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Titel der Dissertation

Functional analysis of epialleles in diploid and tetraploid *Arabidopsis thaliana*

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I. Zusammenfassung

Polyploidie, die Multiplikation des diploiden Chromosomensatzes, tritt bei Pflanzen häufig auf. Die Entstehung polyploider Pflanzen ist oft mit Änderungen ihrer Genom- als auch ihrer Epigenomorganisation verbunden. Die transkriptionelle Geninaktivierung (transcriptional gene silencing, TGS) führt zu kompletten und erblichen Verlust der Expression vorher aktiver Gene. Durch TGS können Epiallele entstehen, die in ihrer DNA Sequenz übereinstimmen, aber unterschiedlich exprimiert werden. Das inaktive Epiallel kann durch epigenetische Wechselwirkungen die aktive Form *in trans* beeinflussen, was zu einer epigenetischen Angleichung der beiden Epiallele führt. Dieser Erbgang folgt nicht den Mendelschen Regeln und ähnelt der Paramutation. Da epigenetische Diversität direkten Einfluss auf die phänotypische Vielfalt und den Wachstumserfolg von Pflanzen hat, sind die zugrundeliegenden, regulatorischen Mechanismen von großer Bedeutung für natürliche Evolution und Pflanzenzüchtung.

Die vorliegende Arbeit beschäftigt sich mit Prinzipien der polyploidie-assoziierten transkriptionellen Geninaktivierung (polyploidy-associated transcriptional gene silencing, paTGS) eines transgenen Resistenzgens (Hygromycinphosphotransferase, *HPT*), das in aktiven oder inaktiven Epiallel-Varianten in *Arabidopsis thaliana* auftritt. Um den paTGS-Mechanismus und die allelische Interaktion zu analysieren, habe ich die genetischen und epigenetischen Merkmale der Epiallele in diploidem und tetraploidem Hintergrund und nach Mutagenese charakterisiert. Die genetische Struktur der Epiallele wurde in Linien mit aktiven, inaktiven und strukturell veränderten *HPT* Genen untersucht. Ich konnte spezifische epigenetische Modifikationen (DNA-Methylierung und Histonmodifikationen) der Epiallele nachweisen. Da Paramutation in Mais im funktionellen Zusammenhang mit regulatorischen RNAs (small RNAs) steht, analysierte ich die Fähigkeit des Transgens, solche RNAs oder Antisense-Transkripte zu generieren. Zusätzlich wurde der Einfluss vieler epigenetischer Mutanten auf das inaktive Epiallel untersucht.

In dieser Arbeit habe ich gezeigt, dass sich die beiden Epiallelformen in ihrer epigenetischen Zusammensetzung entsprechend ihrer Expressionsmuster, unterscheiden. Small RNAs oder Antisense-Transkripte dürften bei der Regulation der Epiallele keine Rolle spielen. Verschiedene strukturelle Änderungen im inaktiven Epiallel führten zur Reaktivierung der *HPT* Expression, was auf einen starken Einfluss der DNA Sequenz, vermutlich über die Chromatinorganisation hindeutet.

II. Abstract

Polyploidy, a condition of containing more than two sets of homologous chromosomes, is frequent among higher plants. The formation of polyploids is often associated with genomic rearrangements and epigenetic changes, such as transcriptional gene silencing (TGS), resulting in heritable loss of gene expression from previously active genes. TGS can lead to the formation of epialleles, which are identical in DNA sequence, but differ in expression states. Silent epialleles can exert non-Mendelian behavior in a paramutation-like interaction, silencing their expressed counterpart *in trans* and thereby leading to functional epigenetic homozygosity. Since epigenetic diversity may create phenotypic diversity and determine plant performance, these regulatory mechanisms are likely important for natural evolution and plant breeding.

In the experimental work for this thesis I investigated the principles of polyploidy-associated transcriptional gene silencing (paTGS) in a model system based on transgenic *Arabidopsis thaliana* lines, containing a resistance marker gene (hygromycin phosphotransferase, *HPT*) in active or inactive epiallelic state. I characterized genetic and epigenetic features of the epialleles in diploid, tetraploid and mutant lines, aiming to identify the mechanisms of paTGS and the allelic interaction. The structure of the epialleles is investigated in expressing and non-expressing lines, as well as in mutant lines with structural rearrangements of the epialleles. I was able to portray the epigenetic state of epialleles including DNA methylation and several histone modifications. Since paramutation in maize is functionally linked with enzymatic amplification of small RNAs, I analyzed the role of the transgene and the RNA features for their ability to produce small RNAs or antisense transcripts. Numerous Arabidopsis mutants affecting well-defined elements of epigenetic regulation allowed further mechanistic investigations of epigenetic phenomena in polyploids.

In summary, I was able to show that the epialleles differ in their chromatin modification according to the differences in their expression states. No evidence for an involvement of small RNAs or antisense transcripts in silencing was observed. However, structural changes within the silent epiallele led to reactivation of the inactive *HPT* gene, indicating strong impact of the sequence composition on epiallelic gene regulation.

1. Introduction

1.1. Background

1.1.1. Polyploidy

Polyploidy, the presence of more than two complete chromosome sets, is frequent among higher plants. One can distinguish between multiplication of a single genome (autopolyploids) or combination of two or more divergent genomes (allopolyploids). Genome analyses have revealed that even seemingly diploid eukaryotic genomes have a polyploid ancestry indicating that polyploidization is an important and obviously beneficial evolutionary step in eukaryotes. Polyploidy has extensive effects on genome organization and transposon activity, but also on gene expression, since e.g. transcriptional gene silencing accompanies polyploid formation and continues over evolutionary time (Figure 1). Early estimates of the polyploid fraction amongst angiosperm species range from 30 to 80% (Stebbins 1966). In fact, modern genome studies suggest that possibly all angiosperm species might be so-called paleopolyploids (Cui *et al.,* 2006). Studies of polyploids shortly after genome duplication have illustrated genetic and epigenetic interactions between redundant genes (Comai *et al.,* 2000; Madlung *et al.,* 2002). Polyploidy has considerable effects on duplicate gene expression as plant genomes harbor evidence of multiple rounds of polyploidization events, often followed by massive silencing and elimination of duplicated genes.

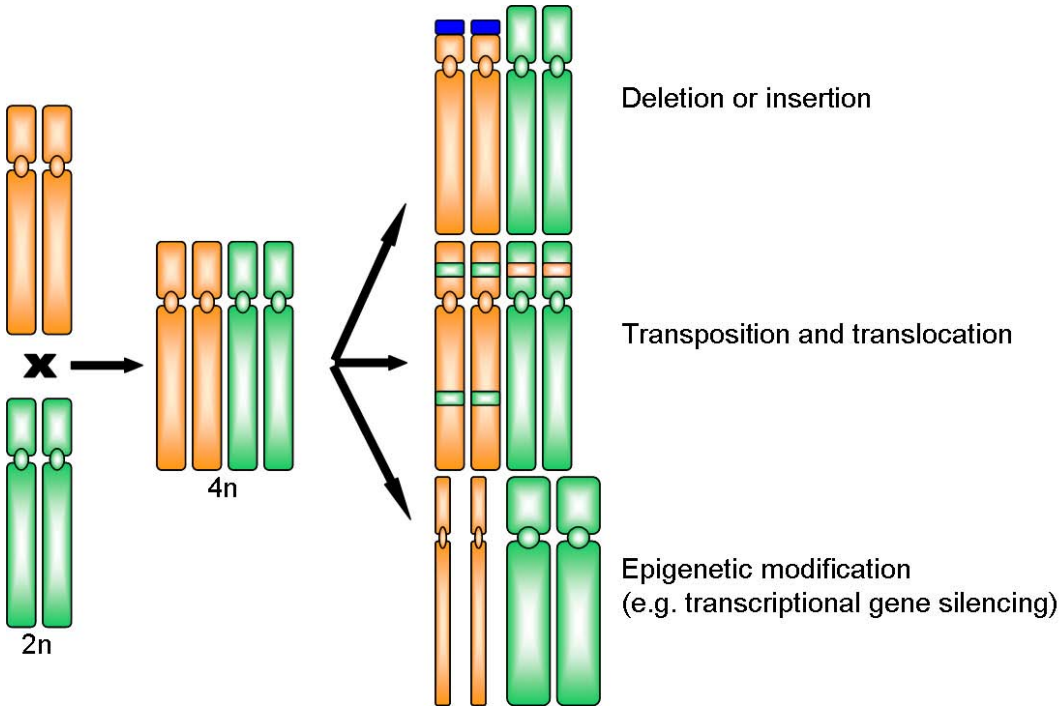


Figure 1. Polyploidy-dependent effects. Genomic modification involves deletion or insertion, translocation and transposition between chromosomes that are derived from different parental species, and epigenetic modification, including repression or activation of gene expression. Orange and green represent two genomes or chromosomes from different parents (adapted from (Chen and Ni 2006)).

Polyploidy has several advantages because the organisms can resort to a higher number of genes and higher maximum number of allelic variants. Gene redundancy, a result of gene duplication, shields polyploids from deleterious effects of mutations. There is evidence showing that genes that are retained in duplicate typically diversify in function to take over the task in different tissues (Adams and Wendel 2005b). There are also other possible mechanisms that could link polyploidy to diversification and adaptation. Heterosis (the increase in performance displayed by hybrids) causes polyploids to be more vigorous than their diploid progenitors. During man-made selection, breeding especially enriched polyploids, resulting in many polyploid crops (such as oilseed rape, bread wheat, and cotton). Polyploidy can facilitate reproduction through self-fertilization or asexual means, allowing the plant to reproduce in the absence of sexual mates (Comai 2005). The major constraints on polyploidy formation are disruptive effects caused by an increase of the genomic content leading to nuclear and cell enlargement and changes in cellular architecture. Polyploids may also be impaired during mitosis and meiosis, as the increased

number of chromosomes may cause instabilities during segregation. It is believed that especially in autopolyploids, bivalent pairing during meiotic prophase I is an adaptation that stabilizes polyploids.

The merging and doubling of two genomes creates novel expression patterns, and changes in gene regulation in polyploids due to epigenetic factors may lead to transcriptional remodeling after genome duplication. Recent studies have demonstrated that many of these effects arise immediately after polyploid formation, whereas others play out over a longer evolutionary timescale (Adams and Wendel 2005b). Key to these insights is the use of newly created, synthetic plant polyploids that mimic natural systems. In this thesis I studied epigenetic effects upon polyploid formation in a system which was previously described as a compelling demonstration of epigenetic remodeling that is associated with autopolyploidization (Mittelsten Scheid *et al.,* 2003). Since epigenetic regulation in polyploid plants provides the background for my thesis, an overview of epigenetic regulatory mechanisms and their major key players will be presented in the following.

1.1.2. Epigenetics

Epigenetics deals with molecular pathways regulating gene expression and packaging of chromosomes stably inherited between cell divisions and across generations. Epigenetic mechanisms often conflict with Mendelian models, explaining non-traditional inheritance patterns, for instance, paramutation (heritable expression changes due to interaction of two alleles) or transgene silencing. The main components of epigenetic regulation in plants are DNA methylation, histone modification, and non-coding RNAs. They involve chemical modifications of DNA and associated histones and structural changes in chromatin organization which will be explained below.

1.1.2.1 Chromatin modifications

A nucleosome consists of approximately 146 bp of DNA wrapped around an octamer containing two copies of each histone (H2A, H2B, H3, and H4). In the interphase nucleus, the linker histones of the H1 class associate with DNA between single nucleosomes establishing a higher level of organization, the 30 nm fibers. Non-histone proteins are responsible for further organization and condensation of the fiber into chromatin or chromosome structure. This fiber is the basic constituent of both euchromatin and heterochromatin. Chromatin is the complex of DNA and associated proteins in the nucleus. The euchromatin, or dispersed open chromatin, consists largely of gene-rich sequences which are actively transcribed. Heterochromatin, which contains mainly silent, non-transcribed parts of the genome, is densely condensated and plays an important role in the organization and proper functioning of genomes. Indeed, nucleosomes themselves can be subjected to positional alterations, thereby affecting the structure and packaging of chromatin and accessibility of DNA followed by a change in gene expression (Kornberg and Lorch 1999; Becker and Horz 2002). Two other factors involved in transcriptional regulation are the density of nucleosomes at the promoter region plus the post-translational modifications of histone tails (Jenuwein and Allis 2001). Beside the histone-based modifications, another important epigenetic mark is deposited as a covalent modification directly on the DNA. DNA methylation is a eukaryotic gene-silencing mechanism that protects the genome (Chan *et al.,* 2005). This mechanism will be described in the next chapter, followed by histone modifications in the subsequent section.

1.1.2.2 DNA methylation

DNA methylation of cytosine plays a crucial role in the regulation of gene expression and the control of genome stability in some but not all higher eukaryotes, mainly in vertebrate animals, plants and some fungi. In plants, cytosines in all sequence contexts can be methylated. They exhibit methylation at CG, CHG and CHH sites, where H is every nucleotide except G (Chan *et al.,* 2005). In the Arabidopsis

genome, about 24% of CG, 6.7% of CHG, and 1.7% of CHH are methylated (Cokus *et al.,* 2008). DNA methylation can be divided into *de novo* methylation and maintenance methylation. mCG (methylated CG) and mCHG (methylated CHG) are referred to as symmetrical methylation sites, as the G residue base-pairs with the C residue during replication, generating a hemimethylated substrate that becomes immediately modified in the new strand by maintenance methyltransferases. This function is provided in plants by *MET1* (methyltransferase 1, a *DNMT1*-like gene) for mCG, and by *CMT3* (chromomethyltransferase 3) for mCHG (Lindroth *et al.,* 2001). A maintenance function for mCHH (methylated CHH) methylation sites, which can only be symmetrical in palindromes of more than three bases, has not yet been identified. This modification is believed to be generated and 'maintained' by *de novo* methylation, carried out in Arabidopsis by *DRM1* and/or *DRM2* (domains-rearranged methyltransferases 1 and 2, *DNMT3* homologs) (Cao and Jacobsen 2002). DNA methylation needs an activated methylgroup, usually in the form of S-adenosyl-methionine, which is transferred by methyltransferases. *HOG1* (homology-dependent gene silencing 1) is crucial for the biochemical pathway providing this factor, leading to defects in global DNA methylation when mutated (Rocha *et al.,* 2005). Epigenetic regulation is potentially reversible; therefore factors removing active or passive marks are needed. There are two possibilities to lose DNA methylation, either passively via dilution of cytosine methylation when maintenance methylation is defect or down-regulated during DNA replication, or by active demethylation which requires enzymatic activity of DNA glycosylases. These are normally involved in base excision repair and play a major role in removing methylated cytosines from DNA. *ROS1* (repressor of silencing 1), *DME* (Demeter), and *DML* (DME-like) are proteins containing DNA glycosylase domains and act in epigenetic regulation antagonistically to DNA methyltransferases (Choi *et al.,* 2002; Gong *et al.,* 2002; Penterman *et al.,* 2007). Methyl-DNA-binding proteins (MBD proteins) are thought to transduce DNA methylation patterns into altered transcriptional activity. MBD proteins play a role in gene silencing and in plant development (Zemach and Grafi 2003). In mammals, MBD proteins bind methylated DNA and perform various functions, such as recruiting histone deacetylases, to reinforce transcriptional silencing, whereas in Arabidopsis only little is known about the functions of MBD proteins.

Recently, genome-wide high-resolution maps of DNA methylation were generated by bisulfite sequencing for the Arabidopsis genome. Integrating this genome-wide map with analysis of transcriptome data and small RNA profiles revealed insights into the global interplay of DNA methylation, small RNAs, and transcription (Cokus *et al.,* 2008; Lister *et al.,* 2008) and contributed to assemble the first “epigenome” of a eukaryote. Like previous studies mapping methylation in Arabidopsis on the basis of microarrays (Zhang *et al.,* 2006; Zilberman *et al.,* 2007), these sequencing studies found extensive DNA methylation throughout the genome. DNA methylation occurs mainly at repetitive elements and plays a critical role in silencing transposable elements. Interestingly, over one third of expressed genes contain methylation also within transcribed regions, whereas only 5% of genes show methylation within promoter regions (Zhang *et al.,* 2006). Genes methylated in transcribed regions are highly expressed and constitutively active, whereas promoter-methylated genes show a greater degree of tissue-specific expression (Zhang *et al.,* 2006). Sequences matching small RNAs are more likely to be methylated than sequences without small RNA matches (Zhang *et al.,* 2006). This correlation between small RNAs and DNA methylation may be supported by RNA-directed DNA methylation (RdDM), which will be described later in the text (Huettel *et al.,* 2006). New studies have shown a more dynamic picture of DNA methylation and gene silencing than previously thought. DNA methylation can be restored after global reduction, thus leading to reestablishing of gene silencing, while a complete remethylation was observed only after several generations (Teixeira *et al.,* 2009). High amounts of siRNAs characterized remethylated loci, whereas loci that remained unmethylated lacked siRNA. Small RNA silencing seems to have a conserved function in epigenetic regulation of transposable elements in pollen, suggesting an important developmental role for reestablishing DNA methylation and reinforcing silencing in the germline (Slotkin *et al.,* 2009). Importantly, extensive DNA demethylation occurs also in the seed endosperm which is accompanied by hypermethylation in the embryo, suggesting another role of small RNAs in regulation of transposable elements during development (Gehring *et al.,* 2009; Hsieh *et al.,* 2009). A recent report implicates an involvement of small RNAs in regulation of imprinting, extending the potential roles of small RNAs on evolution (Mosher *et al.,* 2009). The integrity of plant genomes is maintained by differential cytosine methylation of genes and transposons. It is well known that methylation of promoter sequences can lead to

transcriptional repression, while the function of gene body methylation remains elusive. In Arabidopsis many genes are methylated over their ORF, differentiating genes and transposable elements. While transposable elements are heavily methylated at both CG and non-CG sites, active genes rarely possess non-CG methylation. This leads to the suggestion that non-CG methylation distinguishes RdDM targets like transposons from genes and helps to maintain genome integrity, while genic CG methylation may control transcriptional elongation (Miura *et al.,* 2009) or hide cryptic promoters. Variation in DNA methylation can affect gene expression and be inherited across generations. Complex traits, like flowering time and plant height, can be affected by DNA methylation differences between individuals, demonstrating the importance of integrating epigenetic information in population genetics studies (Johannes *et al.,* 2009). However, chromatin structure is not defined by DNA methylation alone; other decisive factors are histone modifications which will be discussed in the next section.

1.1.2.3 Histone modifications

The modifications of histone tails, such as acetylation, methylation, or phosphorylation, are important in transcriptional regulation, and many of these modifications are stably maintained during cell division and between generations (Jenuwein and Allis 2001). Such modifications mainly correspond to methylation at lysine (K) and arginine (R) residues (Fuchs *et al.,* 2006; Pfluger and Wagner 2007). For the analysis of the different histone modifications (Figure 2), antibodies are invaluable tools. They recognize post-translationally modified amino acids in the context of the surrounding amino acid sequence with high specificity and high sensitivity.

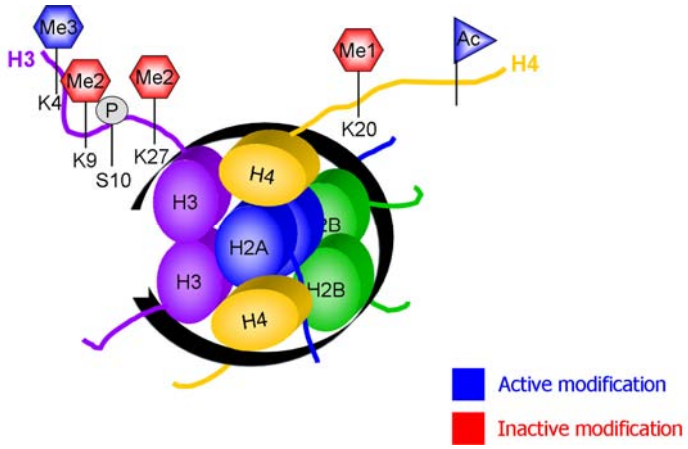


Figure 2. Overview of a nucleosome including sites of histone tail modifications analyzed in this thesis. K: lysine; S: serine; me: methylation; P: phosphorylation; ac: acetylation; H: histone variant.

The position and the combination of the modifications represents a readout mechanism by interacting with numerous binding proteins and thereby encodes information that regulates chromatin properties and gene activity (Jenuwein and Allis 2001). Histone modifications fulfill the same or similar functions in many organisms, ranging from yeast to man, including plants. Histone H3K4 methylation and histone H3 and H4 acetylation are signals for active chromatin in many organisms. However, some details are different in plants. Lysine residues can be mono-, di-, or trimethylated, and this can lead to a different readout of the genetic code. While H3K9 di- and trimethylation are repressive marks in the mammalian system (Lachner *et al.,* 2001), H3K9 trimethylation is found to be associated with euchromatin in Arabidopsis, whereas mono- and dimethylation are heterochromatic marks (Fischer *et al.,* 2006). Histone modification also helps to distinguish between constitutive and facultative heterochromatin. H3K9 methylation marks very stable silent entities (constitutive heterochromatin), while H3K27 methylation is associated with facultative heterochromatin, allowing a change of the chromatic status depending on environmental stimuli and developmental processes.

Histone-modifying enzymes are often encoded by comparatively large gene families, and functional information about most family members is still limited. They might have potentially redundant functions or could be involved in yet unexplored regulatory mechanisms. Enzymes regulating the two most common histone modifications (acetylation, methylation) are among the best studied histone-modifying

enzymes. Histone acetyltransferases (HATs) and deacetylases (HDACs) are antagonistic and thereby reversibly regulate acetylation. The Arabidopsis genome encodes 12 putative HATs and 18 HDACs (Pandey *et al.,* 2002). Thirty-two SET (SU(VAR)/E(Z)/TRX) domain proteins with a possible function in regulating gene expression are found in Arabidopsis (Baumbusch *et al.,* 2001). Besides the catalytic SET domain, the SRA (SET and RING associated) methyl-cytosine-binding domain is common in histone methyltransferases (HMTs) (Johnson *et al.,* 2007). In Arabidopsis, H3K9me2, catalyzed by the HMT KRYPTONITE (*KYP*, also known as *SUVH4*, a *SU(VAR)3-9* homologue) is required for maintenance of non-CG methylation, suggesting a self-reinforcing feedback loop for maintenance of DNA and histone methylation (Jackson *et al.,* 2002). Additionally, a correlation between H3K9me2 and CHG methylation throughout the whole genome was observed recently, strengthening the theory of interdependence between the two chromatin marks (Bernatavichute *et al.,* 2008). Other SRA domain proteins (e.g. *VIM1* or *SUVH2*) enforce the link between DNA and histone methylation. *VIM1* is required for maintenance of centromeric DNA methylation (Woo *et al.,* 2007), while *SUVH2* acts together with *DRM2* in *de novo* DNA methylation in a locus-specific manner (Johnson *et al.,* 2008). *DRM2* and *SUVH2* suggest a link between histone modifications and RdDM.

1.1.2.4 RNA-mediated chromatin modifications

Recent research revealed that much more of the eukaryotic genome than previously assumed is transcribed, with huge amounts of non-coding RNAs (ncRNAs) of unknown function. ncRNA molecules of various sizes appear to play a broad role in the regulation of chromosome organization. There is increasing evidence that the ncRNAs themselves act in establishing and maintaining the epigenetic architecture of eukaryotic genomes. Their functions range from long ncRNAs classically implicated in the regulation of dosage compensation and genomic imprinting, to small ncRNAs which contribute to heterochromatin assembly via the RNA interference (RNAi) pathway (Buhler 2009). In some mammalian cases, long ncRNAs are directly involved in recruiting chromatin factors and thereby directly regulating transcription (Nagano *et al.,* 2008; Pandey *et al.,* 2008; Terranova *et al.,* 2008). In plants, two RNA

polymerase II-related RNA polymerases (Pol IV and Pol V), together with proteins of the RNAi machinery, regulate the generation of long and short ncRNAs involved in epigenetic regulation.

