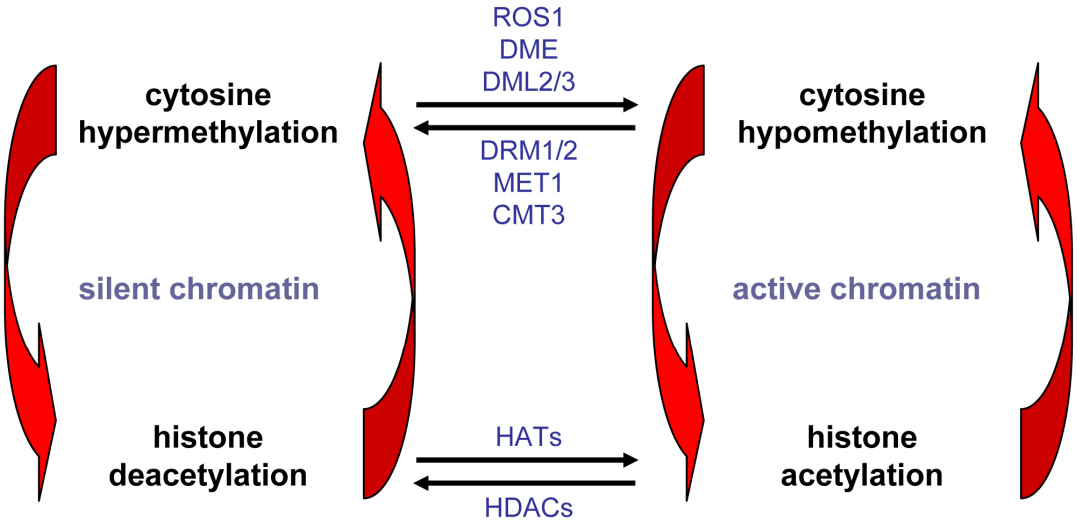

Figure I7. Model of gene silencing and activation. DNA methylation and histone acetylation are each upstream of one another in a self-reinforcing repression or activation cycle. Histone acetylation/deacetylation and DNA methylation/demethylation are both hypothesized to be likely control points for switching between silenced and active states. Proteins able to enzymatically control the respective epigenetic states are depicted in blue.

2. Results

2.1 Phenotypic characterization of *rts*1 mutant plants

2.1.1 Kanamycin resistance phenotypes and their complementation in *rts*1 mutants

As described previously, the *rts*1-1 mutant was identified in a genetic forward screen directed to retrieve mutants defective in RNA-directed transcriptional silencing. This screen was based on a two-component transgene system: a target reporter transgene conferring kanamycin resistance (NOSpro:NPTII) and an unlinked silencer complex encoding a constitutively transcribed inverted repeat of the NOSpro (35S:NOSproIR). The 35S:NOSproIR transgene produces small NOSpro RNAs that transcriptionally silence the NOSpro:NPTII reporter *in trans*, resulting in kanamycin sensitivity (Figure I5). Plants harboring both, target and silencer, are called DT plants (double transformed) in the remainder of this study. If RNA-directed transcriptional silencing is disrupted, mutant plants can be identified by screening for recovery of kanamycin resistance (Aufsatz et al. 2002a; Aufsatz et al. 2002b). As *rts*1-1 exhibits kanamycin resistance, it was tested whether the *rts*1 missens mutants *rts*1-3, *rts*1-4 and *rts*1-5, show similar phenotypes (Figure R1). Seedlings of all genotypes were able to grow under control conditions (i.e. media without kanamycin; Firgure R1A upper panel). On MS plates supplemented with 40 mg/L kanamycin, however, only the *rts*1 mutants were able to grow, showing robust kanamycin resistance compared to DT (Figure R1A lower panel). This resistance is due to reactivation of the NOSpro:NPTII reporter as could be shown by NPTII specific RT-PCR (Figure R1C).

N-terminally multiple tagged (HIS, Express and HA-tag) mutant RTS1 proteins as well as HDA6 were constitutively overexpressed in *rts*1-1 mutant plants (*rts*1-1/35S:HDA6, *rts*1-1/35S:RTS1-3, *rts*1-1/35S:RTS1-4, *rts*1-1/35S:RTS1-5) to examine whether they complement the reactivation phenotype of the NOSpro:NPTII reporter. Transformed seedlings were plated on MS medium containing 40 mg/L kanamycin and scored for kanamycin sensitivity or resistance. The 35S:HDA6 construct can fully complement *rts*1-1 with regard to kanamycin tolerance (Figure R1B). The *rts*1-1/35S:HDA6 seedlings were kanamycin sensitive, caused by a reduction of NPTII transcript levels to DT background levels (Figure R1C). In contrast, all overexpressed RTS1 mutant proteins are unable to complement the *rts*1-1 reactivation phenotype as plants are still kanamycin resistant and show high NPTII transcript levels (Figure R1B and R1C). Therefore, fast and efficient resilencing of the NOSpro:NPTII reporter occurs only in presence of a functional HDA6 protein, mutant proteins lack this ability even when they are overexpressed.

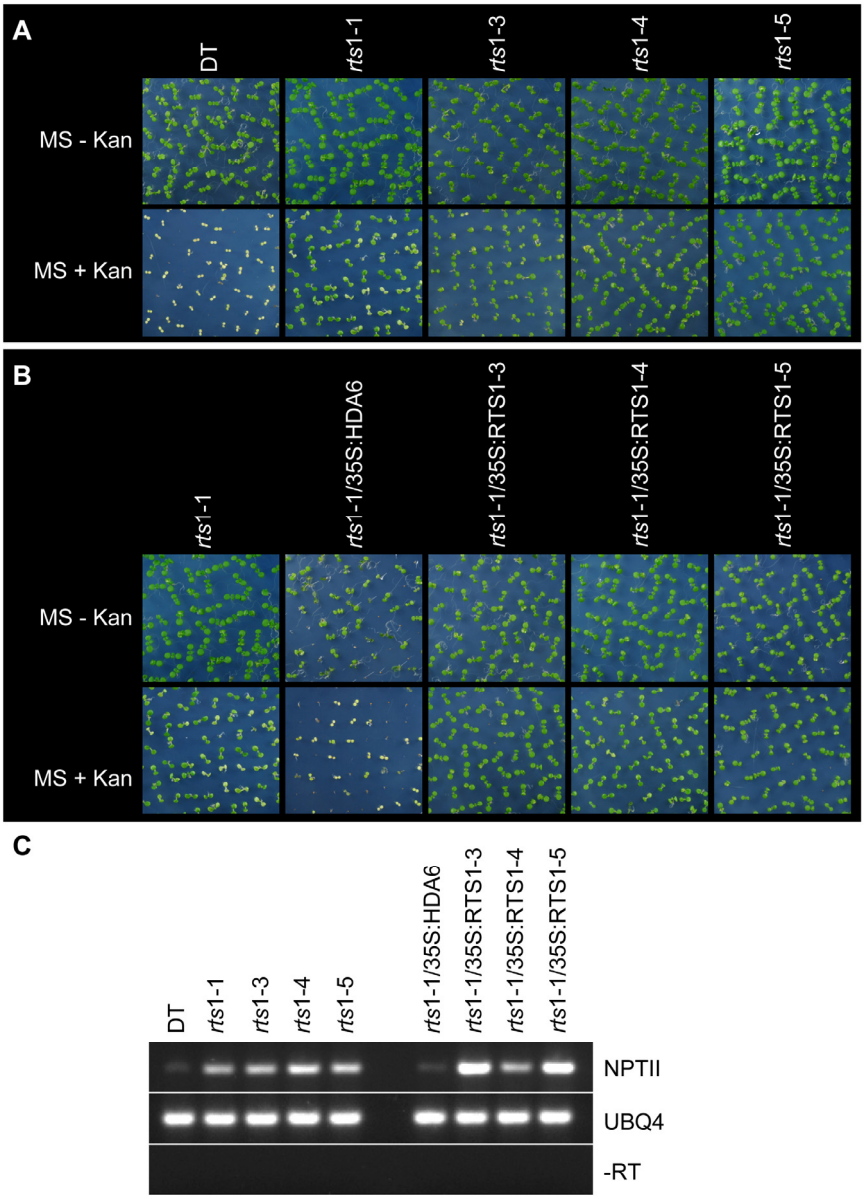

Figure R1. Kanamycin phenotypes of ten days old *rts*1 mutant seedlings. In both (A) and (B) the upper panels show control medium (MS without kanamycin), lower panels show MS medium supplemented with 40 mg/L kanamycin. (A) DT plants are sensitive to kanamycin treatment. All *rts*1 mutants, the *rts*1-1 null allele as well as the three missense mutants *rts*1-3, *rts*1-4 and *rts*1-5, are kanamycin resistant. (B) The kanamycin resistance can be complemented by overexpressing a functional HDA6 allele in the *rts*1-1 mutant background (*rts*1-1/35S:HDA6). Similar overexpression of the *rts*1 missense alleles (*rts*1-1/35S:RTS1-3, *rts*1-1/35S:RTS1-4, *rts*1-1/35S:RTS1-5), however, does not complement kanamycin sensitivity of the *rts*1-1 mutant. (C) RT-PCR showing transcriptional reactivation of the NOSpro:NPTII reporter in *rts*1 mutants, as well as in *rts*1-1 mutants transformed with overexpression-constructs of the missense alleles (*rts*1-1/35S:RTS1-3, *rts*1-1/35S:RTS1-4, *rts*1-1/35S:RTS1-5). Transformation with a constitutively expressed wild-type HDA6 (*rts*1-1/S5S:HDA6) results in resilencing of NOSpro:NPTII to levels similar in DT. Amplification of UBQ4 (At5g20620) was used as loading control. The –RT panel shows PCR of RNA samples without reverse transcription to test for contaminating DNA.

2.1.2 Growth and morphological phenotypes of *rts*1 mutant plants

Most mutants known to be involved in RdDM are reported to show no severe morphological abnormalities, most probably due to high genetic redundancies (www.arabidopsis.org). When grown under long day conditions, *rts*1 mutants do as well not exhibit severe developmental defects, but show a somewhat retarded growth phenotype.

Growth was first examined by measuring the rosette diameter of 27 days old plants. Rosette size does not differ significantly between DT, *rts*1-1, *rts*1-3 and *rts*1-4 plants (Figure R2A and R2B), whereas *rts*1-5 plants show a significant reduction in rosette diameter. When HDA6 is overexpressed in *rts*1-1 mutants (*rts*1-1/35S:HDA6), plants exhibit increased rosettes size. Interestingly, the opposite is found when the mutant RTS1 proteins are overexpressed in the *rts*1-1 background (*rts*1-1/35S:RTS1-3, *rts*1-1/35S:RTS1-4, *rts*1-1/35S:RTS1-5). Here, a significant reduction in rosette diameter is observed. The differences in rosette diameter are caused by dissimilarities in leaf size (Figure R2B). The *rts*1-5 mutant has smaller leaves compared to all other *rts*1 mutants and DT. Generaly, leaves of *rts*1 mutants do not exhibit major differences in morphology accept a slightly serrated phenotype, which is complemented in the *rts*1-1/35S:HDA6 line. It is noteworthy that lines overexpressing the point mutant alleles do not complement leaf serration and exhibit overall reduced leaf size, resulting in a smaller rosette diameter. When plants are allowed to reach maturity and plant height is measured 49 days after sowing, the overall plant stature is not affected. The *rts*1 mutant plants, however, are slow growing and show a reduced final plant size (Figure R2D and R2E). The reduction in plant size is only partially complemented in *rts*1-1/35S:HDA6 lines, which show a slight increase in plant height compared to *rts*1-1 but do not reach the same height as DT plants. The *rts*1-5 mutant plants are the most effected in terms of rosette diameter and leaf size in early stages of development, and also show the strongest phenotype at maturity. They display a dwarf phenotype compared to DT and even to all other *rts*1 mutants.

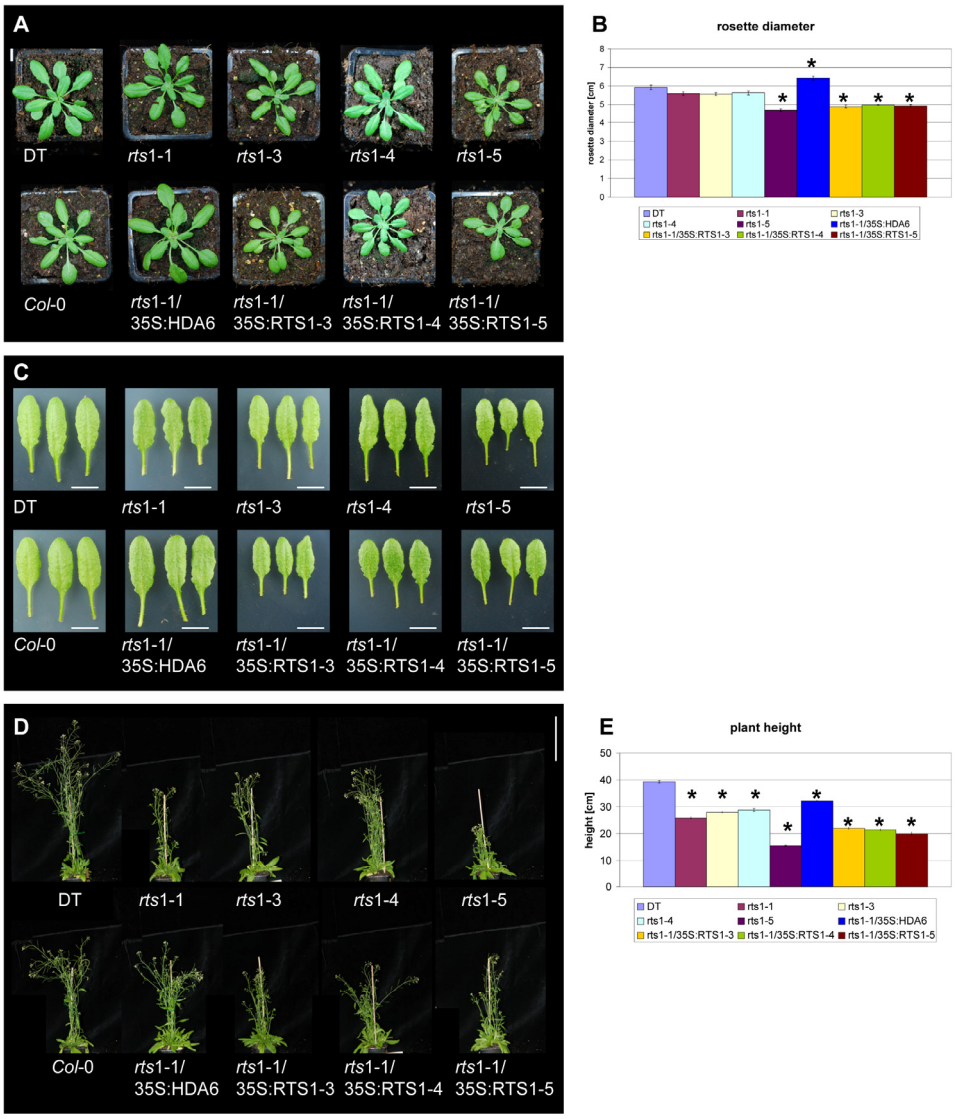

Figure R2. Growth phenotypes of *rts*1 mutants. (A and B) Rosette size and diameter 27 days after sowing. (C) Leaf size and structure of 27 days old plants. The scale bar equals 1 cm. (D and E) Plant statue and height of mature 49 days old plants. In (D) the scale bar in the top right corner equals 10 cm. All plants were grown under standard long day growth conditions. For statistical analysis (B and E), a total number of 10 plants was examined. Error bars represent standard error of the mean. Asteriks indicate significant changes when compared to DT, p-value ≤ 0,05.

2.1.3 Late flowering phenotype of *rts*1 mutant plants

All *rts*1 mutant plants (*rts*1-1, *rts*1-3, *rts*1-4 and *rts*1-5) exhibit a delayed flowering phenotype when compared to DT plants (Figure R3A). This phenotype is partially rescued by overexpressing a functional HDA6 allele in the *rts*1-1 background (*rts*1-1/35S:HDA6).

Flowering time is controlled by several pathways, including the gibberellin, photoperiod, autonomous and vernalization pathways (Boss et al. 2004; Henderson and Dean 2004). One important player in the flowering regulating network is the homeodomain-containing transcription factor FWA. Hypomethylation and ectopic expression of FWA is known to cause late flowering phenotypes (Soppe et al. 2000). The FWA gene contains two tandem repeats around the transcription start site that are necessary and sufficient for silencing via DNA methylation in wild-type plants (Soppe et al. 2000; Kinoshita et al. 2004). In *rts*1 mutants, bisulfite sequencing revealed no significant changes at FWA for CG sites. CHH methylation was slightly reduced in *rts*1-1 and *rts*1-5 mutants, and more drastically in *rts*1-4 mutants, but was close to DT in *rts*1-3 mutants. Methylation at CHG sequence context, which accounts for 13% of potentially methylated Cs within the analyzed sequence, was strongly reduced in all *rts*1 mutants (Figure R3C). This decrease in DNA methylation, however, seems not to be significant to trigger ectopic expression of FWA in all *rts*1 mutants. Transcript levels were assayed by RT-PCR using 3 different primer pairs (Figure R3B) and by analysing genome wide transcription changes via ATH1 microarray (data not shown). Collectively, these results indicate that the observed late flowering phenotype is not due to FWA activation.

Another key regulator of flowering is flowering locus C (FLC), a MADS-box transcription factor that blocks the floral transition by negatively regulating downstream flowering activators like FT and SOC1 (Michaels and Amasino 1999; Sheldon et al. 1999; Michaels and Amasino 2001; Helliwell et al. 2006). Whole transcriptome analysis of *rts*1-1 and *rts*1-5 revealed a significant increase of FLC expression in both mutants. A log fold change of 2,12 (*rts*1-1) and 1,98 (*rts*1-5), respectively, was revealed when compared to DT (Figure R3D). FLC is indeed a direct target of HDA6 since occupancy is approximately 9 times above background levels in *rts*1/35S:HDA6 plants (Figure R3E). Both results suggest that the late flowering phenotype of *rts*1 mutants is FLC dependent.

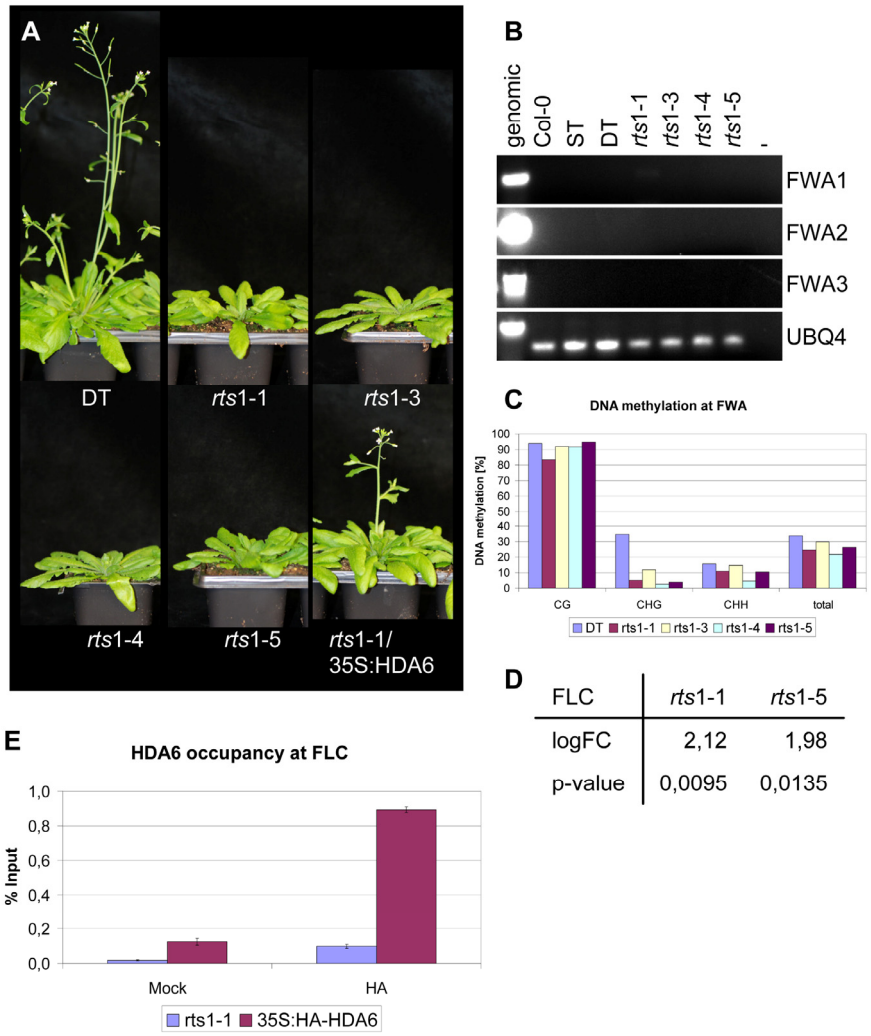

Figure R3. Late flowering phenotypes of *rts*1 mutants. (A) All *rts*1 mutants (42 days old) are delayed in flowering time when compared to DT. Late flowering can be partially rescued by the expression of 35S:HDA6 in the *rts*1-1 background. (B) Late flowering is not caused by ectopic FWA expression. RT-PCR of FWA is shown, using three different primer pairs. Amplification of UBQ4 (At5g20620) was used as loading control. (C) DNA methylation of the FWA promoter in DT and *rts*1 mutants assessed by bisulfite sequencing. Methylation is shown in CG, CHG and CHH (H = A, T or C) contexts, or as total methylation. (D) FLC upregulation (logFC = log fold change) revealed by microarray data of *rts*1-1 and *rts*1-5 mutants. (E) Enrichment of HA-HDA6 in *rts*1-1/35S:HDA6 plants compared to *rts*1-1 plants. Chromatin immunoprecipitation was done from EGS/FA crosslinked plant material with antibodies directed against HA (panel HA), followed by qPCR with FLC specific primers. Results from immunoprecipitation without antibody are shown at the left (panel Mock). Data form the immunoprecipitations were normalized to Input (% Input). The error bars represent the standard deviation of experimental triplicates.

2.2 Release of transcriptional gene silencing at endogenous RdDM targets in *rts*1 mutants

Since HDA6 was shown to be involved in RNA-directed transcriptional silencing of a transgene (Aufsatz et al. 2002b), the ability of *rts*1 mutants to release silencing at endogenous targets was tested. Therefore well characterized RdDM targets, such as *At*SN1, *At*GP1, soloLTR and IG5 were investigated.

*At*SN1 and *At*GP1 are high copy number retrotransposons shown to be derepressed in various silencing mutants (Lippman et al. 2003; Xie et al. 2004; He et al. 2009; He et al.

2009b) For both loci, transcripts were undetectable in DT, but a strong induction could be observed in all *rts*1 mutants. Resilencing occurred when a functional HDA6 allele was overexpressed in the *rts*1-1 background (Figure R4A, mock panel).

The intergenic region IG5 was previously described by Huettel et al 2006. It resides on chromosome 3, initiates in the 3`LTR of a Copia-like retrotransposon and encodes siRNAs (Lu et al. 2005; Huettel et al. 2006). IG5 is transcriptionally induced in all *rts*1 mutants relative to DT. When HDA6 was overexpressed in the *rts*1-1 mutant, resilencing of IG5 occurred only partially (Figure R4A, mock panel).

The soloLTR is a Copia-like retrotransposon which is silenced in wild-type plants by RdDM. When activated in RdDM mutants, the soloLTR functions as a regulatory element that influences the expression of adjacent genes in a bidirectional manner (Huettel et al. 2006). For this reason the genomic soloLTR region was divided into three parts (Figure R4D). The first part covers a 280 bp long region directly downstream of the soloLTR that was shown to be upregulated in several RdDM mutants (*drd*1, *nrpd*2a, *nrpd*1b, *nrpd*1b, *rdr*2), but not in mutants of the CG-specific maintenance DNA methyltransferase MET1 (Huettel et al. 2006). Part 1 will be referred to as “soloLTR” in Figure R4D. Part two spans 282 bp of the 5`UTR of the adjacent RPL18C gene (At5g27850), also shown to be upregulated in RdDM mutants. The third part resides approximately 2 kb downstream of the soloLTR within a neighbouring LINE element (At5g27845) and will be referred to as “soloLINE”. In DT, transcripts from all three regions were undetectable by RT-PCR, indicating that the soloLTR is stably silenced in wild-type plants. Increased transcript levels were detected in all *rts*1 mutants, which were restored to DT levels in the *rts*1-1/35S:HDA6 line (Figure R4B, mock panel). In summary, these results clearly demonstrate that the loss of HDA6 function results in attenuation of transcriptional gene silencing at a number of RdDM target loci. Silencing can be restored, at least partially, by constitutive expression of HA-tagged HDA6, suggesting that the tag does not interfere with the biological function of HDA6.

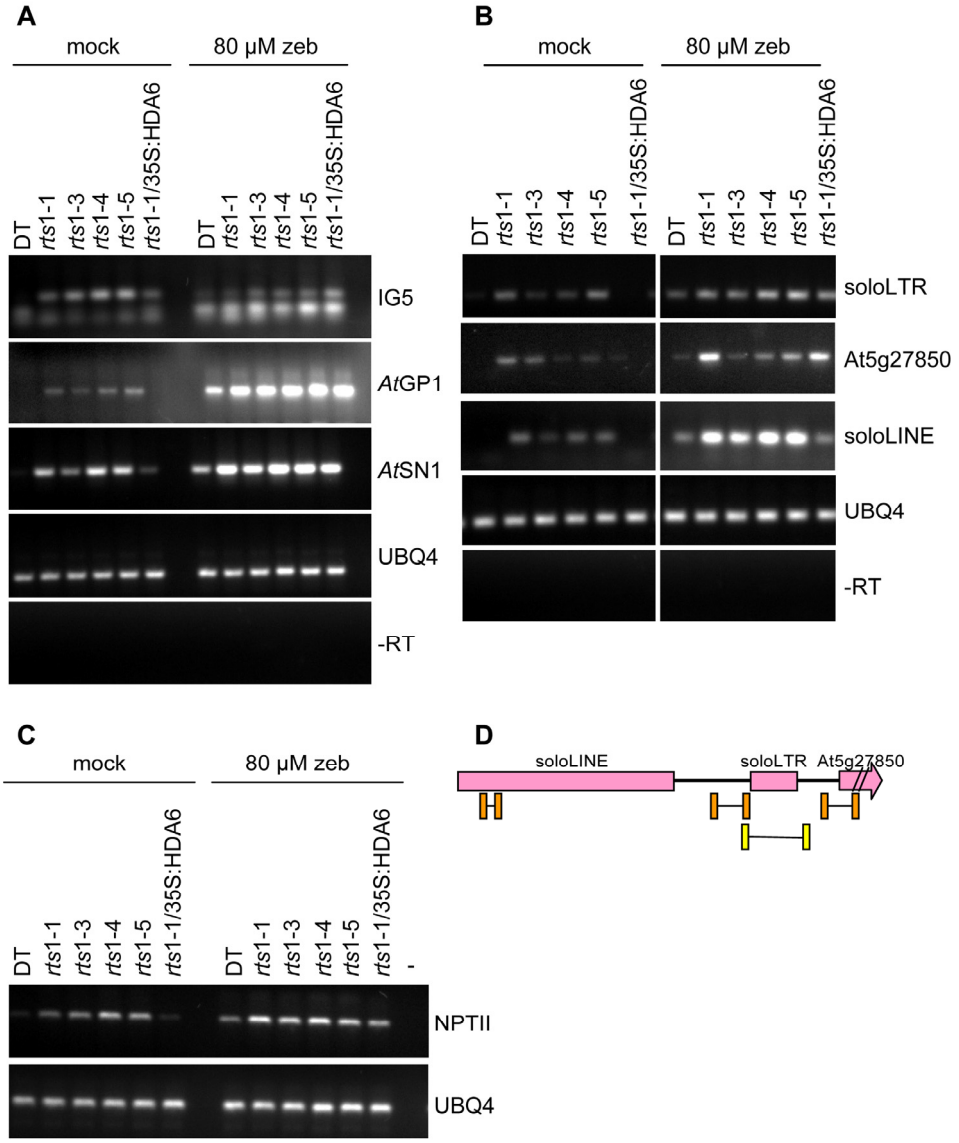

Figure R4. Release of transcriptional gene silencing in *rts*1 mutants. Release of transcriptional gene silencing in *rts*1 mutants before (left panel in A, B, and C, "Mock") and after zebularine treatment with 80 µM zebularine (right panel in A, B, and C, "80 µM zeb"). (A) Reactivation of the intergenic region IG5 and both high copy number retrotransposons *At*GP1 and *At*SN1 are shown. (B) Reactivation of the soloLTR and its upstream (At5g27850) and downstream regions (soloLINE) are shown. (C) Reactivation of the NPTII transgene. In all cases UBQ4 (At5g20620) was used as loading control. (D) Scheme of the genomic region of the soloLTR locus. Pink boxes depict annotated sequences for soloLINE, soloLTR and At5g27850, repectively. Orange boxes indicate RT-PCR primers and yellow boxes label the sequence used for bisulfite analysis.

2.3 Effects on DNA methylation in *rts*1 mutants

To test whether the suppression of silencing in the *rts*1 mutants correlates with a loss of DNA methylation, the DNA methylation status of the NOSpro:NPTII reporter transgene and of the above mentioned endogenous RdDM targets was analyzed by bisulfite sequencing. Bisulfite treatment of genomic DNA results in the conversion of unmethylated cytosine to uracile, whereas methylated cytosines are not converted. At least 20 independent PCR clones per tested locus were analyzed.

At the promoter region of the NOSpro:NPTII transgene, allele-specific effects of *hda*6 mutants on DNA methylation could be observed (Figure R5A). The *rts*1-1 and *rts*1-4 mutants show strong decrease in DNA methylation in all sequence contexts (pattern frequency: 27% CG, 25% CHG, 48% CHH). In contrast, *rts*1-3 and *rts*1-5 mutants show a relatively mild decrease of DNA methylation only in CG context, whereas no changes are detectable at CHG and CHH sites when compared to DT. Silencing of the NOSpro:NPTII transgene, however, is relieved in all *rts1* mutants (Figure R1C and Figure R4C), regardless of the DNA methylation phenotype, suggesting an uncoupling of DNA methylation and transcriptional reactivation at this locus.

For *At*GP1 and *At*SN1, a general decrease of DNA methylation could be observed in all *rts*1 mutants. The SINE retrotransposon *At*SN1 shows a strong decrease in DNA methylation at CG sites, which represent only 7% of potentially methylated cytosines at this locus (Figure R5B and Table R1). Except for *rts*1-4, which shows drastic DNA methylation decrease in all sequence contexts, CHG sites are not as severely affected. No significant changes in DNA methylation status could be observed for CHH methylation, which make 81% of cytosine pattern frequency. The drastic DNA methylation phenotypes seen for the *rts*1-4 allele at the *At*SN1 resembles the situation at the NOSpro:NPTII transgene, where the *rts*1-4 allele also had the most drastic effects. At *At*GP1, only the CHG sequence context, having a frequency pattern of 46 % in the analyzed sequence, is reduced by HDA6 deficiency to levels around 50% (Figure R5C and Table R1). CG and CHH, in contrast, show no or only minor changes in DNA methylation. Since *At*GP1 is reactivated in all *rts*1 mutants (Figure R4A), it can be concluded that reduced DNA methylation at a specific sequence context site can be sufficient for transcriptional reactivation at some loci.

At IG5, total DNA methylation is not drastically altered in all *rts*1 mutants (Figure R5D). However, IG5 shows decreases of CG and CHG methylation, whereas CHH methylation is unaffected. The pattern frequency of potentially methylated cytosines within IG5 is 19% for CG, 22% for CHG and 59% for CHH (Table R1).

In comparison to the above mentioned loci, no significant changes in DNA methylation in the *rts*1 mutants was detected at the soloLTR (pattern frequency: 6% CG, 6% CHG, 88% CHH) (Figure R5E and Table R1). To test how stable the DNA methylation status at the soloLTR is and how significant the slight variations between the different *rts*1 mutant alleles are, the DNA methylation was assessed for 3 to 4 biological independent replicates, including adult leafs and two weeks old seedlings. As can be seen in Figure R5F, the DNA methylation at the soloLTR is stable and highly reproducible. The error bars show the standard deviation among the biological replicates, demonstrating that there is no significant difference with respect to methylation in all cytosine contexts between the DT and the *rts*1 mutant alleles. Since the soloLTR was transcriptionally activated in all *rts*1 mutants (Figure R4B), DNA methylation *per se* is not sufficient to maintain silencing of this locus when HDA6 function is compromised.

In summary, the results suggest both, locus- and allele-specificity in *hda*6 mutants with regard to effects on DNA methylation. Compromised HDA6 function can lead to reactivation coupled to a loss of DNA methylation (e.g. *At*SN1, *At*GP1). More striking, however, is the observation made at the transgenic NOSpro:NPTII reporter and at soloLTR. Here, a methylation independent release of silencing could be observed (for some alleles at NOSpro:NPTII and for all alleles at the soloLTR), suggesting that HDA6 suppresses transcription directly by histone deacetylation without indirect effects on DNA methylation. This places HDA6 either upstream or separating its functions to be independent of DNA methylation.

Table R1: Cytosine pattern frequency at different loci.

pattern frequency	CG [%]	CHG [%]	CHH [%]
NOSpro	27	25	48
soloLTR	6	6	88
IG5	19	22	59
*At*SN1	7	12	81
*At*GP1	24	46	30

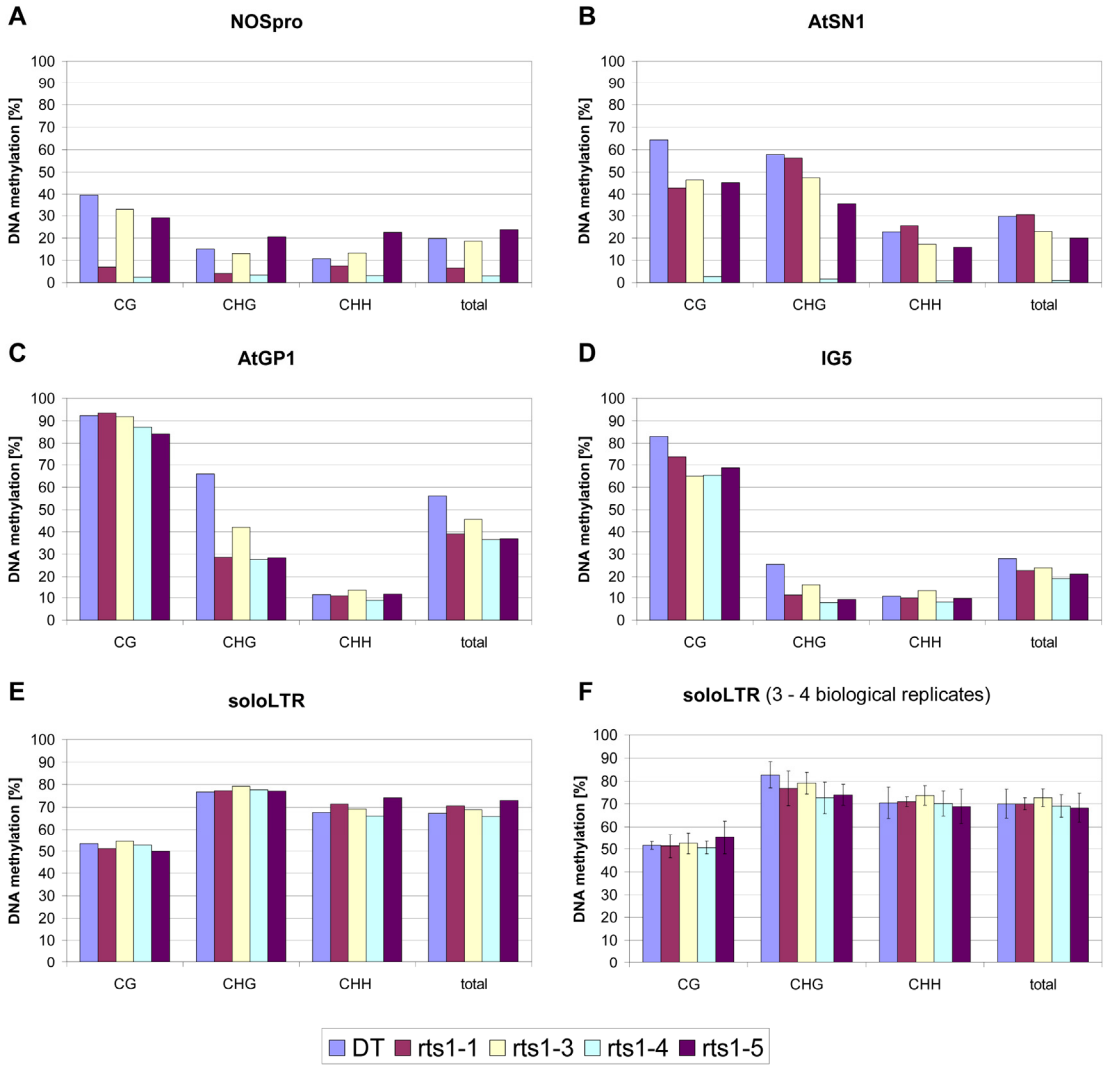

Figure R5. DNA methylation at several loci in wild-type and *rts*1 mutant plants. Levels of DNA methylation were assessed by bisulfite sequencing. Graphs represent the percentage of methylated cytosines in different sequence contexts (CG, CHG and CHH), as well as total DNA methylation levels. (A-E) Methylation data of at least 20 individual clones from pooled two weeks old seedlings. (F) Methylation data of the soloLTR from three to four biological replicates, including two weeks old seedling and mature leaves. For each biological replicate at least 15 individual clones were sequenced.

2.4 The DNA demethylating drug zebularine increase TGS derepression in *rts*1 mutants

The transcriptional silencing of loci that are controlled by the RdDM pathway coincides with DNA methylation within their promoter regions. Therefore, a decrease of DNA methylation can cause transcriptional reactivation of normally silent loci (Depicker and Montagu 1997; Meins et al. 2005). All RdDM target loci mentioned above showed strong transcriptional reactivation in *rts*1 mutants. Some of them, however, did not exhibit accompanying changes in DNA methylation (e.g. soloLTR) or showed allele-specific DNA methylation phenotypes (e.g. NOSpro:NPTII). To investigate whether artificially induced loss of DNA methylation would act additive with loss of HDA6 in the transcriptional reactivation

of those loci, plants were grown in the presence of zebularine. Zebularine was shown to efficiently block DNA methylation transiently in any sequence context and to reactivate silent genes (Cheng et al. 2003; Baubec et al. 2009). Plants were grown either for two or four weeks on 80 µM zebularine and hypersensitivity to the drug was scored (Figure R6). After only two weeks of zebularine treatment all plants had reduced growth compared to mock but no differences could be observed between *rts*1-1 and wild-type plants (Figure R6A). After four weeks of exposure, however, *rts*1-1 plants were severely affected in growth compared to wild-type plants, implying additive effects of misregulated DNA methylation and loss of HDA6 catalyzed histone deacetylation on plant growth (Figure R6B). The notion that loss of HDA6 function negatively affects plant growth in addition to DNA methylation is supported by the fact that growth is restored to that of wild-type plants in *rts*1-1/35S:HDA6 plants. In order to exclude growth stress related differences among genotypes and because Baubec and coworker showed a significant decrease of DNA methylation after only two weeks zebularine treatment (Baubec et al. 2009), DNA methylation status and transcriptional reactivation was further assessed upon two weeks of zebularine treatment.

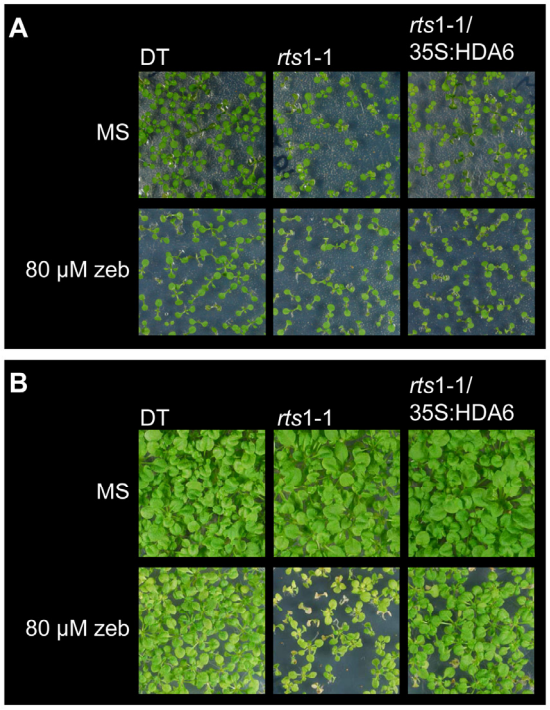

Figure R6. Growth phenotypes of *rts*1-1 and wild-type plants on 80 µM zebularine. (A) No severe hypersensitivity to zebularine treatment could be observed for two weeks old *rts*1-1 mutant seedlings when compared to DT or *rts*1-1/35S:HDA6 plants. (B) *Rts*1-1grown for four weeks on zebularine exhibited a clear hypersensitivity phenotype that could be complemented in *rts*1-1/35S:HDA6 plants.

Zebularine treatment of *rts*1 mutants resulted in significantly increased reactivation of all RdDM loci compared to mock grown plants (Figure R4). Transcription was also increased in DT and *rts*1-1/35S:HDA6 compared to "mock" treatment, indicating that DNA methylation has a slight additive effect to HDA6 function in silencing of these loci. Since zebularine also increased transcript levels of the NOSpro:NPTII transgene, it was tested whether this results in elevated kanamycin resistance of zebularine treated DT and *rts*1-1/35S:HDA6 seedlings (Figure R7). When plants were grown on media supplemented with 40 mg/L kanamycin plus low zebularine concentrations (40 µM and 60 µM; Figure R7 panels C and D), DT and *rts*1-1/35S:HDA6 plants were still kanamycin sensitive. Addition of higher zebularine concentrations (80 µM zebularine; Figure R7 panel E), resulted in visible kanamycin resistance of both DT and *rts*1-1/35S:HDA6 plants. Interestingly, the kanamycin resistance of DT plants is more pronounced then that of *rts*1-1/35S:HDA6 seedlings, indicating that overexpression of functional HDA6 renders plants more resistant to DNA demethylation dependent loss of transcriptional gene silencing.

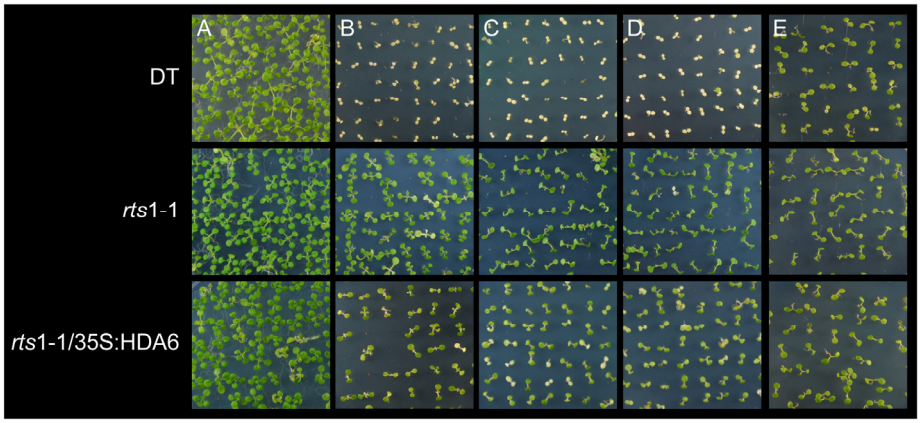

Figure R7. High zebularine concentrations result in efficient derepression of the NOSpro:NPTII transgene silencing. Two weeks old seedlings of indicated genotypes were grown on MS medium (A). MS medium supplemented with 40 µg/mL kanamycin (B), with 40 µg/mL kanamycin + 40 µM zebularine (C), 40 µg/mL kanamycin + 60 µM zebularine (D) or 40 µg/mL kanamycin + 80 µM zebularine (E).

To verify that reactivation of the tested loci really resulted from the zebularine induced inhibition of DNA methylation, methylation levels were assessed in representative methylated chromosomal regions. Since Baubec and coworkers demonstrated significantly reduced DNA methylation levels at the FWA promoter after zebularine treatment (Baubec et al. 2009), this sequence was included as a positive control. Figure R8 shows the DNA methylation status of selected loci before and after zebularine treatment. DNA methylation at the FWA promoter was reduced in both DT and *rts*1 mutants when compared to non-treated seedlings, demonstrating that the zebularine treatment was successful (Figure R8A). Interestingly, DNA

methylation was more affected in *rts1* mutants than in wild-type plants, which might be due to already slightly decreased DNA methylation levels in the mutants (see CHG methylation in Figure R8A "Mock"; Figure R3C). Contradictory results were obtained for *At*GP1 (Figure R8B), where DNA methylation was significantly reduced in DT seedlings upon zebularine treatment, but not in *rts*1 mutants. Surprisingly, no significant changes in DNA methylation were observed for soloLTR and IG5 in all genotypes (Figure R8C and R8D). As already mentioned, soloLTR is a regulatory element influencing the expression of the adjacent genes in a bidirectional manner (Huettel et al. 2006). Upon zebularine treatment, both adjacent genes (soloLINE downstream and At5g27850 upstream) and the soloLTR showed increased transcription levels relative to mock treatments in both wild-type seedlings and *rts*1 mutants (Figure R4B). Since there is no detectable change in DNA methylation at the soloLTR regulator element itself, this induction upon zebularine treatment seems to be DNA methylation independent, as was already shown for mock treated seedlings in *rts*1 mutants (Figure R4B, R5E and R5F).

These results show that DNA methylation can be decreased by zebularine treatment only in a locus specific manner and can vary between DT and *rts*1 mutants. Independent of the DNA methylation status, however, zebularine induces increased TGS derepression at all tested loci. This methylation independent transcriptional reactivation might be stress induced, since zebularine treated plants show transient and dose-dependent growth inhibition (Figure R6) (Baubec et al. 2009). As zebularine is known to form covalent complexes with DNA methyltransferases (Zhou et al. 2002), the increased TGS derepression might be an effect of genotoxic stress.

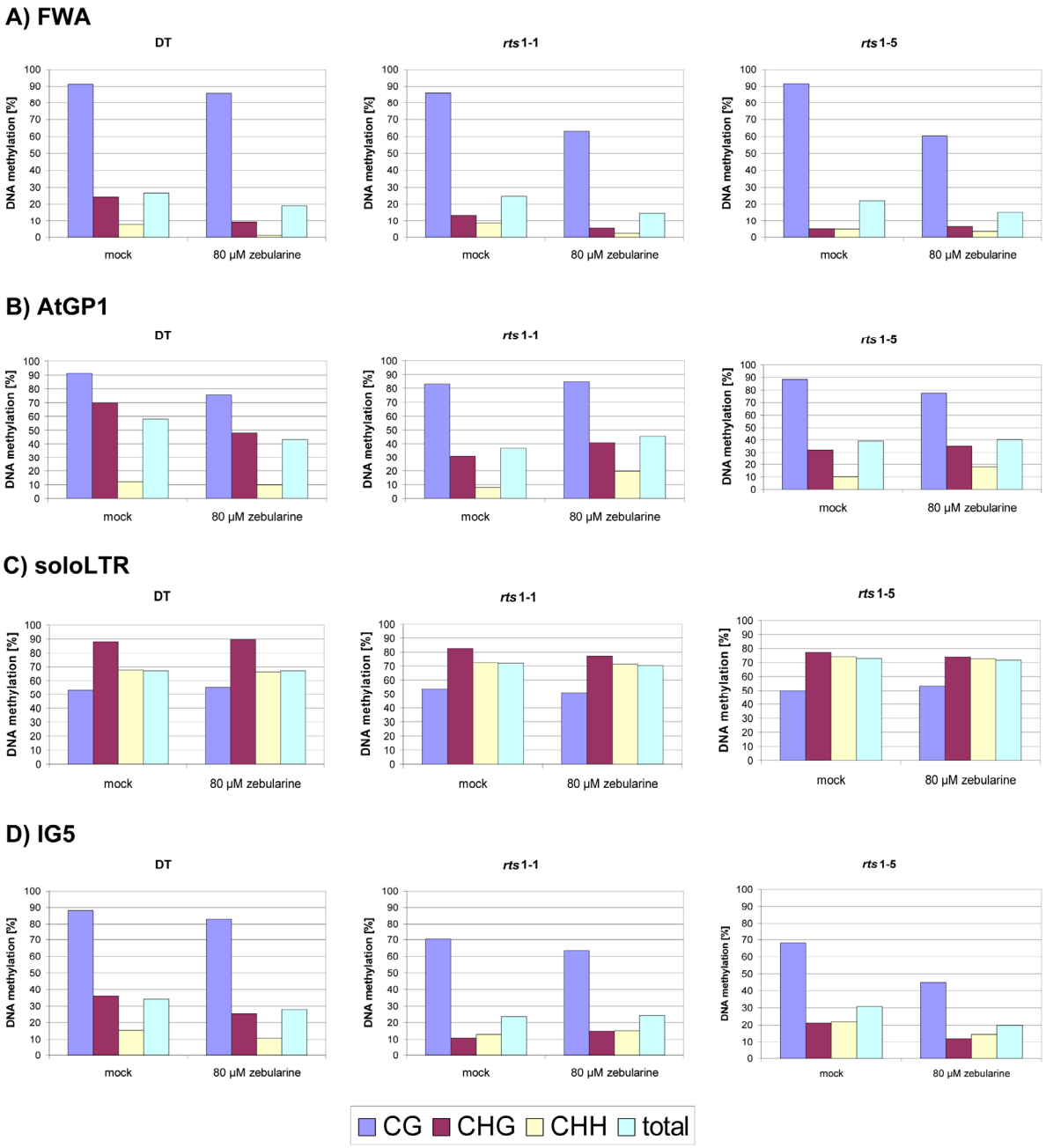

Figure R8. DNA methylation at selected RdDM loci before and after zebularine treatment of wild-type, *rts*1-1 and *rts*1-5 seedlings. Seedlings were grown for two weeks on plates containing 80 µM zebularine or on mock control plates. The DNA methylation status of FWA (A), *At*GP1 (B), soloLTR (C), and *IG5* (D) was assessed by bisulfite sequencing of at least 15 independent clones. Graphs represent the percentage of methylated cytosines in different sequence contexts (CG, CHG and CHH), as well as total DNA methylation levels.

2.5 Histone acetylation in *hda*6 mutants

In addition to DNA methylation, epigenetic regulation of transcription involves histone modifications at N-terminal histone tail residues. To assay histone modifications at RdDM targets and intergenic sequences, chromatin immunoprecipitation (ChIP) combined with quantitative PCR was used. Selected loci were analyzed as triplicates and each result was normalized to values obtained by ChIP with antibodies against unmodified histone H3 (% H3). Mock ChIP reactions done without antibodies revealed background signals. Antibodies specific for the euchromatic histone marks histone 3 lysine 9 and 14 diacetylation (H3K9/14ac2) and histone 3 lysine 4 trimethylation (H3K4me3) and specific for the heterochromatic methylation marks histone 3 lysine 9 dimethylation (H3K9me2) and histone 3 lysine 27 monomethylation (H3K27me1) were used. Additionally a histone 3 (H3) specific antibody, binding to H3 independent of tail modifications, was used to analyse nucleosome density. ChIP was done in *rts*1-1 and in *rts*1-5 mutants, since they showed opposing DNA methylation phenotypes at the transgenic NOSpro:NPTII reporter (Figure R5A).

Analysis of nucleosome occupancy was done by measuring H3 abundance relative to input signal. (% Input; Figure R9). Except for the intergenic region IG5, a relatively stable H3 distribution could be observed for the different genotypes. Whereas in DT and both mutants, *rts*1-1 and *rts*1-5, nucleosome density was relatively equal, it was significantly decreased when a functional HDA6 allele was overexpressed in the *rts*1-1 mutant (*rts*1-1/35S:HDA6). At the IG5 locus, however, the lowest nucleosome density was observed for DT, which was strongly increased in *rts*1-1, *rts*1-5 and *rts*1-1/35S:HDA6 (Figure R9C). Since nucleosome density has a direct influence on the histone modification readout, all values obtained for antibodies directed against modified histones were normalized to H3 abundance (% H3).

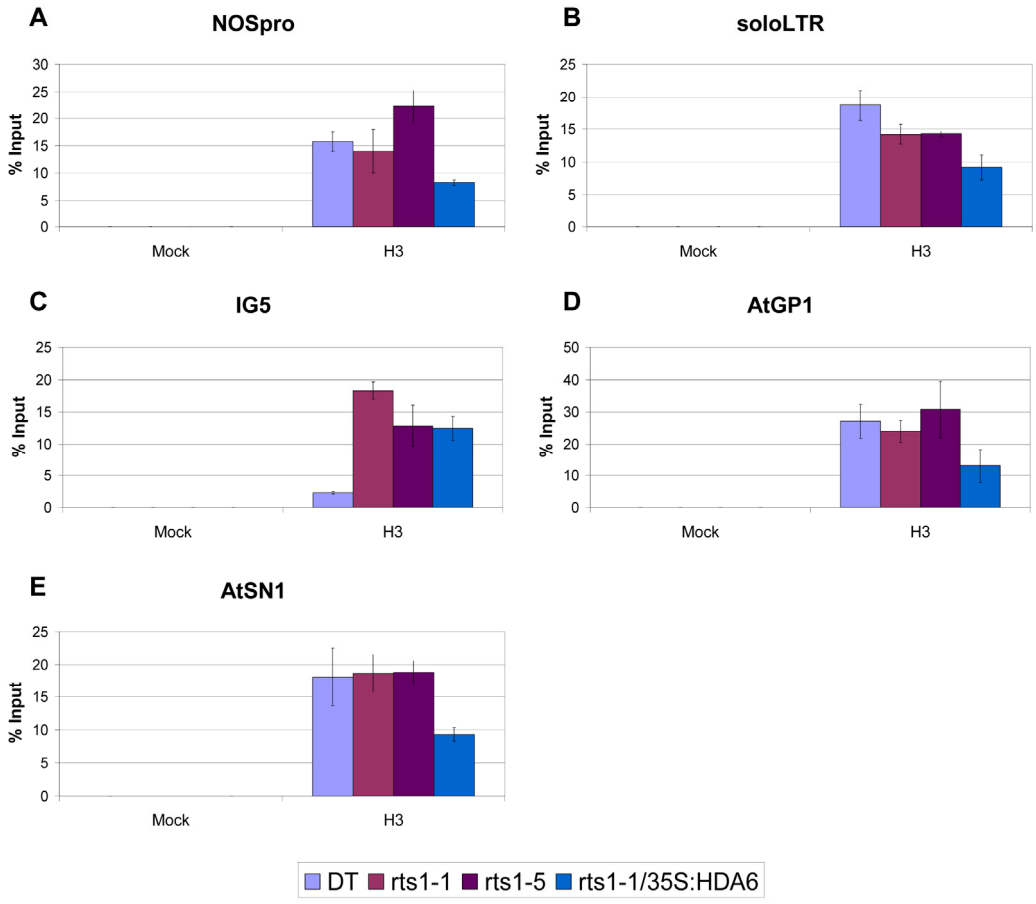

Figure R9. Chromatin immunoprecipitation with an antibody directed against histone H3. Immunoprecipitated DNA was quantified by qPCR in triplicates. The error bars display the standard deviation. Data was normalized to input (% Input).

Using the antibody specific for H3K9/14ac2, increased acetylation relative to DT levels could be observed at all loci in *rts*1-1 and *rts*1-5 mutants (Figure R10), which fits the expectations with regard to transcriptional activation of the loci in the mutants. Since HDA6 activity is compromised in both mutant alleles (Rakic 2010), loci become reactivated (Figure R4) by marking their chromatin as transcriptionally active. The increase in histone acetylation is complemented in the *rts*1-1/35S:HDA6 line (Figure R10). This correlates with the resilencing seen at the transcriptional level for all tested loci (Figure R4).