A nuclear process in plants, in which small interfering RNAs (siRNAs) direct the cytosine methylation of complementary DNA sequences, is called RNA-directed DNA methylation (RdDM). RdDM induces *de novo* methylation of cytosines in all sequence contexts (CG, CHG, and CHH) at the region of siRNA-DNA sequence homology (Matzke *et al.,* 2009). Genome-wide DNA methylation analysis revealed that about 30 % of methylated cytosines in *Arabidopsis thaliana* are directed via siRNAs (Cokus *et al.,* 2008; Lister *et al.,* 2008). Double-stranded RNAs (dsRNAs) generated by RNA-dependent RNA polymerase 2 (*RDR2*) serve as precursors for Dicer-like 3 (*DCL3*)-dependent processing of 24-nt siRNAs. Pol IV is predicted to generate initial RNA transcripts that are substrates for *RDR2*. siRNAs are loaded onto an Argonaute 4 (*AGO4*)-containing RISC (RNA-induced silencing complex) that targets *DRM2* (a *de novo* DNA methyltransferase) to RdDM target loci. Pol V transcription requires the putative chromatin-remodeling protein *DRD1* (defective in RNA-directed DNA methylation 1) to physically associate with intergenic loci. Nascent RNA transcripts from target loci are generated by Pol V, and these transcripts help recruiting complementary siRNAs and the associated RdDM effector complex to target loci in a transcription-coupled DNA methylation process (Figure 3) (Matzke *et al.,* 2009). One of the well-studied functions of RdDM is transposon silencing leading to genome stability. Increasing evidence is found that RNA-mediated silencing is implicated in development, stress response and natural epigenetic variation (Martienssen *et al.,* 2008; Matzke *et al.,* 2009; Slotkin *et al.,* 2009).

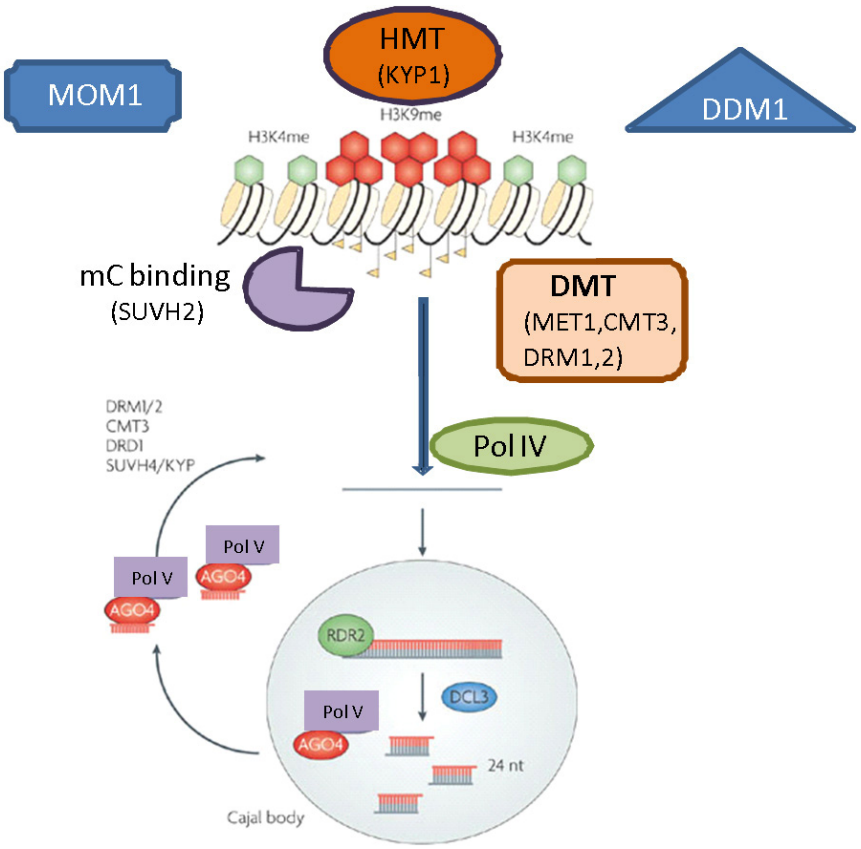


Figure 3. Factors involved in epigenetic regulation. A summary of genes investigated during this thesis is presented. The factors are indicated at the site of their action. RdDM and chromatin remodeling in Arabidopsis can involve small RNAs formed through a pathway including RNA-dependent RNA polymerase 2 (*RDR2*), Dicer-like 3 (*DCL3*), RNA polymerase IV (Pol IV). Effector complexes containing small RNAs, Argonaute 4 (*AGO4*) and RNA polymerase V (Pol V) direct DNA and chromatin modifications through the activities of many factors, including Domains Rearranged Methylase 1 and 2 (*DRM1* and *DRM2*), Chromomethylase 3 (*CMT3*), Defective in RNA-directed DNA methylation 1 (*DRD1*) and histone methyltransferase (*HMT*) SU(VAR)3-9 homologue 4 (*SUVH4*, also knows as Kryptonite, *KYP*). Other factors regulating the chromatin state are Morpheus' Molecule 1 (*MOM1*) or chromatin remodeling enzyme Defective in DNA methylation 1 (*DDM1*), proteins binding methylated cytosines (*SUVH2*, SU(VAR)3-9 homologue 2) or maintenance DNA methyltransferase Methyltransferase 1 (*MET1*). Figure adapted from (Chapman and Carrington 2007).

1.1.2.5 Other components of epigenetic regulation

Non-histone proteins have a broad impact on regulating chromatin structure. Other chromatin proteins regulating transcription involve PcG proteins (Polycomb group) and trxG proteins (trithorax group) (Schuettengruber *et al.,* 2007). While PcG proteins

suppress gene expression, trxG proteins are required to maintain an active state. They were initially identified in Drosophila as factors maintaining the cellular memory. Several PcG proteins have been found in plants that control many aspects of plant development. PcG proteins are part of large complexes that act as HMTs, targeting predominantly H3K27, whereas trxG proteins are involved in complexes targeting H3K4 methylation. SWI2/SNF2 chromatin remodeling factors are able to perform ATP-dependent nucleosome displacement. They are known to be involved in losening DNA-histone interactions and therefore affect nucleosome positioning. So far, only two out of 40 SWI2/SNF2 homologues encoded in the Arabidopsis genome have been functionally linked to regulation of transcription. *DDM1* (decreased DNA methylation 1) and *DRD1* were shown to be involved in regulating DNA methylation (Jeddeloh *et al.,* 1999; Kanno *et al.,* 2005). DDM1 maintains TGS at silent transposons and repetitive loci, and *DDM1*-deficient mutants show severe developmental and morphological defects, which appear only after several generations due to epimutations and insertional mutagenesis of reactivated transposons (Johannes *et al.,* 2009). *MOM1* (Morpheus' Molecule 1) was shown to be required for the heritable maintenance of transcriptional gene silencing (TGS) but unlike other epigenetic regulators it regulates selected loci in a DNA methylation-independent manner (Amedeo *et al.,* 2000; Caikovski *et al.,* 2008). Chromatin assembly factor (CAF) is involved in reestablishment of chromatin after replication and repair. Loss of functional CAF complex causes morphological anomalies, DNA damage and releases TGS from different loci, thereby linking repair and epigenetic regulation. It contains the subunits *FAS1* and *FAS2* (Fasciata 1 and 2) and the MSI1 protein (Multicopy Suppressor of IRA1) (Kaya *et al.,* 2001; Hennig *et al.,* 2003; Ono *et al.,* 2006). Similarly, *BRU1* (Brushy 1) and *RPA2* (replication protein A2) were described to function in DNA repair and replication, as well as in transcriptional gene silencing (Takeda *et al.,* 2004; Elmayan *et al.,* 2005).

1.2. Hypothesis

1.2.1. Formation of stable epialleles and polyploidy-associated transcriptional gene silencing (paTGS)

Epigenetic regulation is a widespread mechanism controlling gene expression in eukaryotes. It affects endogenous genes, as well as intruding sequences like transposons, transgenes, and viruses. Epigenetics is contributing to genome stabilization, regulation of development and morphology, and it has strong impact as evolutionary factor. Transcriptional gene silencing (TGS) is a heritable complete loss of gene expression from previously active genes. TGS can occur spontaneously in the genome or be targeted via double-stranded homologous transcripts (Matzke and Birchler 2005; Wassenegger 2005). Silencing is accompanied by chemical modifications of DNA and associated histones, followed by structural changes of chromatin. Polyploidization of genomes is often correlated with changes in genomic organization and gene expression. Differences in gene expression are frequently accompanied by extensive and rapid genetic and epigenetic changes which may lead to silencing at the transcriptional level due to interaction between the combined genomes (Osborn *et al.,* 2003; Adams and Wendel 2005b). Hybridization and polyploidization represent drastic events and challenges for the plant, requiring genome reorganization but leading to increased diversity. Additionally, polyploidy is suggested to help a population to overcome a bottleneck situation (Rapp and Wendel 2005). Therefore, the genetic and epigenetic modifications in polyploids are interesting in basic plant biology and may likely apply also during plant adaptation and breeding.

Only little is known about which factors are involved in the connection between polyploidy and epigenetic changes. Naturally occurring polyploids can be synthetically “reconstructed” to study polyploidy-associated genome rearrangements or gene expression changes without effects on the DNA sequence. These gene expression changes have been shown to be of epigenetic nature, as for example in the case of nucleolar dominance in allopolyploids that combine two different parental genomes. Nucleolar dominance is known to transcriptionally silence one parental set

of rDNA genes after hybrid formation and was recently shown to be regulated via RdDM (Lawrence and Pikaard 2004; Preuss *et al.,* 2008). Studies of allopolyploids have contributed a lot of insight into polyploidy-related epigenetic processes. However, allopolyploidy inherently changes two parameters at once and does not allow separating the effects of chromosome number change versus hybrid formation. Chromosome rearrangements are primarily thought to be connected with incompatible genomes in freshly formed allopolyploids.

Based on a well established system, the effects of paTGS in Arabidopsis were studied during this thesis. The system used is based on autopolyploids and has the advantage of restricting the changes to one parameter, namely chromosome number. Therefore, it is possible to focus on gene expression changes in polyploids, like the pronounced silencing of a marker gene due to epigenetic modifications in autotetraploids. I used tetraploid plants which were previously generated from the diploid *Arabidopsis thaliana* line C, homozygous for a transgenic hygromycin phosphotransferase resistance gene (*HPT*) (Mittelsten Scheid *et al.,* 2003) (Figure 4). After polyploidization, different expression states of the resistance gene were found in the autotetraploid derivatives. While some plants had resistant progeny and an active version of the *HPT* gene (RRRR), other lines had sensitive progeny and an inactive *HPT* copy (SSSS). These two expression states result from epigenetic changes within the transgene. Since both lines had identical DNA sequence at the same genomic location but differed in their expression states, the respective types were called epialleles. The epiallelic differences were also stably maintained in diploid derivatives (RR or SS) of the respective active or inactive tetraploid progenitors obtained after repeated backcrossing to diploid plants. Resistance or sensitivity to hygromycin was maintained even after several generations of self-pollination, indicating a very stable mechanism of maintenance of the respective expression state. A comparison of inactive and active status of the gene in diploid and tetraploid plants with regard of their chromatin features permits addressing the role of chromosomal location and gene structure.

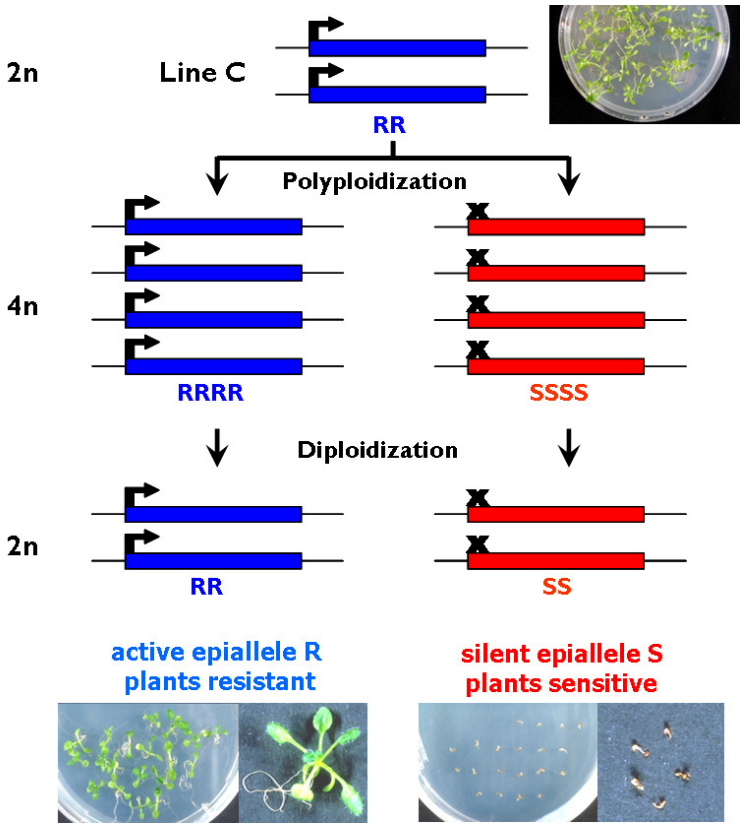


Figure 4. Schematic representation of the generation of tetraploids and diploid derivatives. The diploid *Arabidopsis thaliana* line C ecotype Zürich containing the hygromycin phosphotransferase resistance gene was used to generate tetraploid lines. After polyploidization two different expression states were observed, active (RRRR) and silent (SSSS). The diploid derivatives were also stably maintained in their respective expression state (RR, SS).

Epialleles can be used as traits for genetic analysis upon polyploidization. Partial analysis of DNA methylation at the affected sequence had shown that it was inversely correlated with the expression state (Mittelsten Scheid et al. 2003), but a detailed analysis of DNA methylation status of *HPT* epialleles was still missing. This question was addressed during this thesis by bisulfite sequencing. Other heterochromatic features may play a role in epiallelic regulation. Therefore I analyzed histone modifications associated with active and silent *HPT* by chromatin immunoprecipitation. The nature of the transgene, coding for a selectable marker, allowed to screen for recovery of gene expression via forward or reverse mutational approaches (Milos 2006; Baubec 2009). Both mutant screens were applied in the lab, and I expanded the reverse genetic screen by including RdDM mutants and double mutants. Additionally, the epiallelic chromatin features (including DNA methylation and histone modifications) were analyzed in material from the reverse genetic

screen, addressing following questions: Would those epigenetic factors regulate the *HPT* gene? Would the marker gene remain silent in the mutant background? The forward mutant screen (Baubec 2009) resulted in two classes of elements involved in paTGS. *Trans*-acting mutations modulate HPT gene expression by affecting other genes outside the transgene, whereas *cis*-factors change details of structure and sequence of the transgene itself. This thesis describes these *cis*-mutations in the *HPT* locus, induced by T-DNA transformation in the forward-directed mutant screen. I present a detailed comparison of these new epialleles and their features involved in regulating their epigenetic state. The fact that the *cis*-mutants restored *HPT* expression from a previously very stable silent epiallele could be expected to give some hints as to which alterations in gene structure of the locus and which chromatin features determine its epigenetic state and make it susceptible to paTGS.

1.2.2. Epialleles and their paramutation-like interaction

Beside of the stability of each epiallele and their respective chromatin modifications, another interesting feature had been observed in epialleles. The inactive gene exerted a dominant epigenetic interaction over its active homologue when they were combined in the same tetraploid (Mittelsten Scheid *et al.,* 2003). The *trans*-inactivation led to heritable gene silencing persisting after segregation from the inactivating epiallele, resembling the phenomenon of paramutation (Stam and Mittelsten Scheid 2005). The initial formation of the silent epiallele in the freshly generated polyploid line and also the paramutation-like event in the tetraploid hybrids, indicating ongoing establishment of silent alleles, were observed only in tetraploids, arguing that ploidy-dependent epigenetic regulatory mechanisms exist in Arabidopsis. To investigate the role of structural and epigenetic components in establishing paTGS and *trans*-silencing, I used tetraploid *cis*-mutants to address the question of structural involvement in the silencing phenomenon. A mechanism like this can lead to functional epigenetic homozygosity of alleles and, thus, to conversion of new alleles into traits expressed in early polyploid generations. It is likely that such interactions contribute to rapid adaptation and evolution of polyploid plant species. The *HPT* marker gene offers a reliable and easy assay to investigate epigenetic changes in paTGS and can provide information on similar regulation of endogenous

sequences. Insight into the role of the *cis*-components is expected to help in understanding the mechanism of paTGS maintenance at the transgenic locus. Finally, the identified features may be involved in the paramutation-like interaction and establishing of silencing and are anticipated to add to our understanding about adaptation and evolution of polyploid plant species.

1.3. Aim of this thesis

Polyploidy is associated with many genetic and epigenetic changes in the genome, including establishment and maintenance of TGS, which can lead to the generation of genetically identical epialleles differing in their epigenetic as well as in their expression state. The aim of this work was the detailed molecular and epigenetic characterization of epialleles based on a transgenic marker. More specifically, I performed a detailed analysis of DNA methylation and histone modification in active and silent epialleles, in diploid and tetraploid genotypes, and in the background of mutations in DNA methylation, histone modification, chromatin remodeling and RdDM pathways. I set off to investigate molecular factors involved in paTGS including sequence and structural details of mutagenesis-derived epialleles to get insight in the structural basis behind maintenance of paTGS. Further, by crossing tetraploid active and inactive lines carrying these epialleles, I investigated whether and how the structure of these epialleles would affect the paramutation-like interaction. Additionally, the sequence modifications in the *cis*-mutants made a distinction between epialleles possible, and I could follow their genetic and epigenetic fate after segregation, demonstrating that the *trans*-silencing is correlated with an increase of DNA methylation and paTGS.

2. Results

Differential expression levels of genes can be due to different amounts of transcription components, differences in the non-transcribed regulatory sequences, influence from neighboring sequences, or different chromatin packaging. While it was unlikely that the (morphologically indistinguishable) lines would differ in the basic elements of the transcription machinery, and initial studies did not show evidence for an involvement of neighboring sequences, it was known that the HPT epialleles were differentially methylated at certain restriction enzyme recognition sites (Mittelsten Scheid *et al.,* 2003). Analysis of epigenetic and structural factors of the epialleles might give valuable hints which parts of the locus determine its epigenetic state and make it susceptible to paTGS. However, a detailed analysis of chromatin organization at the epialleles and the neighboring sequences, as well as a thorough structural analysis of the epialleles were missing and therefore addressed during this thesis.

The *HPT* insertion was identified to be located on chromosome 3 (Figure 5a) in an AT-rich intergenic region (Mittelsten Scheid *et al.,* 2003). The transgene was inserted at nucleotide position 20306465 between genes At3g54800 and At3g54810, in inverse orientation with regard to their transcriptional direction (Mittelsten Scheid *et al.,* 2003) (Figure 5b). The insert is flanked by gene At3g54800, a pleckstrin homology (PH) domain-containing protein with unknown function. On the other side the insert is located close (approximately 1 kb distant) to gene At3g54810, which is known as *BME3* (Blue Micropylar End 3), a low expressed GATA zinc finger transcription factor influencing Arabidopsis seed germination (Liu *et al.,* 2005).
Previous investigations (Mittelsten Scheid *et al.,* 2003) and currently available epigenetic data from genome-wide screens (Cokus *et al.,* 2008; Lister *et al.,* 2008) indicate that the genomic localization itself is unlikely to influence the epigenetic behavior as no prominent epigenetic modifications are present at the site of insertion (Figure 5b). Different browsers providing epigenetic genome-wide data allowed a detailed analysis of the insertion site in the Arabidopsis genome on single nucleotide level. I was able to verify the previous statements showing that the transgene is inserted in an intergenic region with low levels of epigenetic modifications.

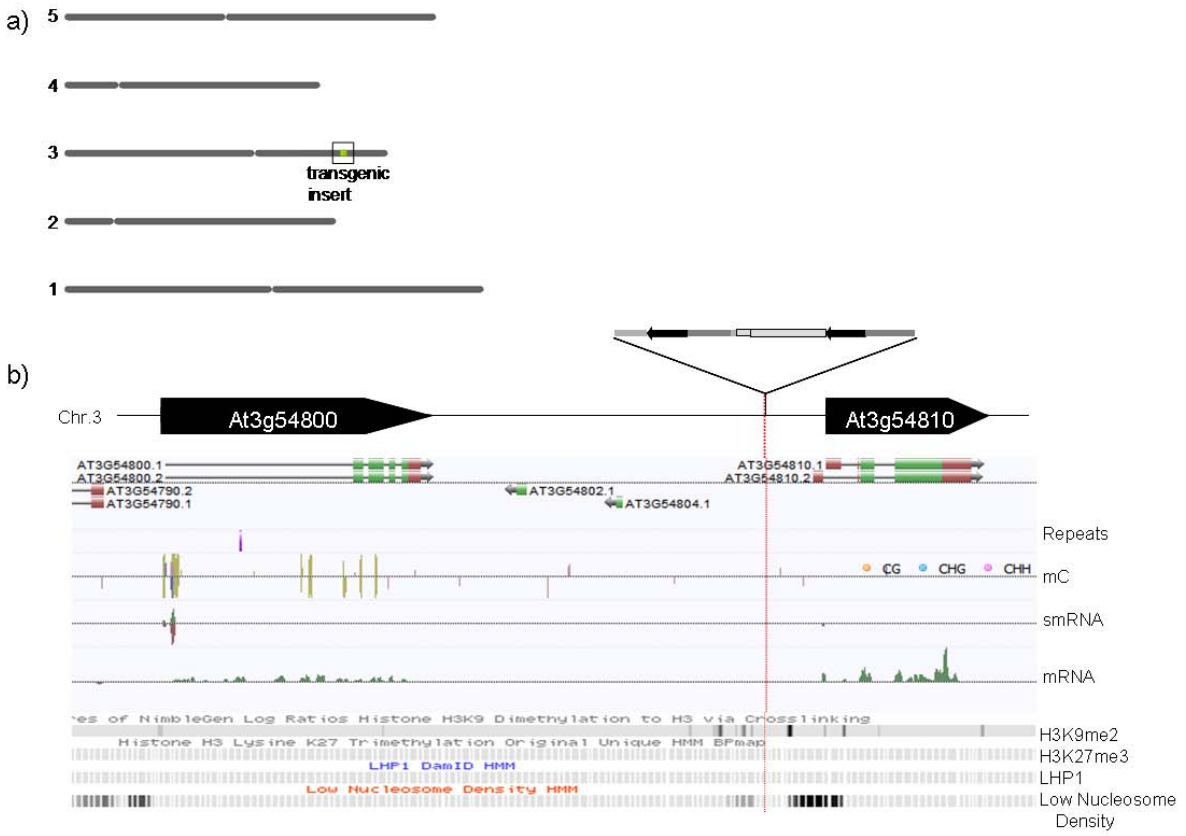


Figure 5. The site of transgenic insertion. a) Transgene integration site on chromosome 3 of *Arabidopsis thaliana*, ecotype Zürich (www.arabidopsis.org). b) Genetics and epigenetics of regions flanking the transgene. (AnnoJ: http://neomorph.salk.edu/epigenome.html, UCSC Genome Browser: http://epigenomics.mcdb.ucla.edu/BS-Seq).

2.1. The composition of the epiallele

2.1.1. The epiallelic structure

The *Arabidopsis thaliana* transgenic line C ecotype Zürich was generated by PEG-mediated direct gene transfer of the plasmid pGL2 containing the hygromycin phosphotransferase gene fused to the 35S promoter of the Cauliflower Mosaic Virus. The plasmid was transformed to mesophyll protoplasts and has been described in detail (Mittelsten Scheid *et al.,* 1991). Upon transformation, the 35S promoter and adjacent vector sequence became duplicated and this resulted in two tandem repeats interspersed with *HPT* and a truncated 35S terminator which lost its termination function (Figure 6). To assist in transformation, bovine carrier DNA was

used in the initial transformation procedure and mosaics of this carrier DNA were also integrated in the insertion site. In total, the transgenic insert harboring the resistance gene is 4 kb in size. The insertion occurred without a loss of plant genomic DNA.

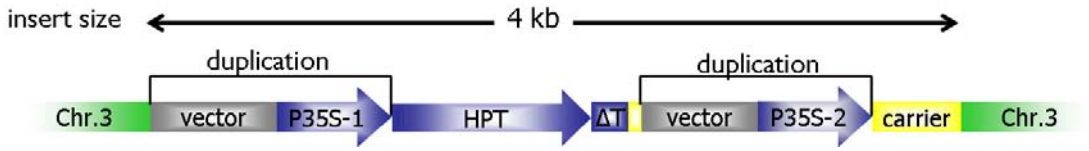
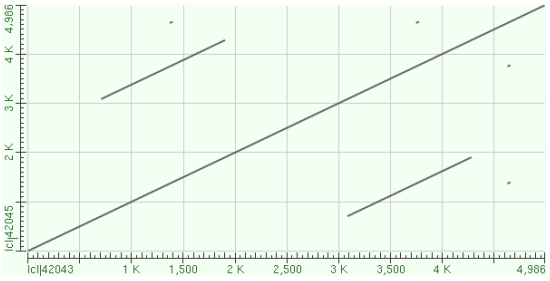


Figure 6. The structure of the transgenic insert. The transgene consists of a duplication of vector (grey) and 35S promoter sequence, a single copy of the hygromycin phosphotransferase resistance (*HPT*) gene, a truncated 35S terminator (blue) and some bovine carrier DNA (yellow).