When using an antibody specific for the euchromatic mark H3K4me3, the mutant alleles show opposing effects. Only in *rts*1-5 an increase of H3K4me3 is observed consistently for all loci. In contrast, *rts*1-1 shows no significant changes or even decreased H3K4me3 levels. In *rts*1-1/35S:HDA6 plants, H3K4me3 levels are approximately the same as in DT plants (Figure R10).

For the heterochromatic marks H3K9me2 andH3K27me1, a decrease was observed for all tested loci in both mutants compared to wild-type plants. This again fits the transcriptional

reactivation phenotype observed by RT-PCR (Figure R4). When a locus becomes reactivated due to HDA6 deficiency, the histone acetylation increases, whereas the heterochromatic marks H3K9me2 and H3K27me1 decrease. Upon resilencing in the *rts*1-1/35S:HDA6 line, acetylation marks decrease to DT levels, H3K9me2 and H3K27me1 become restored, with H3K27me1 being even more efficiently restored than in DT plants.

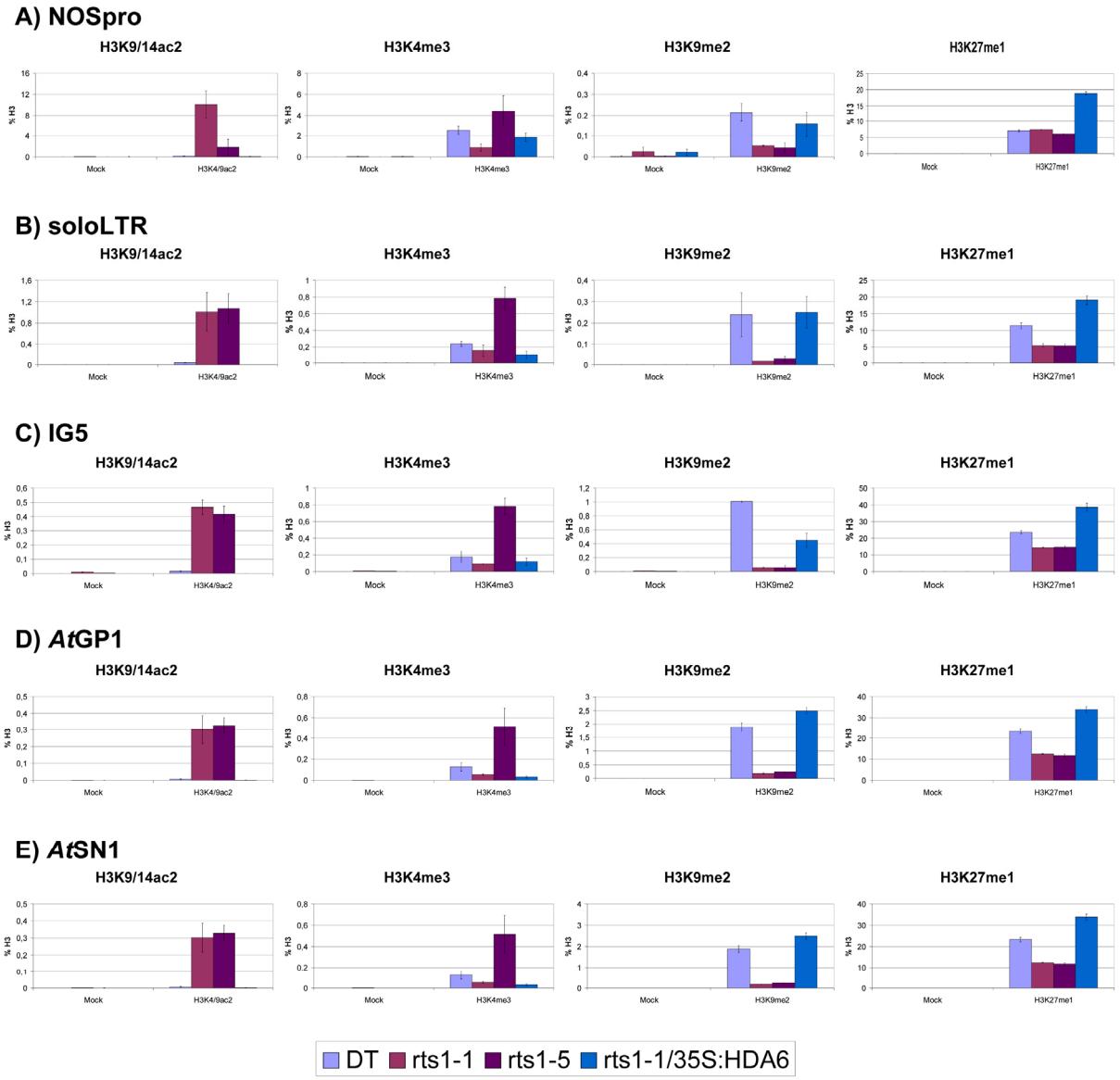

Figure R10. Assessment of histone modififcation at selected RdDM target loci in wild-type, *rts*1-1 and *rts*1-5 plants. Immunoprecipitated DNA was quantified by qPCR in triplicates. The error bars display the standard deviation. Data was normalized to H3 occupancy (% H3). Antibodies specific for histone 3 lysine 9/14 diacetylation (H3K9/14ac2), histone 3 lysine 4 trimethylation (H3K4me3), histone 3 lysine 9 dimethylation (H3K9me2), and histone 3 lysine 27 monomethylation (H3K27me1) were used.

Taken together, these results show a correlation between transcriptional reactivation and histone 3 acetylation levels. Next to histone acetylation, it also seems that at least the repressive H3K9me2 and H3K27me1 marks are influenced by HDA6 deficiency, suggesting

an interplay between these regulatory modifications. The fact that the active chromatin mark H3K4me3 shows opposing behaviour in *rts*1-1 and *rts*1-5 mutants could indicate that histone acetylation, and not H3K4me3, is the euchromatic mark with a major role in rendering loci transcriptionally active.

2.6 HDA6 occupancy at different endogenous loci

2.6.1 Establishing a dual crosslinking protocol for HDA6 chromatin occupancy

Using the common formaldehyde (FA) crosslinking step no sufficient amounts of immunoprecipitated tagged HDA6 was detected after ChIP (Figure R11 and R12). Formaldehyde has a short crosslinking spacer arm, spanning approximately 2°A, and is therefore not effective to examine proteins that are indirectly associated with DNA. Previous studies in yeast showed that RPD3-like HDACs were insufficiently immunoprecipitated from formaldehyde crosslinked samples. Efficiency could be increased by a dual crosslinking protocol using dimethyl adipimidate (DMA) (Kurdistani and Grunstein 2003). To overcome the possibility that HDA6 was not efficiently crosslinked to the DNA, the two step crosslinking protocol previously published for yeast RPD3-like histone acetylases (Zeng et al. 2006) was optimized for *Arabidopsis* (see Materials and Methods). Crosslinking was tested with two different crosslinking agents, namely DMA and ethylene glycolbis(succinimidyl succinate) (EGS). Both agents are membrane permeable and have longer spacer arms then formaldehyde.

Since there is no HDA6 specific antibody commercially available, two plant sources expressing differently tagged HDA6 were used. The first lines, already described above, overexpressed wild-type and mutant HDA6 in the *rts*1-1 background as N-terminally multiple tagged proteins (HIS, Express and HA-tag) and will be referred to as 35S:HA-HDA6, 35S:HA-RTS1-3, 35S:HA-RTS1-4 and 35S:HA-RTS1-5, respectively. The second set of tagged HDA6 lines was a kind gift of C. Pikaard (Indiana University, USA). Those lines express HDA6 C-terminally tagged with FLAG driven by its native promoter in the *axe*1-5 mutant background. For immunoprecipitation 10 µg anti-HA antibody (Covance, MMS-101P) or 10 µg anti-FLAG antibody (SIGMA, F1804) were used.

In ChIP experiments with the FLAG-tagged lines, no significant qPCR signal could be detected in any case (Figure R11). The underlying reason for this might be either masking of the FLAG epitope during the crosslinking procedure or low protein expression levels due to use of the native HDA6 promoter.

For the HA-tagged lines, the FA/DMA crosslinking protocol did not reveal stable pull down efficiencies (Figure R12). When compared to single formaldehyde crosslinking, a roughly three fold HDA6 occupancy increase at the NOSpro:NPTII transgene could be detected. However, no signal increase could be observed at the soloLTR. In contrast, the FA/EGS crosslinking procedure increased HDA6 occupancy signals 22 to 25 fold at both loci compared to FA alone. Even though the negative control signal (*rts*1-1) increased as well, an approximate four fold enrichment over *rts*1-1 could stably be detected at both loci (Figure R12).

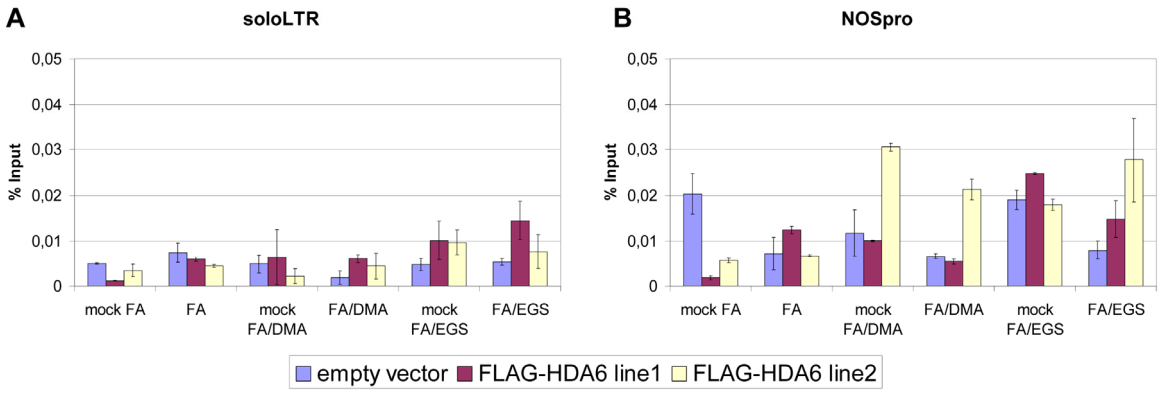

Figure R11. Efficiency of different crosslinking protocols for ChIP of FLAG-HDA6 at selected RdDM loci. Immunoprecipitated DNA was quantified by qPCR in triplicates. The error bars display standard deviation. Data was normalized to Input (% Input). Two weeks old seedlings were crosslinked either with formaldehyde (FA) alone or additionally with DMA (FA/DMA) or EGS (FA/EGS).

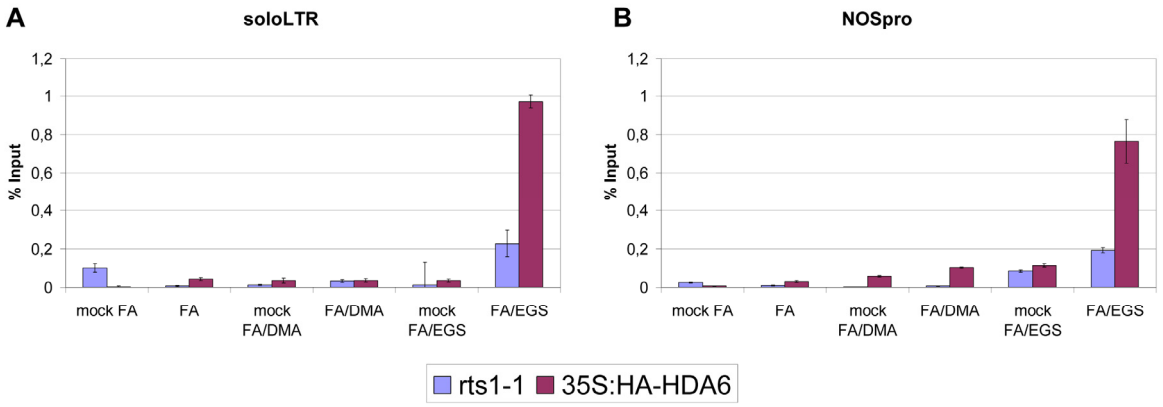

Figure R12. Efficiency of different crosslinking protocols for ChIP of HA-HDA6 at selected RdDM loci. Immunoprecipitated DNA was quantified by qPCR in triplicates. The error bars display standard deviation. Data was normalized to Input (% Input). Two weeks old seedlings were crosslinked either with formaldehyde (FA) alone or additionally with DMA (FA/DMA) or EGS (FA/EGS).

In conclusion, the HA-tagged lines were used in combination with the FA/EGS crosslinking protocol to study HDA6 occupancy at further loci.

2.6.2 HDA6 occupancy at RdDM target loci

To asses whether the loci reactivated in *rts*1 mutants (NOSpro:NPTII, soloLTR, IG5, *At*SN1, *At*GP1) are direct HDA6 targets, binding of HDA6 to the chromatin was analyzed with experimental parameters successfully evaluated in the previous section. N-terminally multiple tagged (HIS, Express and HA-tag) lines overexpressing wild-type and mutant HDA6 proteins (35S:HA-HDA6, 35S:HA-RTS1-3, 35S:HA-RTS1-4 and 35S:HA-RTS1-5) in the *rts*1-1 mutant background were used. The qPCR data were subjected to two normalization steps: Data was first normalized to input signal, followed by subtraction of *rts*1-1 background levels. Values obtained for 35S:HA-HDA6 were arbitrarily set one. HDA6 association with all tested loci (Figure R13) could be detected, indicating a direct role of HDA6 in silencing of those loci. For the mutant proteins encoded by the *rts*1 missense alleles, the ability to associate with the analyzed loci varied highly. HA-RTS1-3 and HA-RTS1-5 occupancy were always strongly decreased relative to that of HA-HDA6, indicating that RTS1-3 and RTS1-5 recruitment is affected. For HA-RTS1-4 decreased recruitment ability could be observed at all loci. However, the occupancy phenotype varied from almost no recruitment (Figure R13B and D) to reduced occupancy levels (approximately 50%) compared to HA-HDA6 protein (Figure R13A, C and E).

The variation in association to target loci might be due to different expression levels of the mutant proteins. When total protein extracts from the analyzed plant lines were subjected to Western blot analysis using HA specific antibody, high variations in protein amount could be detected (Figure R14). For normalization, the same blot was probed with an anti-histone H3 antibody. HA-RTS1-3 and HA-RTS1-5 showed significantly lower expression levels, while HA-RTS1-4 expression was reduced only to minor extend relative to HA-HDA6. These differences in protein levels probably directly affect IP efficiency of these proteins, which in turn will be reflected in the qPCR output.

In summary, only the HDA6 mutant protein RTS1-4 can still be recruited to its target site, however, to varying degrees and in a locus specific manner. Both the RTS1-3 and RTS1-5 proteins are severely affected in their recruitment abilities. Taking into account that the RTS1-4 mutant is enzymatically inactive (Rakic 2010), its defect in gene silencing deficiency can rather be attributed to loss of activity than to gross defects in recruitment of the encoded protein.

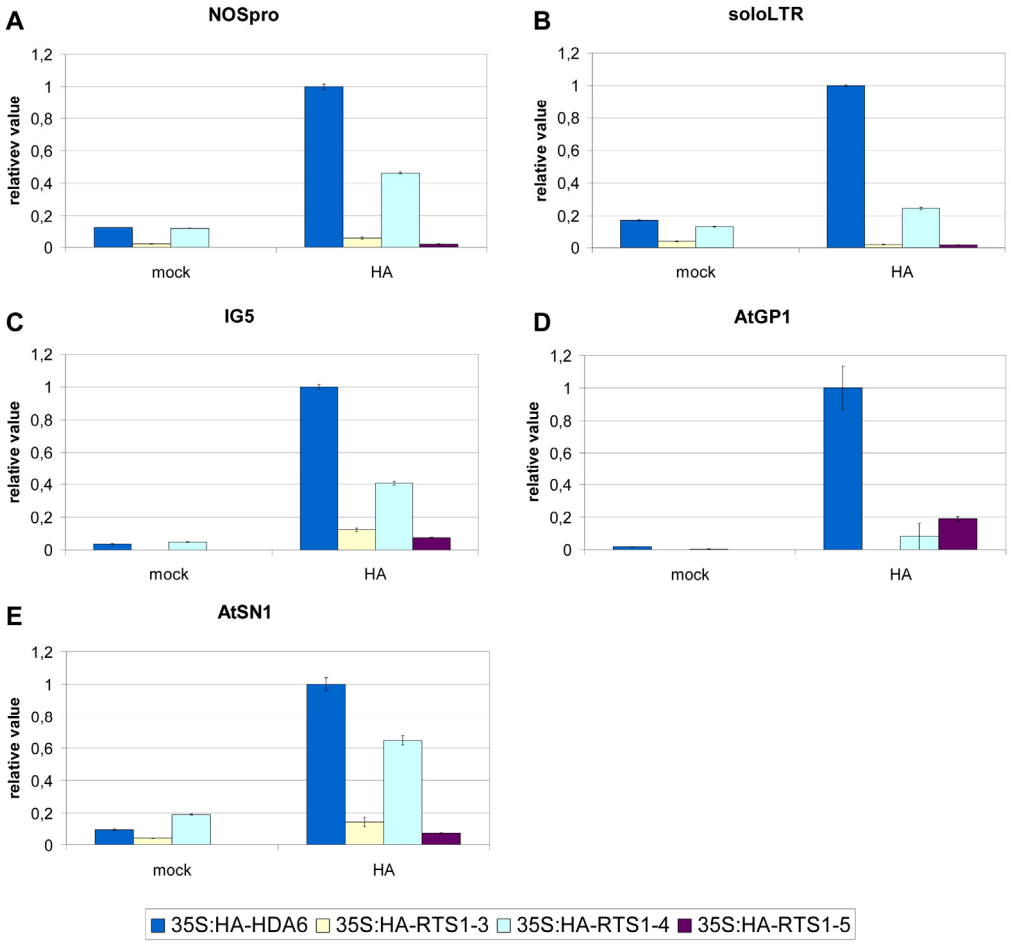

Figure R13. Occupancy of wild-type and mutant HA-tagged HDA6 proteins at selected RdDM loci. Immunoprecipitated DNA was quantified by qPCR in triplicates. The error bars display standard deviation. Data was normalized to Input, background values from the *rts*1-1 control line were subtracted and values obtained for 35S:HA-HDA6 were arbitrarily set one. 35S:HA-RTS1 values were normalized accordingly. Two weeks old seedlings were crosslinked with EGS and FA.

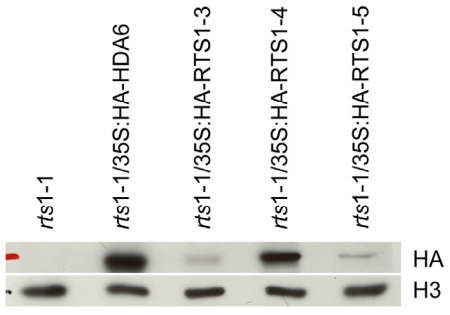

Figure R14. Protein levels of wild-type and mutant HA-tagged HDA6 proteins in the transgenic lines used in this study. Total proteins were extracted from two weeks old seedlings and subjected to Western blot analysis with anti-HA antibody (HA) or anti-histone H3 antibody (H3). The latter served as a loading control.

2.7 DNA damage

2.7.1 *Rts*1-1 mutants increase sensitive to DNA damaging agents

Previous studies demonstrated that several *Arabidopsis* mutants affecting TGS (*bru*1, *fas*1, *fas*2, *rpa*2) were sensitive to methylmethane sulfonate (MMS), suggesting that these genes have functions in DNA repair (Takeda et al. 2004; Elmayan et al. 2005; Kapoor et al. 2005). Similarly in this study, *rts*1 mutant plants were found to be hypersensitive to the DNA demethylating drug zebularine (Figure R6; M. Rosa, unpublished results), which was also shown to induce DNA damage via binding to DNA methyltransferases that are covalently attached to the DNA (Zhou et al. 2002).

In order to determine whether mutations in HDA6 influence DNA repair, the sensitivity of *rts*1-1 mutant and DT seedlings to the DNA damaging agents MMS (Lundin et al. 2005) and cis-Dichlorodiammine platinum(II) (cisplatin) was compared. Both, cisplatin, which is an intra- and interstrand DNA crosslinking agent, and MMS, a radiation mimicking monofunctional alkylating agent, induce DSBs during DNA synthesis (Ulm et al. 2001; Gong et al. 2002; Abe et al. 2005). These DNA lesions are repaired predominantly by homologous recombination, which is one of the major repair pathways during S-phase. (Abe et al. 2005; Frankenberg-Schwager et al. 2005; Osakabe et al. 2006). DT, *rts*1-1 and *rts*1-1/35S:HDA6 seeds were sown on MS medium supplemented with different concentration of MMS or cisplatin and grown under long day conditions. Sensitivity was scored after two weeks by comparing growth and root length of the plants.

Both drug treatments inhibited growth of all genotypes when compared to seedlings grown on unsupplemented control MS medium (Figure R15A and R16A). Plants grown on cisplatin were smaller compared to control plants, however, no significant differences were observed among the genotypes to different concentrations of the drug. All genotypes exhibited similar root lengths upon treatment with 20 µM and 30 µM cisplatin (Figure 15C). Therefore, *rts*1-1 mutants are not hypersensitive to cisplatin.

Upon treatment with MMS, however, *rts*1-1 seedlings were smaller than DT seedlings and their root length was significantly shortened (Figure R16). This hypersensitivity phenotype of the *rts*1-1 mutation could be rescued by overexpressing functional HDA6 in the mutant background (*rts*1-1/35S:HDA6, Figure R16). In contrast, the root length hypersensitivity phenotype could not be rescued, when mutant HDA6 proteins were overexpressed in *rts*1-1 plants (Figure R16C). This suggests that not the presence of the protein *per se*, but the enzymatic activity of HDA6 is needed in order to make plants more resistant to DNA damage.

In summary, these results indicate that HDA6 affects nuclear genome stability and has an important role in certain DNA damage repair processes *in planta*.

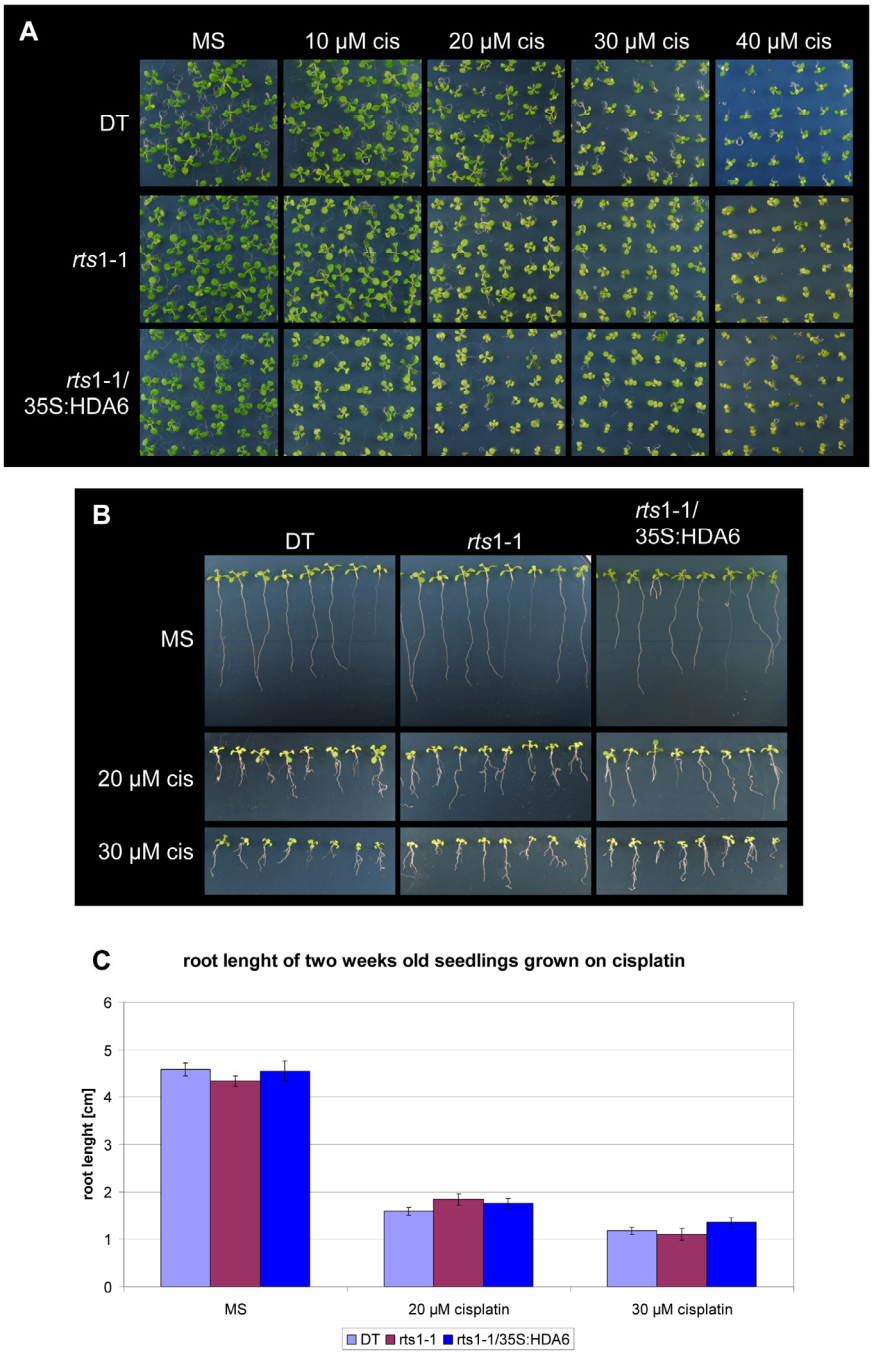

Figure R15. Phenotypes of wild-type, *rts*1-1 and *rts*1-1/35S:HDA6 seedlings grown on cisplatin. Seedlings were grown on MS medium and four different cisplatin concentrations (A). Root lenght was compared after growing seedlings for two weeks on 20 µM and 30 µM cisplatin (B and C). n=24, error bars represent the standard error of the mean (C).

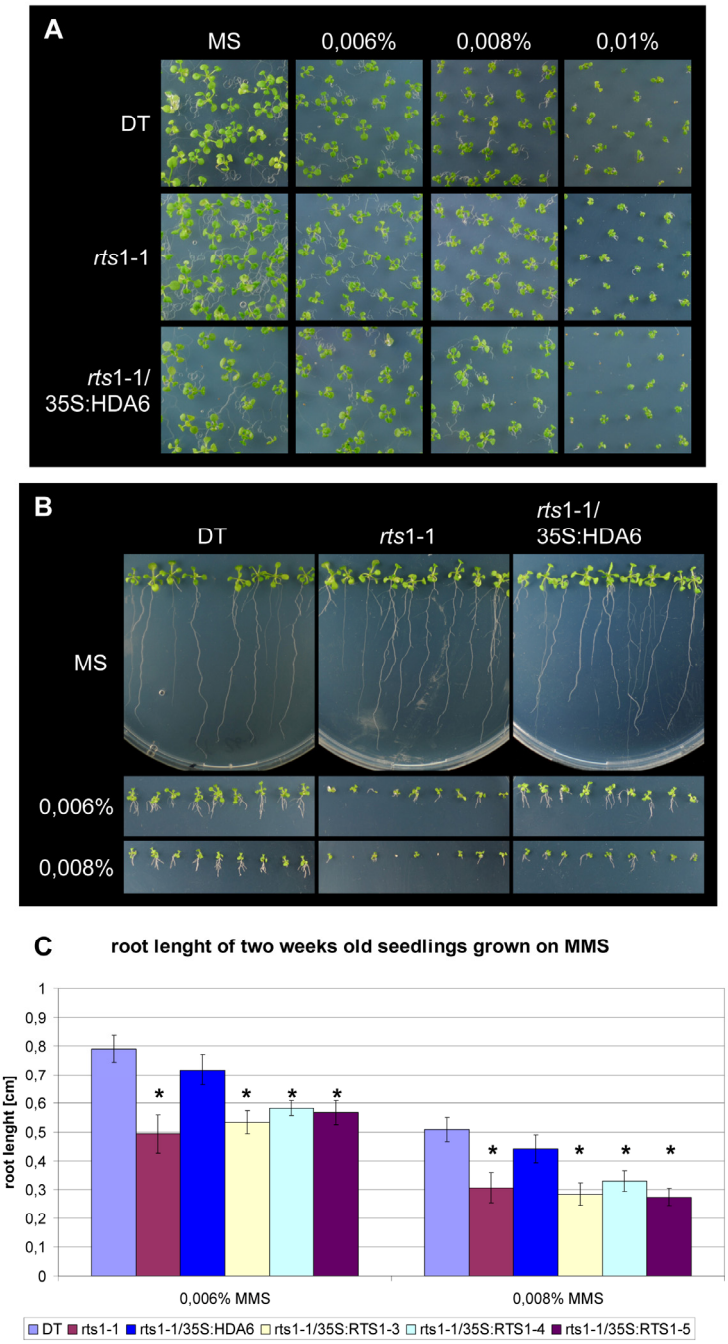

Figure R16. Phenotypes of wild-type, *rts*1-1 and *rts*1-1/35S:HDA6 seedlings grown on MMS. Seedlings were grown on MS medium and three different MMS concentrations (A). Root lenght was compared after growing seedlings for two weeks on 0,006 % and 0,008 % MMS (B and C). Analysis of *rts*1-1/35S:RTS1-3, *rts*1-1/35S:RTS1-4 and *rts*1-1/35S:RTS1-5 plants were included in root length measurements. n=24, error bars represent the standard error of the mean. Asteriks indicate significant changes when compared to DT, p-value ≤ 0,05 (C).