2.1.2. The epiallelic sequence

The complete loss of expression in the hygromycin-sensitive line could have been also due to a mutation of the transgene, blocking transcription. My detailed sequence analysis excluded any genetic change. Proof-reading PCR and sequence analysis after back-cloning of the transgene allowed comparison of the sequences of the active and the silent epialleles. Sequences from cloned epiallelic DNA sequences of RR and SS were analyzed via alignment and dot plot (Figure 7). In the dot plot analysis, the duplication of the vector-promoter region is clearly recognizable. These methods revealed 100 % sequence identity, proving that the active and inactive epialleles are isogenic.

a)



b)

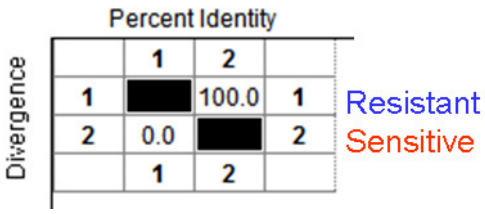


Figure 7. Sequence identity between inserts in the silent and resistant lines. a) Dot plot matrix view of RR vs. SS obtained from “Blast 2 Sequences”. It shows 100 % sequence identity, no gaps, and the duplication within the sequence. b) Sequence distances of RR vs. SS were analyzed in Meg Align (Laser Gene package).

2.1.3. Transcriptional activity in active and silent epialleles

The differences in hygromycin resistance in sensitive and resistant plants are due to different expression states of the epialleles. The active epiallele has strong transcriptional activity whereas the silent form is transcriptionally inactive. This was already shown by northern blotting and transcriptional run-on assays (Mittelsten Scheid *et al.,* 2003). For a more detailed analysis of the epialleles and their expression states in wild type compared to mutant background I applied a real-time PCR approach. The results for wild type epiallelic expression levels are shown in Figure 8. As a control, diploid and tetraploid lines of wild type Zürich without the resistance gene were used. On the one hand, resistant plants (blue) show very high *HPT* expression levels, on the other hand no expression of the silent epialleles was detected, neither in diploid nor in tetraploid plants.

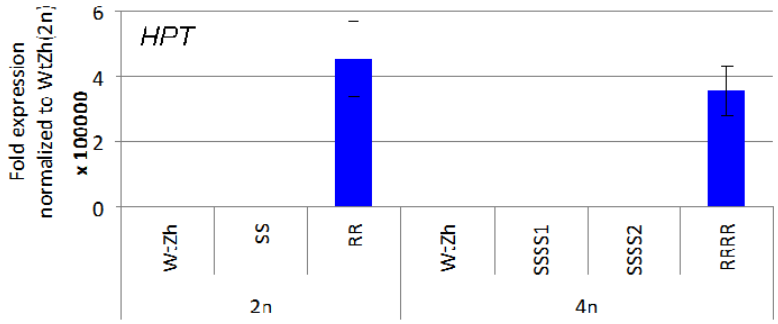


Figure 8. *HPT* real-time PCR data from diploid and tetraploid Arabidopsis ecotype Zürich with or without (wild type, WtZh) the resistance gene. Data were normalized to WtZh (2n) and *eIF4a* was used as an unaffected reference gene. Error bars represent standard deviation of experimental triplicates.

2.1.4. Chromatin modification at active and silent epialleles

Since the active and inactive epialleles were isogenic, but still different in their expression states, a possible explanation for the two expression levels was suspected to lay in differential chromatin modification, leading to stable transcriptional expression changes. DNA methylation and other epigenetic modifications involved in transcriptional gene regulation in plants are widely studied and shown to be involved in regulation and maintenance of genetic information in a

dynamic manner (reviewed in (Chan *et al.,* 2005; Fuchs *et al.,* 2006)). To investigate the role of chromatin modification in epiallelic stability, I performed a detailed and comprehensive epigenetic analysis of the epialleles using different types of experiments to analyze DNA methylation and chromatin modifications in active and inactive versions.

2.1.4.1 DNA methylation at the epiallele

Previous studies had provided preliminary evidence for differences in cytosine methylation levels at the *HPT* between active and inactive versions of the epialleles (Mittelsten Scheid *et al.,* 2003). To specify this connection, analysis by methylation-sensitive Southern blots was performed (Figure 9). Genomic DNA from three week-old seedlings and approximately six months-old stable callus culture was digested with the methylation-sensitive restriction enzyme *Hpa*II and hybridized to a probe detecting the open reading frame of *HPT*. Data from previous studies could be confirmed, showing very stable and strong DNA methylation in silent epialleles apparent as a high molecular weight band, whereas the active versions (RR and RRRR) with low levels of DNA methylation could be cut by *Hpa*II and therefore resulted in a low molecular band at around 250 bp on the Southern blot.

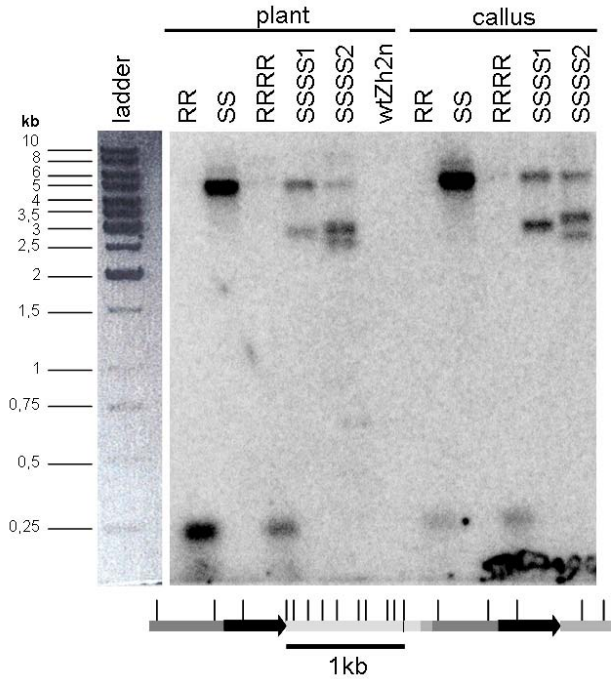


Figure 9. Methylation analysis of epialleles. Southern blot analysis of genomic DNA from either diploid or tetraploid epialleles or wildtype Zürich (wtZh2n), from three week-old seedlings (plant) or callus tissue, cut with the methylation-sensitive restriction enzyme *Hpa*II (CCGG) and hybridized to a probe spanning the open reading frame (*HPT*, 1 kb). Enzyme recognition sites are indicated by dashes in the graph.

The Cauliflower Mosaic Virus 35S promoter (P35S) is one of the best-studied promoters constitutively active in many plant systems (Guilley *et al.,* 1982). A specific transcription factor (ASF1) and its binding sites in P35S were identified in 1989 (Benfey *et al.,* 1989; Lam *et al.,* 1989). Later, scientists described a connection between epigenetic gene inactivation and cytosine methylation in the transcription factor binding site of the 35S promoter in Arabidopsis (Inamdar *et al.,* 1991). In order to study cytosine methylation of P35S on the single nucleotide level, I performed bisulfite sequencing within the epialleles. Complete chemical conversion in all bisulfite-treated DNA samples was certified using diagnostic primers in a region known to be free from methylation (Hetzl *et al.,* 2007). Subsequently, fully converted DNA was amplified with a bisulfite-specific primer set with degenerate bases at cytosine positions, to avoid bias towards modified or non-modified genomic templates. The obtained amplicons were cloned, sequenced and then analyzed with the software tool CyMATE, which was established during this thesis (Hetzl *et al.,* 2007). First, I analyzed the tandemly repeated copies of the 35S promoter. Both promoters were analyzed in all epigenotypes, by sequencing 5 clones for SS and 10 clones for SSSS. Analysis in RR and RRRR was restricted to 2 clones each, as they were nearly completely unmethylated and therefore uniform. Methylation patterns in S lines were not identical and represented epigenetic heterogeneity with respect to all three classes of potential methylation sites. As can be seen from the graphical output shown in Figure 10a, the epialleles can be distinguished based on their DNA methylation level. In active versions of the epiallele (R), both promoter copies are nearly completely depleted from methylated cytosines, while the inactive lines (S) are substantially hypermethylated in both promoter regions (Hetzl *et al.,* 2007). No significant difference in methylation between sequences derived from diploid or tetraploid plants was detectable (Table 1). In the 35S promoter there are two binding motives for the transcription factor ASF1 tandemly organized and separated by 7 bp. As described previously, binding efficiency of ASF1 to its binding motif – TGACG – is methylation-sensitive (Inamdar *et al.,* 1991). Methylation differences in the epialleles are especially pronounced at the transcription factor binding sites (Figure 10b). Corresponding to the data obtained by Southern analysis R lines were completely unmethylated allowing the transcription factor to initiate active transcription, whereas S lines had at least one of the two ASF1 copies methylated leading to complete silencing of *HPT* transcription.

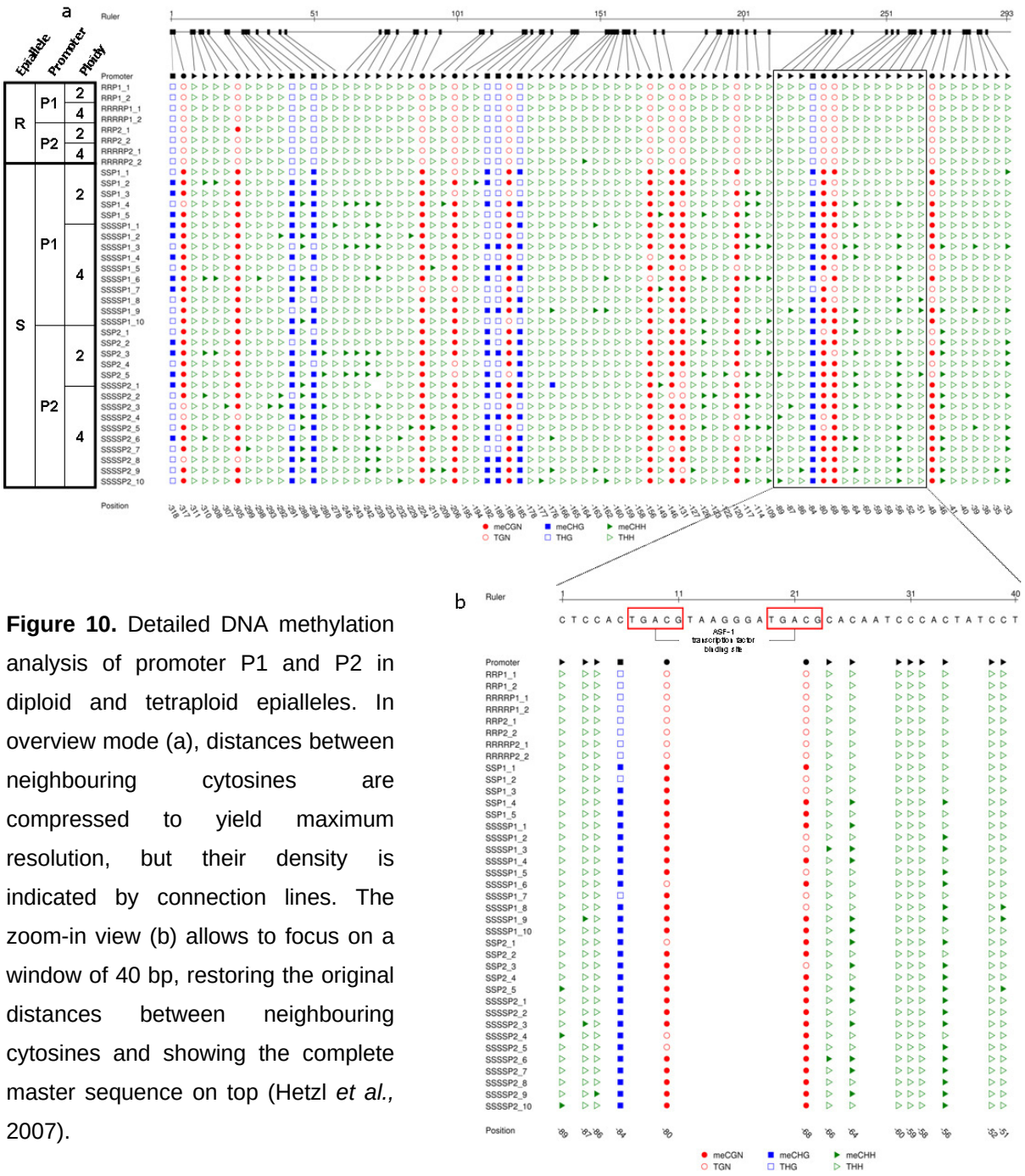


Figure 10. Detailed DNA methylation analysis of promoter P1 and P2 in diploid and tetraploid epialleles. In overview mode (a), distances between neighbouring cytosines are compressed to yield maximum resolution, but their density is indicated by connection lines. The zoom-in view (b) allows to focus on a window of 40 bp, restoring the original distances between neighbouring cytosines and showing the complete master sequence on top (Hetzl *et al.*, 2007).

P1	mC (%)	mCGN (%)	mCHG (%)	mCHH (%)
RR	0,0	0,0	0,0	0,0
RRRR	0,0	0,0	0,0	0,0
SS	23,1	83,3	60,0	6,4
SSSS	26,0	85,9	58,6	10,0

HPT down	mC (%)	mCGN (%)	mCHG (%)	mCHH (%)
RR	2,8	3,6	8,0	0,0
RRRR	3,3	3,6	8,0	1,0
SS	62,0	98,2	89,0	30,2
SSSS	51,9	92,9	76,0	19,1

P2	mC (%)	mCGN (%)	mCHG (%)	mCHH (%)
RR	0,6	4,2	0,0	0,0
RRRR	0,6	0,0	0,0	0,8
SS	28,7	81,7	71,4	12,9
SSSS	31,3	87,5	73,1	14,6

Table 1. General methylation at epialleles. Relative values in percentages are represented for all epigenotypes analysed in diploid and tetraploid state. P1, promoter upstream of the *HPT* gene; *HPT* down, region downstream of the *HPT* gene; P2, promoter duplication downstream of *HPT (Hetzl et al., 2007)*.

Data obtained from the methylation-sensitive Southern blot suggested strong DNA methylation spanning the whole *HPT* gene (Figure 9). Thus, I decided to expand the detailed bisulfite methylation analysis to other regions within the epialleles, namely a region downstream of the *HPT* gene including a small piece of carrier DNA and partially the vector duplication (Figure 11). A significant difference between the epialleles could also be detected. Sequences from active epialleles in both ploidy versions showed strong hypomethylation, whereas the silent versions in diploid and tetraploid lines were highly methylated in all sequence contexts. Like in the promoter regions, no significant difference in the methylation pattern between diploids and tetraploids could be observed (Table 1).

Based on the data presented here and some less detailed bisulfite analysis of additional regions (i.e. carrier and *HPT*, data not shown) I conclude that the active epialleles (R) are nearly unmethylated throughout the whole region, whereas the silent epialleles (S) are strongly hypermethylated all over the region.

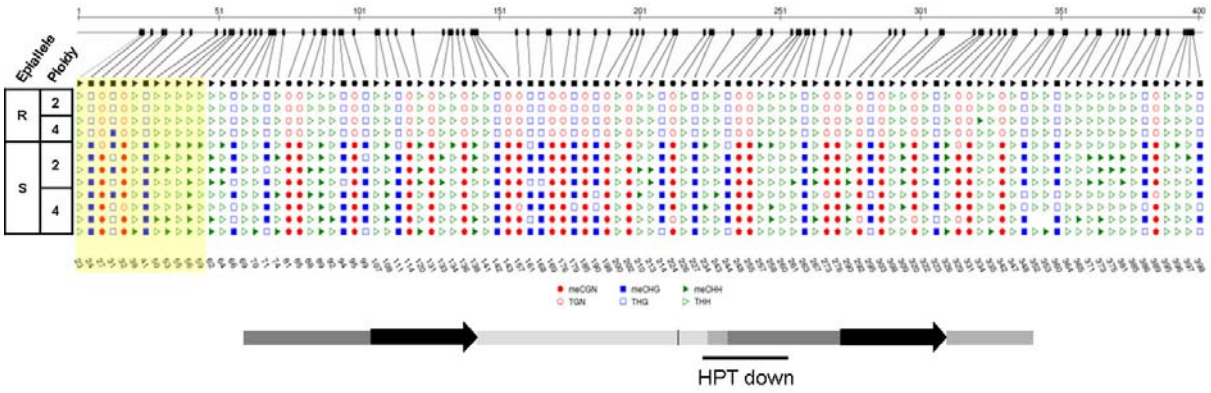


Figure 11. Detailed DNA methylation analysis of the region downstream of the *HPT* gene (HPT down) in diploid and tetraploid epialleles. Area indicated in yellow corresponds to the carrier region between T35S and vector sequence.

2.1.4.2 Histone modifications at the epiallele

To extend the analysis of chromatin features beyond DNA methylation levels I performed chromatin immunoprecipitation with material from the diploid and tetraploid lines with differently expressed epialleles. Initially, I tested different antibodies (i.e. H3K4me3, H3K9me2, H3S10ph, H3K27me2, H4K20me1, H4ac) and screened for the corresponding marks within the epialleles. Modifications which

normally appear in active chromatin are lysine 4 trimethylation on histone H3 and acetylation of histone H4. Further, serine 10 phosphorylation of histone H3 may have some function in transcriptional activation of genes in plants (Fuchs *et al.,* 2006). Typical histone marks in heterochromatin are lysine 9 and 27 dimethylation at histone H3 and lysine 20 monomethylation at histone H4 (Fuchs *et al.,* 2006). I compared the precipitated DNA after ChIP via PCR analysis spanning different regions along the whole epiallele (Figure 12). While some antibodies (like those against H4K20me1, H4ac, and H3S10ph) gave either only weak signals or no difference between the epialleles (and were excluded from further experiments), I found significant differences between the lines concerning type and level of other modifications along the whole transgenic insert. While expressing lines are marked by euchromatic signals (mainly H3K4me3), the silenced lines have typical heterochromatic modifications (H3K9me2 and H3K27me2). These marks extend only very little from the transgene into the flanking plant DNA. The differences can be used to identify changes in a mutant background, as I will describe later.

2.2. Stability of the expression state of epialleles

The different expression levels of the epialleles were stably inherited even in backcrosses of tetraploids with a diploid wildtype and subsequent self-pollination to generate diploid lines (Mittelsten Scheid *et al.,* 2003). Over a period of more than six years (corresponding to a minimum of eight generations obtained by self-pollination in the laboratory), no evidence of spontaneous reversions was reported. Even stress treatment, like UV-C irradiation in a dose that induces homologous recombination (Molinier *et al.,* 2005; Pecinka *et al.,* 2009), did not change the expression levels of the epialleles (data not shown). However, plant cells grown in culture exhibit genetic and epigenetic instability upon dedifferentiation (Berdasco *et al.,* 2008; Tanurdzic *et al.,* 2008; Krizova *et al.,* 2009).

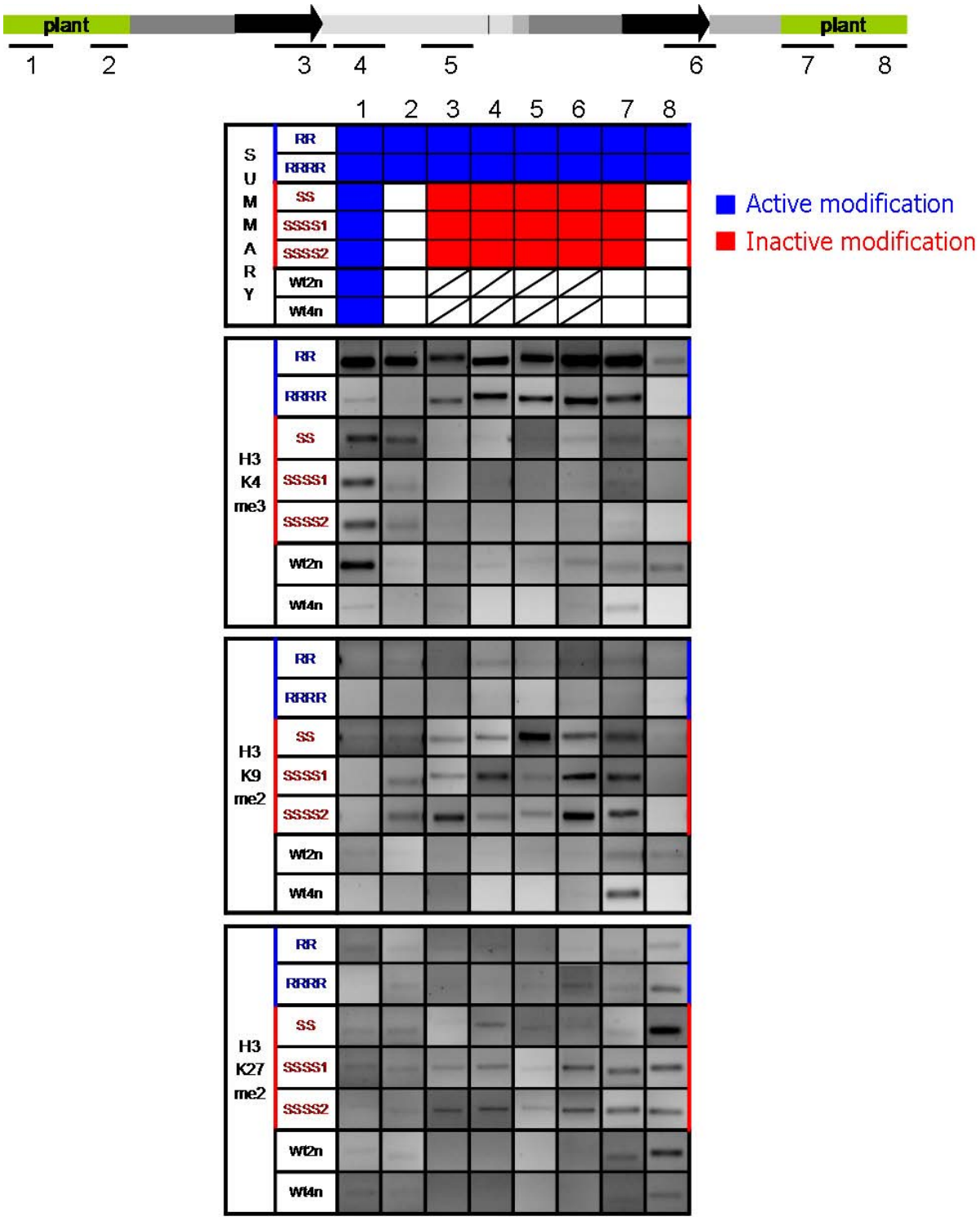


Figure 12. Histone modification at epialleles. Chromatin immunoprecipitation was performed using three antibodies against different histone modifications (H3K4me3, H3K9me2, H3K27me2). Sequences analyzed by semi-quantitative PCR are indicated by numbers. The summary provides an overview of the results shown in detail below (red: inactive modification, blue: active modification, white: equal or no signal). Region 1 corresponds to *BME3*, the closest neighboring gene.

To find out more about the stability of the epialleles upon dedifferentiation, a callus culture was initiated from cotyledons and propagated for six months. Callus tissue was similarly analyzed as described for the differentiated plant tissue. At first the callus culture was screened for its ability to grow on hygromycin selection medium. Several calli containing the active, inactive, or no epiallele were transferred to hygromycin-containing growth medium and maintained under selection for up to 5 weeks. Screening of the plates was performed at several time points. Calli with the active epiallele were resistant whereas calli obtained from silent lines or wild type were dying on selection plates (data not shown). In addition, chromatin modifications and DNA methylation were determined in callus tissue grown on non-selective medium. In the Southern blot with methylation-sensitive restriction enzymes shown in Figure 9, no differences in the methylation level compared to leaf tissue could be detected. Even with the higher resolution of bisulfite sequencing, the methylation levels at the CaMV promoters in plant and callus are fairly similar in diploid and tetraploid silent epialleles (Figure 13a). Moreover, histone methylation data from callus are very similar to those from differentiated plant material. Active epialleles (R) exhibit strong H3K4 trimethylation, whereas the inactive lines (S) are characterized by strong signals of H3K9 and H3K27 dimethylation (Figure 13b). Together, these results indicate similar and stable regulation of epialleles even upon dedifferentiation.