2.7.2 Treatment with DNA damaging agents does not mimic the *rts*1 mutation with regard to releasing TGS of the NOSpro:NPTII reporter

To determine whether the release of TGS is directly related to genomic instability caused by DNA damage, the growth of DT, *rts*1-1 and *rts*1-1/35S:HDA6 seedlings on MS medium containing 40 mg/mL kanamycin and supplemented with 0,004% or 0,006% MMS was tested. Growth on kanamycin and cisplatin was not assessed, since the *rts*1-1 mutant plants were not hypersensitive to this drug (Figure R15). If treatment with MMS releases TGS of the NOSpro:NPTII reporter transgene, the DT and *rts*1-1/35S:HDA6 seedlings should be resistant to kanamycin. In the presence of MMS, however, both DT and *rts*1-1/35S:HDA6 seedlings were still hypersensitive to kanamycin, whereas *rts*1-1 seedlings were resistant and therefore able to grow. These results suggest that treatment with DNA damaging agents does not directly release TGS at the NOSpro:NPTII reporter transgene. It is therefore likely that roles of HDA6 in gene silencing and DNA damage repair are not overlapping.

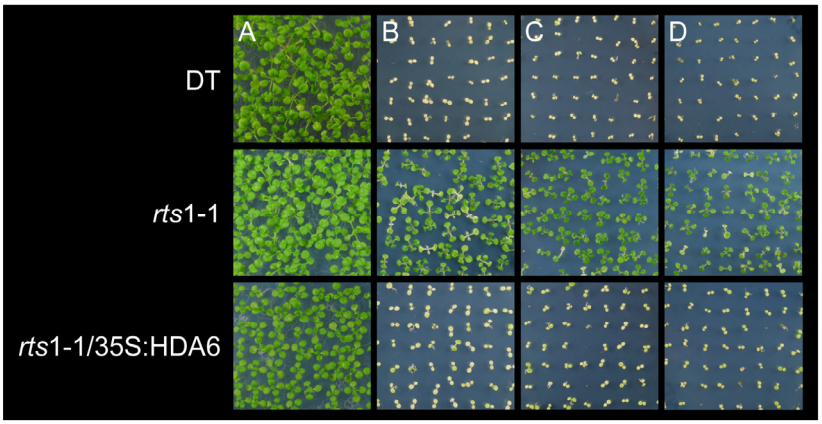

Figure R17. Kanamycin phenotypes of wild-type, *rts*1-1 and *rts*1-1/35S:HDA6 seedlings grown on MMS. Two weeks old seedlings were grown on MS medium (A), MS medium with 40 µg/mL kanamycin (B), MS medium with 40 µg/mL kanamycin and 0,004 % MMS (C) or MS medium with 40 µg/mL kanamycin and 0,006 % MMS (D).

2.8 Microarray analysis

In order to investigate genes differentially regulated between *hda*6 mutants and DT plants, gene expression profiles were compared by using Affymetrix ATH1 microarrays. As mutant lines only *rts*1-1 and *rts*1-5 plants were chosen, because they show opposite methylation phenotypes at the NOSpro:NPTII transgene (Figure R5A). For microarray hybridization, total RNA was isolated in triplicates from aerial parts of pooled five weeks old seedlings grown on MS agar under long day conditions. After verification of total RNA purity and quality, samples were sent to NASC (Nottingham *Arabidopsis* Stock Centre) for cDNA synthesis and hybridization onto Affymetrix ATH1 chips. The raw datasets were analyzed for quality by Fitting Probe Level Models (Bolstad et al. 2005), normalized using Robust Multichip Average (RAM) (Irizarry et al. 2003; Irizarry et al. 2003) and differential gene expression was determined using linear modelling (Wettenhall and Smyth 2004) in Bioconductor/R. Genes with a cut-off p-value ≤ 0,05 and with a ≥ 2-fold change (i.e. ≥ 2 and ≤ -2) were defined as significantly induced or repressed genes, respectively.

2.8.1 Misregulated genes in *rts*1-1 and *rts*1-5 mutants, and their overlap

Statistical analysis revealed 54 probe sets that were specifically upregulated in *rts*1-1 mutants, 61 probe sets specifically induced in *rts*1-5 mutants and 65 probe sets upregulated in both mutants compared to DT plants. Probes sets showing decreased transcript levels were, as expected, found in lower numbers than the upregulated ones. In *rts*1-1 mutants only 8 probe sets were significantly repressed, 11 probe sets in *rts*1-5 mutants and 9 probe sets were downregulated in both *rts*1-1 and *rts*1-5 plants (Figure R18A and R18B). A complete list of misexpressed probe sets can be found in the supplementary data (Table S1 to S6). Using the TAIR database, all misexpressed genes were classified into transposons, protein encoding genes (i.e. “other genes”), genes encoding proteins with unknown function and pseudogenes (Figure R18D and R18E). In all cases, genes belonging to the category “other genes” were the most dominate group of misregulated loci in *rts*1 mutants. Interestingly, transposons, which are the main silencing targets of the RdDM pathway (Matzke and Birchler 2005; Matzke et al. 2007), represented only a minor group of loci being upregulated in *rts*1-1. In *rts*1-5 mutants, however, the transposons represented approximately 1/3 of induced loci. With regard to this apparent underrepresentation of transposable elements among misregulated probe sets it should be mentioned that the ATH1 GeneChip does not cover heterochromatic regions of the genome extensively. Therefore, the regulation of transposons, which mainly reside in heterochromatin, cannot fully be monitored using this GeneChip. Only one pseudogene was

induced in both *rts*1-1 and *rts*1-5 mutants (i.e. "overlap"), suggesting that this group of genes are not major HDA6 targets (Figure R18D). Genes repressed in *rts*1-1 plants, *rts*1-5 plants, or both, were, as already mentioned above, low in number and mainly genes belonging to the category "other genes" (Figure R18E).

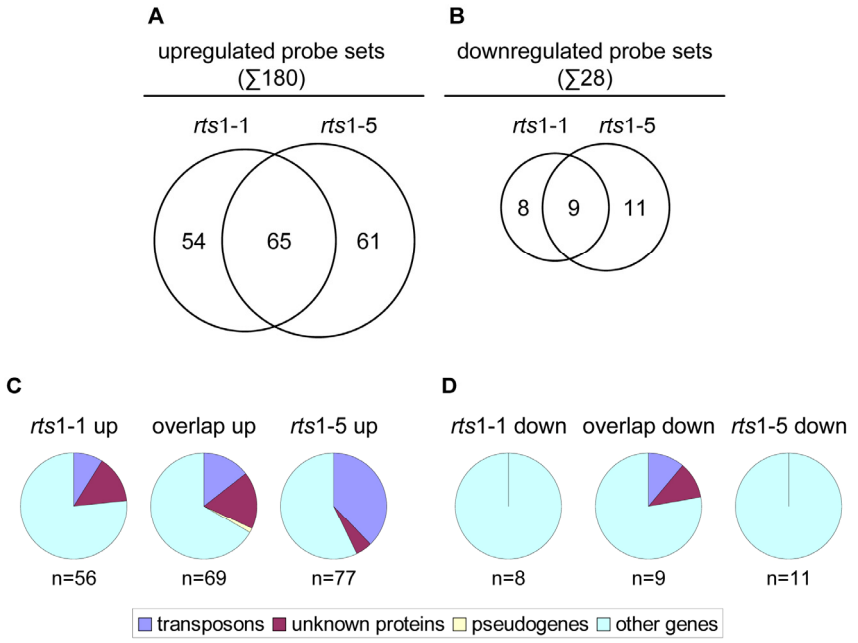

Figure R18. Diagrams showing up- and downregulated genes in *rts*1-1 mutants and *rts*1-5 mutants. Depicted are the number of probe sets upregulated (A) or downregulated (B) in *rts*1-1 and *rts*1-5 plants, and their overlap. The pie charts (C and D) represent the number of genes found to be misexpressed in *rts*1-1, *rts*1-5 or both. Genes were grouped according to annotation into the categories transposons, unknown proteins, pseudogenes and other genes (genes not represented in the before mentioned categories).

In order to confirm the microarray results, RT-qPCR of a random selection of twelve induced loci was done. All loci were indeed upregulated in *rts*1-1 and *rts*1-5 mutants relative to DT, thus confirming the whole transcriptome data. In most cases, full resilencing occurred when a functional HDA6 allele was overexpressed in the *rts*1-1 background (Figure R19).

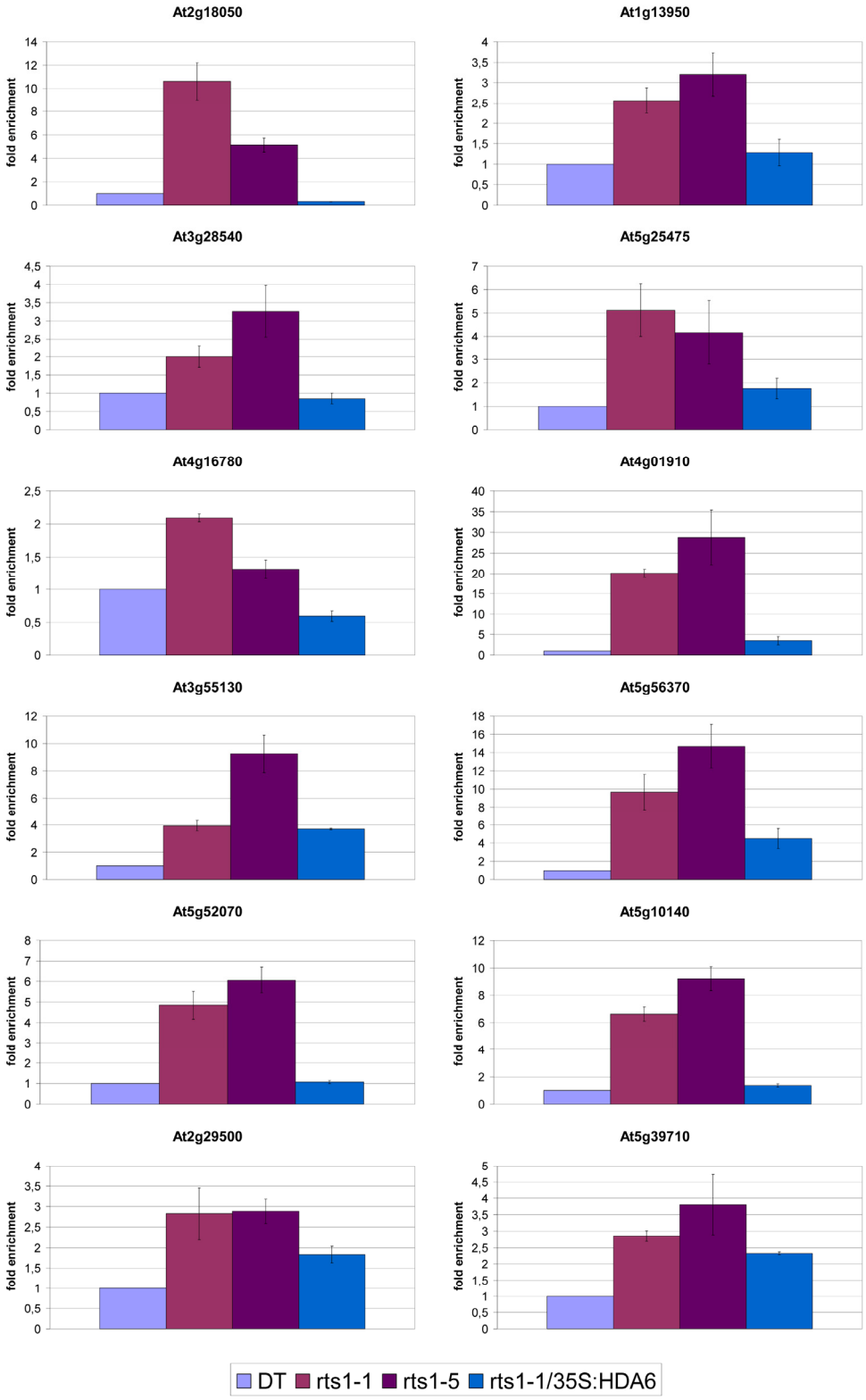

Figure R19. RT-qPCR validation of upregulated genes identified by ATH1 microarray expression analysis. Fold enrichment was calculated with the Pfaffl method, using UBC28 expression for normalization. DT values were arbitrarily set to 1. Error bars represent the standard deviation of experimental triplicates.

HDA6 was shown to be part of the RdDM pathway, which silences transgenes, transpososon and repetitive elements in a siRNA-directed and DNA methylation dependent manner (Murfett et al. 2001; Aufsatz et al. 2002a; Aufsatz et al. 2002b; Probst et al. 2004;

Earley et al. 2006; Earley et al. 2010; To et al. 2011; Yu et al. 2011). Therefore, misexpressed genes were analysed for a possible vicinity to repeats and/or transposable elements, for their DNA methylation status at promoter and 5`UTR sequence in wild-type plants (*Col*-0) and for targeting of their promoter or 5`UTR regions by 24 nt siRNAs. Additionally, genes were grouped according to whether or not they are expressed or silenced in *Col*-0. Analysis was done using the *Arabidopsis* Epigenome Maps ANNO-J (http://neomorph.salk.edu/epigenome/epigenome.html). Figures R20 to R22 show the Venn diagrams of genes upregulated in *rts*1-1, *rts*1-5 or both, respectively.

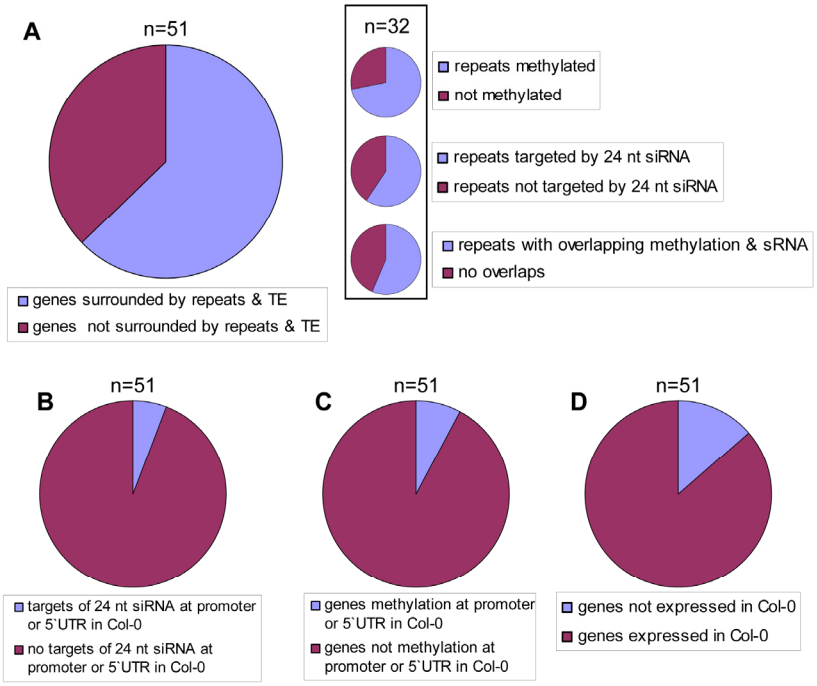

Figure R20. Genes upregulated specifically in *rts*1-1 mutants. All annotated genes except transposons were classified according to whether or not they are surrounded by repeats or transposable elements (A), targets of 24 nt siRNAs (B), methylated at their promoter or 5`UTR sequences (C), and silenced in wild-type plants (*Col*-0) (D). In case of genes surrounded by repeats or transposable elements (A), those were further subdivided based on whether or not surrounding repeats and TEs are methylated, targeted by 24 nt siRNAs or both. The analysis was done using the *Arabidopsis* epigenome maps from ANNO-J.

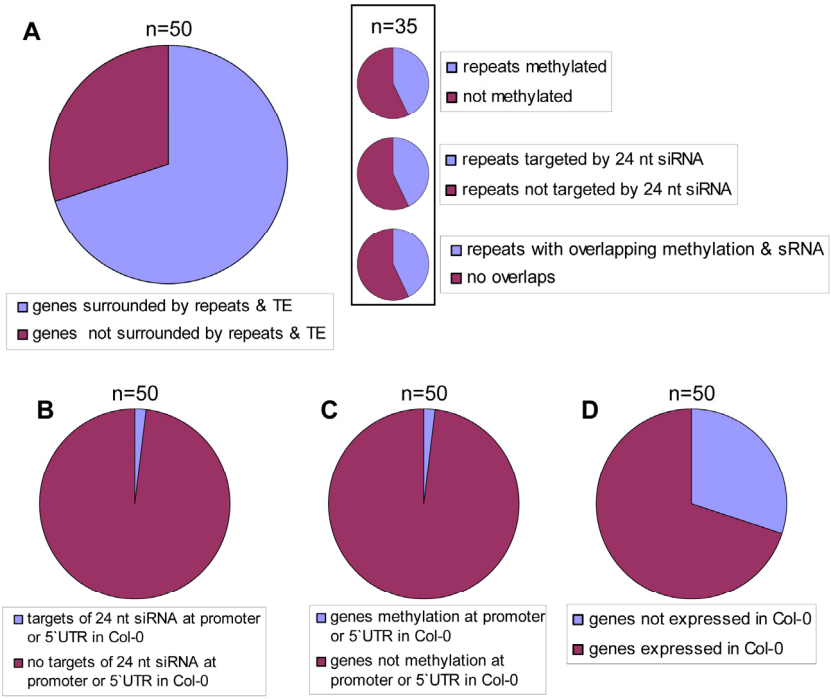

Figure R21. Genes upregulated specifically in *rts*1-5 mutants. All annotated genes except transposons were classified according to whether or not they are surrounded by repeats or transposable elements (A), targets of 24 nt siRNAs (B), methylated at their promoter or 5`UTR sequence (C), and silenced in wild-type plants (*Col*-0) (D). In case of genes surrounded by repeats or transposable elements (A), those were further subdivided based on whether or not surrounding repeats and TEs are methylated, targeted by 24 nt siRNAs or both. The analysis was done using the *Arabidopsis* epigenome maps from ANNO-J.

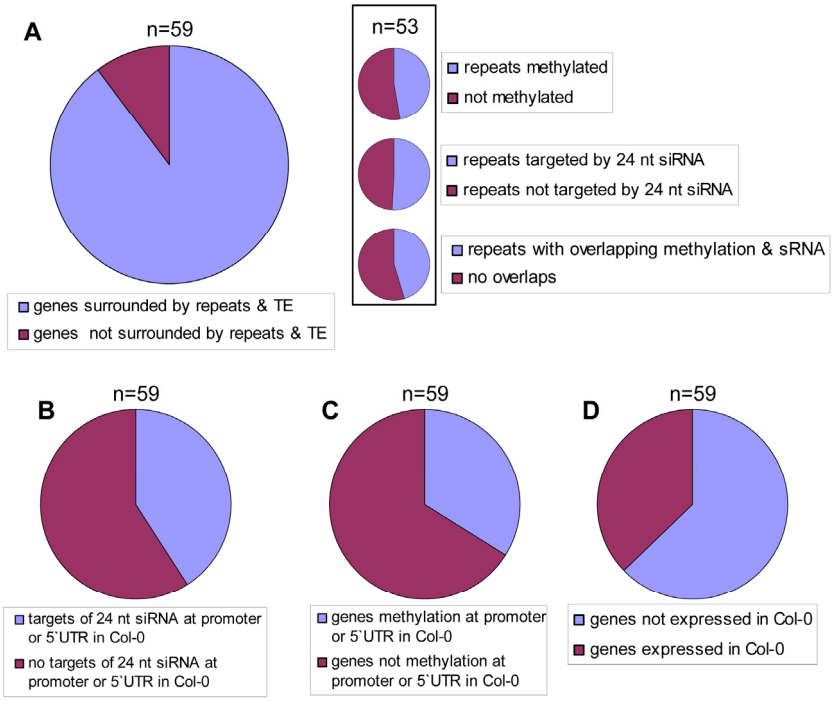

Figure R22. Genes upregulated in both *rts*1-1 and *rts*1-5 mutants. All annotated genes except transposons were classified according to whether or not they are surrounded by repeats or transposable elements (A), targets of 24 nt siRNAs (B), methylated at their promoter or 5`UTR sequence (C), and silenced in wild-type plants (*Col*-0) (D). In case of genes surrounded by repeats or transposable elements (A), those were further subdivided based on whether or not surrounding repeats and TEs are methylated, targeted by 24 nt siRNAs or both. The analysis was done using the *Arabidopsis* epigenome maps from ANNO-J.

In all cases (i.e. *rts*1-1 plants, *rts*1-5 plants, and overlap), most induced genes were surrounded by or located within repeats and/or transposons. When close to the promoter or the 5`UTR of the induced gene, the repeats and transposons were analysed for cytosine methylation and presence of 24 nt siRNAs. Interestingly, a large fraction of these elements is indeed methylated and targeted by 24 nt siRNAs in wild-type (Figure R20A, R21A and R22A), rendering them potential RdDM targets. It is likely that those genes were only induced due to the release of silencing of the neighbouring repeats and/or transposons in *rts*1 mutants. As a consequence, these elements could potentially act as promoters and/or enhancers for the neighbouring genes, causing transcriptional induction. It cannot be excluded therefore that the observed induced expression is rather a secondary than a direct effect of HDA6 deficiency. However, also genes not obviously surrounded by repeats and transposons were upregulated, and these genes could indeed be direct targets of HDA6.

Most of the genes overexpressed in *rts*1-1 and *rts*1-5 mutants are not methylated and/or targets of 24 nt siRNA at their promoter and/or 5`UTR in wild-type plants. Additionally, many of these genes are expressed in wild-type plants and therefore not obvious targets of silencing pathways (Figure R20B-D, R21B-D, R22B-D). This, however, does not exclude a requirement for HDA6 at those loci, as HDA6 could be needed for a balanced expression of these genes.

2.8.2 Functional annotation of misregulated genes using gene ontology categorization

To obtain a global view of transcriptional changes between *rts*1-1, *rts*1-5 and DT plants, the respective gene lists were analyzed for the enrichment of gene ontology (GO) terms using the GO Annotation tool (Berardini et al. 2004), available at TAIR (www.arabidopsis.org). Annotations are classified into “cellular components” (CC), “biological processes” (BP) and “molecular functions” (MF). Pie charts showing the distribution of gene ontologies for the sets of misexpressed genes in *rts*1-1 mutants, *rts*1-5 mutants, and their overlap, are depicted in Figure R23.

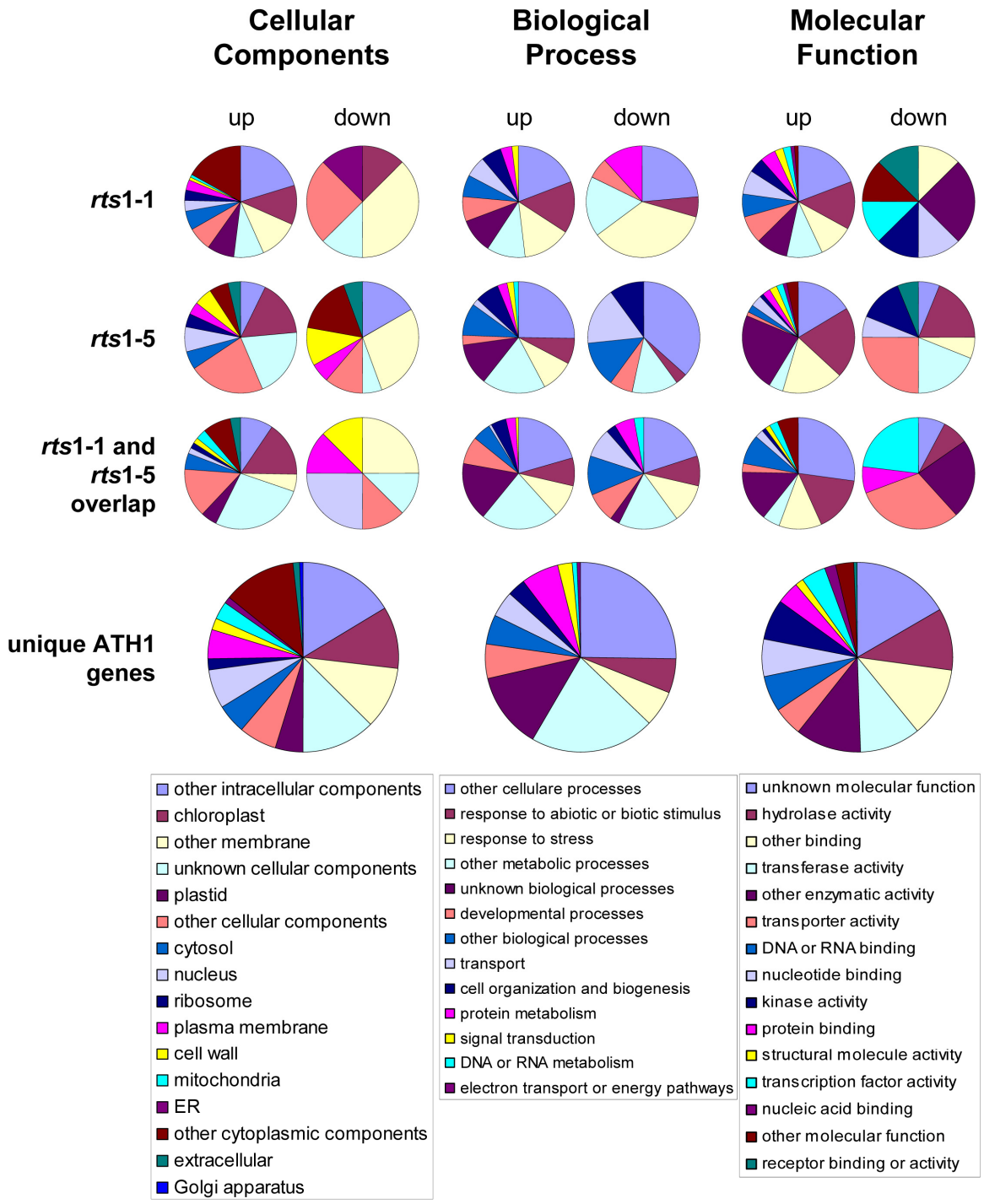

Figure R23. Gene ontology annotation of genes misregulated in *rts*1-1 mutants, *rts*1-5 mutants or both. The gene ontology (GO) annotation was done using the TAIR database and is grouped in "cellular components", "biological function" and "molecular function" of genes misregulated in either *rts*1-1, *rts*1-5 or both datasets. The *rts*1-1 and *rts*1-5 overlap represents genes not present in the single *rts*1-1 and *rts*1-5 datasets. For comparison the GO of unique ATH1 genes is included.