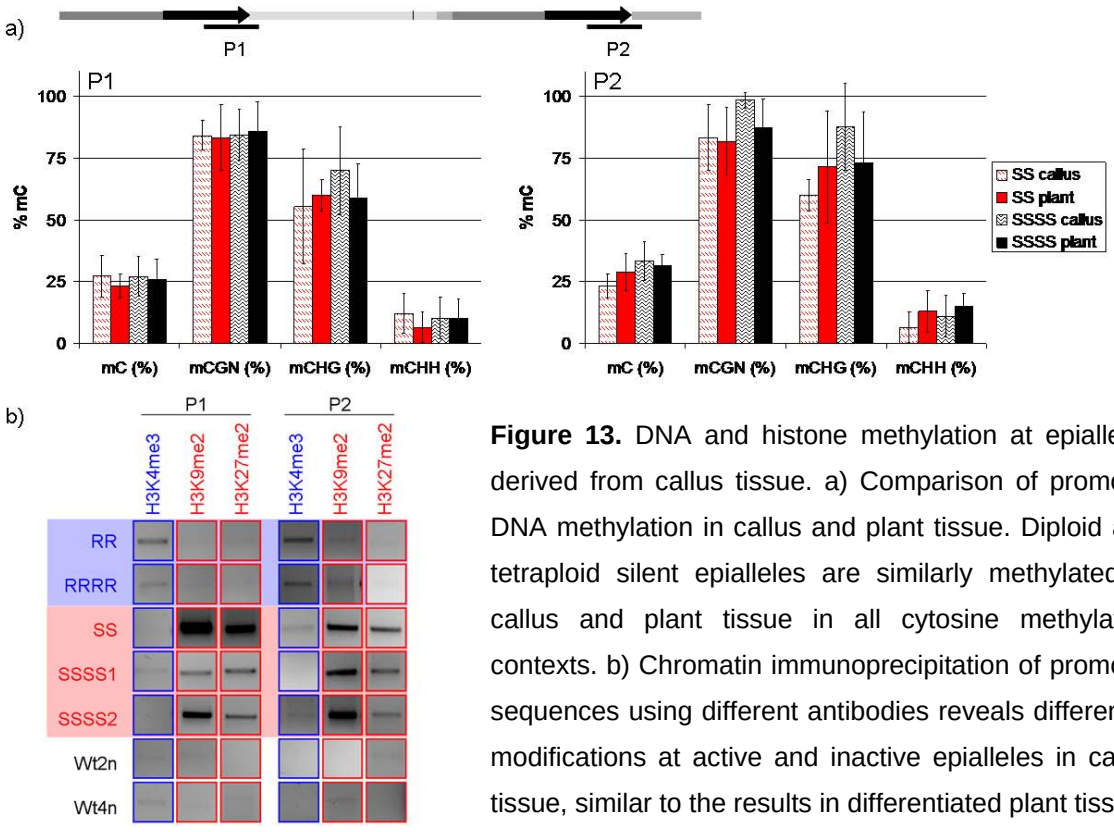


Figure 13. DNA and histone methylation at epialleles derived from callus tissue. a) Comparison of promoter DNA methylation in callus and plant tissue. Diploid and tetraploid silent epialleles are similarly methylated in callus and plant tissue in all cytosine methylation contexts. b) Chromatin immunoprecipitation of promoter sequences using different antibodies reveals differential modifications at active and inactive epialleles in callus tissue, similar to the results in differentiated plant tissue.

2.3. Epigenetic factors involved in maintaining paTGS

Transcriptional gene silencing depends on a variety of components involved in DNA methylation, histone modification, chromatin remodeling, RNA-based transcriptional silencing and other factors with not yet assigned molecular function. To find out which of these known factors were involved in paTGS, a reverse mutant screen was performed in the lab (Milos 2006). This screen was carried out by introducing the diploid silent *HPT* epiallele into the background of already characterized epigenetic mutants. We asked whether the *HPT* marker is reactivated in homozygous mutants and would therefore lead to hygromycin resistance on selection medium. For the mostly recessive mutants, this should occur for the first time in the segregating F2 populations when one or two copies of the silent epiallele would be combined with homozygous mutant genotypes in 3/16 among all plants. Among the material available at the beginning of this thesis, no resistant plants were observed in any cross (Milos 2006). A partial resistance was observed only in F4 of crosses with the *ddm1* mutation (Mittelsten Scheid *et al.,* 2003). Therefore, F2 populations from all crosses were genotyped and plants homozygous for the respective mutation and for the *HPT* insert were identified and selfed to generate corresponding F4 progeny.

Although no hygromycin resistance was detected in any other F4 generation than that of *ddm1*, the material was used to further analyze the chromatin features at the epialleles with different molecular techniques. Two scenarios were possible. Either the mutated gene would have an effect on the modification at the *HPT* epiallele without restoration of resistance, or the mutated gene would not even affect the modification at the epiallele. To investigate these two possibilities I performed a detailed epigenetic analysis of the epiallele in mutant background. Unexpectedly, I found different transcriptional regulation between promoter 1 and promoter 2. While transcription of P1, which is driving the *HPT* resistance gene, is activated only partially by the introduced mutations (Figure 14a), the second copy of the promoter (P2) changes its activity in many more mutations and to a larger extent (Figure 14b). *HPT* re-expression is detectable only in *ddm1*, a chromatin remodeler, *met1*, a DNA methyltransferase, and very small amounts in *mom1-2*, another modifier of TGS (Figure 14a). As resistance on selection medium was only observed in *ddm1* mutant background which has by far the highest transcript level among all mutants, it seems

that all other mutations do not cause P1 reactivation beyond the threshold of transcription necessary to obtain at least partially resistant plants. Figure 14b indicates the differences in the two promoter copies. The P2 transcript is reactivated in mutants of DNA methyltransferases (*met1-3*, *cmt3*, *drm1,2*), in a chromatin remodeler (*ddm1-5*), in the RNA-dependent silencing pathway (*drd1*, *ago4*, *rdr2*) and mutations in a TGS modifier of unknown function (*mom1-2*). The higher levels of carrier transcript in *met1-3* and *ddm1-5* background may be due to additional read-through activity of *HPT* transcript driven by P1. As we (Figure 14c) and other labs (Zheng *et al.,* 2007) have noticed some alteration in adjacent gene promoters caused by the 35S promoter, we included analysis of *BME3* expression (the gene upstream of the epiallele) in the mutant background. No significant increase of *BME3* gene expression compared to wild type Zürich or the line containing the silent epiallele could be observed (Figure 14c).

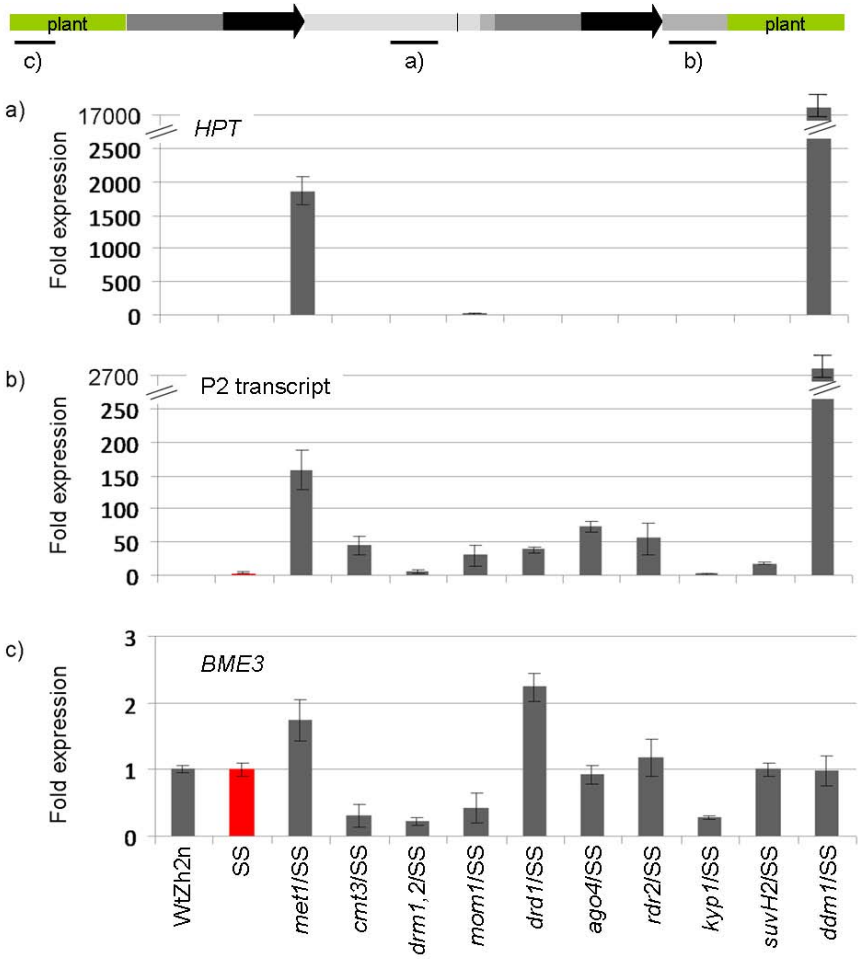


Figure 14. Expression levels in epigenetic mutant background. Data are normalized to SS, and the reference gene is *eIF4a*. Error bars represent standard deviation from technical triplicates.

Some mutants involved in RNA-based transcriptional silencing had not been included in the initial screen. Therefore I extended the screen by introducing the diploid silent *HPT* epiallele in RNAi mutants covering the RNA-dependent RNA polymerase IV (NRPD1, NRPD2/NRPE2) and RNA-dependent RNA polymerase V (NRPE1, NRPD2/NRPE2) pathway. Screening of homozygous mutants on hygromycin selection medium in the F2 and F4 generation showed no restoration of resistance in the mutant background. There is no impact of the RNAi machinery on the expression levels of *HPT*, P2 transcript or *BME3* (Figure 15).

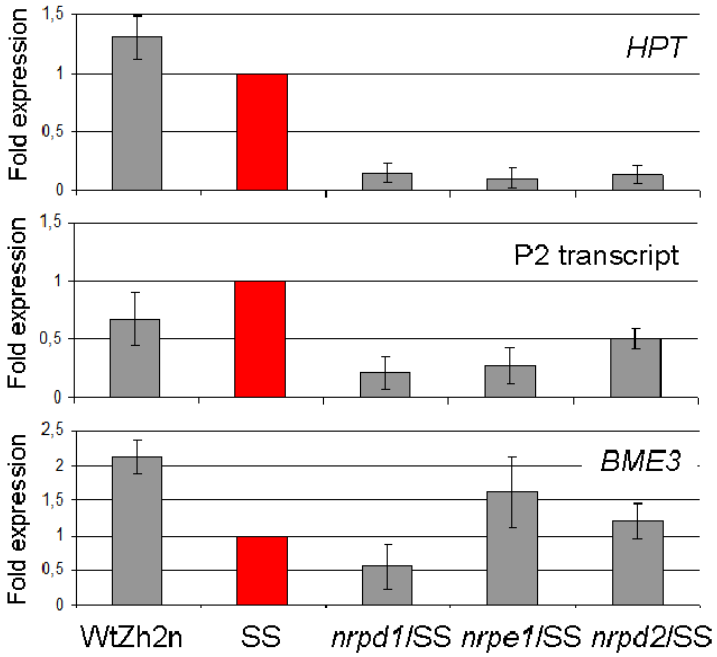


Figure 15. Levels of *HPT*, carrier and *BME3* expression in the background of RNAi mutants. Data are expressed as the average fold expression over *eIF4a* relative to SS *HPT* expression in technical triplicates.

For a detailed analysis of chromatin changes in the mutant background, I performed a Southern blot with methylation-sensitive enzymes on genomic DNA of the previously mentioned mutants with the introgressed *HPT* allele (Figure 16). DNA methylation-sensitive restriction enzymes *Hpa*II and *Msp*I and radioactive probes specific for the *HPT* with or without promoter sequence showed that DNA methylation was slightly changed in *met1-3*, *cmt3*, *kyp1*, *suvH2* and heavily altered in *ddm1-5* (Figure 16a, b). Hybridization with the HPT probe alone or with the P35S+HPT probe allowed distinguishing between sites in the coding region and the promoter,

respectively. For a better illustration, bands specific for the 35S promoter are indicated by a red arrow (Figure 16a, b). As an additional control, a probe specific for the Arabidopsis centromeric 180 bp repeats was applied (Vongs *et al.,* 1993). Those repeats are highly methylated and practically not expressed in wild type plants, but become hypomethylated and transcribed in *met1* or *ddm1* mutants (Vongs *et al.,* 1993; Mittelsten Scheid *et al.,* 1998). As expected, methylation of centromeric repeats is strongly affected in *met1-3* and *ddm1-5* (Figure 16c) and in *cmt3* and *kyp1* at CHG sites (Figure 16d).

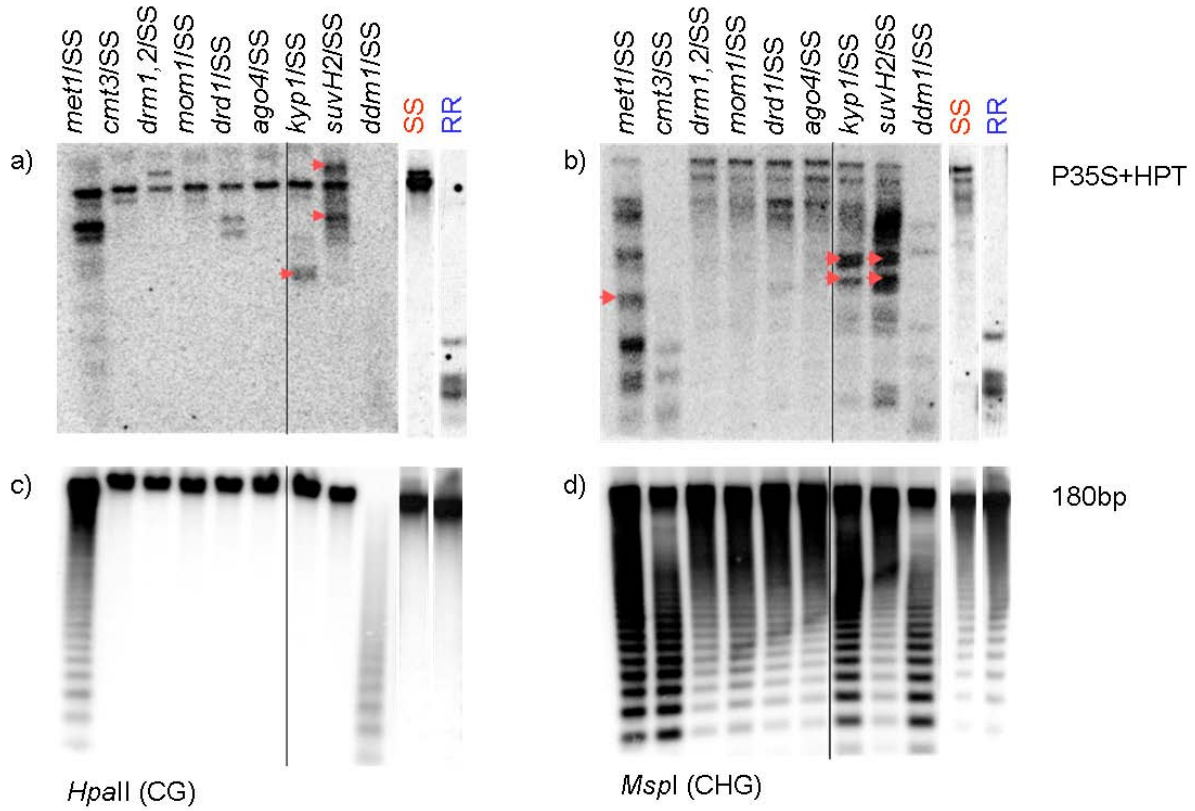


Figure 16. Southern blot after digest with methylation-sensitive enzymes of DNA from known epigenetic mutants with introgressed *HPT* epiallele. Methylation-sensitive restriction enzyme *Hpa*II (blocked by mCG and mCHG) was used in a) and c), *Msp*I (blocked by mCHG, cuts mCG) in b) and d). Probes for hybridization in a) and b) P35S+*HPT,* c) and d) 180 bp centromeric repeats probe.

The different degrees of DNA demethylation requested the application of bisulfite conversion and subsequent sequencing to obtain a more detailed view of the DNA methylation around the promoter duplication in mutant background. For each line, 5 different bisulfite clones were sequenced and analyzed (Figure 17). A drastic decrease in methylation in any pattern could be detected in *ddm1-5*, where CG

methylation was decreased by about 70 % in both promoters. Methylated CHGs were reduced by more than two thirds in P1 and P2, and the asymmetric DNA methylation (CHH) was affected in P1 but not in P2. *cmt3*, a mutation in a maintenance DNA methyltransferase specific for CHG methylation, showed significant decrease in CHG methylation in promoter 1 and 2. All the other tested mutations had no effect on DNA methylation of the silent epiallele.

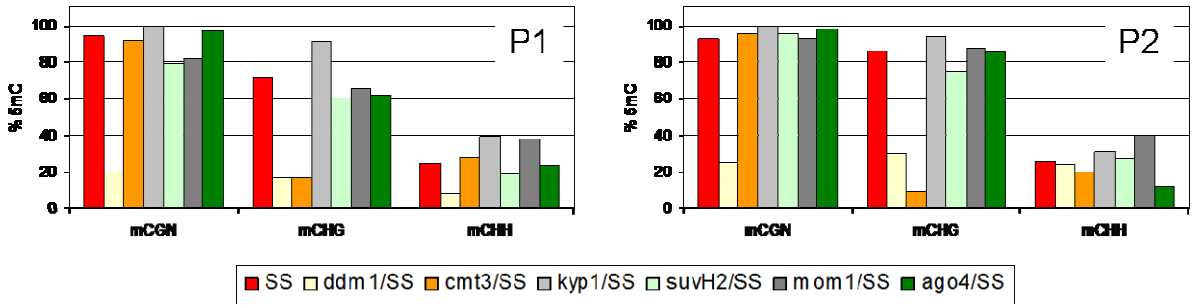


Figure 17. Percentage of methylated cytosines at the two promoter copies (P1 and P2) in epigenetic mutant background.

To complete the overall picture of the epiallele in mutant background, I performed chromatin immunoprecipitation with antibodies against histone H3 methylation. I could show that the reactivation of the silent epiallele in the *ddm1-5* background was due to a combination of loss of DNA methylation as well as a switch in histone modification. After propagating the previously silent epiallele over 4 generations in *ddm1-5* homozygous mutant background, the histone modification was switched from methylation on lysine 9 to a strong methylation on lysine 4 of histone H3 (Figure 18b). In *kyp1* (also known as *suvH4*, a histone methyltransferase) background, histone modification at the epiallele was affected in P2 but not in P1 or *HPT* (Figure 18c). Additional controls at known targets of *kyp1* showed that the mutation was effective there, as the expected reduction in lysine 9 methylation was obvious at retrotransposons in the mutant background. Therefore, I suggest that P1 histone methylation of the HPT epiallele is regulated by a different epigenetic mechanism or via a different set of HMTs. The other previously mentioned epigenetic mutants did not show any changes that were detectable by chromatin immunoprecipitation at the silent epiallele when compared to non-mutant background (data not shown).

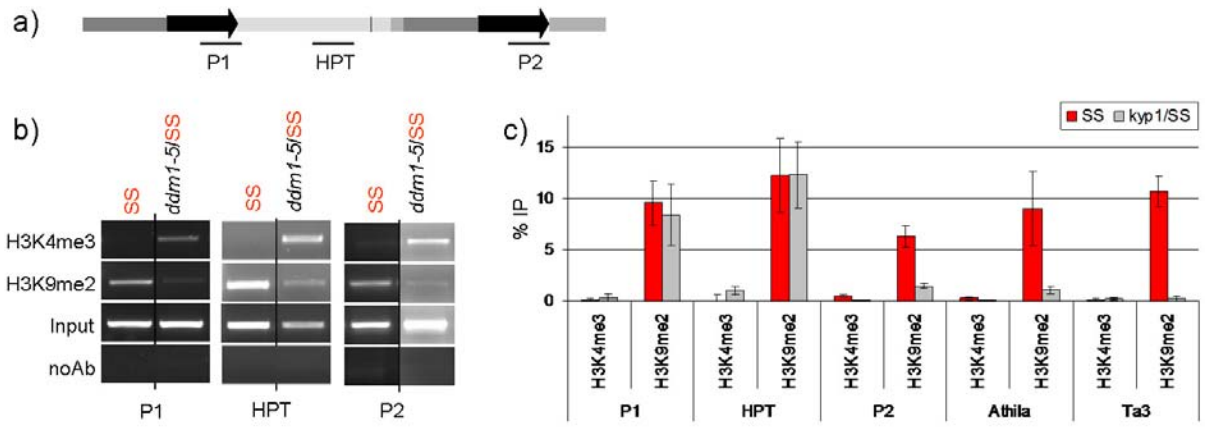


Figure 18. Abundance of histone H3 modifications at the epiallele and other genomic targets in epigenetic mutant background. a) Schematic representation of the epiallele. b) Semiquantitative PCR on ChIP samples revealed a switch from H3K9me2 to H3K4me3 within the epiallele in *ddm1-5* mutant background. c) qRT-PCR of ChIP samples from *kyp1* mutants shows reduction of methylation in P2 and control targets in the genome (*Athila* and *Ta3* are retrotransposons known to be affected by the *kyp1* mutation (Ebbs *et al.,* 2005)) but unchanged levels in P1 and *HPT*.

In *ddm1-5* mutant background I could detect a combined loss of DNA and histone methylation in the epiallele. It seems likely, that both modifications need to be removed in order to reactivate the silent epiallele as a single mutation affecting either DNA methylation or histone modification alone did not lead to reactivation of *HPT* expression. Therefore I generated double mutants harboring two single mutations of either the DNA methylation or histone modification pathway in SS background. A combination of *drm1* and *drm2* with *suvH4/kyp1* was not possible due to genetic linkage. The double mutants merging defects in DNA methylation (*cmt3*, *drm1* and *drm2*) and/or histone modification (*suvH2*, *kyp1*) were screened for hygromycin resistance in homozygous state in the F2 and/or F4 generation. However, also the double mutants did not show any sign of hygromycin resistance (Figure 19).

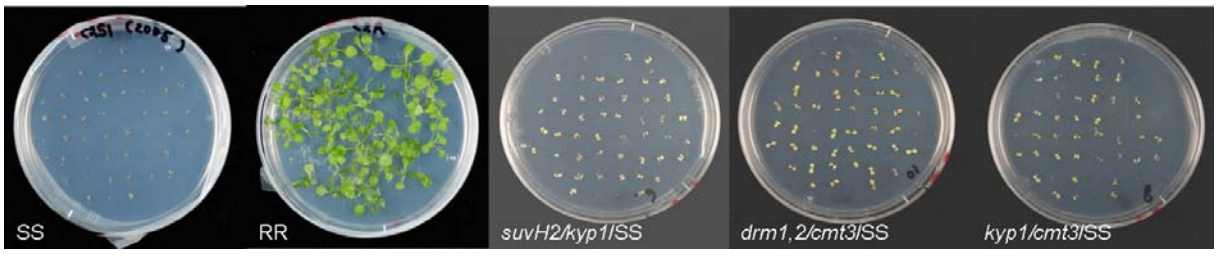


Figure 19. No resistance to hygromycin was observed in double mutants combining mutations in DNA methylation (*cmt3*, *drm1,2*) and histone modification (*kyp1*, *suvH2*) pathways. Data presented for *suvH2/kyp1*/SS, *drm1,2/cmt3*/SS, *and kyp1/cmt3*/SS are from homozygous plants in F4 generation.

Another way to screen for the involvement of regulatory epigenetic pathways is the combination of loss-of-function mutants with epigenetic drugs. Drugs can be easily applied by adding them to the growth medium during germination and subsequent growth. The applicable dose range and effectiveness of zebularine (a DNA methyltransferase inhibitor) and DZNep (3-deazaneplanocin A, a S-adenosyl-L-homocysteine hydrolase (SAHH) inhibitor) were established previously in the lab (Baubec *et al.,* 2009a; Baubec *et al.,* 2009b). While zebularine leads to DNA demethylation (Zhou *et al.,* 2002), DZNep was shown to affect histone methylation (Miranda *et al.,* 2009), but it also reduces DNA methylation (Baubec et al, submitted). To obtain conditions where both pathways (DNA methylation and histone methylation) are affected, single mutants carrying the silent *HPT* were grown on plates containing the before-mentioned drugs. Seedlings were harvested 3 weeks later (Figure 20), and gene expression analysis was performed by qRT-PCR (Figure 21). Inhibition of global methylation at histones and DNA via DZNep led to a strong reactivation of the silent epiallele in SS, while RR, the active epiallele, did not show any major expression changes after drug application (Figure 21). *nrpd2*/*nrpe2*/SS was hypersensitive to DZNep (indicated by a red arrow in Figure 20), therefore it was excluded from further analysis. *ago4*/SS seedlings on DZNep plates were small and had some growth defects, therefore the seemingly strong reactivation obtained in DZNep-treated samples may be due to apoptotic effects caused by the drug or by changing the expression of the reference gene. DZNep activated expression of *HPT* and P2 transcript in all mutant backgrounds if plants survived, whereas zebularine acted in a more moderate way together with the mutations. The drug-treated SS showed *HPT* activation to the same extent as *HPT* in mutant background. In summary, the combination of epigenetic mutations and DNA-methylation or SAHH inhibitors does not have synergistic effects on reactivating the silent epiallele.

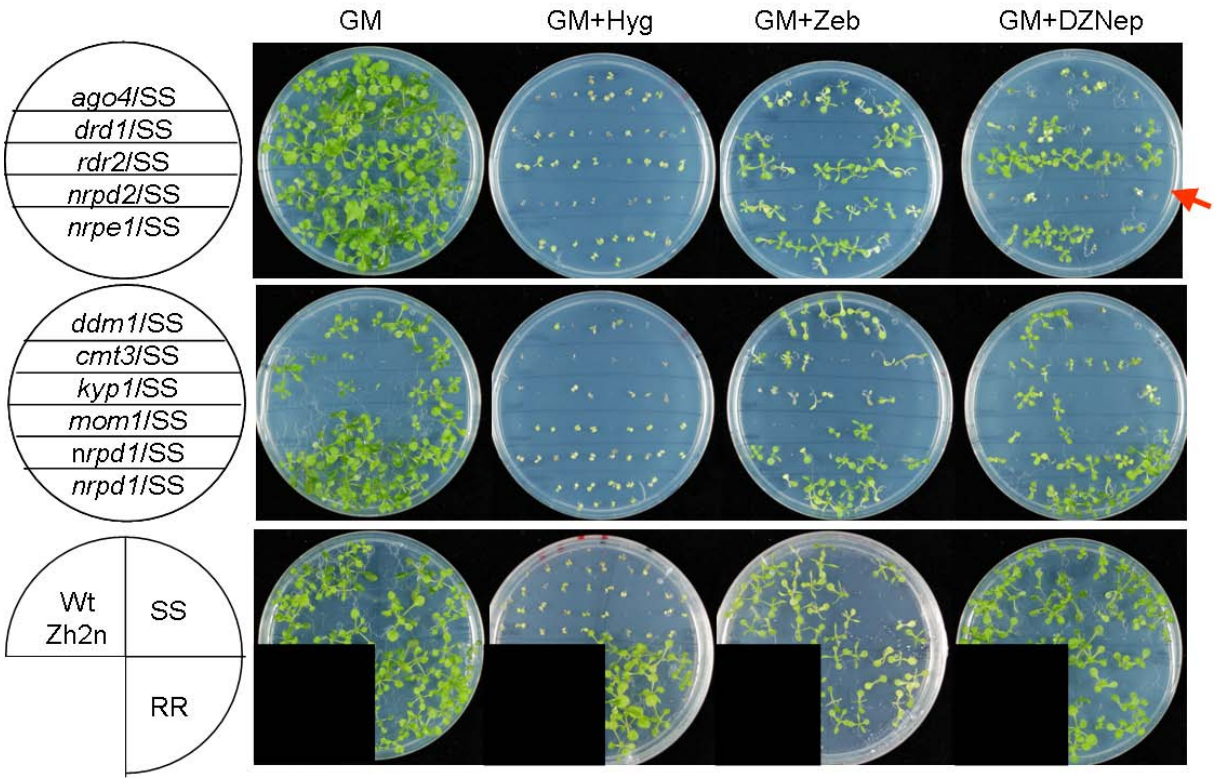


Figure 20. Overview of drug application experiments in mutant background. Plants with homozygous epialleles in mutant background were plated on GM (control), GM+Hyg (10 µg/ml hygromycin), GM+Zeb (40 µM zebularine), or GM+DZNep (2µM DZNep). Wild type Zürich 2n, SS and RR were used as controls.

The fact that only *ddm1* but no other known mutant restored significant transcription from the silent *HPT* epiallele was a strong argument to invest in a forward-directed mutant screen. T-DNA mutagenesis of the diploid line carrying the silent epialleles (previously performed at the Friedrich Miescher Institute for Biomedical Research in Basel, Switzerland) was followed by screening of the M2 progeny for hygromycin resistance. Approximately 20000 T-DNA insertion lines were screened and 20 positive candidates were further characterized in the lab. Several stronger alleles of the *ddm1* mutation and a new *hog1* allele led to resistance in M2 progenies (Baubec 2009). Some of the mutations occurred in the *HPT* gene itself or in close neighborhood, leading to *HPT* reactivation due to changes in DNA sequence and genomic context of the marker gene. In contrast to the mutations occurring in *trans*-acting factors, those mutants with a rearranged *HPT* gene were called *cis*-mutants and will be described in the next chapter.

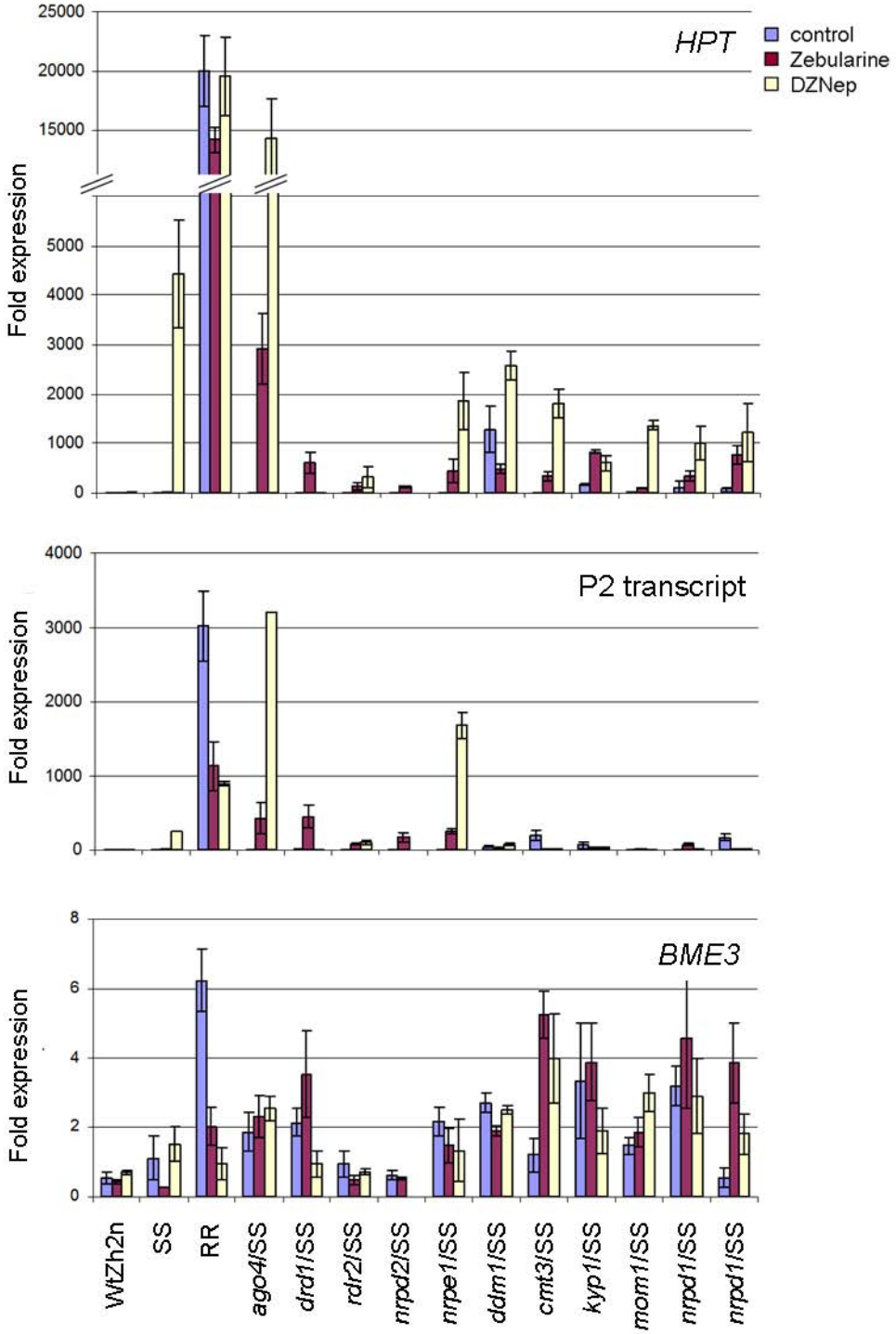


Figure 21. Gene expression analysis after drug application by qRT-PCR. All data are expressed as the average fold-expression over *eIF4a* relative to expression levels in SS. Error bars represent standard deviation of triplicate data.

2.4. Structural impact on maintaining paTGS

In some of the hygromycin-resistant mutants selected in the screen, T-DNA mutagenesis of the diploid silent line led to structural rearrangements within the epiallele. Six M2 candidates are such *cis*-mutants in which deletions or translocations within the silent epiallele caused sufficient reactivation of the *HPT* gene to achieve resistance on selection plates. The genetic changes were expected to give valuable hints to which features of the locus determine stability of its epigenetic state and susceptibility to paTGS.

2.4.1. Diploid *cis*-mutants

The occurrence of *cis*-mutants indicated that DNA structure of epialleles is an important parameter in maintaining paTGS. In order to investigate the role of DNA structure for the *HPT* locus, I performed a detailed genetic and molecular analysis of the *cis*-mutants. While some mutants stably maintained an active epiallele (283, 1146), others showed a rather unstable and variable resistance pattern (Figure 22a). Mutant 279, for example, had 84 % resistant progeny in F4, whereas in the next generation only 8 seedlings out of 100 grew on *HPT* selection medium (Figure 22a). Consistently, *HPT* expression levels detected by qRT-PCR are in perfect correlation with the resistance ratios on selection plates (Figure 22b). *Cis*-mutants with complete resistance had high *HPT* expression levels, whereas lines with incomplete resistance showed lower *HPT* expression. As previously mentioned, I could detect differences in expression of a gene located upstream of the transgene, depending on the expression state of the epiallele. This gene, called *BME3*, is also affected by changes in epiallelic expression in the *cis*-mutants. *BME3* is up-regulated in all six *cis*-mutants compared to wild type or the silent epiallele (Figure 22b). The P2 transcript, driven by the second copy of the P35S promoter in the active epiallele was missing or only very weakly expressed in *cis*-mutants, suggesting that structural change after mutagenesis would have affected the 3'-region of the epiallele.

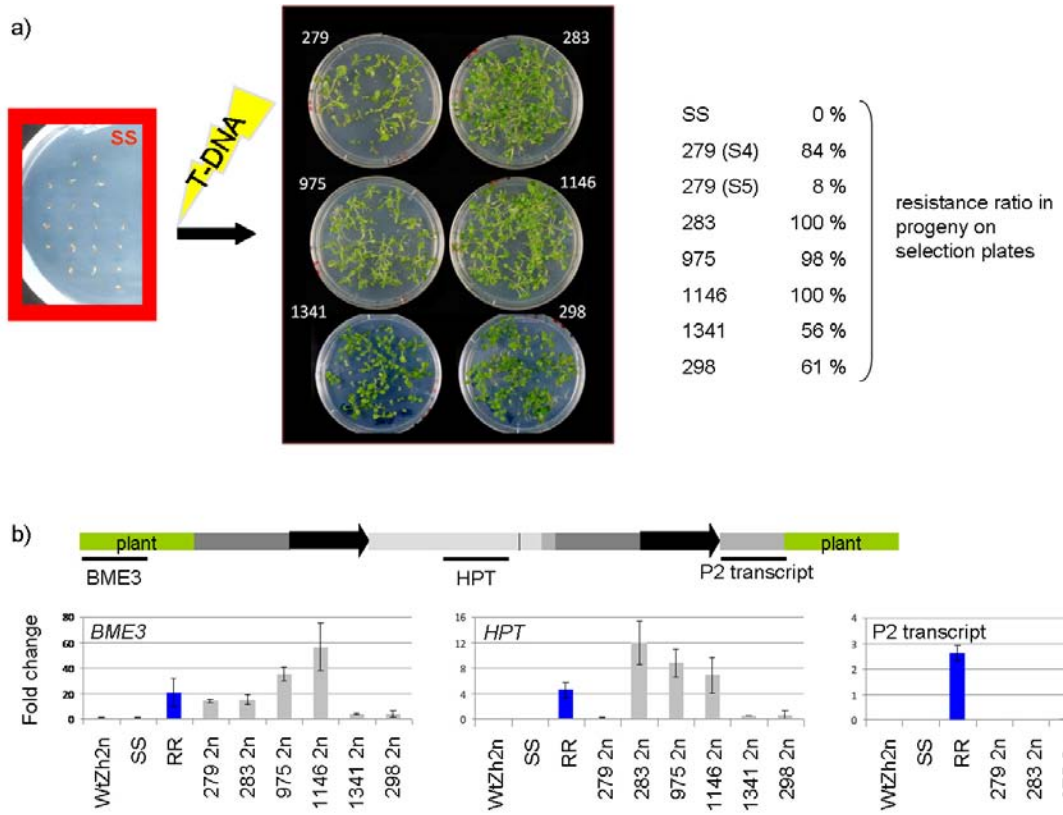


Figure 22. Resistance screening in *cis*-mutants after T-DNA mutagenesis of the diploid silent line (SS). a) Resistance ratios after hygromycin selection in S4 (and S5) generation. S4: 279 (S4), 283, 975, 1146, 1341, 298; S5: 279 (S5). b) RT-PCR data for *HPT* expression, P2 transcript and the upstream endogenous gene *BME3* in wild type (WtZh), the control lines (SS, RR) and *cis*-mutants (279 (S5); 283, 975, 1146, 1341, 298 (all S4)).

A detailed structural analysis of all *cis*-mutants was performed by Southern and northern blot analysis, backcloning the transgene from mutant genomic DNA and transcript cloning, followed by sequence analysis on DNA and RNA level. Southern blot data (Figure 23 a-c) revealed differently sized *HPT* bands between *cis*-mutants and the original epiallele, indicating major genomic rearrangements within the epiallele in *cis*-mutants. In Figure 23a, genomic DNA was digested with *Hind*III which cuts only in sites flanking the epiallele. In the diploid silent (SS) and active (RR) line the expected size of 4582 bp was detected, whereas the size varies in the *cis*-mutants. This variation could have been due to an insertion or deletion of DNA after T-DNA mutagenesis. In line 1341 a larger fragment in addition to the normal sized band (indicated by an arrow) was detected by Southern analysis (Figure 23a). As sequence analysis of 1341 excluded a point mutation in the restriction enzyme recognition site, a change in DNA methylation may cause this change in size, as

*Hind*III is methylation-sensitive (McClelland and Nelson 1992). Additional restriction digests of genomic DNA from *cis*-mutants, SS and RR with another enzyme cutting within the epiallele (*Eco*RV) confirmed the suspected structural changes. The results shown in Figure 23b and c suggest structural changes in the 3' region, whereas the *HPT* part seems to be unaffected, as expected by the selection for hygromycin resistance. The expected band of the first promoter (*Eco*RV: 2660 bp) is unchanged in all mutants, while the smaller band with the second copy is shifted up- or downwards in some but not all *cis*-mutants. Northern blot analysis of *cis*-mutants indicated a change in *HPT* transcript length (Figure 23d). As mentioned before and shown in Figure 8, the silent line (SS) does not transcribe *HPT*, while the resistant line (RR) has a strong transcript of 3 kb in size, which is longer than expected, due to a dysfunctional 35S terminator, transcriptional read through and termination of transcription outside of the transgenic insertion (Figure 23d and Figure 24). In addition to the main band, less abundant *HPT* transcripts of different smaller sizes are also visible in RR plants. The length of the main transcript in *cis*-mutants is strongly reduced, but still shows a ladder pattern. The shortened *HPT* transcript suggested deletions within the epiallele in *cis*-mutants leading to reactivation of the resistance gene.

To find out more about the *HPT* transcripts in *cis*-mutants I characterized them by 3'-RACE (rapid amplification of cDNA 3' ends). This experiment confirmed the assumption of deletions and rearrangements on the basis of RNA. A summary is shown in Figure 24. The mutants 279 and 283 revealed huge deletions spanning the entire duplicated region (vector and P35S-2), including the carrier sequence and some plant sequences. The deletions of 1146, 1341, and 298 are restricted to the carrier sequence. The 3' end of the transcript in mutant 975 covers a sequence from 1.2 kb upstream of the epiallelic insertion site, indicating a possible translocation.

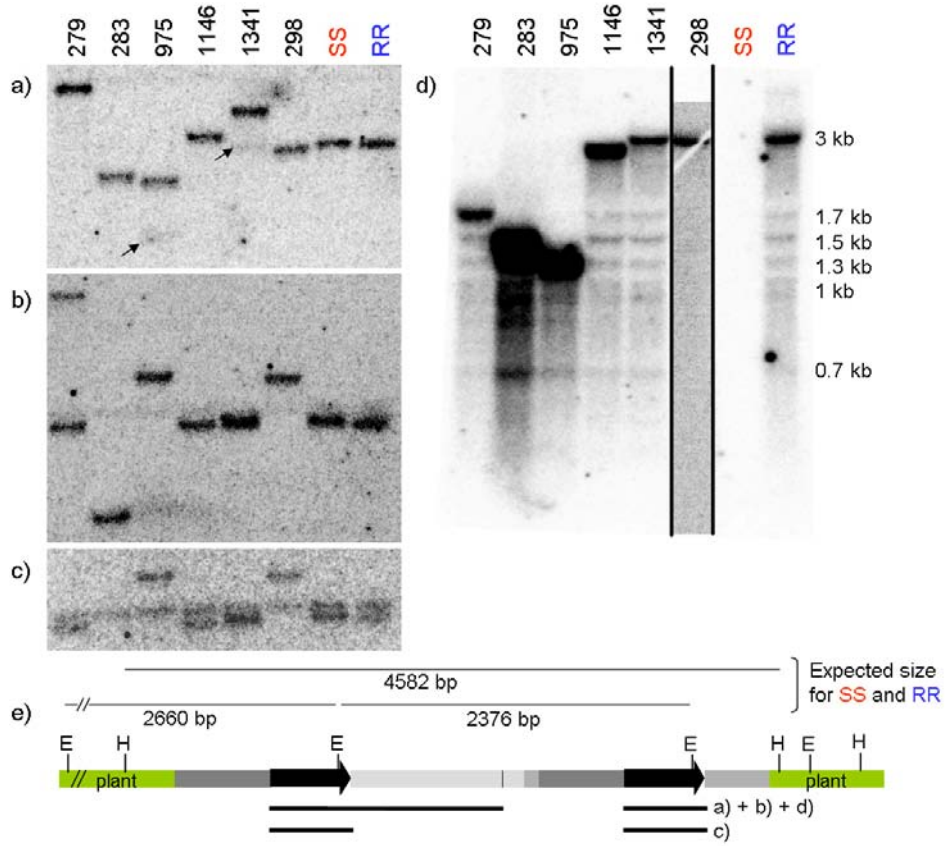


Figure 23. Structural analysis of *cis*-mutants. a-c) Southern blot data from different digests of genomic DNA. a) *Hind*III and b) *Eco*RV: P35S+*HPT* probe; c) *Eco*RV: P35S probe. d) RNA transcript length analysis by northern blotting, using P35S+*HPT* probe. e) Schematic representation of expected band sizes in epialleles after restriction digest and Southern analysis.

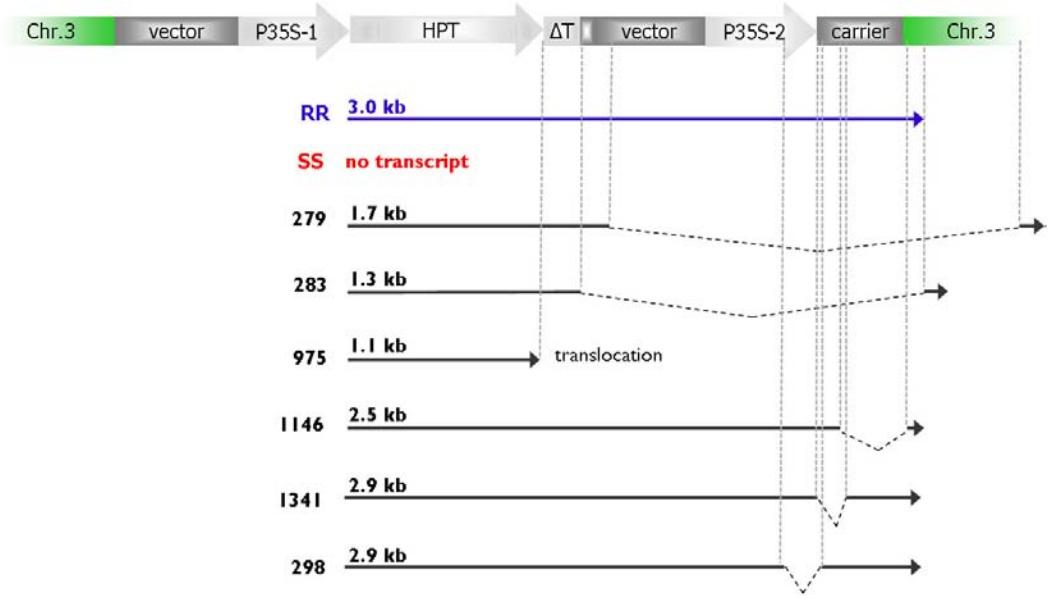


Figure 24. Transcript composition of *cis*-mutants compared to the silent (SS) and active (RR) epiallele. All *cis*-mutants show deletions spanning either the whole duplicated region or small parts in the carrier sequence. Mutant 975 contains a major rearrangement, due to translocation of a sequence upstream of the epiallele. Transcript size is indicated in kb.

The exact sequence composition of all *cis*-mutants was refined by a detailed DNA analysis via proof-reading PCR, back-cloning and sequencing. An overview of genomic sequences in all *cis*-mutations is shown in Figure 25, with the original sequence composition of the epiallele before T-DNA mutagenesis on top. The graph also includes restriction sites for *Eco*RV and *Hind*III and their respective size, in order to allow a comparison to Southern blot data from Figure 23a-c. For the different *cis*-mutants, the sites where the rearrangements happened are indicated in red. Line 279 shows a large deletion spanning the duplicated region and some sequence of chromosome 3. In total, 4748 bp are deleted in line 279. Another huge deletion (2188 bp) was detected in mutant 283, including the duplicated region and a smaller fraction of plant genomic DNA. Smaller deletions had occurred in 1146 (567 bp within the carrier), 1341 (65 bp within the carrier), and 298 (143 bp covering P35S-2 and carrier). The sequence of 975 shows a large recombination event, starting 1.2 kb upstream of the transgenic insertion. The recombined sequence covers a region starting 1.2 kb upstream of the insert and ending just before P35S-1. This sequence is copied into the region directly downstream of the *HPT* gene and stops at P35S-2. It seems that the sequence identity between P35S-1 and P35S-2 induced this large recombination event. These data show that all *cis*-mutants exhibit rearrangements within the epiallele, followed by a change in gene expression leading to hygromycin resistance. Some *cis*-mutants lost large parts of the duplicated sequence, including the second copy of the promoter (279, 283), while others were reactivated upon only small sequence deletions. Additionally, there is little or no overlap between the deletions in the *cis*-mutants, for example the deletion in 298 overlaps only 6 bp with 1341, while it does not overlap with 1146. Taken together, I can reject the hypothesis that the duplication within the epiallele is responsible for the silencing, as the duplicated promoter is still completely (975, 1146, 1341) or partially present (298) in the *cis*-mutants.