In order to quantify GO categories significantly overrepresented in the misexpressed gene sets, they where compared to the GO categorization of uniquely annotated genes represented on the ATH1 gene chip. Significant enrichment was determined by the hypergeometric test with a cut-off p-value ≤ 0,05. Table R2 summarizes the GO term counts of up- and downregulated genes in the respective mutants. The asterisks indicate GO categories significantly enriched compared to the ATH1 gene set. The following GO terms were enriched in the set of upregulated genes in both mutants, including the overlap between *rts*1-1 and *rts*1-5 plants: "other intracellular components" (CC) and "other cytoplasmic components" (CC). The term "other metabolic processes" (BP) was only enriched in the single *rts*1-1 and *rts*1-5 data sets without the overlap. Interestingly, the GO category "response to stress" (BP) is equally overrepresented in the *rts*1-1 up- and downregulated gene sets, indicating a major role for HDA6 in stress response pathways. Additionally, the terms "response to abiotic and biotic stimulus" (MF), "cell organization and biogenesis" (BP), "plastid" (CC) and "cytosol" (CC) are significantly enriched in the *rts*1-1 upregulated gene set. No GO category of the "molecular function" (MF) classification was enriched among genes misregulated in *rts*1-1 mutants. In *rts*1-5 plants, the terms "unknown cellular components" (CC), "plasma membrane" (CC), "unknown molecular function" (MF), "transferase activity" (MF), "DNA or RNA binding" (MF), "other cellular processes" (BP), "unknown biological processes" (BP), "developmental processes" (BP) and "protein metabolism" (BP) were significantly overrepresented in the upregulated gene set, and "other membrane" (CC), "other biological processes" (BP) and "transport" (BP) in the downregulated gene set. For the overlapping gene sets, only "transcription factor activity" (MF) was overrepresented in the downregulated gene set, whereas an total of nine GO categories was overrepresented in the upregulated gene set. No genes belonging to the following GO terms were found in the analyzed sets: "Golgi apparatus" (CC), "electron transport or energy pathways" (BP), and ER (CC), the latter with the exception of the set of downregulated genes in *rts*1-1 plants (Table R2).

Table R2: GO term gene count of up/downregulated genes

		rts 1-1		*rts* 1-5		overlap	
Cellular Component	total[1]	up	down	up	down	up	down
other intracellular components	3662	15 *	0	4 *	1	4 *	0
chloroplast	2536	7	1	7	0	7	0
other membrane	2888	10	2	0	4 *	2 *	2
unknown cellular components	4356	9	1	9 *	1	17	1
plastid	1037	7 *	0	0	0	1	0
other cellular components	2275	9	2	10	2	9	1
cytosol	1501	7 *	0	2	1	3	0
nucleus	2075	4	0	4	0	1 *	2
ribosome	394	2	0	2	0	1	0
plasma membrane	1679	4	0	2 *	1	0	1
cell wall	514	1	0	3	1	1	1
mitochondria	912	1	0	0	0	2	0
ER	356	0	1	0	0	0	0
other cytoplasmic components	2950	14 *	0	3 *	1	3 *	0
extracellular	377	0	0	2	1	2	0
Golgi apparatus	209	0	0	0	0	0	0
Molecular Function							
unknown molecular function	5616	15	0	13 *	1	22 *	1
hydrolase activity	2549	9	0	10	2	9	1
other binding	3235	6	1	10	1	7	0
transferase activity	2266	4	0	2 *	2	4	0
other enzymatic activity	2814	4	2	8	0	9	2
transporter activity	1123	4	0	1	2	1	2
DNA or RNA binding	1854	4	0	2 *	0	5	0
nucleotide binding	1859	4	1	3	1	1 *	0
kinase activity	1235	2	1	1	1	1	0
protein binding	1173	4	0	2	0	0	1
structural molecule activity	460	2	0	2	0	1	0
transcription factor activity	1402	2	1	2	1	2	3 *
nucleic acid binding	620	1	0	1	0	0	0
other molecular function	965	1	1	3	0	4	0
receptor binding or activity	196	0	1	0	1	0	0
Biological Process							
other cellulare processes	8618	19	3	17 *	6	17 *	5
response to abiotic or biotic stimulus	1792	12 *	1	6	1	5	1
response to stress	1978	12 *	5 *	7	0	7	1
other metabolic processes	7848	14 *	2	11 *	3	21	3
unknown biological processes	6523	13	0	16 *	0	19	1
developmental processes	1745	7	1	2 *	2	5	1
other biological processes	1795	5	0	6	4 *	5	2
transport	1547	6	0	2	3 *	1 *	1
cell organization and biogenesis	1073	6 *	0	4	2	2	1
protein metabolism	2851	5	0	3 *	0	3 *	2
signal transduction	999	3	2 *	2	0	1	0
DNA or RNA metabolism	307	0	0	1	0	0	1
electron transport or energy pathways	254	0	0	0	0	0	0

1: total unique genes represented on the ATH1 GeneChip

*: significantly enriched compared to ATH1 gene categorization (hypergeometric test, p-value: <0,05)

2.8.3 The chromosomal distribution of misregulated genes in *rts*1 mutants

To get insights into the chromosomal distribution of genes misregulated in the tested *rts*1 mutants, the scattering of respective locations was displayed on the according chromosomes with the chromosome map tool available at TAIR (www.arabidopsis.org). All up- and downregulated genes show an unbiased distribution among the chromosomes (Figure R24). Even though they seem to be relatively evenly scattered on the single chromosomes, some upregulated genes appear to form clusters or cluster-like structures on chromosome 5 in both *rts*1-1 and *rts*1-5. These clusters are shown in enlarged view (Figure R24) using the NCBI map viewer tool (http://www.ncbi.nlm.nih.gov/projects/mapview/). In both cases, the clusters show high levels of repetitive elements (Figure R24, enlarged window, second row), sequences enriched in CpG islands (Figure R24, enlarged window, third row) and high DNA methylation levels in *Col*-0. (http://neomorph.salk.edu/epigenome/epigenome.html; data not shown).

2.8.4 Expression profile of misregulated genes in *hda*6 mutants

To analyze tissue specific expression patterns of the misregulated genes the reference expression database and meta-analysis system Genevestigator (Zimmermann et al. 2004) (www.genevestigator.ethz.ch) was used. Based on their expression profiles in different tissues types, probe sets were clustered by Pearson correlation (Figure R25). In the upregulated *rts*1-1 probe set many genes have a ubiquitously high expression pattern, whereas genes upregulated in *rts*1-5 show either ubiquitous or very restricted and tissue specific expression profiles (Figure R25A). For example, the probe sets boxed in red are only expressed in callus tissue or cell culture. Most genes upregulated in both *rts*1-1 and *rts*1-5 show relatively low overall expression, except for some ubiquitously expressed genes. A small subset of genes is expressed in a tissue specific manner, e.g. exclusively in protoplasts, root-specific tissues, dermis or endosperm and seedcoat (Figure R25A, boxed in green). Genes downregulated in all three probe sets show ubiquitous expression patterns (Figure R25B).

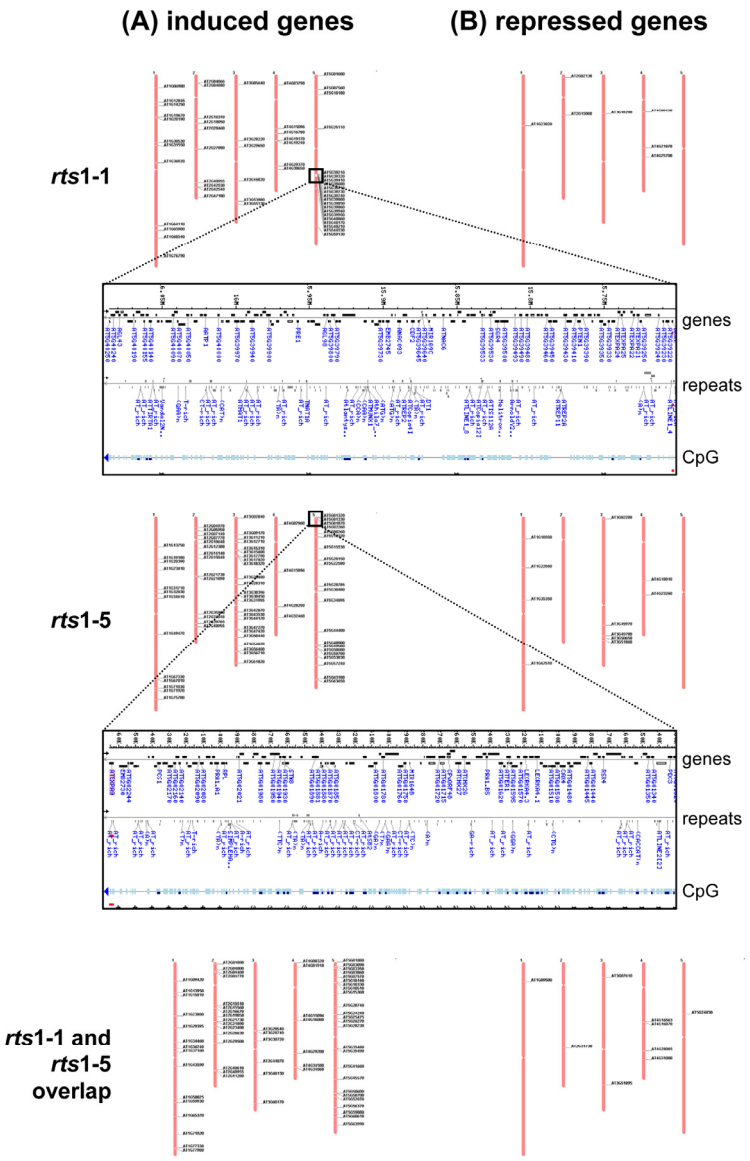

Figure R24. Chromosomal distribution of misregulated genes. Induced (A) and repressed (B) genes in either *rts*1-1 mutants, *rts*1-5 mutants or in both mutants. For depicting the chromosomal distribution, the chromosome map tool of the TAIR database was used (http://www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp). Enlarged views are shown with the NCBI map viewer tool (http://www.ncbi.nlm.nih.gov/projects/mapview/).

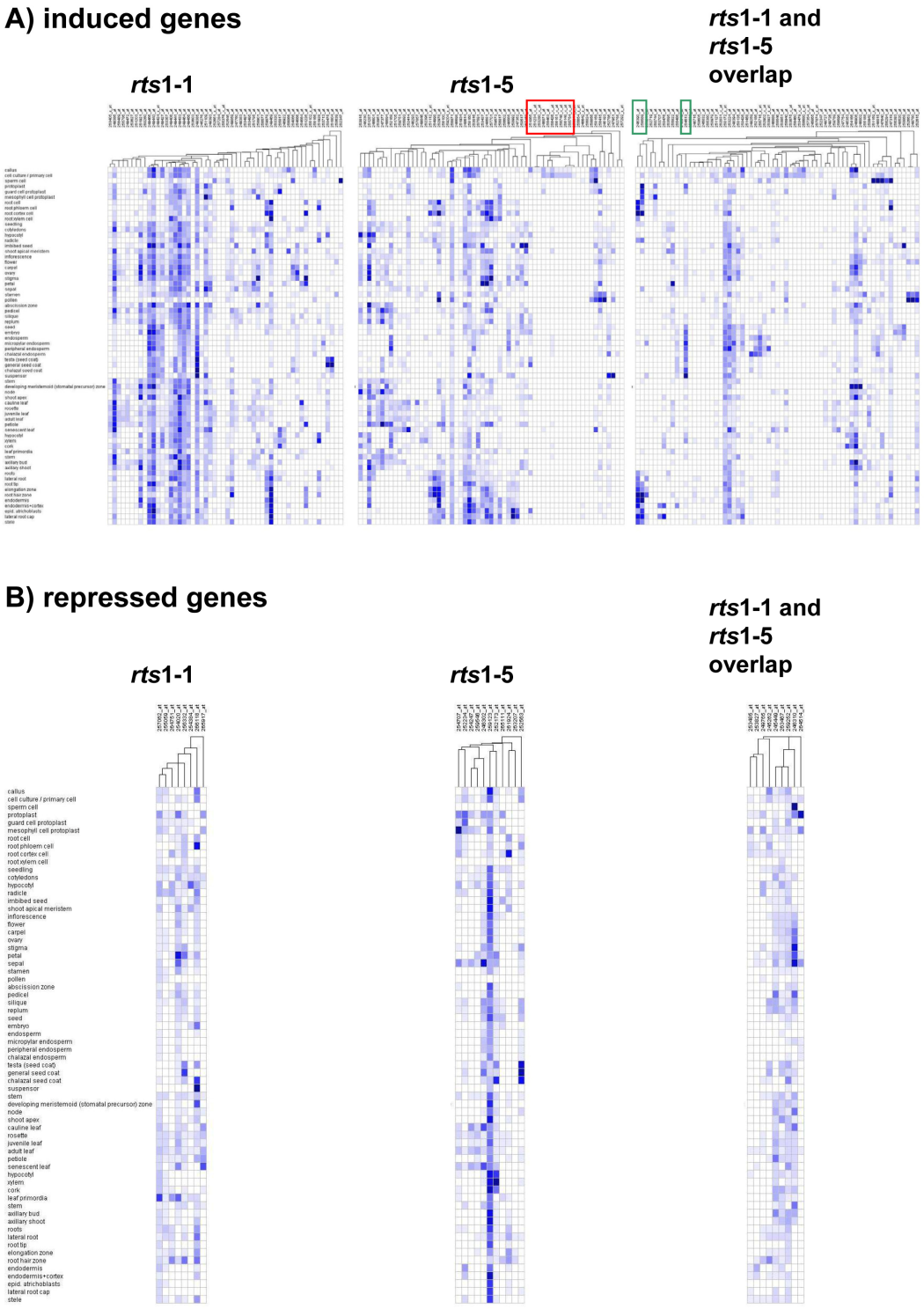

Figure R25. Hierarchical clustering of up- (A) and downregulated (B) genes in *rts*1-1 mutant, *rts*1-5 mutants or both, compared to DT. Clustering was done using Pearson coefficient gene correlation in Genevestigator (www.genevestigator.ethz.ch) according to tissue specific gene expression. Top lables show the gene tree with respective probe sets, right labeling indicates the tissue types. Dark blue colour represents high expression,white colour indicates no expression.

2.8.5 Comparison of the *rts*1-1 upregulated gene set with publicly available transcriptome data

Genes upregulated in the *rts*1-1 mutant were compared to different publicly available transcriptome datasets for potential overlaps. Transcriptome profiles used are listed in Table R3.

Table R3: Publicly available transcriptome data used

mutants	approach	reference
*rdm*4	Affymetrix *Arabidopsis* ATH1 GeneChip	He et al. 2009
*rdr*2, *ddc**	GeneChip *Arabidopsis* tiling array set	Kurihara et al. 2008
*met*1, *ddc*	self designed genome-wide tiling-array set	Zhang et al. 2006
*met*1, *ddc*, *rdd**	mRNA sequencing	Lister et al. 2008
HDA6-RNAi	Affymetrix *Arabidopsis* ATH1 GeneChip	Yu et al. 2011
*axe*1-5	Aligent's Whole *Arabidopsis* Gene Expression Microarray	To et al. 2011
*rts*1-1	Affymetrix *Arabidopsis* ATH1 GeneChip	O. Popova, unpublished results

**ddc*: *drm*1-2 *drm*2-2 *cmt*3-11 triple mutant, *rdd*: *ros*1-3 *dml*2-1 *dml*3-1 triple mutant

As mentioned already above, 119 probe sets, equal to 125 genes, are upregulated in *rts*1-1 mutant (Figure R18). The increase in gene number over probe set number is due to binding of several probes to multiple targets, such as duplicated or homologous genes. Since most of the public transcriptome data provide gene lists with TAIR accession IDs, the total gene set (125 genes) and not the probe set list was used for comparison. From the 125 genes upregulated in the *rts*1-1 mutants, 72 genes were found to be upregulated in at least one of the above mentioned transcriptome profile (Zhang et al. 2006; Kurihara et al. 2008; Lister et al. 2008; He et al. 2009b; To et al. 2011; Yu et al. 2011), hence confirming that those genes are directly or at least indirectly misexpressed upon silencing defects (Figure R26).

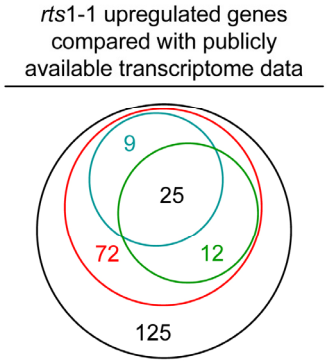

Figure R26. Genes upregulated in *rts*1-1 mutants compared to publicly available transcriptome data. A comparative analysis of genes upregulated in *rts*1-1 plants (black circle) that are also induced in other *hda*6 mutants (green circle) or different silencing mutants (red circle) is shown. The blue circle represents the number of genes confirmed by only one different transcriptome approache.

From these 72 genes, 34 were upregulated in only one of the used transcriptome profiles, whereas 38 genes were found in two or more profiles used. Interestingly, 37 genes from the 72 genes were found to be specifically regulated by HDA6 (Table R4) as they were found to be upregulated in the transcriptome profiles of *axe*1-5 (To et al. 2011), *rts*1-1 (Popova, unpublished results) or the RNAi-HDA6 line (Yu et al. 2011) but not in those of other silencing mutants (Figure R26).

Table R4: Genes specifically induced upon *hda* 6 deficiency.

id	description
AT1G77330	similar to 1-aminocyclopropane-1-carboxylate oxidase
AT1G13950	EUKARYOTIC ELONGATION FACTOR 5A-1
AT1G29395	COLD REGULATED 314 INNER MEMBRANE 1
AT5G39730	AIG2-like (avirulence induced gene) family protein
AT4G34580	CAN OF WORMS1
AT5G39410	Saccharopine dehydrogenase
AT2G21730	CINNAMYL ALCOHOL DEHYDROGENASE HOMOLOG 2
AT1G71920	HISTIDINE BIOSYNTHESIS 6B
AT5G07570	glycine/proline-rich protein
AT2G47180	GALACTINOL SYNTHASE 1
AT2G27880	AGO5
AT5G01600	ATFER1
AT1G65370	TRAF-like family protein
AT4G01910	Cysteine/Histidine-rich C1 domain family protein
AT1G19670	CHLOROPHYLLASE 1
AT5G07560	GLYCINE-RICH PROTEIN 20
AT5G63990	Inositol monophosp
AT3G53980	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
AT2G18050	HIS1-3
AT4G34860	ALKALINE/NEUTRAL INVERTASE B
AT5G03860	MALATE SYNTHASE
AT2G29500	HSP20-like chaperones superfamily protein
AT4G29370	Galactose oxidase/kelch repeat superfamily protein
AT5G45570	Ulp1 protease family protein
AT3G44070	Glycosyl hydrolase family 35 protein
AT5G56370	F-box/RNI-like/FBD-like domains-containing protein
AT5G60610	F-box/RNI-like superfamily protein
AT4G00320	F-box/RNI-like superfamily protein
AT1G34460	CYCLIN B1;5
AT5G28230	pseudogene
AT2G16340	unknown protein
AT1G36920	unknown protein
AT1G15010	unknown protein
AT1G77960	unknown protein
AT2G20460	transposable element gene
AT2G20460	transposable element gene
AT3G29650	transposable element gene

That only approximately 58% of upregulated genes (72 out of 125) were found to be misexpressed in other silencing mutants and only 30% (37 out of 125) could be confirmed to be specifically induced by *hda*6 deficiency might have several reasons. First of all one has to take into consideration that the transcriptome profiling was done using different approaches and techniques e.g. ATH1 chips, Tiling array, mRNA sequencing. All this methods have different euchromatic and heterochromatic coverage. And second, plant material, age, growth conditions and statistical analysis methods were not identical among the assays.

Genes specifically induced upon HDA6 deficiency are listed in Table R4 and include four genes encoding unknown proteins (At2g16340, At1g36920, At1g15010, At1g77960), three transposable elements (At2g20460, At2G20460, At3g29650) and one pseudogene (At5g28230). Interestingly, a large fraction of genes are involved either in stress response (At1g29395, At5g39730, At2g47180, At5g01600, At1g19670, At2g29500) or metabolic processes (At1g77330, At1g71920, At5g63990, At4g34860, At5g45570, At3g44070), highlighting a broad HDA6 function in processes other then genome defense.

To gain insights in the functions of genes upregulated specifically by *hda*6 deficiency or other silencing mutants, the gene lists were analysed for enrichment of gene ontology (GO) terms as described above. The distribution of gene ontologies for the induced genes sets are depicted in Figure R27. Table R5 summarises GO categories significantly overrepresented in *hda*6 and silencing mutants compared to the whole genome categorization for “cellular components” (CC), “biological process” (BP) and “molecular function” (MF). For genes specifically induced upon HDA6 deficiency, no enrichment could be detected for both cellular component (CC) and molecular function (MF) GO category. However, “unknown biological processes” (BP) and, interestingly, “response to both stress and abiotic and biotic stimulus” (BP) was significantly enriched in this gene list, highlighting a function of HDA6 in the control of stress responses. Genes upregulated in *rts*1-1 and silencing mutants other than *hda*6 showed GO enrichment for “unknown cellular component” (CC), “other binding” (MF), “other cellular processes” (BP) and “unknown biological processes” (BP). None of the following GO terms were overrepresented in the analyzed sets: “ER” (CC), “Golgi apparatus” (CC), “receptor binding activity” (MF), “DNA and RNA metabolism” (BP) and “electron transport or energy pathways” (BP).

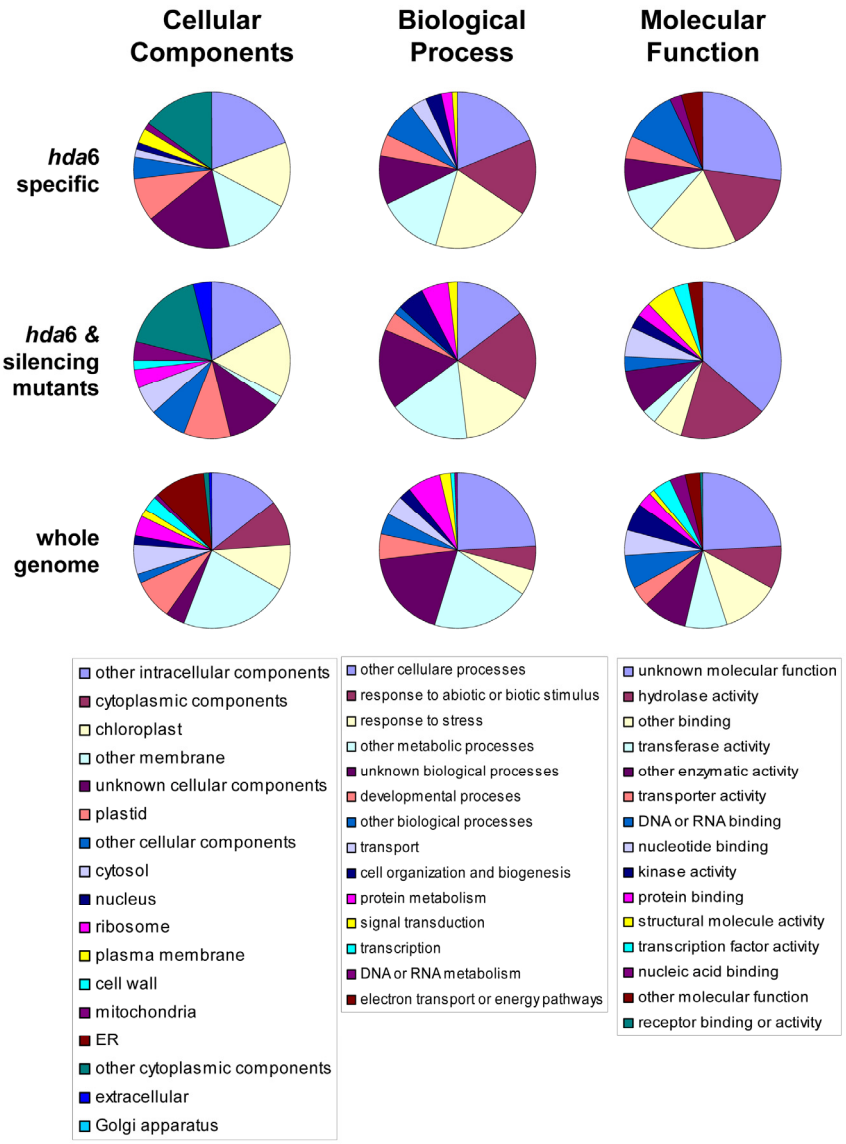

Figure R27. Gene ontology annotation of genes upregulated in *rts*1-1 mutants and either different *hda*6 mutants or silencing mutants. The gene ontology (GO) annotation was done using the TAIR database and is grouped in "cellulare components", "biological function" and "molecular function". The upper panel shows the GO annotation of genes upregulated in *rts*1-1 mutants and different *hda*6 mutant lines obtained from publicly available datasets. The middle panel shows the GO annotation of genes upregulated in *rts*1-1 mutants as well as in different silencing mutants other then *hda*6. The lower panel shows the whole genome GO annotation.

Table R5: GO term gene count of genes upregulated in *rts* 1-1 and different transcriptome approaches

		transcriptome comparison	
Cellular Component	total[1]	*hda* 6 specific	silencing mutants
other intracellular components	4244	7	6
chloroplast	2934	4	4
other membrane	3326	5	1
unknown cellular components	9724	12	6 *
plastid	1112	3	3
other cellular components	3605	3	4
cytosol	1641	2	3
nucleus	2434	1	0
ribosome	473	0	2
plasma membrane	1823	2	0
cell wall	555	0	1
mitochondria	1105	1	2
ER	407	0	0
other cytoplasmic components	3278	5	6
extracellular	439	0	2
Golgi apparatus	229	0	0
Molecular Function			
unknown molecular function	10885	12	12
hydrolase activity	2932	5	5
other binding	4513	7	1 *
transferase activity	2513	3	1
other enzymatic activity	3202	3	2
transporter activity	1250	1	0
DNA or RNA binding	2877	2	1
nucleotide binding	2127	0	1
kinase activity	1352	0	1
protein binding	1307	0	1
structural molecule activity	537	0	2
transcription factor activity	1681	0	1
nucleic acid binding	1466	1	0
other molecular function	1147	2	1
receptor binding or activity	232	0	0
Biological Process			
other cellulare processes	10932	13	5 *
response to abiotic or biotic stimulus	2070	7 *	4
response to stress	2307	8 *	4
other metabolic processes	9931	11	8
unknown biological processes	11376	9 *	9 *
developmental processes	2219	2	2
other biological processes	2076	4	1
transport	1795	3	0
cell organization and biogenesis	1236	2	1
protein metabolism	3926	2	3
signal transduction	1182	1	1
DNA or RNA metabolism	376	0	0
electron transport or energy pathways	283	0	0

1: whole genome classification (TAIR)

*: significantly enriched compared to whole genome gene categorization (hypergeometric test, p-value: <0,05)

The genomic distribution of genes upregulated either specifically in *hda*6 mutants or in other silencing mutants was assessed as described above and is depicted in Figure R28. Both gene lists showed an unbiased and even distribution over individual chromosomes. No clustering, as described for *rts*1-1 and *rts*1-5 upregulated probe sets (Figure R24), could be observed.

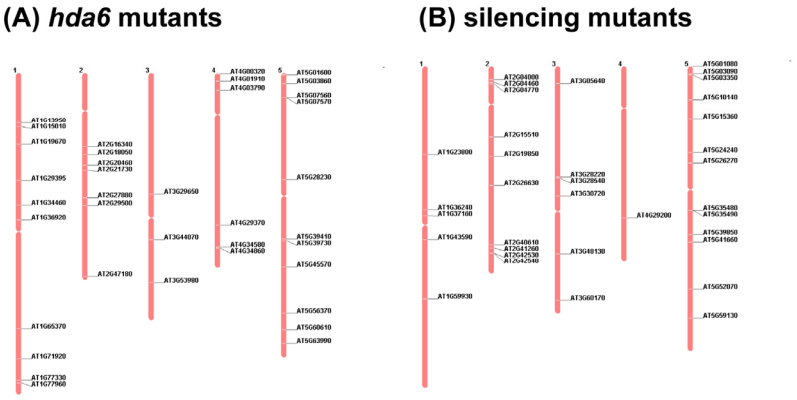

Figure R28. Chromosomal distribution of genes found to be upregulated in different whole transcriptome approaches. (A) genes upregulated specifically in *hda*6 mutants (*rts*1-1, *axe*1-5) and HDA6 knockdowns (HDA6-RNAi lines). (B) genes upregulated in *rts*1-1 mutants as well as different silencing mutants (*rdm*4, *rdd*, *ddc* or *met*1).

3. Discussion

3.1 Late flowering phenotype of *rts*1 mutants

The timing of floral transition is genetically controlled by a network of four major flowering pathways in *Arabidopsis*: the photoperiod pathway (which counts day lengths), the vernalization pathway (which senses prolonged cold temperature), the gibberellin pathway (gibberellins) and the autonomous pathway. These different pathways converge at the regulation of integrator genes, which play crucial roles in flower time regulation. Such common downstream target genes like SOC1 (suppressor of overexpression of constans 1) and FT (flowering locus T) in turn activate floral-meristem-identity genes such as LEAFY and APETALA1, causing the production of flower primordia. Well characterized central players in these pathways are FWA and FLC (Boss et al. 2004; Putterill et al. 2004).

FWA, a homeodomain-containing transcription factor first identified based on its ability to delay flowering, is presumed to affect flowering through the photoperiod pathway (Koornneef et al. 1998). When expressed ectopically, FWA is causal for a delayed floral transition (Fujimoto et al. 2011) by inhibiting the function of FT through direct interaction (Kakutani 1997; Soppe et al. 2000; Ikeda et al. 2007). The silencing of FWA depends on cytosine methylation of the SINE-related tandem repeats in its 5`region. These tandem repeats are source of siRNAs which epigenetically silence the FWA gene via the RdDM pathway (Cao and Jacobsen 2002; Chan et al. 2004; Lippman et al. 2004; Chan et al. 2006). Even though HDA6 was shown to be a major player in the RdDM pathway (Aufsatz et al. 2002b) and *hda*6 mutants exhibit a delayed flowering phenotype (Figure R3A), no epigenetic switch in FWA expression could be detected in *rts*1 mutants (Figure R3B and R3C).

Another key component in the regulatory network of flowering control is the MADS-box transcription factor FLC, which is a negative regulator of floral transition (Michaels and Amasino 1999; Sheldon et al. 1999). Microarray analysis revealed ectopic FLC expression in *rts*1 mutants (Figure R3D), suggesting that this misexpression is causal for the observed late flowering phenotype. Furthermore, the late flowering phenotype could partially be complemented in *rts*1-1 mutant lines overexpressing a functional HDA6 allele. These results suggest that HDA6 is involved in floral transition pathways by epigentically controlling FLC expression. FLC expression can be independently repressed either via the vernalization pathway in response to a prolonged cold exposure or constitutively via the autonomous pathway (Michaels and Amasino 2001). Until now, there are seven genes known to be involved in the autonomous pathways: FCA (Macknight et al. 1997), FLD (flowering locus D) (He et al. 2003), FPA (Schomburg et al. 2001), FVE (Ausin et al. 2004), FY

(Simpson et al. 2003), LD (luminidependens) (Lee et al. 1994; Lim et al. 2004) and FLK (flowering locus K) (Lim et al. 2004; Mockler et al. 2004).

In *fld* and *fve* mutants, in contrast to other autonomous mutants including *fca*, *fpa* and *ld*, histones H3 and H4 are both hyperacetylated at FLC, indicating that FLD and FVE participate in the deacetylation as components of an HDAC complex to regulate FLC expression (He et al. 2003; Ausin et al. 2004; Kim et al. 2004). FLD is a plant homolog of the human KIAA0601/LSD1. KIAA0601 was shown to be a component of human histone deacetylase 1,2 (HDAC1/2) corepressor complexes which are involved in gene repression by deacetylation of histone residues (Humphrey et al. 2001; Hakimi et al. 2003). Interestingly, FLD is also involved in H3K4 demethylation and deacetylation of FLC chromatin causing FLC downregulation and onset of flowering (He et al. 2003; Jiang et al. 2007; Liu et al. 2007). Furthermore, a recent study in *Arabidopsis* could show that HDA6 physically interacts *in vitro* and *in vivo* with FLD, indicating that these two proteins act in the same protein complex (Yu et al. 2011).

FVE encodes a nuclear protein with six WD domains and was previously annotated as *At*MSI4 (Ausin et al. 2004). FVE/*At*MSI4 is a plant homolog of yeast MSI (multicopy suppressor of IRA1) and the mammalian retinoblastoma-associated proteins RbAp46 and RbAp48 (Kenzior and Folk 1998; Kim et al. 2004). MSI1-like proteins have been identified to occur in complexes promoting the assembly and modification of chromatin (Qian et al. 1993; Parthun et al. 1996; Taunton et al. 1996; Verreault et al. 1996; Ach et al. 1997; Jullien et al. 2008). For example, the orthologs MSI1, RbAp48 and p55 are components of chromatin assembly complexes (CAF) in yeast, humans and Drosophila, respectively (Tyler et al. 1996; Verreault et al. 1996; Kaufman et al. 1997). Furthermore, RbAp48 and its homolog RbAp46 together with HDAC1 are components of the mSin3A complex (Hassig et al. 1997). In *Arabidopsis,* MSI1 orthologs are represented by a small gene family of five MSI-like genes. By phylogenetic analysis these proteins can be clustered into two main clades, suggesting that there is only limited functional redundancy between them. One clade is constituted of *At*MSI1, *At*MSI2 and *At*MSI3. Clade 1 members are more closely related to animal sequences than *At*MSI4 and *At*MSI5, which form the second clade (Hennig et al. 2003; Ausin et al. 2004). *At*MSI4 was shown to participate in a protein complex repressing FLC transcription through a histone deacetylase mechanisms by interacting with retinoblastoma protein (Ausin et al. 2004). *At*MSI1, like *At*MSI4, can interact with retinoblastoma related proteins and the histone deacetylase HDA1 in *Arabidopsis* (Hennig et al. 2003) and was recently copurified with tagged HDA6 (Rakic 2010, PhD thesis). Even though morphological studies and

phylogenetic analysis suggest that there is only a limited functional redundancy between *At*MSI1 and *At*MSI4, it cannot be excluded that some functions overlap. Our results strengthen the idea that HDA6 associates with multiple MSI1-like proteins in *Arabidopsis* and acts together with FVE and FLD to regulate FLC expression by histone deacetylation.

3.2 Release of TGS in *rts*1 mutants and the crosstalk between its associated epigenetic marks

In addition to the NOSpro:NPTII transgene, four endogenous RdDM target loci were examined for their epigenetic status. While all *rts*1 mutants showed a release of transcriptional gene silencing relative to DT, not all loci, however, exhibited detectable changes in cytosine methylation (Figures R4 and R5).

3.2.1 HDA6 and its possible links to the MET1/DDM1 and CMT3/KYP pathway

For the high copy number retrotransposons *At*SN1 and *At*GP1, as well as for the single copy intergenic region IG5, reactivation correlated with a loss of cytosine methylation (Figure R5). At *At*SN1, a significant decrease of CG methylation was observed, whereas CHG and CHH methylation were only marginally affected. The *rts*1-4 mutant, however, showed strong cytosine demethylation in all sequence contexts. Transcriptional reactivation of *At*GP1 and IG5 could be correlated with significant losses of cytosine methylation at CHG sites. In addition, IG5 exhibited decreased CG methylation levels. Even though cytosines in the CHH sequence context had the highest pattern frequency (Table R1) in all of the analyzed loci, those sites were least effected by *hda*6 deficiency, suggesting a specific role of HDA6 in the maintenance of symmetric CG and CHG methylation. Both symmetric methylation marks are, once established, maintained throughout DNA replication even in the absence of a constant siRNA trigger (Jones et al. 2001; Aufsatz et al. 2002a).

CG sites are efficiently maintained by the DNA methyltransferase MET1 (Jones et al. 2001) in a pathway proposed to involve HDA6 (Aufsatz et al. 2002b; Aufsatz et al. 2004) and DDM1 (Mittelsten Scheid et al. 1998; Gendrel et al. 2002; Johnson et al. 2002; Lippman et al. 2004). MET1 mutants were discovered in screens for global loss of DNA methylation and for the release of TGS (Vongs et al. 1993; Kankel et al. 2003; Saze et al. 2003) and found to be dispensable for RdDM initiation, but absolutely necessary for TGS maintenance (Jones et al. 2001). That DNA methylation requires the action of additional proteins besides DNA methyltransferases was evident when mutants with decreased DNA methylation (*ddm*) were discovered and further characterized (Vongs et al. 1993). DDM1 encodes a member of the

SNF2/SWI2 family, binds to nucleosomes and promotes chromatin remodelling in an ATP-dependent manner (Brzeski and Jerzmanowski 2003). DDM1 is required to maintain DNA and H3K9 methylation levels and is responsible for transposon and transgene silencing (Jeddeloh et al. 1998; Gendrel et al. 2002; Johnson et al. 2002; Lippman et al. 2003). In addition to MET1 and DDM1, CG methylation is also controlled by VIM1 (variant in methylation 1) (Woo et al. 2007; Woo et al. 2008), a SRA (SET- and RING-associated) domain methylcytosine-binding protein. VIM1 has overlapping functions in the maintenance of global CG methylation and epigenetic transcriptional silencing with its homologs VIM2 and VIM3 (Woo et al. 2008). It is suggested that VIM1 acts at the DNA methylation–histone interface to maintain heterochromatin. Another gene required for the maintenance of CG methylation is HDA6 (Murfett et al. 2001; Aufsatz et al. 2002b; Probst et al. 2004), placing HDA6 together with MET1, DDM1 and potentially VIM1 in one pathway. Interestingly, next to HDA6, a second histone deacetylase, HDT1, was shown to be required for cytosine methylation maintenance at the rRNA gene promoter (Lawrence et al. 2004). Whether and how these players act together in one pathway or they function in separate complexes or pathways that converge on CG methylation maintenance is still unclear.

Since H3K9 acetylation and H3K9 methylation are mutually exclusive (Lawrence et al. 2004), it is likely that HDA6 and/or HDT1 potentially deacteylate H3K9 prior to H3K9 methyltransferase action. After H3K9 has been methylated, it is recognized by chromodomain-containing proteins. In many organisms, H3K9me is bound by orthologs of the mammalian HP1 (Heterochromatin protein 1). In *Arabidopsis*, however, the HP1 ortholog LHP1 (Like heterochromatin protein 1) is mainly associated with euchromatic regions marked by H3K27me3, although it can also bind to H3K9me2/me3 (Zemach et al. 2006; Turck et al. 2007; Exner et al. 2009). Hence, a chromodomain protein fulfilling the canonical role of HP1 still remains to be identified. Evidence from mammals shows, however, that in a subsequent step DNA methyltransferases interact with HP1 (Lehnertz et al. 2003; Fuks et al. 2003a). Even though this still needs to be shown for *Arabidopsis* DNA methyltransferases, it is possible that MET1, the ortholog of mouse Dnmt1 (Finnegan and Dennis 1993), is recruited via a yet to be identified protein with HP1 function to methylate CG residues. This would highlight a mechanism by which DNA methylation is specified by histone deacteylation and subsequent histone methylation. Since VIM1, a methylcytosine-binding protein, was also shown to interact with methylated CG and CHG (Woo et al. 2007; Woo et al. 2008), a second pathway, either distinct or overlapping with the one described above, is likely to exist. It is known for mammals that methylcytosine-binding proteins (MBP) associate with both histone

deacteylases (Jones et al. 1998; Nan et al. 1998; Tatematsu et al. 2000) and histone methyltransferases (Fuks et al. 2003b), thereby bridging DNA metylation and histone modifications. VIM1, however, lacks such an MBD domain and binds methylcytosines through a region encompassing the SRA domain. It is known that mammalian proteins with SRA domains, like Np95 and NIRF, can recruit histone deacetylases (Unoki et al. 2004), thereby connecting DNA methylation and histone modification (Rottach et al. 2010). It can therefore be proposed that VIM1 binds to methylated DNA, alters histone modifications by recruiting histone deacetylases which in turn make the histones accessible for histone methyltransferases. In both proposed pathways, the SNF2/SWI2 family member DDM1 would be needed to remodel the chromatin in order to make it accessible for the above mentioned proteins. Either way, both discussed mechanisms highlight the importance of histone deacetylase activity in maintenance of DNA methylation status and epigenetic silencing.

Maintenance of CHG methylation in *Arabidopsis* is controlled by a pathway distinct from that regulating CG methylation (Gruenbaum et al. 1981). CHG methylation maintenance requires the concerted action of CMT3 (Lindroth et al. 2001) and the histone methyltransferase KYP (kryptonite) (Jackson et al. 2002). CMT3 encodes a plant specific DNA methyltransferase containing a chromodomain and a bromo-adjacent homology (BAH) domain (Henikoff and Comai 1998; Callebaut et al. 1999; McCallum et al. 2000) and is a key determinant for maintaining CHG methylation and epigenetic gene silencing (Bartee et al. 2001; Lindroth et al. 2001). KYP is a SU(VAR)3-9 homolog that methylates H3K9 *in vitro* (Jackson et al. 2002), and *kyp* mutants mimic *cmt*3 mutant phenotypes in showing reduced levels of CHG methylation and transcriptional reactivation of endogenous retrotransposons (Jackson et al. 2002). In a pathway involving CMT3, KYP and potentially HDA6, H3K9 would first needed to be deacetylated by HDA6, since H3K9ac and H3K9me are, as already mentioned above, mutually exclusive histone marks (Lawrence et al. 2004). H3K9 would then be methylated by the action of KYP, and the resulting H3K9me mark would subsequently be recognized and transmitted down to the level of DNA methylation. A HP1-like intermediate might be unnecessary for CMT3 targeting to chromatin, since the chromodomain of CMT3 interacts, at least *in vitro*, with the N-terminal tail of H3 simultaneously methylated at both H3K9 and H3K27 (Lindroth et al. 2004). Therefore, CHG methylation would be controlled by histone deacetylation and methylation and by interaction of CMT3 with methylated H3K9. As CMT3 binds histone H3 only when both H3K9 and H3K27 are simultaneously methylated,

H3K27 methylation by an unknown enzyme might provide a combinatorial histone code for CMT3 recruitment.

3.2.2 Uncoupling of transcriptional reactivation and DNA methylation in *rts*1 mutants

At the NOSpro:NPTII transgene, allele specific methylation phenotypes were observed upon HDA6 deficiency. Despite a decrease in cytosine methylation for *rts*1-1 and *rts*1-4, DNA methylation was unaltered in both *rts*1-3 and *rts*1-5 (Figure R5). Since the silencing of the transgene was suppressed in all *rts*1 mutants (Figure R4), *rts*1-3 and *rts*1-5 mutants show an uncoupling of DNA methylation and transcriptional reactivation. The reasons for the observed allele specific effects on DNA methylation are currently unclear. However, small RNAs for this transgene are provided *in trans* by an unlinked silencer transgene (Aufsatz et al. 2002a). This is in contrast to all other endogenous loci analyzed, where the silencing sRNAs are derived *in cis*, rendering the loci source and target of small RNAs, respectively. The soloLTR is reactivated in all *rts*1 mutants without changes in DNA methylation (Figure R4, Firgure R5E and R5F). Therefore, the epigenetic state of the soloLTR seems to be primarily controlled by HDA6 dependent histone deacetylation rather then by a switch in DNA methylation patterning.

In recent years, the crosstalk between TGS release and DNA methylation has been under tremendous investigation, showing that release of silencing does not necessarily correlate with decreased DNA methylation levels (Amedeo et al. 2000; Takeda et al. 2004; Elmayan et al. 2005). For example, BRU1 (brushy 1), also described as MGO3 (Guyomarc'h et al. 2004) and TSK (Suzuki et al. 2004), was shown to stochastically reactivate transgenes without any influence on respective cytosine methylation levels (Takeda et al. 2004). Even though BRU1 harbors two conserved domains predicted to be involved in protein-protein interaction, the protein structure *per se* does not allow any speculations about its function (Takeda et al. 2004). Additionally to its reactivating phenotype, it was shown that BRU1 mutants show altered global and locus specific histone acetylation levels and that BRU1 is required for a correct transition to flowering (Guyomarc'h et al. 2006).

Another gene able to release silencing without detectable changes in cytosine methylation is a major nuclear single stranded DNA binding protein called RPA2 (DNA replication protein A2) (Bochkarev and Bochkareva 2004; Elmayan et al. 2005). In contrast to the majority of genes identified in the control of TGS (e.g. *ddm*1, *met*1 or *mom*1), mutant alleles of both genes, BRU1 and RPA2, as well as HDA6 are hypersensitive to the DNA damaging agent MMS (Takeda et al. 2004; Elmayan et al. 2005; Kapoor et al. 2005; this

study). These results thus suggest that the function of BRU1, RPA2 and HDA6 in the control of epigenetic states might be closely associated with processes involving DNA repair and/or replication. This novel link will be discussed further in section 3.4.

In addition to *bru*1 and *rpa*2, mutations in MOM1 (morpheus` molecule 1) release silencing of clustered transgenes (Amedeo et al. 2000), transposons (Steimer et al. 2000; Habu et al. 2006) and 5S rRNA genes (Vaillant et al. 2006) in a DNA methylation independent manner. MOM1 has an incomplete ATPase/helicase domain homologous to that of SWI2/SNF2 chromatin remodeling proteins (Amedeo et al. 2000) and is closely related to the CHD3/Mi-2 chromatin remodeling proteins found in higher eukaryotes (Caikovski et al. 2008). Taking into consideration that target loci reactivated in *mom*1 (Numa et al. 2009) and *rts*1 (Figure R10) mutants are associated with reduced levels of H3K9me2 within their promoter regions, histone marks but not cytosine methylation seem to be the major switches controlling the transcriptional activity of (at least some) respective target loci.

Further hints that TGS does not primarily rely on DNA methylation, but can be regulated by independent pathways, originate from studies employing two adjacent transgene targets: Pro_{RD29A}:LUC and Pro_{35S}:NPTII. The Pro_{RD29A}:LUC transgene is regulated by the RdDM pathway, since mutations in RDM2, NRPD1, NRPE1, NRPD2a, HEN1 and DRD1 released silencing only of this transgene, but not of the neighboring Pro_{35S}:NPTII transgene. The Pro_{35S}:NPTII locus is most likely regulated by a "spreading heterochromatin pathway" independent of RdDM and matching siRNAs (Gong et al. 2002; Kapoor et al. 2005; Xia et al. 2006; Wang et al. 2007; He et al. 2009). Interestingly, only mutations in HDA6 and AGO4 caused reactivation of both transgenes, suggesting that these factors control TGS in both siRNA-directed DNA methylation dependent and independent pathways (He et al. 2009).

In summary, it can be concluded that TGS relies on both DNA methylation dependent and independent pathways and that HDA6 is a major player in both. In case of the DNA methylation independent pathway, the major switch in transcriptional states seems to be histone acteylation, which influences histone methylation levels as described above.

3.3 Zebularine and its effect on transcriptinal reactivation

In this study it could be shown that the treatment with the DNA demethylating agent zebularine increases transcriptional reactivation in DT plants and *rts*1 mutants. Not in all cases, however, this reactivation was accompanied by loss of cytosine methylation (Figure R4, R8).

Zebularine is a cytidine analog containing a 2-(1H)-pyrimidinone ring that was originally developed as a cytidine deaminase inhibitor, because it lacks an amino group on

position 4 of the ring (Kim et al. 1986; Laliberte et al. 1992). In contrast to other DNA demethylation drugs like aza nucleosides, zebularine is stable in aqueous solution and minimally toxic when used *in vivo* (Cheng et al. 2003). Zebularine exerts its demethylation activity by stabilizing the binding of DNA methyltransferases to the DNA, blocking the methylase activity and decreasing the dissociation of the enzyme, thereby trapping it and preventing its activity even at remote sites (Hurd et al. 1999; Champion et al.). Effective DNA demethylation that correlated with increased transcriptional activity could be observed in various organisms and cell types upon zebularine treatment (Cheng et al. 2003; Cheng et al. 2004; Lee et al. 2004; Scott et al. 2007; Baubec et al. 2009).

As depicted in Figure R8, DNA demethylation effects after zebularine treatment were only observed at the FWA promoter, and, for DT plants, also at the retrotransposon *At*GP1. In contrast, no decrease in cytosine methylation could be detected at the soloLTR and the intergenic region IG5. Despite the lack of DNA demethylation in some cases, however, all loci were strongly upregulated (Figure R4). This is in contrast to previously published results, where effective DNA demethylation was reported after zebularine exposure (Cheng et al. 2003; Cheng et al. 2004; Baubec et al. 2009). The underlying reasons for the unchanged DNA methylation levels at soloLTR and IG5 remain obscure. It should be noted, however, that DNA remethylation is a constant process acting in parallel to the cytosine demethylation activity of zebularine. Even though it was shown that zebularine increases the covalent DNA binding of DNA methyltransferases, it was also reported that zebularine fails to irreversible crosslink those DNA - DNA methyltransferase complexes (Champion et al. 2010).

One can only speculate that if the soloLTR and IG5 were first demethylated, causing the increased transcription of these loci, they were effectively remethylated afterwards without causing immediate transcriptional shutdown, or that both de- and remethylation processes were acting in parallel, keeping DNA methylation status in balance. Results favoring the first hypothesis originate from a study in T24 cells, which is a human urinary bladder carcinoma cell line. Bender and coworker showed that remethylation of genes that were demethylated after 5-Aza-CdR was efficient and that rates of remethylation are rather time dependent and not generally restricted to the S phase of the cell cycle. Furthermore, that study revealed that actively transcribed regions are faster remethylated then genes usually not transcribed and that transcription through CpG islands does not inhibit their remethylation (Bender et al. 1999). Supposing that this mechanism can be transferred to plants, the soloLTR and IG5 loci indeed could have become demethylated first and efficiently remethylated

afterwards, whereas their transcription stayed elevated as remethylation would not immediately counteract transcription.

Even though it was thought until recently that DNA methylation cannot be restored in plants once it has been severely compromised (Richards 2006; Henderson and Jacobsen 2007; Mathieu et al. 2007), Teixeira and coworkers demonstrated the existence of an efficient RNAi machinery protecting against loss of DNA methylation. This remethylation process, however, is usually progressive over several generations and, in case of reactivated transposable elements, is associated with their resilencing (Teixeira et al. 2009). Taking this into consideration, a decrease of DNA methylation by zebularine followed by a fast remethylation at both the soloLTR and IG5 seems unlikely. Therefore, it appears more plausible that at both loci zebularine action was prevented or less effective due to yet unknown reasons.

It has been reported recently, that abiotic stress treatments can cause derepression of silenced loci in a DNA methylation independent manner (Lang-Mladek et al. 2010; Pecinka et al. 2010). As zebularine was shown to induce genotoxic stress (Lee et al. 2004; Ruiz-Magana et al. 2011), the increased reactivation could also be explained by a stress induced release of TGS.

3.4. A genetic link among epigenetic gene silencing and DNA damage reponses

The analyzed HDA6 mutant allele *rts*1-1 was found to be hypersensitive to the DNA damage inducing agents MMS (Figure R16) and zebularine (Figure R6). MMS is a radiation mimicking monofunctional alkylating agent that induces DNA DSBs (Ulm et al. 2001; Gong et al. 2002; Abe et al. 2005). DSBs are repaired predominantly by homologous recombination as one of the major repair pathways during S-phase. (Abe et al. 2005; Frankenberg-Schwager et al. 2005; Osakabe et al. 2006). As shown in Figure R16, *rts*1-1 hypersensitivity and impaired rooth lenght can be complemented by *rts*1-1 mutant lines constitutively expressing a tagged and functional HDA6 allele, suggesting that HDA6 deficiency is causing the observed phenotype. Furthermore, *rts*1-1 mutant plants are sensitive to zebularine (Figure R6; M. Rosa, unpublished results), a drug shown to efficiently block DNA methylation transiently in any sequence context (Baubec et al. 2009). Recent studies showed that zebularine causes induction of DNA damage and caspase-dependent apoptosis in Jurkat, CEM-6 and MOLT-4 leukemia T-cell lines (Ruiz-Magana et al.) as well as reduced growth rate, frame shifts and base substitutions in *E.coli* (Lee et al. 2004). Therefore, the hypersensitivity of *rts*1-1 to zebularine and MMS, and the ability of a functional, overexpressed HDA6 allele to complement this phenotype (Figure R6) strengthen the idea that HDA6 is involved in DNA damage response pathways. Additionally, *rts*1-1 mutant plants are also hypersensitive to

bleocin (inducer of DSBs) and mitomycin C (crosslinking of DNA strands), supporting its important role in DNA damage response (M. Rosa, unpublished results). Considering, that other TGS mutants, such as *ddm*1, *mom*1 or *met*1 show unchanged resistance to DNA damaging treatments, HDA6 seems to have a dual role as guardian of both epigenetic information and genomic stability.

Next to HDA6, several other genes were shown to coordinate the TGS machinery with DNA repair and/or replication. As already mentioned above, BRU1 and RPA2 deficiency causes hypersensitivity to MMS and the release of silencing of transgenes and transposon-related sequences in a stochastic frequency (Takeda et al. 2004; Elmayan et al. 2005; Kapoor et al. 2005). Furthermore, TEBICHI (TEB), a gene encoding a putative helicase and DNA polymerase domains-containing protein is required for normal DNA replication progression and for correct gene expression during development (Inagaki et al. 2009). Although the molecular details of how these factors contribute to the diverse processes is still unclear for plants, data involving human cells demonstrate that chromatin restoration after DNA damage challenges epigenetic stability and involves the histone chaperone chromatin assembly factor 1 (CAF-1) (Polo et al. 2006).

The human CAF-1 complex consists of three subuntis, namely p150, p60 and p48 (Smith and Stillman 1989) and is evolutionarily conserved. Orthologs have been described in vertebrates, yeast, insects and plants. CAF-1 is thought to mediate the deposition of H3 and H4 histones onto replicating DNA (Smith and Stillman 1989; Shibahara and Stillman 1999; Tagami et al. 2004) and to be involved in chromatin assembly after nucleotide excision repair (Ridgway and Almouzni 2000). Yeast cells lacking CAF-1 activity are impaired in gene silencing at the mating type locus and at telomeres (Enomoto et al. 1997; Monson et al. 1997; Zhang et al. 2000). In *Arabidopsis* the respective orthologs are encoded by FAS1, FAS2, and, most likely MSI1 (Kaya et al. 2001; Hennig et al. 2003). Interestingly, both FAS1 and FAS2 mutants are hypersensitive to MMS and show stochastic upregulation of transgenes and transposons, which is not accompanied by global changes in DNA methylation (Takeda et al. 2004; Ono et al. 2006). MSI1 is part of several protein complexes: It interacts with the retinoblastoma-related protein RBR1 (Ach et al. 1997; Jullien et al. 2008) and is a subunit of the PRC2 (Polycomb repressive complex 2)-like complexes, including the EMF2- and the FIS2 complexes. Together with FAS1 and FAS2, MSI1 was also shown to be a subunit of the CAF-1 complex, as already mentioned above (Kaya et al. 2001). Interestingly, MSI1 copurifies with tagged HDA6 (Rakic 2010), highlighting a role of HDA6 in different developmental processes besides RdDM. As already discussed for HDA6, FAS1 and FAS2,

MSI1 is also required for an efficient maintenance of chromatin structure in a DNA methylation independent manner (Hennig et al. 2003).