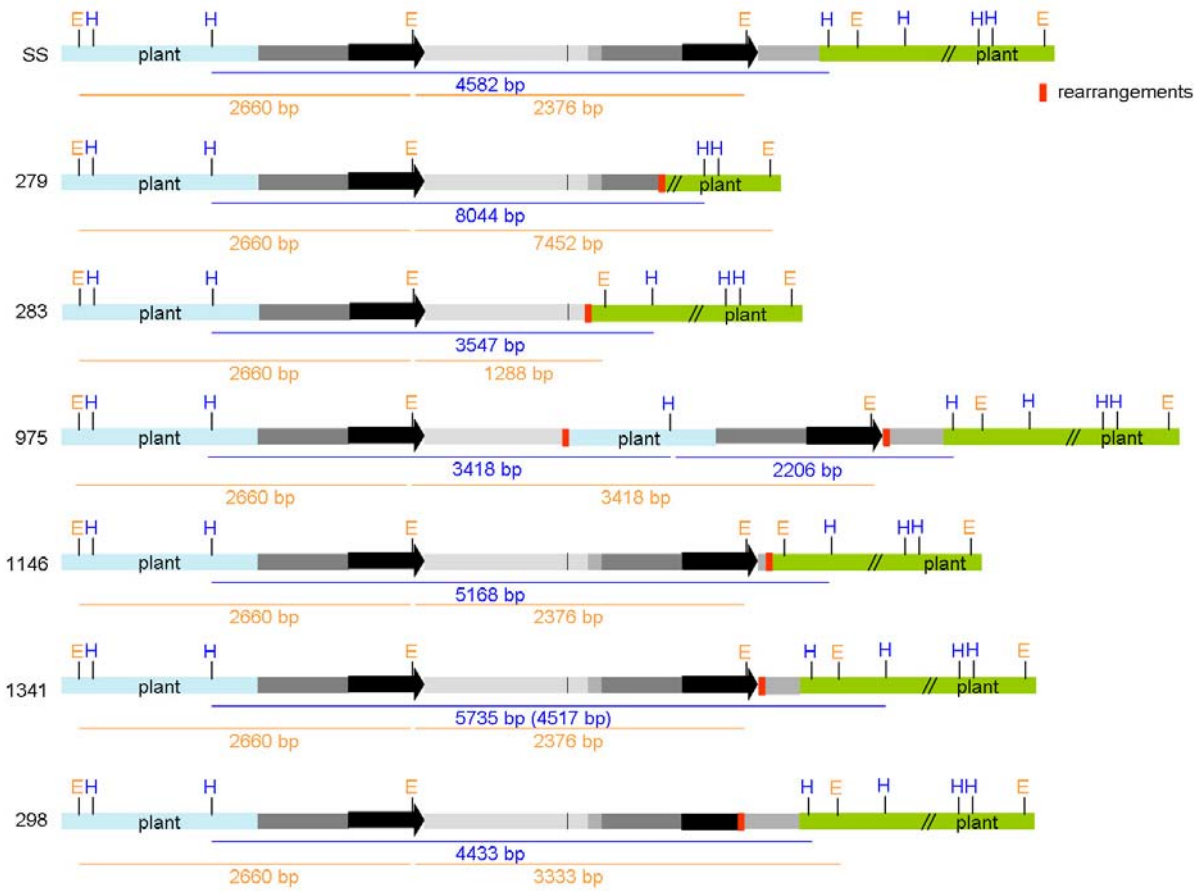


Figure 25. Overview of DNA sequence composition of *cis*-mutants. SS represents the original silent epiallele before T-DNA mutagenesis. *Cis*-mutants: 279, 283, 975, 1146, 1341, 298. The sites of rearrangements are indicated in red. Restriction sites (H: *Hind*III, in blue, E: *Eco*RV, in orange) and expected pattern size for Southern blot detection are shown.

After knowing the exact sequence of *cis*-mutants, I addressed the epigenetic behavior of the rearranged epialleles. The chromatin state of four *cis*-mutants was probed via chromatin immunoprecipitation using antibodies against H3 lysine 4 trimethylation or H3 lysine 9 dimethylation and subsequent analysis by quantitative PCR (Figure 26a). Three mutants switched from the silent modification (high levels of H3K9me2) present in their progenitor (SS) to an active pattern (H3K4me3) in P35S-1 and *HPT*, only one mutant (279) kept very high levels of H3K9me2 in combination with a noteworthy increase of H3K4me3. At the same time, bisulfite sequencing gave a detailed cytosine methylation analysis on P35S-1 in SS and *cis*-mutants. Several clones per line were sequenced, and results are summarized in Figure 26b. While the overall cytosine methylation does not seem to be reduced in *cis*-mutants, a detailed comparison of methylation at the transcription factor binding site (indicated with asterisks) between SS and *cis*-mutants showed a reduction of methylation in *cis*-

mutants. This reduction is strongly pronounced at mutants 279, 283, 975, and 1341. Additionally to the detailed bisulfite methylation analysis, a Southern blot with a methylation-sensitive restriction enzyme (*Hpa*II) was performed (Figure 26c). The differences between the silent and resistant lines, in diploid and tetraploid state are perfectly visible. Moreover a switch in *cis*-mutants from the silent high molecular band to an unmethylated and thus digested state is obvious in all *cis*-mutants. Tetraploid *cis*-mutants, discussed later in the text, give similar patterns to diploid *cis*-mutants. These observations confirm the active chromatin state of *cis*-mutants correlating the reactivation of resistance gene after T-DNA mutagenesis with changes in chromatin modifications.

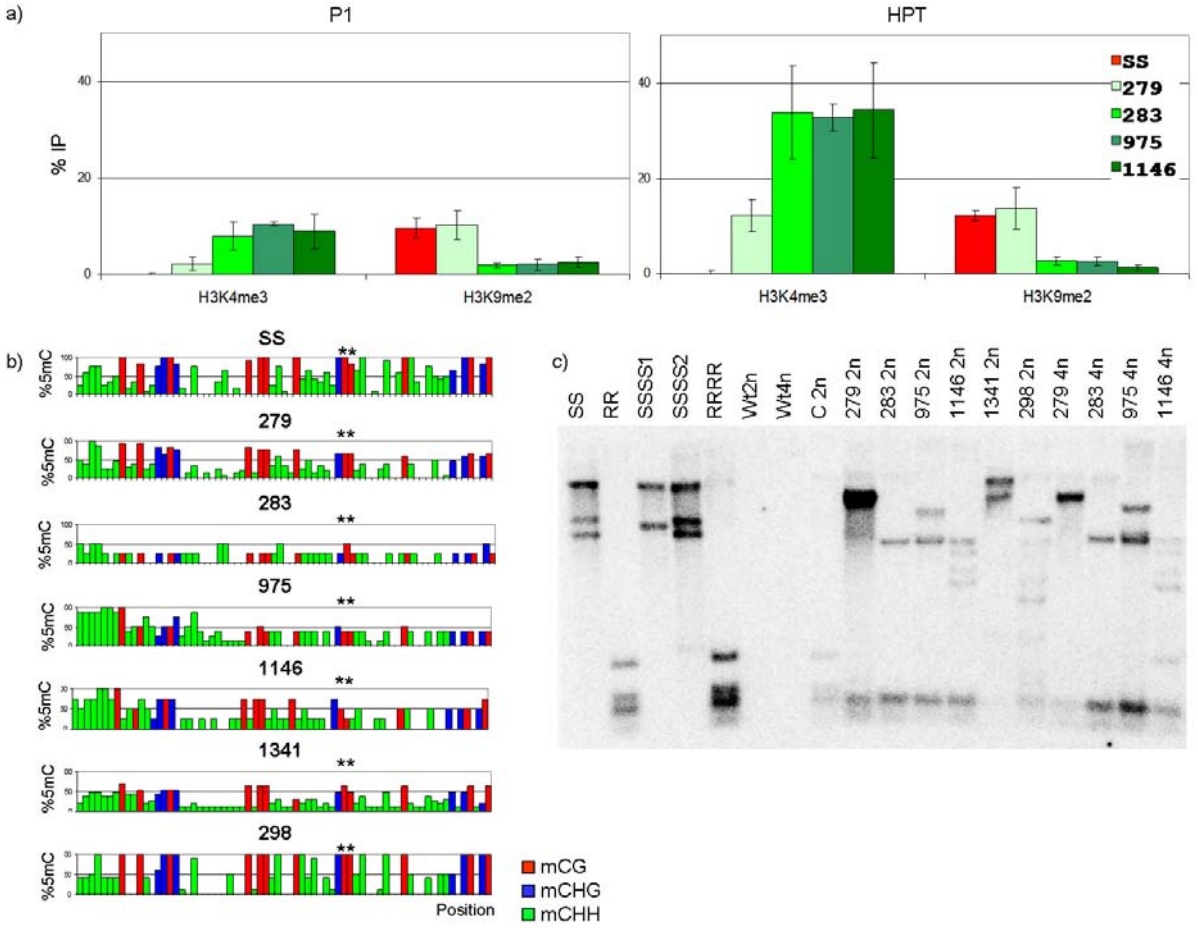


Figure 26. Chromatin modification changes in *cis*-mutants. a) Chromatin immunoprecipitation results of P35S-1 (P1) and *HPT*, calculated relative to input. b) Bisulfite sequencing of P35S-1 in SS and *cis*-mutants. The transcription factor binding site is labeled with an asterisk (m: methylated). Number of sequenced clones: SS: 9; 279: 8; 283: 4; 975: 8; 1146: 4; 1341: 8; 298: 6. c) Southern blot. Genomic DNA digested with a methylation-sensitive enzyme (*Hpa*II, blocked by mCG methylation) and probed with P35S+HPT. Tetraploid *cis*-mutants are addressed in chapter 2.4.2.

The following experiments determine whether *cis*-mutants might have additional effects *in trans* or whether the *HPT* gene reactivation would be due to or influenced by other mutations outside of the transgene. To investigate genome-wide effects on DNA methylation, hydrolyzed DNA from the *cis*-mutants was subjected to high pressure liquid chromatography (HPLC). All lines, including diploid and tetraploid *HPT* lines, as well as *cis*-mutants were tested for their cytosine methylation levels (Figure 27a). The overall cytosine methylation varied between 5 and 8 % which is in the range of values published for Arabidopsis (Cokus *et al.,* 2008; Lister *et al.,* 2008). A chromatin remodeler mutant (*ddm1*), known to have a very drastic decrease in the methylation level was used as a control.

To exclude a *trans*-effect in the *cis*-mutants, a repetitive and transcriptionally silent marker gene coding for ß-glucuronidase TS-GUS (=L5, (Morel *et al.,* 2000)), responding to many epigenetic mutants (Elmayan *et al.,* 2005), was introgressed into the background of the *cis*-mutants, SS and wild type Zürich. While the positive control with TS-GUS in the background of *ddm1-5* showed strong staining, screening for reactivation of the marker in the *cis*-mutants did not reveal any GUS expression (Figure 27b). These observations confirm that *cis*-mutants are unlikely to have hidden *trans*-acting effects beside the described reactivation of the resistance gene upon the structural rearrangements within the epiallele.

In summary, the previously silent epiallele in the *cis*-mutants was reactivated due to a wide range of structural rearrangements, likely caused by T-DNA mutagenesis of the diploid silent progenitor (SS). There is a good correlation between resistance ratio, *HPT* expression level, and chromatin modifications, since strong *HPT* resistance is associated with higher *HPT* expression, loss of inactive chromatin features (H3K9me2) and acquisition of active chromatin marks (H3K4me3). Even though the DNA methylation change is not as pronounced as the switch in histone modification, one can see a significant decrease of DNA methylation at the transcription factor binding sites in the promoter region.

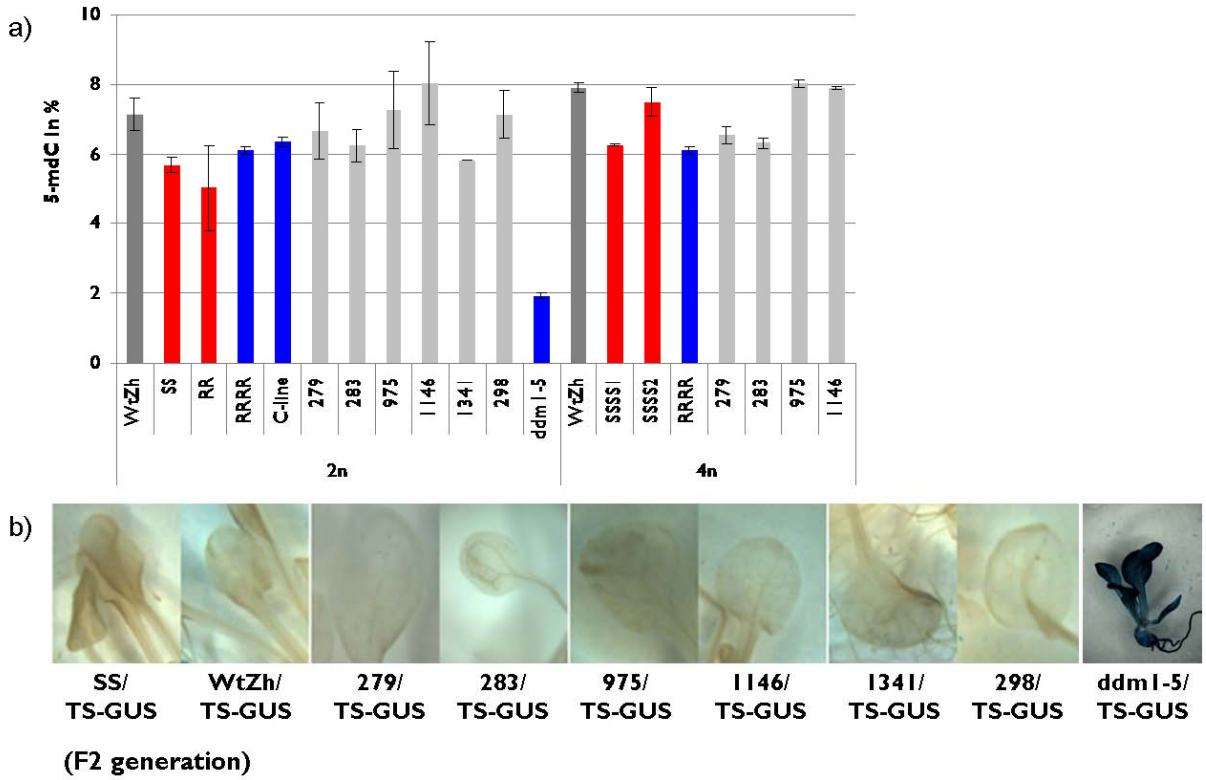


Figure 27. Assay for genome-wide effects of *cis*-mutants. a) Genome-wide cytosine methylation levels were measured by HPLC in diploid and tetraploid lines. A mutant Arabidopsis line (*ddm1-5*) with known effects on genome-wide methylation levels was used as a control. b) TS-GUS in the background of homozygous c*is*-mutants in F2 generation. As a control mutant *ddm1-5* showed reactivation of transcriptional silent GUS.

2.4.2. Tetraploid *cis*-mutants

The stable silencing of the inactive epiallele in some tetraploid lines and the epiallelic interaction between silent and active epialleles in tetraploids suggested a correlation between polyploidy and TGS. Since the structure of the epiallele 3' end had shown to be important for silencing, I wanted to elucidate the impact of epiallelic composition and structural effects on paTGS in a polyploid background and generated tetraploid derivatives from the diploid *cis*-mutants. Young seedlings of the diploid *cis*-mutants were treated with the microtubule-disassembling drug, colchicine, to generate tetraploid derivatives. The success of the treatment was monitored by flow cytometry of nuclei from the colchicine-treated progeny (Figure 28a) and chromosome counting (Figure 28b). Tetraploid progeny of four *cis*-mutants was propagated to the next generation and used for further experiments.

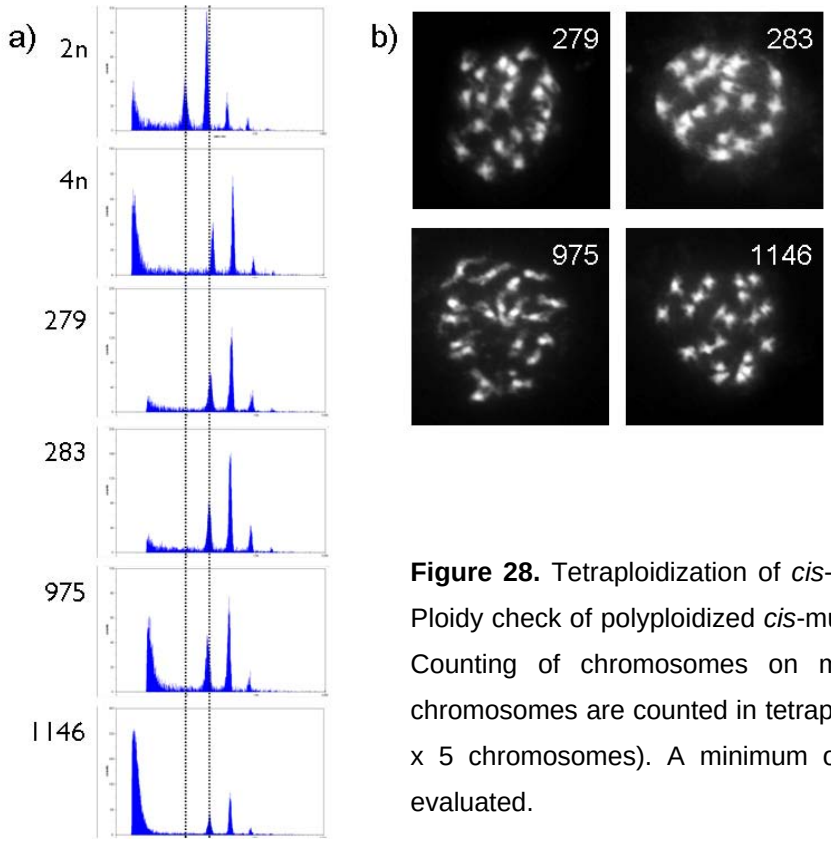


Figure 28. Tetraploidization of *cis*-mutants with colchicine. a) Ploidy check of polyploidized *cis*-mutants by flow cytometry. b) Counting of chromosomes on metaphase plates. Twenty chromosomes are counted in tetraploid *Arabidopsis thaliana* (4 x 5 chromosomes). A minimum of 10 nuclei per line were evaluated.

To determine whether the polyploidization affected resistance, I performed hygromycin selection of tetraploid *cis*-mutants. This revealed stable and complete maintenance of actively expressed *HPT* in two (283, 1146) out of four *cis*-mutants (Figure 29a). Two lines were less stable. Line 279 (with 35 % resistance) was generated from the diploid 279 S4 generation with 84 % hygromycin resistance, and the progenitor of 975 (90 % resistance) was a diploid S4 generation displaying 98 % resistance. In short, only lines which were 100 % resistant in their diploid progenitors maintained full resistance also in the tetraploid state. Comparing the resistance ratios to the *HPT* expression values (Figure 29b) one can see a strongly reduced *HPT* level in 279, whereas 283 *HPT* expression is even higher than in RRRR. 975 and 1146 display similar *HPT* levels, even though they have slightly different resistance levels. With the exception of 975, P2 transcript expression is not detectable as already shown in their diploid progenitors. Interestingly, all four tetraploid *cis*-mutants lost their enhancer function for *BME3*, as *BME3* transcript levels are pending around the wild type amount.

Methylation analysis of tetraploid *cis*-mutants by Southern blot (Figure 26c) produced a mixed pattern of digested and high molecular weight DNA, in a similar ratio as in the diploid progenitor. The methylation density seems to be similar in the two ploidy states, as the detected bands equal in size between diploid progenitor and tetraploid progeny of *cis*-mutants. The high level of DNA methylation in line 279 remains high also in the tetraploid, and there is no evidence for gain of new methylation in those lines that had lost it in the diploids. Thus, one can conclude that the DNA methylation was not affected after the change of ploidy.

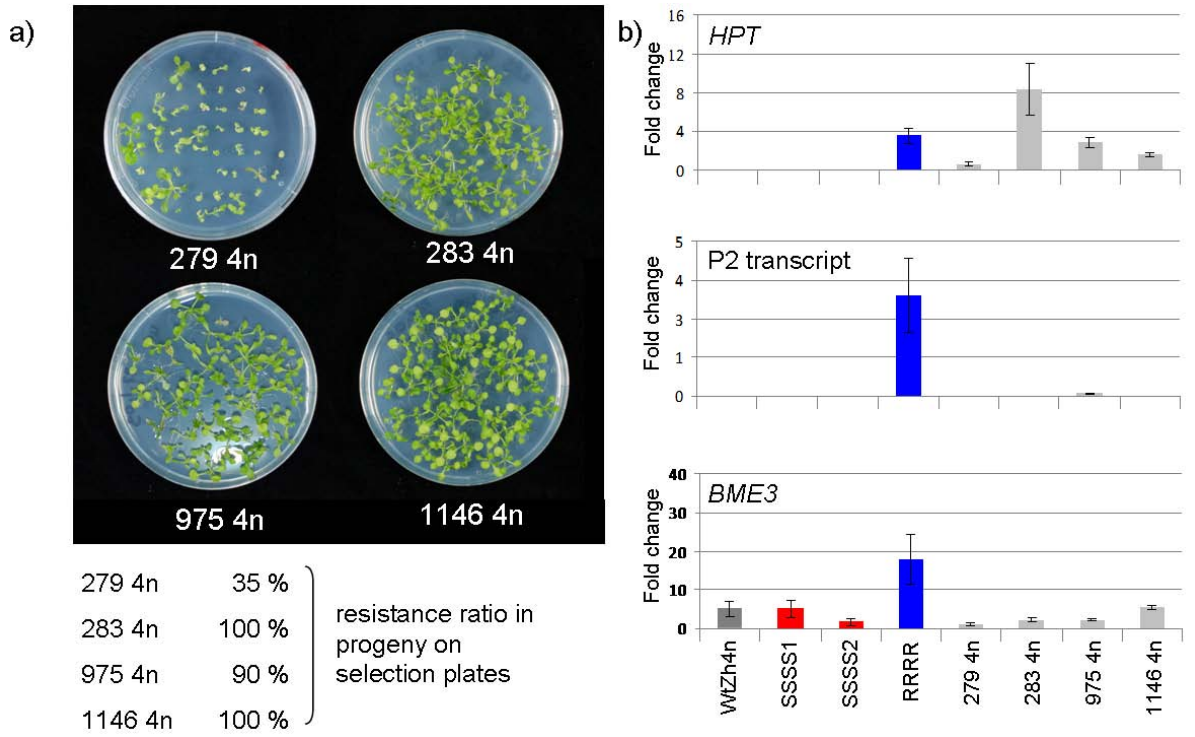


Figure 29. *Cis*-mutants after tetraploidization. a) Resistance ratio after hygromycin selection. b) *HPT*, *BME3*, and carrier expression levels of tetraploid lines (WtZh, SSSS1, SSSS2, RRRR, 279, 283, 975, 1146).

The observations presented for tetraploid *cis*-mutants confirmed the data shown for diploid *cis*-mutants. Reactivation of the silent epiallele due to structural rearrangements is maintained in tetraploid state, but only when the line has already shown full resistance in the diploid progenitor. *HPT* expression levels correlate with the hygromycin selection data, and associated DNA methylation levels scored via Southern blot connect the genetic data with epigenetic observations.

2.5. Impact of epiallele structure on transcripts

Since structural differences in *cis*-mutants reactivated the silent epiallele, I decided to investigate following questions. What is the link between genomic sequence, resulting transcripts, and the effects of epiallele structure which lead to reactivation? Why and how is transcription reestablished? Is a certain region of the epiallele connected to transcriptional regulation? To address these questions I first analyzed the polyadenylation of *HPT* transcripts. In addition, I screened for antisense transcripts and possible small RNAs generated from the epiallele. Additionally, I investigated the role of P35S-2 in regulating paTGS, as one part of the epiallele structure, namely the duplication of the 35S promoter, may play a role in maintaining transcriptional silencing.

2.5.1. Polyadenylation of *HPT* transcript

The expressing epiallele produced an abundant *HPT* transcript of about 3 kb, with additional smaller transcripts. To elucidate the molecular nature of those smaller transcripts, poly(A)-RNA was purified from total RNA. By northern blot analysis, total RNA and poly(A)-RNA were compared, and the result indicated that all *HPT* transcripts are polyadenylated (Figure 30a, d). Even the P2 transcript generated from the second promoter gives a strong band in the poly(A)-RNA-enriched fraction upon northern blot detection (Figure 30b, e). Due to the deletions spanning the 3’ region of the epiallele in 279, 283, 1146, and 298, no P2 transcript could be detected. U6 RNA, a non-polyadenylated RNA-Polymerase III-dependent RNA acted as a control for the purity of the poly(A)-RNA fraction.

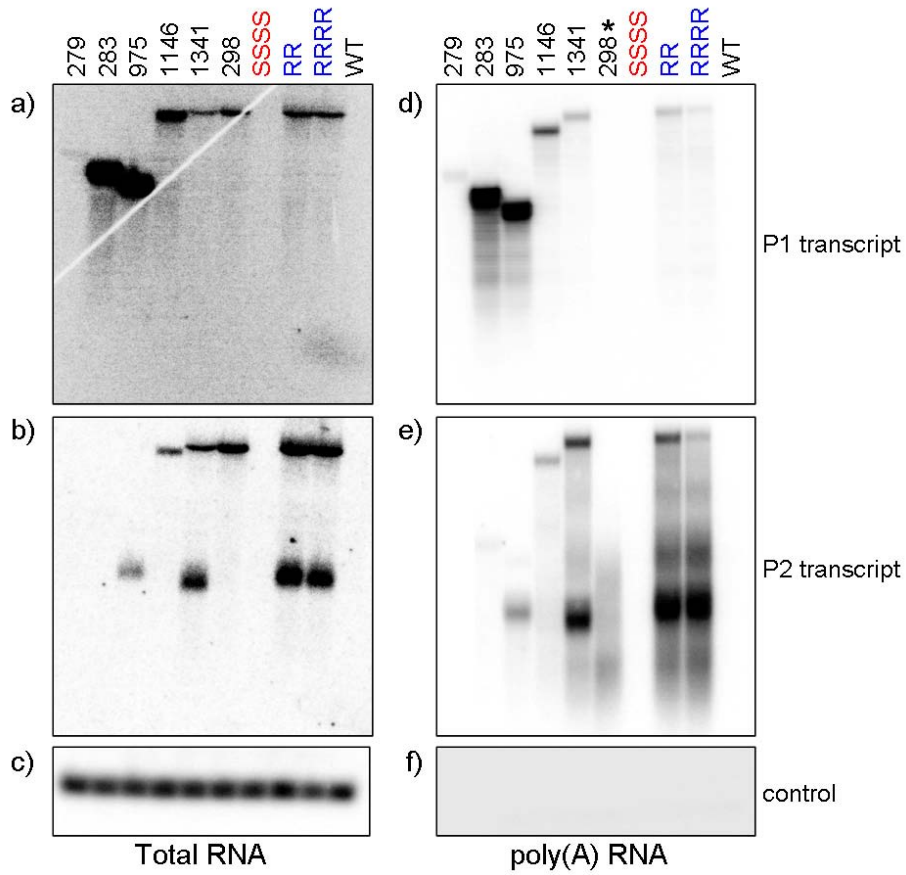


Figure 30. Northern blot analysis comparing total RNA to poly(A)-RNA in *cis*-mutants and active and silent epialleles. a, d) probe: P35S+*HPT*. b, e) probe: P2 transcript. c, f) probe for the non-polyadenylated U6 transcript, to exclude contamination with total RNA. WT = wild type Zürich 2n.
* Poly(A)-RNA from 298 was degraded, and therefore excluded from analysis.

2.5.2. "Carrier" has no promoter function

There was no or only little overlap between the individual deletions of *cis*-mutants, but the region affected in all cis-mutants was covering the so-called carrier sequence of non-plant/non-vector DNA inserted downstream of the second promoter. A Blast search indicated that it is very likely that the region transcribed by P2 was derived from calf thymus carrier DNA used during transformation (Karesch *et al.,* 1991). No function could be assigned to this sequence, but it was not excluded that the carrier sequence had some unknown function involved in maintaining silencing. Aberrant transcripts from cryptic promoters are able to cause transcriptional silencing (Eike *et al.,* 2005). Possibly the carrier had some hidden promoter function producing RNA which is complementary to the sense RNA transcribed from P35S-1 and P35S-2.

This would lead to the generation of double stranded RNA regions creating small interfering RNAs. To investigate a possible promoter function exhibited by the carrier, I cloned the full length carrier sequence in sense and anti-sense orientation in front of a GUS reporter in the pCBK04 vector. *Agrobacterium tumefaciens* strains containing these constructs were used to transform an Arabidopsis Col-0 cell suspension culture, followed by a screen for GUS expression. The positive control under regulation of the P35S promoter drove GUS expression and generated blue cells after GUS staining, whereas the carrier sequence showed no promoter function, neither in sense nor antisense orientation (Figure 31). These observations make a strong promoter function of the carrier sequence unlikely.

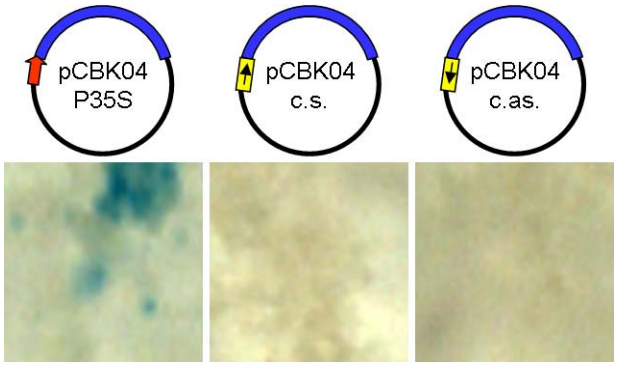


Figure 31. The carrier fragment has no promoter function. The carrier sequence was cloned in sense (c. s. = carrier sense) and anti-sense (c. as. = carrier anti-sense) orientation in front of a GUS reporter gene. The original pCBK04 vector under regulation of the P35S promoter provided a positive control. *Agrobacterium tumefaciens* was transformed with the appropriate vectors and cell suspension culture (Col-O) was transfected. GUS staining was performed on 1 ml of transformed cells.

2.5.3. No anti-sense transcript within the epiallele

Although the carrier did not show promoter activity, it is still possible that an anti-sense transcript is produced from a different region of the epiallele. To investigate this possibility, I screened for antisense transcripts within the epiallele. The complete epiallele sequence was divided in four pieces and cloned in an expression vector and strand-specific RNA was generated with either T7 or SP6 polymerase. These controls checked for strand-specific detection during northern blot hybridization, in parallel to total RNA from the plant lines. No specific antisense transcripts from the epiallele could be detected (Figure 32). The faint bands on the blots are also visible

in the slots with wild type RNA and resemble the ribosomal RNA pattern, indicating some unspecific detection. With this experiment, I could exclude the possibility of significant anti-sense RNA involved in regulation of paTGS.

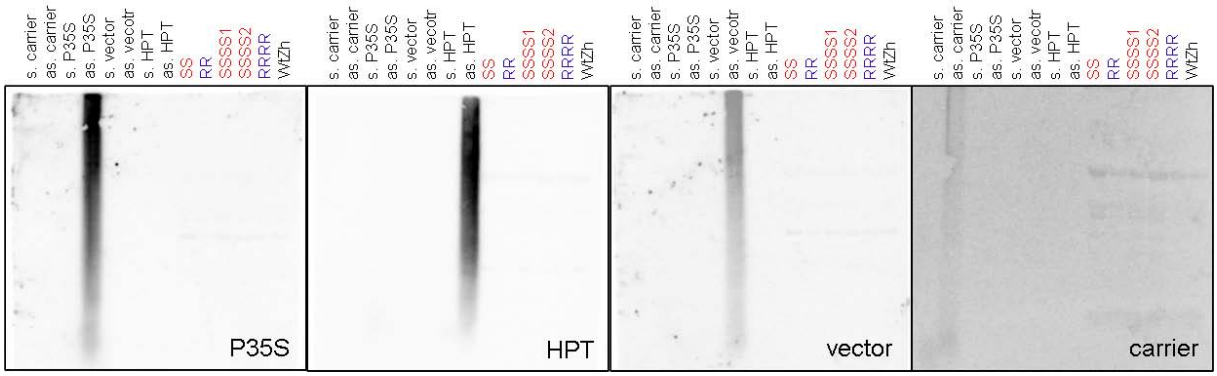


Figure 32. No anti-sense transcript within the epiallele. Northern blot analysis with labeled strand-specific oligonucleotides homologous to different regions of the epiallele (P35S: P35S-F, HPT: HM3F, vector: CF9, carrier: CF5). Sense and anti-sense RNA homologous to different parts of the epiallele and included in the blots as controls were generated by T7 or SP6 polymerase transcription of the respective sequences cloned in the pGEM-T easy vector (Promega).

2.5.4. No evidence for involvement of small RNAs in paTGS

A common phenomenon of silencing on the transcriptional level is the involvement of small RNAs and RdDM. To investigate a possible role of small RNAs in paTGS of epialleles, I performed several experiments. I hybridized a northern blot with small RNA enriched from the total RNA fraction. Hybridization included oligo-probes as well as ribo-probes for the different regions of interest of the epiallele. None of the applied probes gave evidence for the existence of specific small RNAs generated from active or sensitive lines (Figure 33). Additionally, the reverse mutant screen was extended to include mutants of the Pol IV and Pol V pathway. The silent epialleles were brought in the background of RNAi mutants and screened for resistance on hygromycin. No reactivation could be observed and *HPT* expression levels did not change in mutant background (Figure 15). Further, P2 transcript and *BME3* expression levels were not affected by RNAi mutants. These experiments do not give any evidence for a regulation of maintenance of paTGS of epialleles via the RNAi pathway.

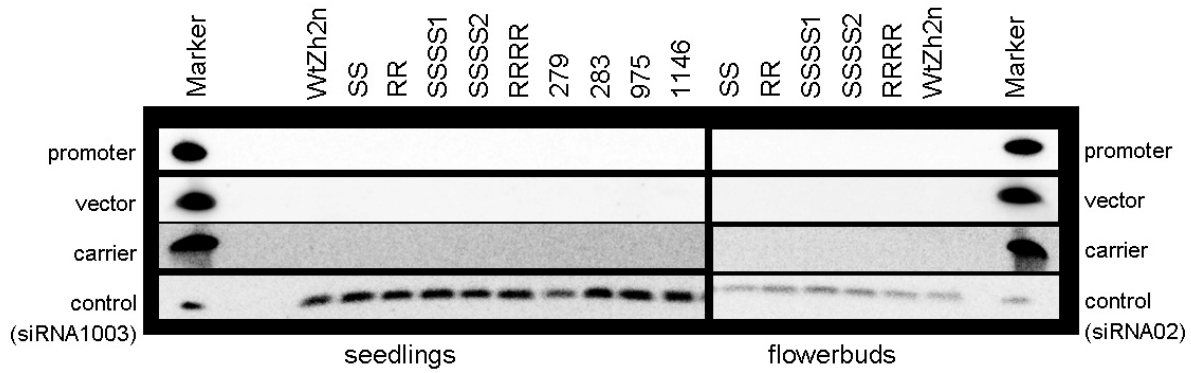


Figure 33. No evidence for an involvement of the RNAi pathway in maintenance of paTGS. Northern blot on small RNAs extracted from seedlings or flower buds from lines with active or inactive epialleles were hybridized with mixtures of 6 end-labeled oligos homologous to each of the regions. Wild type Zürich (WtZh2n) provided a negative control, antisense oligos as positive controls. Endogenous siRNAs (siRNA1003: seedlings, siRNA02: flower buds) were used as loading controls.

2.5.5. Role of the second promoter in regulating paTGS

Although the second copy of the 35S promoter was not deleted in some *cis*-mutants, it might still have played an important role in maintaining silencing. Interestingly, the second promoter copy was missing in mutants 279 and 283, but they differed in their expression patterns over generations. HPT in line 283 was stably expressed in all generations, while 279 exhibited re-silencing over generations. These differences led me to question the role of the second promoter in regulation of paTGS. The *cis*-mutant line 279 showed a hygromycin resistance ratio of up to 84 % in the S4 generation. However, this resistance ratio was not maintained in subsequent generations. S5 progeny have a severely decreased resistance rate, and S6 progeny from one selected progenitor was fully sensitive (Figure 34a). 279 was fully sensitive (0 % resistance) in the S6 generation, while 283 stayed fully resistant. With the complete re-silencing of 279, I asked how stable the newly established silent state was. Southern blot analysis with methylation-sensitive restriction enzymes (*Hpa*II) revealed a decrease of unmethylated cytosines, parallel to the loss of resistance (Figure 34b). Beside the changes in DNA methylation, the analysis of histone modifications gave interesting results: while levels of H3K9me2 in SS and 279 were equal, a strong difference was visible for the active H3K4me3 modification (Figure 34c). Early generations of 279 showed high levels of H3K4me3 at P35S-1 (P1) as

well as at the *HPT* gene, compared to SS, but this active modification was again lost in the re-silenced S5 generation. These data suggest an interesting connection between loss of expression with a decrease of active chromatin modifications. Together, these data support the idea of a double control mechanism of the epiallele via DNA methylation and histone modifications.

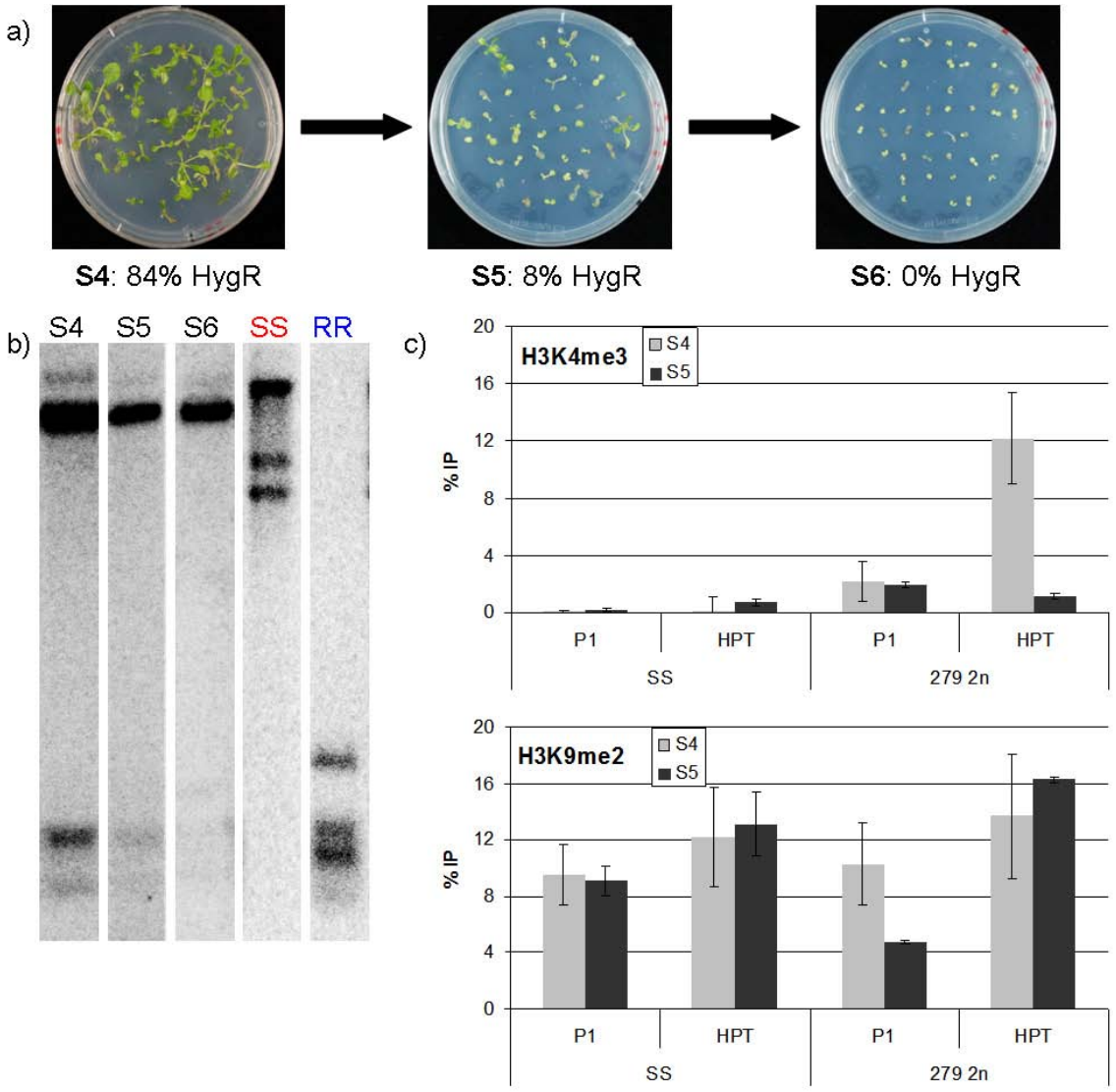


Figure 34. Silencing in the previously expressing *cis*-mutant 279 over generations. a) Comparison of hygromycin resistance between generations. b) Increase of DNA methylation over generations shown by Southern analysis after digest with methylation sensitive restriction enzyme *Hpa*II and probing with P35S+*HPT*. c) Chromatin immunoprecipitation of two different generations using antibodies specific for histone H3 lysine 4 trimethylation or H3 lysine 9 dimethylation. Quantitative PCR was performed in triplicates for SS and 279 for P35S-1 (P1) and *HPT*. Data are relative to input and error bars represent standard deviation of triplicate data.

The re-silencing process of 279 and its total sensitivity in S6 generation led me to examine whether it is possible to reactivate the silenced *HPT* gene in the absence of the second promoter. The re-silenced *cis*-mutant 279 in the late generation was treated with epigenetic drugs, releasing either DNA methylation via zebularine, or depleting DNA and histone methylation via SAHH-inhibition by DZNep (Figure 35a). Three weeks-old seedlings were pooled and tested for *HPT* expression levels after drug treatment, revealing that *HPT* expression could be more easily reactivated in the absence of the second promoter, as *HPT* levels were much higher in 279 compared to SS. *BME3* expression remained unchanged after drug application, suggesting independent regulation. There are two possible scenarios for *HPT* reactivation after drug application. Either the silent 279 (S6) becomes reactivated easier because it was already active in previous generations, or P35S-1 is easier to control in the absence of P35S-2. As the second promoter is unaffected in some of the other *cis*-mutants, and structural analyses of the epiallele did not reveal a special function of the duplicated region, it is more likely that the stronger reactivation after drug treatment of 279 (S6) compared to SS is due to differences in their epigenetic modifications. Such differences in epigenetic regulation might cause the divergent reactivation upon drug application.

In summary, the analysis of DNA and RNA of epialleles in the *cis*-mutants revealed that all epialleles produced a complex pool of polyadenylated *HPT* transcripts. The non-plant derived carrier sequence is not involved in transcription of aberrant transcripts, and no antisense RNAs within the epiallele could be detected. I could not find any evidence for an involvement of RNA in regulating maintenance of paTGS, failing to detect small RNAs or to reactivate the silent epiallele in RNAi mutants. Although low levels of small RNAs may have been below detection limit, or RNA silencing components may be redundant, it is likely that factors other than those maintain the stable silencing by directing chromatin modifications towards the epiallele. The duplicated promoter is deleted in some but not all *cis*-mutants, therefore it seems rather unlikely that the duplication itself is involved in transcriptional regulation. Interestingly, in the absence of the second promoter in 279 inactivation of *HPT* transcription could be observed combined with a decrease of active histone modifications. The other *cis*-mutants maintained their transcriptional active state correlated with active histone modification marks. Together, these

observations suggest a role for chromatin modifications in transcriptional regulation of epialleles.

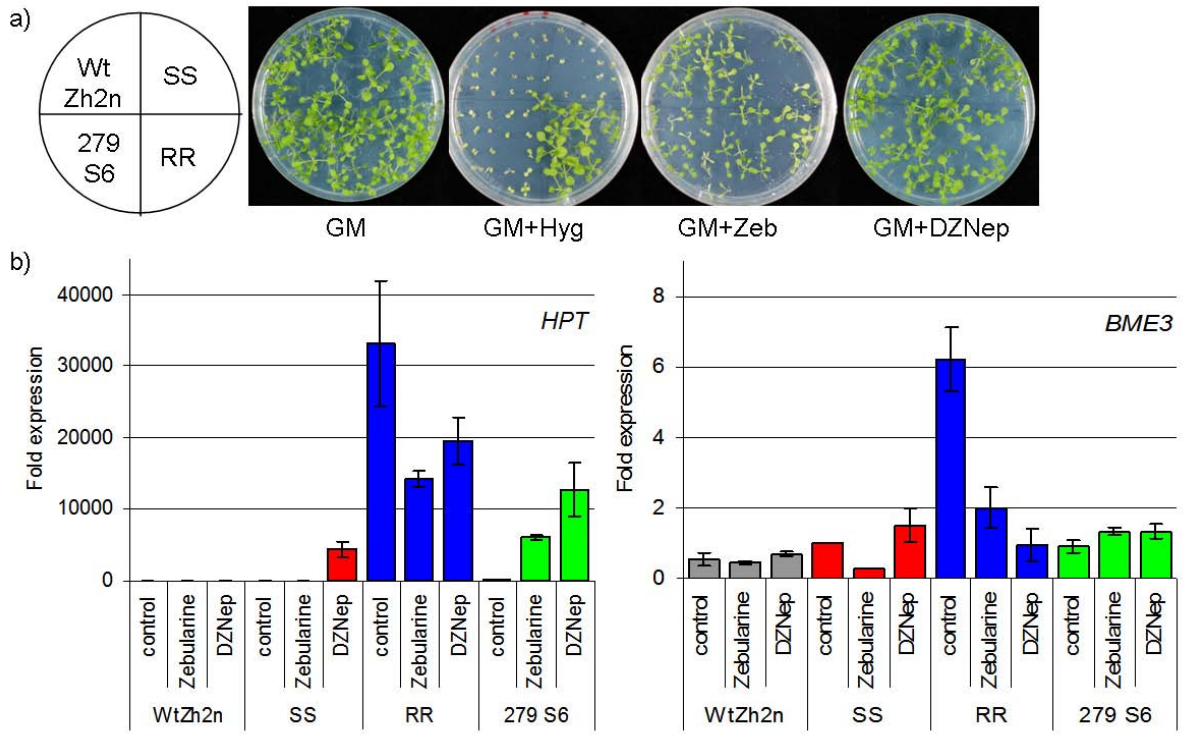


Figure 35. Expression levels in 279 compared to control lines after drug treatment. a) Seeds (S6 generation) from a diploid silent 279 plant were plated on GM (control), GM+Hyg (10 µg/ml hygromycin), GM+Zeb (40 µM Zebularine), or GM+DZNep (2 µM DZNep). Wild type Zürich 2n (Wt), SS and RR were used as controls. b) Gene expression analysis after drug application. All data are expressed as the average fold-expression over *eIF4a* relative to expression levels in SS control. Error bars represent standard deviation of triplicate data.

2.6. Paramutation-like behavior of epialleles

Epialleles used in this study showed a paramutation-like behavior in the F2 generation after crossing sensitive and resistant plants, leading to silencing of the previously active gene and therefore providing a suitable model to study paTGS establishment. There is evidence from other paramutation models (Alleman *et al.,* 2006; Sidorenko and Chandler 2008) that small RNAs can be involved in *trans*-silencing. To challenge the possible involvement of small RNAs, I screened for small RNAs with northern blotting of RNA prepared from tissue where the interaction is

assumed to occur (Figure 36). There is no evidence for small RNAs homologous to the tested regions (promoter, vector, carrier) of the epiallele, with the same limitation of interpretation as stated for the small RNA blots before.

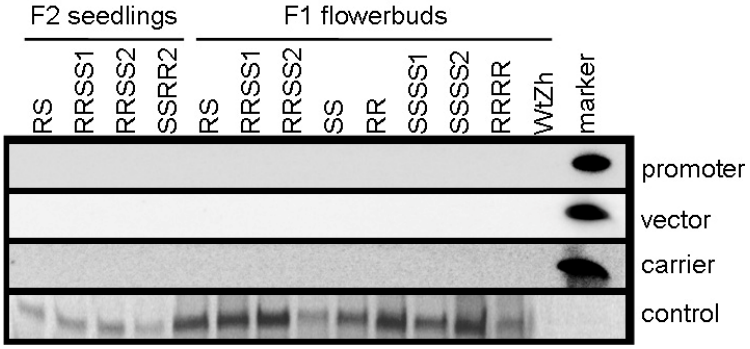


Figure 36. No evidence for an involvement of RNAi pathway in establishment of paTGS. Northern analysis on small RNAs extracted from flower buds or seedlings from lines with active or inactive versions of the epiallele and tetraploid crosses between epialleles. Wildtype Zürich (WtZh) was used as control. Endogenous siRNAs (siRNA1003, siRNA02) were used as loading controls.

2.6.1. Structural impact on paramutation-like behavior

While sequence identity between active and silent versions of the *HPT* attributed the different expression levels unambiguously to epigenetic features, it hampered following the fate of the epialleles upon crossings. Secondary paramutation, exerted from the paramutated to a fresh paramutable allele and an important feature of classical paramutation systems (reviewed in (Chandler and Stam 2004)), could not be proved, since the epialleles could not be distinguished any more in hybrid progeny (Mittelsten Scheid *et al.,* 2003). The rearranged active *HPT* genes of the *cis*-mutants, still allelic to the silent copy S, provided a genetic mark and offered an opportunity to address secondary paramutation.