The studies on BRU1, FAS and RAP2, together with results on HDA6 presented here, not only demonstrate the involvement of these factors in the maintenance of epigenetic states regulating gene expression, but also their functions in DNA replication and repair. The identification of MSI1 as an interaction partner of HDA6 (Rakic 2010) further highlights the possibility that HDA6 actually has broader functions in plant development and strengthens the link of HDA6 with DNA replication/repair processes, like already shown for FAS1 and FAS2. In summary, all studies discussed here provide strong evidence that the contribution of BRU1, RPA2, FAS, MSI1 and HDA6 to the establishment and maintenance of epigenetic states intimately linked with the control of gene expression is closely associated with DNA replication and DNA damage response pathways.

3.5 Transcriptome analysis of *rts*1 mutants

Changes in gene expression of endogenous genes have been reported to occur in numerous mutants of diverse epigenetic regulators (Zhang et al. 2006; Kurihara et al. 2008; Lister et al. 2008). In order to analyze whether the HDA6 mutants *rts*1-1 and *rts*1-5 have similar effects on global gene expression, transcriptional changes were assessed by Affymetrix ATH1 expression arrays.

3.5.1 The nature of genes upregulated in *rts*1 mutants suggests a global role of HDA6 as transcriptional regulator

The microarray analysis showed that the expression of 119 probe sets (corresponding to 125 genes) in *rts*1-1 and 126 probe sets (corresponding to 146 genes) in *rts*1-5 were upregulated more than 2 fold. A total of 65 probe sets (corresponding to 69 genes) were found to be induced simultaneously in both *rts*1-1 and *rts*1-5, identifying them as potential candidates regulated by HDA6 (Figure R18A and B). Interestingly, the majority of overexpressed genes are not transposons but genes with either unknown or annotated function (Figure R18C). The ATH1 expression array, however, does by far not represent all known transposons and retroelements present in the *Arabidopsis* genome, therefore target specificity of HDA6 with regard to these elements cannot be excluded due to the small subset of probes available on the array.

When upregulated genes were analyzed for vicinity to repeats or transposons, most of the upregulated genes are in close proximity to or even overlapping with repeats and/or

transposons. Additionally, a large fraction of these elements are probably RdDM targets, as they are methylated and targeted by 24 nt siRNA in wild-type (*Col*-0). As already described for the soloLTR, it is possible that these elements act as promoters and/or enhancers, even in a bidirectional manner (Huettel et al. 2006). Therefore, it is likely that those genes were only found to be upregulated because the silencing of the nearby repeats and transposons was suppressed in the *rts*1 mutants. Genes not to be found in close proximity to transposable elements or repeats were also analysed for the methylation status and siRNA targeting in wild-type. Surprisingly, all except one gene found in the overlap (AT5G15360) were neither methylated nor targets of 24 nt siRNA in *Col*-0, suggesting that expressional regulation of these genes occurs independent of DNA methylation and siRNAs, but is HDA6 dependent in a direct or indirect manner.

Gene ontology (GO) analysis of the upregulated gene sets did only detect an overlapping significant enrichment for the cellular component (CC) terms "other intracellular components" and "other cytoplasmic components" (Table R2). That no other term was significantly enriched in both *rts*1-1 and *rts*1-5 datasets as well as in the overlap might reflect the fact that a high proportion of *Arabidopsis* coding genes are still uncharacterized and thus not assigned to any GO term. Interestingly, GO analysis for genes upregulated in *rts*1-1 showed enrichment of the biological process (BP) terms "response to abiotic and biotic stimulus", "response to stress" and "other metabolic processes". For example, genes involved in salt stress response (At4g30650, At5g39730), cold response (At2g42540, At2g42530, At4g30650), oxidative stress (At5g59080) or genes induced upon ABA treatment (At1g29395, At2g18050) as well as a gene involved in embryo development (At5g39710) were found to be upregulated in *rts*1-1. This fits well with published results, since different research groups could show an involvement of HDA6 in flowering regulation, ABA and jasmonate response, salt stress response and repression of embryonic properties after germination (Tanaka et al. 2008; Wu et al. 2008; Chen et al. 2010; Chen and Wu 2010; Yu et al. 2011). In addition, HDAC involvement in diverse biological processes was also shown in yeast and fungi. For example, the yeast RPD3-large complex is essential for regulated gene expression upon heat stress (Ruiz-Roig et al.), and is generally required for proper expression of both induced and repressed environmental stress response genes under multiple stress conditions (Sharma et al. 2007; Alejandro-Osorio et al. 2009). HdaA, a major class 2 histone deacetylase of *Aspergillus nidulans*, affects growth under oxidative stress conditions by regulating the expression of enzymes vital for the cellular antioxidant response (Tribus et al. 2005). In *Arabidopsis*, the best studied histone deacetylase HDA19 is a global regulator of

gene expression in development and stress responses (Tian and Chen 2001; Tian et al. 2003; Tian et al. 2005; Zhou et al. 2005). Given the highly similar gene expression profile and sequence similarity between HDA19 and HDA6 (Hollender and Liu 2008), it is not surprising that HDA6 was found to be involved in many of the same processes regulated by HDA19. Recent studies further indicate that HDA6 and HDA19 may play redundant roles in modulating seed germination and salt stress responses, as well as ABA- and salt stress-induced gene expression (Chen et al. 2010; Chen and Wu 2010). HDA6 also plays a critical role in regulating cold acclimation processes that confer freezing resistance (To et al. 2011b). The microarray results presented in this study together with findings documented in the literature indicate that HDA6 induced histone modifications modulate different response pathways in *Arabidopsis*, assigning HDA6 and most likely other RPD3-like HDACs as global regulators of gene expression.

3.5.2 HDA6 and its possible role as transcriptional activator

The general accepted idea that a HDAC acts mainly as a transcriptional repressor is supported by this study, as the number of induced genes exceeds those being repressed in the *rts*1 mutants. Only a small set of genes was found to be significantly downregulated in *rts*1-1 and *rts*1-5 datasets, and their overlap (Figure R18B). The most obvious explanation for the repression of gene activity could be that HDA6 might target transcriptional repressors. However, studies in yeast and animal cells indicate that histone deacetylation can also be required as a transcriptional activation signal. Deletion of the yeast HDA1 and RPD3 histone deacetylases caused downregulation of certain genes, which showed also rapid downregulation upon treatment with the HDAC inhibitor TSA, indicating that yeast HDACs have alternative roles as transcriptional activators (Rundlett et al. 1996; Bernstein et al. 2000). In mouse cells, HDAC1 deficiency causes an increase rather than a loss of silencing at specific gene subsets, highlighting a novel function of HDAC1 as a transcriptional (co)activator (Zupkovitz et al. 2006). Additionally, SIN3, an evolutionarily conserved corepressor that exists in different complexes with histone deacetylases, was suggested to be a dual function protein as it can negatively and positively regulate transcriptional activities (Nawaz et al. 1994). The *Arabidopsis* genome encodes a family of six SIN3 homologues (Bowen et al. 2010). Interestingly, our lab identified two SIN3 homologues, SNL1 and SNL3, as HDA6 interaction partners (Rakic 2010). A recent study of *Arabidopsis* HDA19, also known to interact with SNL3 in yeast two hybrid studies (Song et al. 2005), indicates that HDA19 provides negative as well as positive control of transcriptional regulation (Tian et al.

2005). Together, these studies suggest that gene regulation by histone acetylation and deacetylation is more dynamic than thought before and that HDA6, maybe as the enzymatic component of a SNL1/3 complex, may also possibly function as gene activator.

3.5.3 Transcription profiling of *hda*6 and other silencing mutants

Different publicly available transcriptome profiles of HDA6 mutants and/or knockdowns were used for comparison with the *rts*1-1 upregulated gene set (Table R3). Popova et al (unpublished results) found 71 genes to be upregulated using the same *hda*6 mutant allele as in this study (*rts*1-1). In contrast, To et al. 2011 (*axe*1-5) found 563 genes and Yu et al. 2011 (HDA6-RNAi) 441 genes to be upregulated upon *hda*6 deficiency. In this study, 125 genes were found to be induced in *rts*1-1 mutants (Figure R26; Table S1 and S5). The number of genes overlapping between this study and the other transcriptome data sets was surprisingly low, since only 33 genes were also found by To et al. 2011 (*axe*1-5), and 36 genes overlapped with the HDA6-RNAi data set from Yu et al. 2011. Only 31 genes were in common between the *rts*1-1 data sets from this study and Popova et al (unpublished). This low rate of overlap and general the high variance in number of genes found to be upregulated is probably due to the differences in transcriptome profiling approaches (ATH1 GeneChip, tiling array, mRNA sequencing), different ecotypes (*rts*1-1 and *axe*1-5 are in the *Col*-0 background, whereas the HDA6-RNAi line is in the *Ws* background), differences in growth conditions, plant age and material as well as used statistical analysis methods.

A large fraction of *hda*6 specific upregulated genes have major functions in response to abiotic or biotic stimulus and/or stress (Table R5). For example, the following genes were also found to be induced upon pathogen responses during geminivirus infection: At2g47180**,** At2g47180, At5g39730, At1g29395, At1g19670, At5g39410, At5g01600, At1g65370, At1g19670, At5g63990, At2g18050 (Ascencio-Ibanez et al. 2008). At1g29393, which encodes an integral membrane protein in the inner envelope of chloroplasts, provides freezing tolerance and its expression is induced by short term cold treatment, water deprivation, and abscisic acid treatment (www.arabidopsis.org). Transcripts of At2g47180 (GolS1) are induced in response to methyl viologen, an oxidative damage inducing agent. Plants overexpressing GolS1 have increased tolerance to salt, chilling, and high light stress (www.arabidopsis.org). Interestingly, this gene was also found to be induced upon TSA treatment (Tai et al. 2005) as well as drought and heat stress (Taji et al. 2002; Panikulangara et al. 2004). Another gene, At2g29500, is annotated to be involved in responses to heat, high light intensity, hydrogen peroxide and oxidative stress (www.arabidopsis.org). Taken together, these results highlight

broad HDA6 function in processes other than RdDM, as for example in responses to stressful environments (discussed in 3.5.1).

In addition, the data set of genes upregulated in *rts*1-1 mutants was compared to gene expression profiles created from other silencing mutants (*rdm*4, *rdr*2, *ddc*, *met*1 and *rdd*) (Table R3), resulting in a low overlap of 35 genes in total (Zhang et al. 2006; Kurihara et al. 2008; Lister et al. 2008; He et al. 2009b). Interestingly, 23 out of these 35 upregulated genes (65%) overlap with genes silenced by the CG-specific DNA methyltransferase MET1 (data not shown), suggesting a concerted action of both genes during silencing and CG methylation maintenance. A recent study could also reveal an important functional connection between HDA6 and MET1. Using a GeneChip *Arabidopsis* tiling array for *axe*1-5 and *met*1-3 mutants, a significant overlap of genes silenced by HDA6 and MET1 could be detected (To et al. 2011). As already discussed above (section 3.2.1), the authors propose that HDA6 regulates locus-directed heterochromatin silencing in cooperation with MET1, possibly as a recruiter or as a component of the MET1 silencing machinery, thus forming the foundation of silent chromatin structure for subsequent non-CG methylation.

4. Material and methods

4.1 Plant material and growth conditions

In this study, all *Arabidopsis thaliana* plant lines used were in the background of ecotype Columbia (*Col*-0). Plants double transformed (DT) with both the silencer (inverted NOSpro repeat) and the target (NOSpro:NPTII) were used as wild-type control in experiments using *rts*1 mutants. All *rts*1 mutant plants had the genetic background of DT. Lines constitutively expressing multiple tagged wild-type and mutant HDA6 proteins had the *rts*1-1 mutant background. DT and *rts*1 genotypes were checked regularly by PCR and sequencing approaches with primers listed in Table M2.

For plants grown sterile, seeds were surface sterilized with 1 mL 70% EtOH/0,05% Triton X-100 for 20 min with constant shaking, followed by 10 min incubation with 100% EtOH. Seeds were air dried and plated on MS agar either unsupplemented or supplemented with respective chemicals. After stratification for 48 h at 4°C, plants were grown at 21°C in a 16 h light/ 8 h dark cycle. For scoring NOSpro:NPTII reactivation and complementation analyses, seedlings were selected on 40 mg/mL kanamycin. DNA damage inducing chemicals cisplatin and MMS and the demethylating drug zebularine were added to respective concentrations.

For plants grown directly on soil, seeds were sterilized in 1:2 diluted bleach (commercially available, DanKlorix)/0,1% Triton X-100 for 10 min and washed at least three times with ddH_2O. After stratification for 48 h at 4°C in 0,8% Phytoagar, seeds were sown on soil and grown at 21°C in a 16 h light/ 8 h dark cycle.

4.2 RNA extraction, RNA clean up and Reverse Transcription

100 mg seedlings were frozen in liquid nitrogen and grinded with beads to a fine powder. RNA extraction was done using TriFast (peqGold TriFast, PeqLab) according to manufactures protocol. Subsequently, RNA was DNaseI treated (Qiagen, according to manufactures protocol), followed by further clean up and concentration employing the RNeasy MiniElute Cleanup Kit (Qiagen, according to protocol). The quality of the RNA was checked on 1,2% formaldehyde agarose gels (protocol according to RNeasy Mini Handbook, Qiagen). A second DNaseI digest was done, using 1 u DNaseI /1 µg RNA (Fermentas, according to protocol). Reverse transcription was done using 200 to 500 ng of RNA, random hexamer primers and M-MulV reverse transcriptase (Fermentas, according to manufacturer's protocol).

4.3 Polymerase Chain Reaction (PCR)

PCRs were done in a 20 µL reaction volume using either TrueStart™ Hot Start *Taq* DNA Polymerase (Fermentas) or, in case of amplifying bisulfite treated DNA, Advantage 2 Polymerase Mix (Clonetech) according to manufactures protocols. Except for Colony PCR (annealing temperature 55°C) and bisulfite PCRs (annealing temperature 50°C), the annealing temperatures were chosen according to the melting temperatures of each primer pair.

4.4 Chromatin Immunoprecipitation (ChIP)

4.4.1 Crosslinking for histone modifications

1,5 g of leaf tissue was harvested and washed twice in PBS. Samples were vacuum infiltrated in 1% formaldehyde/PBS for 10 min, subsequently crosslinking was quenched by vacuum infiltration in presence of 135 mM final glycine concentration. Tissue was washed twice in PBS and once with ddH_2O, dried in paper towels and frozen in liquid nitrogen.

4.4.2 Crosslinking for HDA6 occupancy

For crosslinking, 1,5 g tissue was harvested and washed twice in PBS. Samples were vacuum infiltrated for 15 min with either 10 mM DMA/PBS or 2 mM EGS/PBS. Afterwards tissue was washed twice with PBS followed by 10 min vaccum infiltration with 1% formaldehyde/PBS. Subsequently, crosslinking was quenched by vacuum infiltration in presence 135 mM final glycine concentration. Tissue was washed twice in PBS, once with ddH_2O, dried in paper towels and frozen in liquid nitrogen.

4.4.3 Chromatin preparation

The frozen crosslinked plant material was homogenized in 50 mL falcon tube with beads to a fine powder. 30 mL of freshly prepared extraction buffer 1 was added and samples were allowed to thaw on ice with occasional shaking until the solution was homogenous. The homogenate was filtered through one sheet of miracloth into a new 50 mL falcon tube and centrifuged at 4000 rpm for 20 min at 4°C (Eppendorf centrifuge 5810R). The resulting pellet was resuspended in 1 mL extraction buffer 2 by pipetting, transferred to an eppendorf tube and centrifuged at 13000 rpm for 10 min at 4°C. Meanwhile 300 µL extraction buffer 3 were added to a fresh 1.5 mL eppendorf tube. The pellet was resuspended in 300 µL extraction buffer 2, carefully layered over the 300 µL extraction buffer 3 and centrifuged at 13000 rpm for 1 h at 4°C. The pellet was resuspended in 500 µL freshly prepared nuclei lysis buffer and

10 µL of the suspension were set aside on ice for being checked on an agarose gel. Samples were sonicated for 10 min with intervals of 10 sec high power and 45 sec rest (Bioruptor, Diagenode) and then centrifuged at 13000 rpm for 10 min at 4°C. The supernatant was transferred to a new 1.5 mL tube and either snap frozen in liquid nitrogen for storage at -80°C or immediately processed. Before freezing, 10 µL of sample were set aside on ice for being checked on an agarose gel. The sample aliquots (before and after sonication) were separated by electrophoresis on an 1,5% agarose gel. In sonicated samples, the DNA should be shifted and more intense compared to untreated samples and range around 200-2000 bp, centering around 500 bp. All centrifugation steps were done with the Biofuge fresco from Heraues if not indicated differentially.

4.4.4 Immunoprecipitation (IP)

Chromatin concentration was measured at 260 nm using a NanoDrop spectrophotometer and sample input for IP was set equal according to the measured concentrations. The samples were diluted with ChIP dilution buffer to a total volume of 5 mL. Protein A or protein G beads preabsorbed with salmon sperm DNA (Millipore) were washed three times with 1 mL ChIP dilution buffer using 110 µL (for histone modification ChIP) or 170 µL (for occupancy ChIP) of beads per sample. For assessing histone modifications Protein A agarose beads were used, while Protein G agarose beads were used for HDA6 occupancy ChIPs. After washes, beads were diluted with ChIP dilution buffer to obtain a 4x slurry. 140 µL of equilibrated beads (4x slurry) were added to the diluted sample and incubated for preclearing for 1 h at 4°C rotating. Afterwards, the samples were centrifuged at 3000 rpm for 2 min at 4°C to pellet the beads. The supernatant was split equally according to numbers of IPs to be done (number of antibodies plus mock control). For the Input control, 1/10 of used sample volume was set aside at -20°C. The chromatin solution was incubated with appropriate antibodies and beads over night rotating (all antibodies used are listed in Table M1). For antibodies specific to modified histones, 80 µL of equilibrated beads (4x slurry) were used and total sample volume was adjusted to 1.5 mL with ChIP dilution buffer. For occupancy ChIPs 200 µL of equilibrated beads (4x slurry) were used and total sample volume was adjusted to 10 mL. After o/n incubation, the samples were centrifuged at 3000 rpm for 2 min at 4°C. The supernatant was discarded and the beads were washed with 1 ml of respective washing buffer as follows: 1x low salt wash buffer, 1x high salt wash buffer, 1x LiCl wash buffer and 2x TE buffer. After adding washing buffers, the samples were rotated for 5 min at 4°C, followed by centrifugation at 3000 rpm for 2 min at 4°C to pellet the beads. Subsequently, the chromatin

was eluted by adding 250 µL elution buffer. Samples were vortexed and incubated for 15 min at 65°C. After pelleting the beads by centrifugation at 3000 rpm for 2 min at 4°C, the elution was repeated. Both elutes were combined and subjected to reverse crosslinking by adding 20 µL 5 M NaCl and o/n incubation at 65°C. The stored aliquots for the input control were diluted with elution buffer to a final volume of 480 µL and treated equally as the samples during reverse crosslinking. After reverse crosslinking, the resulting DNA was purified using the Promega Wizard® SV Gel and PCR Clean-Up System (according to manufacturer's protocol). DNA elutes were stored at -20°C until further use for qPCR analysis. All centrifugation steps were done with the Biofuge fresco from Heraues if not indicated differentially.

4.5 Bisulfite Sequencing

4.5.1 Genomic DNA isolation, restriction digest and DNA clean up

1 g of plant tissue was frozen in liquid nitrogen and grinded with beads to a fine powder. DNA was isolated using the Qiagen DNeasy Plant Maxi Kit according to manufactures protocol. The DNA was quantified by measuring concentration at 260 nm with a NanoDrop spectrophotometer. For the restriction digest, 2 µg of genomic DNA was digested with HindIII (Fermentas) o/n according to the manufacturer`s protocol. The digested DNA was cleaned up using the Promega Wizard® SV Gel and PCR Clean-Up System (according to manufacturer's protocol). The DNA concentration was then assessed again as described above.

4.5.2 Bisulfite treatment

For the bisulfite reaction, 500 ng of HindIII digested genomic DNA was used. For the conversion and the subsequent clean up, the Qiagen EpiTect Bisulfite Kit was used according to manufacturers protocol.

4.5.3 PCR amplification of target sequences and conversion control

As conversion control, a region from At1g30490 (Phavoluta), that was previously shown to be unmethylated in the 15th exon in *Col*-0 (Bao et al. 2004) was used. The Phavoluta sequence was first amplified with primers spanning 592 bp (PHAV1F + PHAV1R). 10 µL of the resulting PCR product were analyzed by agarose gel electrophoresis. If PCR product was clean and of expected size, the rest of the PCR reaction was diluted and used as template for another amplification cycle using a second set of primers (PHAV2F + PHAV2R) to obtain a

smaller fragment. Again 10 µL of the PCR product were analyzed on agarose gel, the rest was saved for ligation. Target regions of interest were amplified by PCR with primers listed in Table M2. As described for the conversion control, PCR product were analysed by agarose gel electrophoresis and the rest was saved for ligation. In PCRs, the annealing temperature was set to 50°C and at least 30 amplification cycles were done.

4.5.4 Cloning of PCR amplified sequences

2 µL of undiluted PCR products were used for ligation. All ligation reactions were done using the Promega pGEM T-easy vector system according to protocol in a 20 µL reaction volume. Ligations were done o/n at 16 °C. For transformation, 10 µl of ligation reaction were used. Transformed *E. coli* cells were plated on LB + 50 mg/L Amp + X-Gal plates and incubated o/n at 37°C. Positive clones were screened by Colony PCR using T7 and SP6 primers (Table M2). 10 µL of PCR products were analysed on agarose gels, the rest was diluted 1:20 to 1:50, depending on concentration, and kept for sequencing.

4.5.5 Sequence analysis

2 µL of diluted PCR product was sent for sequencing with either T7 or SP6 sequencing primer. Sequencing was kindly done by the service department using the ABI 3730 DNA Genetic Analyser. Sequences were analysed using DNA Star Lasergene software, ClustalW2 Multiple Sequence Alignment (EMBL-EBI) and CyMATE (Hetzl et al. 2007).

4.6 Quantitative Polymerase Chain Reaction (qPCR)

QPCR was employed for quantitative analysis of cleaned genomic DNA after Chromatin Immunoprecipitation (ChIP-qPCR) and for validation of different gene expression levels (RT-qPCR) among mutants. All primers used were designed using Primer3Plus online software (Untergasser et al. 2007) and are listed in Table M2. Primers used for RT-qPCR were spanning intron exon boarders whenever possible to avoid contamination by genomic DNA.

4.6.1 Primer optimization

Two different approaches for primer optimization were used. For ChIP-qPCR, primers were tested using genomic DNA in a temperature gradient ranging from 65°C to 55°C. Additionally, $MgCl_2$ concentrations were optimized for each primer pair. Negative controls were included, using H_2O instead of genomic DNA as a template. Melting curves of PCR

products were analysed for product specific melting peaks and samples were analysed on agarose gels. The setting given rise to the lowest C_t-value was chosen within all conditions having only one specific melting curve peak and no visible primer dimers on agarose gels.

For RT-qPCR, random hexamer primed cDNA was diluted in ten fold series ranging from 1:10 to 1:100.000 and subjected to a qPCR run employing a fixed annealing temperature of 60°C. $MgCl_2$ concentrations were optimized only if standard setting (3 mM $MgCl_2$) did not give reasonable results. After verifying clean PCR products by metling curve and agarose gel analysis, C_t-values were blotted against the respective cDNA dilutions. The slope of the trendline was used to calculate the efficiency according to formular (1).

$$(1)\ efficiency[\%] = ((10^{\frac{-1}{slope}}) - 1) \cdot 100$$

For this study the efficiency tolerance range was set from 90% to 110%. The correlation coefficient R^2 was set to be at least 0,990. Only primer pairs fulfilling these requirements were used for transcription studies. All tests were done in duplicates.

4.6.2 qPCR analysis

All qPCRs were run in a total reaction volume of 15 µl using the SensiMix™ SYBR & Fluorescein Kit (Bioline, according to manufactures protocol). Triplicates were pipetted in 96-well plates, run in the iQ5 real-time PCR detection system (Biorad) and analyzed with iQ5 optical system software (Biorad) and Excel (Microsoft). Data for ChIP assays were first normalized to Input. For occupancy ChIP, the *rts*1-1 background was subtracted, values obtained for 35S:HA-HDA6 were arbitrarily set one and 35S:HA-RTS1 values were normalized accordingly. For histone modification ChIP, all values were normalized to H3 occupancy. RT-qPCR data was analyzed for fold enrichment (R) according to the Pfaffl methode (Pfaffl 2001) with formular (2) and (3).

$$(2)\ \log efficiency[E] = 10^{\frac{-1}{slope}}$$

$$(3)\ R = \frac{(E_{target})^{\Delta C_{target}(control-sample)}}{(E_{ref})^{\Delta C_{ref}(control-sample)}}$$

4.7 SDS-PAGE, Western Blot and immunodection

Total proteins were extracted from two weeks old seedlings using TriFast (peqGold TriFast, PeqLab) and protein quantity was assessed with the Lowry total protein kit from Sigma (Total Protein Kit, Micro Lowry, Peterson's Modification, Sigma TP0300) according to manufactures protocol.

Protein samples were cooked in Laemmli buffer for at least 5 min, cooled down and subsequently loaded on 12% polyacrylamid/SDS gels. Gels were run with 80 to 100 V. After separation, proteins were electroblotted to Hybond-P membranes (Amersham) using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) for 1 h with 100 V. For immunodetection, membranes were briefly washed in 1x PBS/0.05% Tween-20 (v/v) and blocked in 1x PBS/1% milk powder (w/v) at RT for 1 h. Subsequently, membranes were incubated with the first antibody o/n at 4°C. After washing for at least 3x 5 min, the membranes were incubated with the secondary antibody conjugated to horseradish peroxidase at RT for 1 h (secondary antibodies are listed in Table M1). Following washing, signals were visualized using an enhanced chemiluminescence detection kit (LUMI-Light, Roche). All incubation and washing steps were done with constant agitation.

4.8 Microarray analysis

Total RNA was isolated in triplicates from aerial parts of pooled 5 weeks old seedlings grown on MS agar under long day conditions using TriFast (peqGold TriFast, PeqLab) according to manufactures protocol. After TriFast extraction, RNA was cleaned without further DNaseI treatments using the RNeasy MiniElute Cleanup Kit (Qiagen, according to protocol). RNA quality and quantity was checked on 1,2% formaldehyde agarose gels (protocol according to RNeasy Mini Handbook, Qiagen) and NanoDrop, respectively. RNA samples were sent to NASC (Nottingham Arabidopsis Stock Centre) for cDNA synthesis and hybridization onto Affymetrix ATH1 chips. The raw datasets were analyzed for quality by Fitting Probe Level Models (Bolstad et al. 2005), normalized using Robust Multichip Average (RAM) (Irizarry et al. 2003; Irizarry et al. 2003) and differential gene expression was determined using linear modelling (Wettenhall and Smyth 2004) in Bioconductor/R. Genes with a cut-off p-value $\leq 0{,}05$ and with a $\geq$ 2-fold change (i.e. ≥ 2 and ≤ -2) were defined as significantly induced or repressed genes, respectively.

Table M1. Antibodies used in this study.

Antibody	used amount/dilution per IP or Western blot (concentration*)	cat. number	supplier
anti-H3	5 µL (5 µg)	ab1791	Abcam
anti-H3K9/14ac2	5 µL (5 µg)	17-615	Millipore
anti-H3K4me3	7.5 µL	04-745	Millipore
anti-H3K9me2	10 µL (10 µg)	ab1220	Abcam
anti-H3K27me1	4 µL	17-643	Millipore
anti-HA	10 µL (10 µg)	MMS-101P	Covance
anti-FLAG	10 µL (10 µg)	F 1804	Sigma
anti-mouse	1:20000	31444	Pierce
anti-rabbit	1:15000	111-035-008	Jackson Immuno Research

* available concentration according to manufacturer

Table M2. Primers used in this study.

Gene	Primer name	Sequence (5` - 3`)
genotyping		
*rts*1-1 allele	IND220-F IND220-R	GATTCTGAGTGAGAGACGGAG AGCCATACGGATCCGGTGAGG
*rts*1-3 allele	HDA6/F3 HDA6/R3	CTGCAGTTGCTGTTGGAG AAGAGACACCAAACCATC
*rts*1-4 allele	HDA6.RTS1-4SEQ HDA6.RTS1-3SEQ3	ATCGTGCTAGGGATTCTGGAG CTTATCCTCTCCATATCTTTG
*rts*1-5 allele	HDA6/F2 HDA6.RTS1-3SEQ3	CCATGGAGATGGAGTGGA CTTATCCTCTCCATATCTTTG
NOSpro:NPTII transgene untransformed control	KKWT-F KKWT3-2	ATGGACATCCCCGGCAAATG CATTGTACTGCTCTGCTTGATACTGCTTGA
NOSpro:NPTII transgene transformed control	TargF TargR	ATGCCATCTCCATCAACGTC TTTCTGACGTATGTGCTTAG
35S:IR-NOSpro transgene untransformed control	Sil5 Sil3	GAGATAGTGGAGCAATCTCTGAGATG TTCATACGAGACCCTCTGTTTTGGC
35S:IR-NOSpro transgene transformed control	5`Hyg 3`Hyg	GTCCTGCGGGTAAATAGCTGG CGTCTGCTGCTCCATACAAGC
sequencing		
pGEM T-easy vector	T7 SP6	TAATACGACTCACTATAGGG ATTTAGGTGACACTATAG

Table M2. Continued.

Gene	Primer name	Sequence (5` - 3`)
RT-PCR primer		
NOSpro:NPTII	RT-NOSp-F (JP1930) RT-NOSp-R (JP1881)	CGGATCCGGGGGGATCGTTT TGACAACGTCGAGCACAGCTGCGCAA
AtSN1	RT-AtSN1-F RT-AtSN1-R	ACCAACGTGTTGTTGGCCCAGTGGTAAATC AAAATAAGTGGTGGTTGTACAAGC
soloLTR	RT-SoloLTR-F RT-SoloLTR-R	AACTAACGTCATTACATACACATCTTG AATTAGGATCTTGTTTGCCAGCTA
AtGP1	RT-GP1-F RT-GP1-F	ACAGTGCCACAGTTGAGCAG CAGAAAAATACTCGGTGCCAAT
IG5	RT-IG5-F RT-IG5-R	TCGCTTGAATCTAATACTTGTGTGC CGTAAGTGCTTTTCGGACATTACAA
UBQ4	UBQ4F-144 UBQ4R-358	TTACGAAGGCGGTGTTTTTC GCTCAGGATGAGCCATCAAT
Bisulfite primer		
Phavoluta	PHAV1F PHAV1R PHAV2F PHAV2R	GTGYAGATYTGTTTGGAGYTGATTY TTTAATATCTAACATAACCAACCTTT GGAYYATAGTGATGYYATAT GTG TATCATCAACAACTTTCCACACC
soloLTR	BS-SLTR-boF BS-SLTR-boR	AYTGTATATYTYAATTATGAGG TTARCTRRCAAACAARATCCTAA
NOSpro	BS-NOSp-BF BS- NOSp-BR	TTCTTCRCCCACCCCRAATTC GGYAGGAGYAAGGTGAGATGA
AtSN1	BS-AtSN1-TF BS-AtSN1-TR	AAAATAAGTGGTGGTTGTAYAAG TAACTTTCRACTCCCATAARTAAC
IG5	BS-IG5-TF BS-IG5-TR	AYTTTYYGGYYAAATTYYAG ACTATCCTRARAATATTCTR
AtGP1	BS-GP1-F BS-GP1-F	AYAGTGYYAYAGTTGAGYAG CARAAAAATACTCRRTRCCAAT
FWA	BS-FWA-TF BS-FWA-TR	AAAGAGTTATGGGYYGAAG CRRRAACCAAAATCATTCTCTAAACA

Gene	Primer name	Sequence (5` - 3`)
ChIP primer		
soloLTR	CH2-soloLTR-F	AGCTGGCAAACAAGATCCTAA
	qCHIP-Solo-R2	ACAGAGAAAAAGAGAAGAGAAAGAGAAAG
NOSpro	CH-NOSp-F	GAGAATTAAGGGAGTCAC
	CH-NOSp-R	TCGTCCTGCAGTTCATTC
AtSN1	CH-AtSN1-F	CCAGAAATTCATCTTCTTTGGAAAAG
	CH-AtSN1-R	GCCCAGTGGTAAATCTCTCAGATAGA
IG5	CH3-IG5-F	ATTGGGCTTGCTGAGTTG
	CH3-IG5-R	GAGGGAGATAGGATTTTGCAG
AtGP1	RT-GP1-F	ACAGTGCCACAGTTGAGCAG
	RT-GP1-F	CAGAAAAATACTCGGTGCCAAT
At5g10140	FLC-F	TGAGACTGCCCTCTCCGTGA
	FLC-R	TCAACAAGCTTCAACATGAGTTCG
qPCR primer		
UBC28	UBC28qF	TCCAGAAGGATCCTCCAACTTCCTGCAGT
	UBC28qR	ATGGTTACGAGAAAGACACCGCCTGAATA
At5g10140	FLC-F	TGAGACTGCCCTCTCCGTGA
	FLC-R	TCAACAAGCTTCAACATGAGTTCG
At1g13950	EIF5A-F	CGACGAGGAGCATCACTTTG
	EIF5A-R	TGCAGGGACGATTTTTGATG
At2g18050	HIS1-3-F	TAACGAGGCAGCAGGACAAG
	HIS1-3-R	GGCTGTCTCGCCTTCTTCAC
At2g29500	HSP-F	CGTGTGGAGAGATCGAGTGG
	HSP-R	CAGCCTTAGGCACCGTAACA
At3g28540	AAA ATPase1-F	TTTGCCTTACGCGGTTGGTT
	AAA ATPase1-R	CTTGCATCCCTCGCTGCTTT
At3g55130	ATWBC19-F	CGCGTTTATGGTGGTGCAAG
	ATWBC19-R	GAAGACTCGCCGGAGATCCA
At4g01910	DC1-F	TGTAATGCTTGCGGGCTGAA
	DC1-R	GCGAAACACGGTGATCATGC
At4g16780	ATHB2-F	TTTGGTTTCAGAACAGACGAGCA
	ATHB2-R	TCGCAGTCTACCTCCGTTTGC
At5g25475	DNAbinding-F	ACCCCTGCCTCTTCCTTTGC
	DNAbinding-R	CCTTTTTGTCACCGCTTGCAC
At5g52070	Agenet-F	TTGTCGTCGTTCATGATTCTTGG
	Agenet-R	ATCCAACCGTGGACGCAAAT
At5g39800	60Srelated-F	TCCCTGATCTCACCGGCTTT
	60Srelated-R	TGTTGACTTGTATCGGGCATTGA
At5g56370	Fbox2-F	TGCTCAAACATTCCCCGAAA
	Fbox2-R	CGAGCTTGGTTCCTCCCAAT
At5g39710	EMB2745-F	TGGGCAAACCCACATCAATTC
	EMB2745-R	TCTTCGGCGAGGATTTGAGC

4.8 Buffers

4.8.1 SDS-PAGE and Western

10x SDS-PAGE Running Buffer	0.25 M Tris-HCl pH 8.3, 2 M Glycine, 1% SDS
4x lower buffer	1.5 M Tris-HCl pH 8.8, 0.4% SDS
4x upper buffer	0.5 M Tris-HCl pH 6.8, 0.4% SDS
10x transfer buffer	0.25 M Tris-HCl pH 8.3, 1.94 M Glycine
for 1 x transfer buffer add	10% MeOH and 0.01% SDS
10x PBS	137 mM NaCl, 2 mM KH_2PO_4, 2.7 mM KCl, 100 mM Na_2HPO_4

4.8.2 ChIP

extraction buffer 1	0.4 M sucrose, 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 5mM ß-mercaptoethanol, Protease Inhibitors Tablets (Roche)
extraction buffer 2	0.25 M sucrose, 10 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 1% Triton X-100, 5mM ß-mercaptoethanol, Protease Inhibitors Tablets (Roche)
extraction buffer 3	1.7 M sucrose, 10 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 0.15% Triton X-100, 5mM ß-mercaptoethanol, Protease Inhibitors Tablets (Roche)
nuclei lysis buffer	50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, Protease Inhibitors Tablets (Roche)
ChIP dilution buffer	1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.0, 167 mM NaCl
elution buffer	1% SDS, 0.1 M $NaHCO_3$
low salt wash buffer	150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 8.0
high salt wash buffer	500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl pH 8.0
LiCl wash buffer	0.25 M LiCl, 1% Nonidet P-40, 1% Sodium Deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0
protease inhibitors	100mM PMSF, Complete Mini Protease Inhibitor Cocktail tablets (Roche)

4.9 Chemicals

zebularine	Z4775, Sigma-Aldrich
methylmethane sulfonate (MMS)	M4016, Sigma-Aldrich
cisplatin	P4394, Sigma-Aldrich
dimethyl adipimidate (DMA)	D8138, Sigma-Aldrich
ethylene glycolbis(succinimidyl succinate) (EGS)	E3257, Sigma-Aldrich

5. Abbreviations

ABA	abscisic acid
Ac	acetylation
At	*Arabidopsis thaliana*
bp	basepair
cDNA	complementary DNA
Cis	cisplatin
Col-0	*Arabidopsis thaliana*, ecotype Columbia
DDR	DNA damage response
DBS	double strand breaks
DMA	dimethyl adipimidate
DNMT	DNA methyltransferase
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DT	double transformed plants (silencer and target transgene)
EGS	ethylene glycol bis[succinimidylsuccinate]
EMS	ethyl methanesulfonate
ESR	environmental stress response
FA	formaldehyde
GO	gene ontology
H	every nucleotide except G
H1	histone 1
H2	histone 2
H3	histone 3
H4	histone 4
HAT	Histone acetyltransferase
HDAC	Histone deacetylases
HDACi	Histone deacetylase inhibitor
HKMT	histone lysine methyltransferase
IR	inverted repeat
JA	jasmonic acid
K	lysine
Kan	kanamycin antibiotic
kb	kilobase
LTR	long terminal repeat
me	methylation
miRNA	microRNA
MMS	methylmethane sulfonate
mRNA	messenger RNA
NAD+	nicotinamide adenine dinucleotide
NOSpro	nopaline synthase promoter
NPTII	neomycinphosphotransferase II
nt	nucleotide
o/n	over night
PRMT	protein arginine methyltransferase
PTGS	posttranscriptional gene silencing
R	arginine
rDNA	ribosomal DNA
RdDM	RNA-directed DNA methylation
RNA	Ribonucleic Acid
RNAi	RNA interference

rRNA	ribosomal RNA
S	silencer transgene
siRNA	small interfering RNA
ssRNA	single stranded RNA
T	target transgene
TGS	transcriptional gene silencing
TSA	trichostatin A
VIGS	virus-induced gene silencing
zeb	zebularine

Gene, protein and mutant abbreviations are explained in the text.

6. Supplementary Data

Table S1. Probe sets upregulated in *rts*1-1 mutants.

probe set ID	logFC	id	description
249456_at	1,52	AT5G39410	Saccharopine dehydrogenase
249427_at	1,85	AT5G39850	Ribosomal protein S4
250475_at	1,03	AT5G10180	ARABIDOPSIS SULFATE TRANSPORTER 68
263320_at	1,62	AT2G47180	GALACTINOL SYNTHASE 1
249059_at	1,11	AT5G44530	Subtilase family protein
261226_at	1,13	AT1G20190	ATEXPA11
256489_at	1,08	AT1G31550	GDSL-like Lipase/Acylhydrolase superfamily protein
245276_at	1,31	AT4G16780	ATHB2
246922_at	1,05	AT5G25110	SNF1-RELATED PROTEIN KINASE 3.25
262661_s_at	1,54	AT1G14250	GDA1/CD39 nucleoside phosphatase family protein
247760_at	1,44	AT5G59130	Subtilase family protein
264066_at	1,00	AT2G27880	AGO5
251785_at	1,03	AT3G55130	ATWBC19
251109_at	1,66	AT5G01600	ATFER1
249383_at	1,90	AT5G39860	PACLOBUTRAZOL RESISTANCE1
254564_at	1,02	AT4G19170	CAROTENOID CLEAVAGE DIOXYGENASE 4
258901_at	1,79	AT3G05640	Protein phosphatase 2C family protein
261804_at	1,11	AT1G30530	UDP-GLUCOSYL TRANSFERASE 78D1
263497_at	2,61	AT2G42540	COLD-REGULATED 15A
249472_at	1,06	AT5G39210	CHLORORESPIRATORY REDUCTION 7
249433_at	1,01	AT5G39940	FAD/NAD(P)-binding oxidoreductase family protein
259878_at	1,17	AT1G76790	NDOLE GLUCOSINOLATE O-METHYLTRANSFERASE 5
255786_at	1,03	AT1G19670	CHLOROPHYLLASE 1
262347_at	1,21	AT1G64110	DUO1-ACTIVATED ATPASE 1
250639_at	1,07	AT5G07560	GLYCINE-RICH PROTEIN 20
263405_s_at	1,49	AT2G04066 AT2G04080	MATE efflux family protein
263495_at	1,80	AT2G42530	COLD REGULATED 15B
251928_at	1,11	AT3G53980	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
265817_at	1,31	AT2G18050	HIS1-3
249406_at	1,91	AT5G40210	nodulin MtN21 /EamA-like transporter family protein
260260_at	1,03	AT1G68540	CINNAMOYL COA REDUCTASE-LIKE 6
249466_at	1,04	AT5G39740	RIBOSOMAL PROTEIN L5 B
249464_at	1,32	AT5G39710	EMBRYO DEFECTIVE 2745
249393_at	1,06	AT5G40170	RECEPTOR LIKE PROTEIN 54
249385_at	1,16	AT5G39950	THIOREDOXIN H2
253627_at	1,29	AT4G30650	Low temperature and salt responsive protein family
249441_at	1,30	AT5G39730	AIG2-like (avirulence induced gene) family protein
256577_at	1,17	AT3G28220	TRAF-like family protein
249424_s_at	1,09	AT5G39800	Mitochondrial ribosomal protein L27
253713_at	2,38	AT4G29370	Galactose oxidase/kelch repeat superfamily protein
249386_at	1,07	AT5G40060	Disease resistance protein (NBS-LRR class) family
249469_at	1,47	AT5G39320	UDP-glucose 6-dehydrogenase family protein
256066_at	1,10	AT1G06980	unknown protein
263603_at	1,08	AT2G16340	unknown protein
261203_at	1,26	AT1G12845	unknown protein
249443_at	1,12	AT5G39600	unknown protein
252545_at	1,21	AT3G45820	unknown protein
254566_at	1,13	AT4G19240	unknown protein
261921_at	1,06	AT1G65900	unknown protein

Table S1. Continued.

probe set ID	logFC	id	description
256166_at	1,56	AT1G36920	unknown protein
257284_at	1,10	AT3G29650	transposable element gene
255376_x_at	1,44	AT4G03790	transposable element gene
255102_s_at	1,03	AT1G78095 AT4G08680	transposable element gene
263367_at	1,94	AT2G20460	transposable element gene

Table S2. Probe sets downregulated in *rts*1-1 mutants.

probe set ID	logFC	id	description
266118_at	-1,10	AT2G02130	LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 68
257062_at	-1,13	AT3G18290	EMBRYO DEFECTIVE 2454
265917_at	-1,37	AT2G15080	RECEPTOR LIKE PROTEIN 19
254020_at	-1,17	AT4G25700	BETA CAROTENOID HYDROXYLASE 1
256332_at	-1,04	AT1G76890	encodes a plant trihelix DNA-binding protein
254384_at	-1,76	AT4G21870	HSP20-like chaperones superfamily protein
255059_at	-1,52	AT4G09420	Disease resistance protein (TIR-NBS class)
264751_at	-1,33	AT1G23020	FERRIC REDUCTION OXIDASE 3

Table S3. Probe sets upregulated in *rts*1-5 mutants.

probe set ID	logFC	id	description
248614_at	1,22	AT5G49560	Putative methyltransferase family protein
251065_at	1,35	AT5G01870	Predicted to encode a PR (pathogenesis-related) protein
251112_s_at	1,18	AT5G01320	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein
		AT5G01330	PYRUVATE DECARBOXYLASE-3
258856_at	1,60	AT3G02040	SENESCENCE-RELATED GENE 3
249894_at	1,97	AT5G22580	Stress responsive A/B Barrel Domain
256415_at	1,22	AT3G11210	SGNH hydrolase-type esterase superfamily protein
245193_at	1,16	AT1G67810	SULFUR E2
255463_at	1,36	AT4G02960	RETRO ELEMENT 2
250517_at	1,39	AT5G08260	SERINE CARBOXYPEPTIDASE-LIKE 35
265893_at	1,02	AT2G15040	RECEPTOR LIKE PROTEIN 18
256871_at	1,24	AT3G26480	Transducin family protein / WD-40 repeat family protein
256602_at	1,17	AT3G28310	Protein of unknown function (DUF677)
246601_at	1,34	AT1G31710	Copper amine oxidase family protein
262978_at	1,01	AT1G75780	TUBULIN BETA-1 CHAIN
249046_at	1,05	AT5G44400	FAD-binding Berberine family protein
263016_at	1,22	AT1G23410	Ribosomal protein S27a / Ubiquitin family protein
251705_at	2,07	AT3G56400	WRKY DNA-BINDING PROTEIN 70
263951_at	1,07	AT2G35960	NDR1/HIN1-LIKE 12
258158_at	1,88	AT3G17790	PURPLE ACID PHOSPHATASE 17
250992_at	1,78	AT5G02260	EXPANSIN A9
252168_at	1,08	AT3G50440	METHYL ESTERASE 10
247352_at	1,18	AT5G63650	SNF1-RELATED PROTEIN KINASE 2H
258160_at	1,10	AT3G17820	GLUTAMINE SYNTHETASE 1.3
264998_at	1,52	AT1G67330	Protein of unknown function (DUF579)
257701_at	1,50	AT3G12710	DNA glycosylase superfamily protein
251287_at	1,05	AT3G61820	AT2G07140
266426_x_at	1,05	AT2G07140 AT3G18320 AT3G44120	F-box and associated interaction domains-containing protein
247377_at	1,16	AT5G63180	Pectin lyase-like superfamily protein
246071_at	1,85	AT5G20150	SPX domain gene 1 (SPX1)
256017_at	1,58	AT1G19180	JASMONATE-ZIM-DOMAIN PROTEIN 1
251934_at	1,14	AT3G54070	Ankyrin repeat family protein
252786_at	1,12	AT3G42670	CHROMATIN REMODELING 38 (CLASSY 1)
246565_at	1,14	AT5G15530	BIOTIN CARBOXYL CARRIER PROTEIN 2
259751_at	1,14	AT1G71030	Encodes a putative myb family transcription factor.
256100_at	1,19	AT1G13750	Purple acid phosphatases superfamily protein
247951_at	2,87	AT5G57240	OSBP(OXYSTEROL BINDING PROTEIN)-RELATED PROTEIN 4C
267158_at	1,41	AT2G37640	EXPANSIN 3
246293_at	1,92	AT3G56710	SIGMA FACTOR BINDING PROTEIN 1
248681_at	1,17	AT5G48900	Pectin lyase-like superfamily protein
253815_at	1,28	AT4G28250	EXPANSIN B3
248230_at	1,41	AT5G53830	VQ motif-containing protein
267591_at	1,91	AT2G39705	ROTUNDIFOLIA LIKE 8
252414_at	1,82	AT3G47420	PHOSPHATE STARVATION-INDUCED GENE 3
262396_at	1,15	AT1G49470	Family of unknown function (DUF716)
253437_at	1,19	AT4G32460	unknown protein
265654_s_at	1,59	AT5G28785	transposable element gene
246698_at	1,74	AT5G30480	transposable element gene
256844_s_at	2,22	AT3G31955	transposable element gene

Table S3. Continued

probe set ID	logFC	id	description
256748_x_at	1,28	AT3G30396	transposable element gene
252755_at	1,23	AT3G43530	transposable element gene
265754_x_at	1,38	AT2G10640	transposable element gene
259573_at	1,19	AT1G20390	transposable element gene
257057_at	1,56	AT3G15310	transposable element gene
266024_at	1,05	AT2G05950	transposable element gene
266151_x_at	1,27	AT2G12300	transposable element gene
266148_x_at	1,87	AT3G31955	transposable element gene
257292_s_at	1,50	AT2G07770 AT3G15600	transposable element gene
246642_s_at	1,81	AT5G34920 AT5G59620	transposable element gene
259204_s_at	1,14	AT1G34610 AT3G09170	transposable element gene
263280_x_at	1,02	AT1G32830 AT2G04970 AT2G14140 AT3G30450 AT3G47270 AT5G34895	transposable element gene
261234_x_at	1,12	AT1G32830 AT2G04970 AT2G14140 AT3G30450 AT3G47270 AT5G34895	transposable element gene

Table S4. Probe sets downregulated in *rts*1-5 mutants.

probe set ID	logFC	id	description
259123_at	-1,48	AT3G02200	Proteasome component (PCI) domain protein
254247_at	-1,27	AT4G23260	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 18
261924_at	-1,25	AT1G22550	Major facilitator superfamily protein
265111_at	-1,15	AT1G62510	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein
252173_at	-1,17	AT3G50650	GRAS family transcription factor
263207_at	-3,24	AT1G10550	XYLOGLUCAN:XYLOGLUCOSYL TRANSFERASE 33
259546_at	-1,72	AT1G35350	EXS (ERD1/XPR1/SYG1) family protein
252563_at	-3,04	AT3G45970	EXPANSIN-LIKE A1
246302_at	-1,18	AT3G51860	CATION EXCHANGER 3
254707_at	-1,21	AT4G18010	MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2
252234_at	-1,13	AT3G49780	PHYTOSULFOKINE 4 PRECURSOR

Table S5: Probe sets upregulated in both *rts*1-1 and *rts*1-5 mutants.

probe set ID	logFC	id	description
265486_at	5,52	AT2G15560	Putative endonuclease or glycosyl hydrolase
245865_at	5,09	AT1G58025	DNA-binding bromodomain-containing protein
248949_at	3,94	AT5G45570	Ulp1 protease family protein
252663_at	2,66	AT3G44070	Glycosyl hydrolase family 35 protein
256940_at	5,51	AT3G30720	QUA-QUINE STARCH (QQS)
253707_at	2,61	AT4G29200	Beta-galactosidase related protein
249727_at	3,62	AT5G35490	ATMRU1
249780_at	3,17	AT5G24240	Phosphatidylinositol 3- and 4-kinase ;Ubiquitin family protein
250605_at	3,61	AT5G07570	glycine/proline-rich protein
262615_at	2,06	AT1G13950	EUKARYOTIC ELONGATION FACTOR 5A-1
263927_s_at	1,55	AT2G21730	CINNAMYL ALCOHOL DEHYDROGENASE HOMOLOG 2
		AT2G21890	CINNAMYL ALCOHOL DEHYDROGENASE HOMOLOG 3
262902_x_at	3,76	AT1G59930	Encodes a maternally expressed imprinted gene
260172_s_at	1,28	AT1G71920	HISTIDINE BIOSYNTHESIS 6B
		AT5G10330	HISTIDINOL PHOSPHATE AMINOTRANSFERASE 1
251127_at	1,96	AT5G01080	Beta-galactosidase related protein
247640_at	1,43	AT5G60610	F-box/RNI-like superfamily protein
248394_at	2,38	AT5G52070	Agenet domain-containing protein
255715_s_at	1,76	AT4G00320	F-box/RNI-like superfamily protein
266393_at	2,47	AT2G41260	Late-embryogenesis-abundant gene
250942_at	2,10	AT5G03350	Legume lectin family protein
246002_at	1,36	AT5G20740	Plant invertase/pectin methylesterase inhibitor superfamily protein
246390_at	2,45	AT1G77330	similar to 1-aminocyclopropane-1-carboxylate oxidase
264513_at	1,24	AT1G09420	GLUCOSE-6-PHOSPHATE DEHYDROGENASE 4
261159_s_at	1,58	AT1G34460	CYCLIN B1;5
248015_at	1,11	AT5G56370	F-box/RNI-like/FBD-like domains-containing protein
266294_at	1,37	AT2G29500	HSP20-like chaperones superfamily protein
266152_s_at	1,22	AT5G10510	AINTEGUMENTA-LIKE 6
256460_at	1,30	AT1G36240	Ribosomal protein L7Ae/L30e/S12e/Gadd45 family protein
250868_at	1,09	AT5G03860	MALATE SYNTHASE
253244_at	1,19	AT4G34580	CAN OF WORMS1
256589_at	2,08	AT3G28740	Encodes a member of the cytochrome p450 family
265188_at	1,09	AT1G23800	ALDEHYDE DEHYDROGENASE 2B
253224_at	1,26	AT4G34860	ALKALINE/NEUTRAL INVERTASE B
250476_at	2,12	AT5G10140	FLOWERING LOCUS C
259789_at	2,81	AT1G29395	COLD REGULATED 314 INNER MEMBRANE 1
247318_at	1,01	AT5G63990	Inositol monophosp
252347_at	1,35	AT3G48130	ribosomal protein L13 homolog
255546_at	1,28	AT4G01910	Cysteine/Histidine-rich C1 domain family protein
263595_at	1,16	AT2G01890	PURPLE ACID PHOSPHATASE 8
264166_at	1,09	AT1G65370	TRAF-like family protein
255822_at	1,29	AT2G40610	member of Alpha-Expansin Gene Family
256596_at	1,01	AT3G28540	P-loop containing nucleoside triphosphate hydrolases superfamily protein
248520_at	2,11	AT5G50600	HYDROXYSTEROID DEHYDROGENASE 1
		AT5G50700	
246906_at	1,18	AT5G25475	AP2/B3-like transcriptional factor family protein
246715_at	1,25	AT5G28230	pseudogene
262186_at	2,82	AT1G77960	unknown protein
260744_at	1,35	AT1G15010	unknown protein

Table S5. Continued

probe set ID	logFC	id	description
250979_at	3,96	AT5G03090	unknown protein
249726_at	2,94	AT5G35480	unknown protein
246888_at	4,50	AT5G26270	unknown protein
266680_s_at	5,80	AT2G19850	unknown protein
249259_at	4,28	AT5G41660	unknown protein
250135_at	2,85	AT5G15360	unknown protein
267074_s_at	2,64	AT2G40955 AT4G15096	unknown protein
245353_at	1,66	AT4G16000	unknown protein
247754_at	1,10	AT5G59080	unknown protein
251458_at	1,38	AT3G60170	transposable element gene
263851_at	2,51	AT2G04460	transposable element gene
265380_at	2,46	AT2G16670	transposable element gene
262031_x_at	1,90	AT1G37160	transposable element gene
265503_at	1,44	AT2G15510	transposable element gene
245032_at	2,84	AT2G26630	transposable element gene
262719_at	3,37	AT1G43590	transposable element gene
257354_x_at	2,18	AT2G23480	transposable element gene
263675_x_at	4,36	AT2G04770	transposable element gene
263479_x_at	3,87	AT2G04000	transposable element gene

Table S6. Probe sets downregulated in both *rts*1-1 and *rts*1-5 mutants.

probe set ID	logFC	id	description
245449_at	-2,42	AT4G16870	transposable element gene
246310_at	-1,03	AT3G51895	SULFATE TRANSPORTER 3;1
259252_at	-1,27	AT3G07610	INCREASE IN BONSAI METHYLATION 1
253485_at	-2,04	AT4G31800	WRKY DNA-BINDING PROTEIN 18
264514_at	-1,40	AT1G09500	similar to Eucalyptus gunnii alcohol dehydrogenase
249765_at	-2,44	AT5G24030	SLAC1 HOMOLOGUE 3
253827_at	-1,45	AT4G28085	unknown protein
245262_at	-1,52	AT4G16563	Eukaryotic aspartyl protease family protei
263467_at	-1,11	AT2G31730	basic helix-loop-helix (bHLH) DNA-binding superfamily protein

7. Acknowledgements

As this is the end of my long journey in obtaining my degree in science, I have to look back at the years as a PhD student. This journey led me through emotional and scientific valleys and heights, and I can certainly say that it shaped me as a person and has led me to where I am now.

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