The silencing effect in crosses between SSSS and RRRR observed in previous experiments was only visible after two generations, and only in tetraploid crosses (Mittelsten Scheid *et al.,* 2003). Therefore, I recapitulated the experiments with several tetraploid *cis*-mutants and crossed them with tetraploid lines (SSSS1, SSSS2, RRRR, and wild type Zürich). The impact of the structural rearrangements on the *trans*-silencing effect was revealed by screening the resistance ratios in the F2

generation of the crosses (Figure 37a). While silent lines and wild type were fully sensitive to hygromycin, also in crosses between them, the tetraploid resistant line showed 100 % resistance. According to Mendelian segregation for a dominant trait, one would expect only one out of 36 plants to be sensitive in tetraploid outcrossed F2 generations. This would correspond to 97.2 % resistance, which was indeed obtained in crosses between wild type and resistant lines RRRR, 283 and 1146, respectively (Figure 37). Exactly the same ratio would be expected in crosses between resistant (RRRR) and silent (SSSS) lines, assuming independent segregation of alleles from heterozygotes. As described before (Mittelsten Scheid *et al.,* 2003), the epialleles in these crosses did not behave as expected, as they showed a severely reduced resistance ratio in F2 generation (RRRR/SSSS1, RRRR/SSSS2) independent of the parental origin of the active and inactive alleles in the cross. By crossing tetraploid *cis*-mutants with tetraploid control lines (SSSS1, SSSS2, RRRR, wildtype Zürich 4n) different levels of resistance were obtained (Figure 37b-d). Line 279 4n which had only 35 % resistant progeny even in homozygous state had an even lower resistance ratio after crossing to the paramutagenic tetraploid silent lines, demonstrating that it is susceptible to the interaction. The cross 279 4n/RRRR resulted in Mendelian segregation of about 97.2 % resistance, indicating that the line 279 4n by itself is not paramutagenic. The cross with wildtype Zürich did not significantly change the low resistance ratio compared to 279 4n homozygotes, so that a dosage effect is unlikely to be responsible for the limited resistance. Line 283 4n, which showed full resistance to hygromycin was not paramutated by SSSS1 or SSSS2 as the results obtained after hygromycin selection were similar to those of crosses with wild type. When crossed to the fully resistant RRRR, the progenies were 100 % hygromycin-resistant. Line 975 4n which was initially only 90 % resistant to hygromycin showed further increased sensitivity in crosses with tetraploid silent lines. In contrast, the cross between 975 4n and wildtype Zürich 4n also led to reduced resistance, probably reflecting the weaker initial resistance level and possible dosage effects. Line 1146 4n which, like line 283 4n, was 100 % resistant to hygromycin and behaved like the RRRR line in the controls, showed when crossed with SSSS1 or SSSS2 reduced resistance.

Altogether, these data suggest the following interesting conclusions:

(1) Large deletions spanning nearly the same region within the epiallele, as in 279 and 283, do not automatically lead to the same *trans*-silencing behavior.

(2) The reduced resistance of line 279 does not exert *trans*-silencing of RRRR.

(3) The *HPT* gene in the resistant line 1146 4n resembles the R epiallele in stability and its paramutable behavior, but differs in a small deletion from S and R, allowing one to follow the fate of the epialleles even in heterozygous state with R or S (see below).

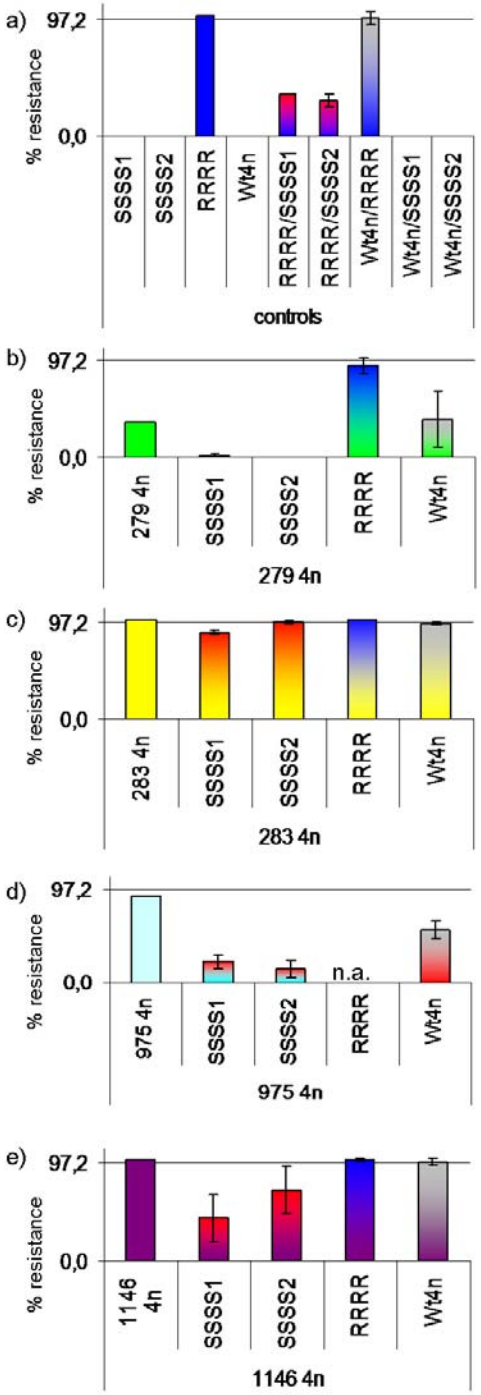


Figure 37. Structural impact on the paramutation-like behavior. a) Controls and control crosses. Tetraploid silent lines (SSSS) and wild type Zürich (Wt4n) are fully sensitive under hygromycin selection while RRRR is 100 % resistant. In crosses with Wt4n, line RRRR showed normal Mendelian segregation in F2 generation, leading to 97.2 % resistant plants, and no resistant plants were observed in crosses between tetraploid silent lines SSSS1 and SSSS2 and Wt4n. b-d) Tetraploid *cis*-mutants (279 4n, 283 4n, 975 4n, 1146 4n) are represented in homozygous tetraploid state and in crosses with SSSS1, SSSS2, RRRR, and Wt4n. n.a.: not analyzed.

To study the interaction of the *cis*-mutants with the S and R epialleles on the molecular level, I investigated the DNA methylation level in the hybrid F1 crossed generation by Southern blot with a probe hybridizing to the promoter and the *HPT* sequence of the epialleles (Figure 38). As shown before, there are 11 *Hpa*II cutting sites in this sequence which give rise to several small pieces in case of unmethylated DNA. This was perfectly visible in the fully active, unmethylated lines RR and RRRR, which resulted in a detection range between 250 and 750 bp. Silent lines appeared as strong high molecular weight bands corresponding to enzyme cutting sites only outside of the highly methylated epiallele. *Cis*-mutants showed variable patterns. While they still had high molecular weight bands corresponding to methylated cytosines, they revealed in addition a strong increase of smaller bands indicating the existence of unmethylated DNA. In crosses, these patterns could be used for "epigenotyping", as indicated in a color code matching size and origin of the bands in the schematic representation underneath the blot (Figure 38). All F1 patterns seem indistinguishable from the overlay of each parental pattern (considering some differences in running distance between different gels) and therefore simply additive, indicating no mutual influence in F1 hybrids.

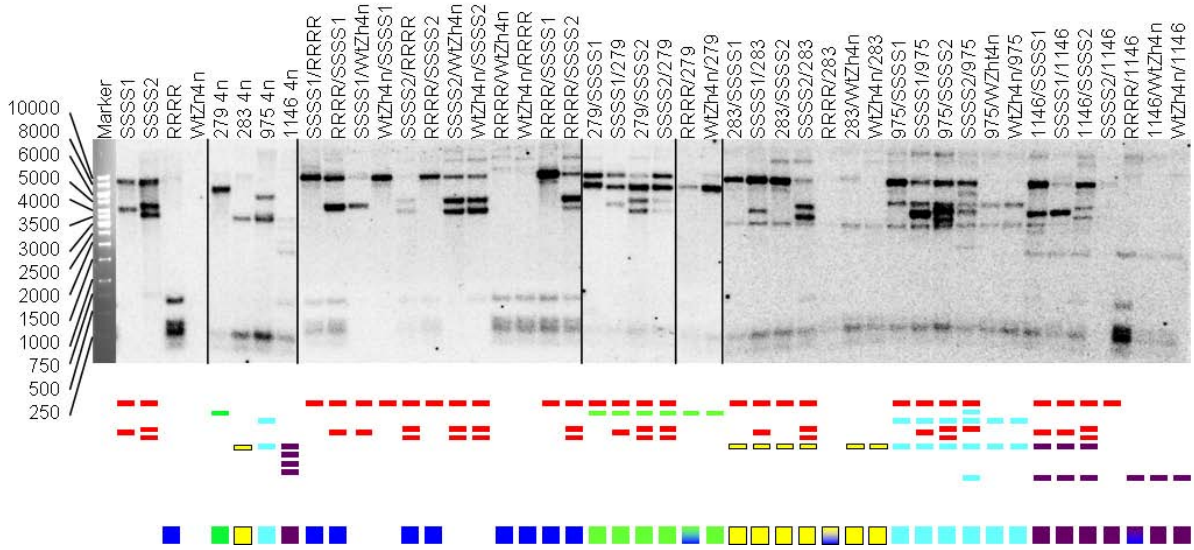


Figure 38. "Epigenotyping" of homozygous lines and heterozygous F1 generations. Southern blot analysis of *Hpa*II (methylation-sensitive restriction enzyme) digested genomic DNA of diploid and tetraploid silent and resistant lines, *cis*-mutants, and F1 generations after crossing silent and active lines with tetraploid *cis*-mutants. Active epialleles show low molecular weight band after methylation-sensitive restriction digest, while silent epialleles show high molecular weight bands. Wild type DNA does not hybridize with the probe. On the bottom of the figure a schematic representation of the detected bands is represented, indicating the origin of the band by a color code. Probe used for hybridization: P35S+*HPT*. For *Hpa*II cutting sites see also Figure 6.

2.6.2. Temperature effects on paramutation-like behavior

Previously it was shown that temperature influences the amount of *trans*-silencing in F2 generations from SSSS and RRRR crosses (Hödl 2007). While low temperatures (16°C) nearly abolished *trans*-silencing between epialleles, high temperatures (27°C) severely increase the *trans*-silencing effects compared to moderate temperature settings (21°C) (Hödl 2007). Searching for these effects in the *trans*-silencing mechanism including the *cis*-mutants, F1 plants from the previously described crosses were grown under two different temperature conditions, 21°C and 27°C. F2 generations were screened for resistance on hygromycin selection media. The resistance ratios are shown in Figure 39. Although a decreased resistance ratio in the progeny of most lines developed at 27°C, thereby confirming the previous findings, the cross 975 4n/SSSS 2 had an unexpected and tremendous increase of resistance. Therefore, *trans*-silencing seems to be affected by temperature changes in a non-consistent way. Since high temperature *per se* represents stressful conditions and can also lead to chromatin changes (Ales Pecinka, personal communication), these effects could be rather indirect and will be addressed in more detail in the discussion.

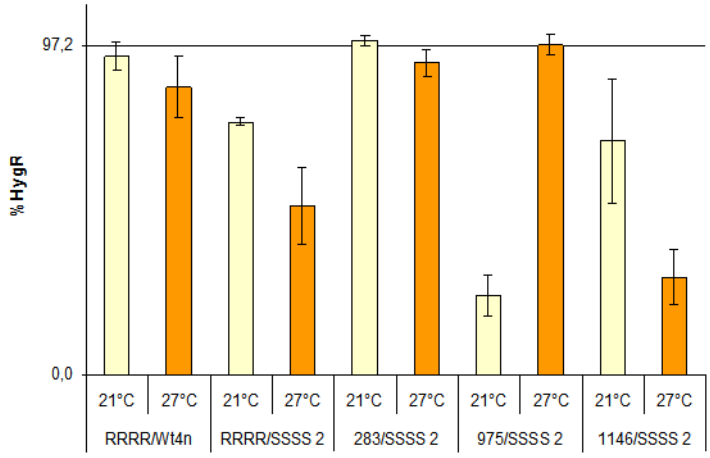


Figure 39. Temperature effects on paramutation-like behavior. Heterozygous tetraploid F1 plants were grown under long-day conditions (16 hours light) at two different temperatures, either at 21°C or 27°C. F2 progeny from three independent F1 plants was screened after 3 weeks for hygromycin resistance. Resistance ratios in percent (%HygR) are presented.

2.6.3. Follow up of paramutation-like behavior

The line 1146 4n, which is fully resistant to hygromycin and shows similar epigenetic configuration to RRRR, was susceptible to *trans*-silencing by SSSS. With the small deletion in the DNA sequence, it was possible to follow the fate of this allele even in the presence of S or R. This allowed me to ask whether an allele that underwent the epigenetic switch by *trans*-silencing could itself become a *trans*-silencer, a final proof for the acquisition of all epigenetic characters from S. Therefore, I identified plants homozygous for the deleted or non-deleted allele, respectively, from crosses of SSSS and 1146 4n. The tetraploid genotypes required an extensive genotyping experiment on 120 individual F2 plants, expecting only one homozygote for each line out of 36 plants. In parallel, I also used the control cross of wild type Zürich 4n (Wt4n) and 1146 4n. I obtained three homozygotes for 1146 4n, two homozygotes for SSSS or Wt4n, and six heterozygotes for each cross used as controls. Expression levels from the identified lines were measured by qRT-PCR for *HPT* and the neighboring *BME3* gene (Figure 40). The homozygous lines from the crosses were labeled as followed: 1146*s or 1146*w indicates 1146 homozygotes segregated from crosses with SSSS or Wt4n, respectively. Wt4n* or SSSS* indicates that this is a wild type or SSSS homozygous line segregated from crosses with 1146 4n. Strikingly, the previously active *HPT* expressing 1146 4n allele (see Figure 40a, *HPT* control) showed strong reduction in 1146*s, while no significant change (rather an increase) of *HPT* level was observed in 1146*w. Expression of *BME3*, the neighboring gene upstream of the epiallele, was correlated with activity of the *HPT* allele, leading to higher *BME3* expression levels in resistant lines. A small reduction of *BME3* expression in 1146*s, pointed to a slight *trans*-silencing effect also for *BME3* (Figure 40b).

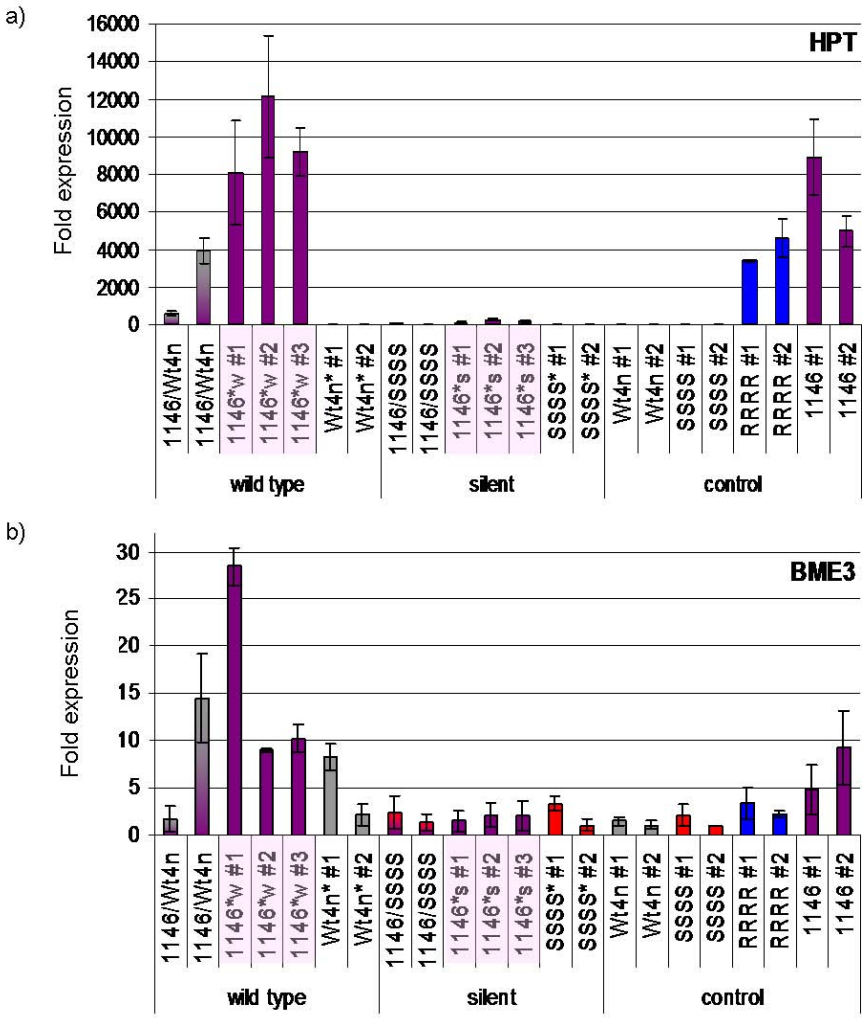


Figure 40. Expression levels of epialleles of homozygous F2 plants. a) Homozygous and heterozygous F2 plants from crosses between 1146 4n and wild type Zürich 4n (Wt4n), or 1146 4n and SSSS. *HPT* expression levels measured by qRT-PCR. b) Expression levels for *BME3* in homozygous or heterozygous F2 generation. All data are expressed as the average fold-expression over *eIF4a* relative to expression levels in SSSS #2. Error bars represent standard deviation of triplicates. F2 heterozygotes: 1146 4n/Wt4n, 1146 4n/SSSS; F2 homozygotes from 1146 4nxWt4n: Wt4n*, 1146*w; F2 homozygotes from 1146 4nxSSSS: SSSS*, 1146*s. Controls are self-propagated homozygous tetraploid lines.

The drastic epigenetic switch at the 1146 allele upon encounter with the S epiallele could also be documented on the molecular level by methylation-sensitive Southern blots. In F2 plants from crosses between 1146 4n and wild type Zürich 4n, hybridizing bands come solely from 1146 4n (Figure 41 left), as wild type does not have any epiallele. In F2 plants from crosses between 1146 4n and SSSS, both epialleles will be detected. No change of methylation in any of the epialleles of F2 plants can be detected after going through heterozygous state with wild type. This is different for F2

plants from the 1146 4n/SSSS cross (Figure 41 right). While in homozygous 1146 #1 no high molecular band was detected, a new band appeared in all three 1146*s (highlighted by a red arrow), in addition to some remaining low molecular bands. This new band represents methylated DNA which appeared after the epiallele of 1146 4n got in contact with the SSSS epialleles, but not in combination with wild type Zürich 4n (compare 1146*w 1-3). In F2 heterozygotes of 1146 4n/SSSS a slight decrease of low molecular weight bands is visible compared to 1146 #1, indicating a slight deviation from the expected additive pattern. These changes in DNA methylation in homozygous 1146*s, together with the clear evidence for secondary paramutation, are an interesting result that opens the way for a better understanding of paTGS and paramutation-like behavior of epialleles in Arabidopsis.

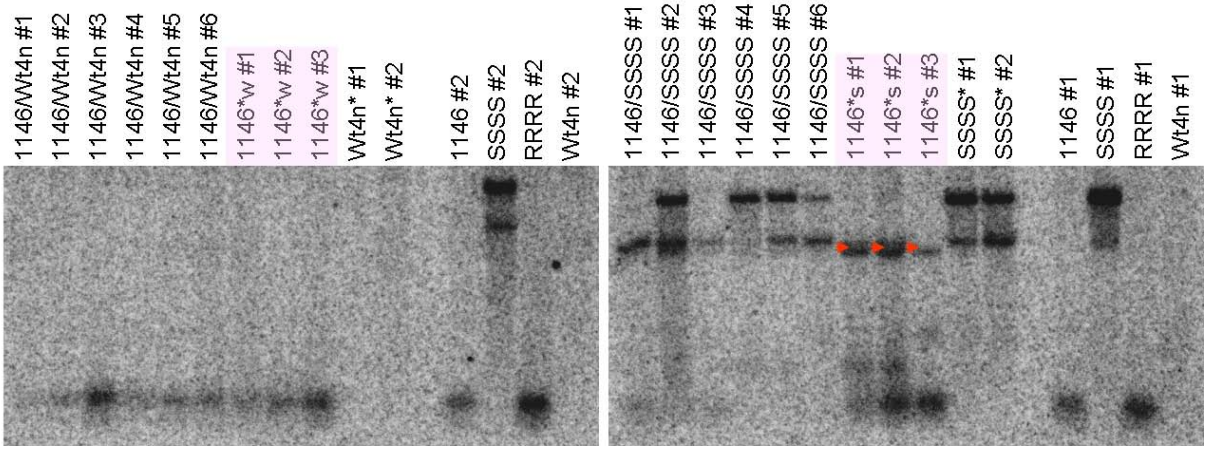


Figure 41. Methylation-sensitive Southern blot analysis of homozygous and heterozygous F2 plants from crosses between 1146 4n and wild type Zürich 4n (left), or 1146 4n and SSSS (right). Genomic DNA was digested with the methylation-sensitive restriction enzyme *Hpa*II. Hybridization was performed with *HPT* probe. Arrows indicate additional bands due to methylation upon trans-interaction of 1146 4n with SSSS.

3. Discussion

This thesis describes the analysis of maintenance and establishment of polyploidy-associated transcriptional gene silencing, and the resulting active and inactive epialleles in diploid and tetraploid Arabidopsis. I used the inactive, silent epiallele to study the impact of structural changes and epigenetic mutations on maintaining transcriptional silencing. This extends the work in the area of polyploidy-associated transcriptional gene silencing (Mittelsten Scheid *et al.,* 1996) by the first complete characterization of active and inactive epialleles in tetraploid, as well as diploid, Arabidopsis lines by structural and epigenetic means. Since the studied epialleles exhibit an unanticipated epigenetic interaction mechanism that resembles paramutation (Mittelsten Scheid *et al.,* 2003), the findings presented in this thesis have significant implications for future research in this field. The data and the plant material described, open the possibility to follow the fate of the paramutagenic and paramutable epialleles in Arabidopsis, comparing their epigenetic states which might give insight to the regulatory mechanism(s) behind paTGS establishment.

3.1. Epialleles involved in paTGS differ in their transcriptional activity and their epigenetic modifications

Allelic diversity causes variation, and epigenetic modifications of alleles with the same DNA sequence can provide a potential source of phenotypic and physiological variation between individuals or within a species. Naturally occurring epialleles are often difficult to exemplify as only a comparison at transcriptional level and of epigenetic data can lead to their elucidation (Rangwala *et al.,* 2006). Nevertheless, there are numerous examples of natural epialleles occurring in mammals (Morgan *et al.,* 1999; Blewitt *et al.,* 2006) and plants (Kalisz and Purugganan 2004). Known plant epialleles have functions in vegetative and seed pigmentation, pathogen resistance, plant development and morphology, as well as flowering time (Kalisz and Purugganan 2004; Shiba and Takayama 2007).

Effects of epiallelic position

In this study I focused on an epiallele with remarkable stability, whose activity is absolutely non-essential for the plant. The major advantage of the applied system is the unbiased propagation or segregation of either the active or inactive state within one Arabidopsis ecotype correlated with stable inheritance of the epialleles to subsequent generations. I performed a detailed analysis of the genetic (DNA sequence) as well as epigenetic (DNA methylation and histone modification) features of this epiallele in *Arabidopsis thaliana* ecotype Zürich. The epiallele is inserted in intergenic region (this thesis; Mittelsten Scheid *et al.*, 2003) and is, therefore, unlikely to cause an insertional mutation or to be by itself affected by local chromatin changes, as described for position effect variegation in *Drosophila melanogaster* where a heterochromatic neighborhood causes gene silencing (Schotta *et al.,* 2003). While it is rather unlikely that the epiallele is affected by its location in the genome, I could observe different effects of the active or inactive epiallele on a gene (*BME3*) located only 1 kb upstream of the insertion. *BME3* encodes for a GATA zinc finger transcription factor and is a positive regulator of Arabidopsis seed germination (Liu *et al.,* 2005). In wild type Arabidopsis, this gene is relatively lowly expressed during the whole plant life cycle (www.genevestigator.com). In lines harboring the inactive epiallele, the expression pattern is unchanged compared to wild type. In active lines *BME3* is upregulated. This may be due to the enhancer activity of the 35S promoter which alters the level and pattern of activity of adjacent genes (Benfey *et al.,* 1989; Zheng *et al.,* 2007).

The CaMV 35S promoter

The CaMV 35S promoter is one of the best studied promoters, and widely applied in driving transgenes in plants. By origin, the promoter regulates transcription of the cauliflower mosaic virus DNA (Guilley *et al.,* 1982), and the promoter confers strong constitutive expression in plants (Benfey *et al.,* 1990). The promoter sequence contains enhancer elements and well defined transcription factor binding sites (Odell *et al.,* 1985; Benfey *et al.,* 1989; Fang *et al.,* 1989; Lam *et al.,* 1989; Lam and Chua 1989; Pauli *et al.,* 2004). Many silencing phenomena in association with the CaMV

35S promoter and in combination with the presence of duplicated sequences and DNA methylation were reported (Mittelsten Scheid *et al.,* 1991; Vaucheret 1993; Vaucheret 1994; Park *et al.,* 1996; Mishiba *et al.,* 2005; Daxinger *et al.,* 2008). The transcription factor recognizing a duplicated binding motif (TGACG) in the 35S promoter is named activation sequence factor 1 (ASF-1) (Lam *et al.,* 1989; Krawczyk *et al.,* 2002). It turned out that lack of cytosine methylation at TGACG is crucial for promoter activity (Inamdar *et al.,* 1991).

Epigenetic modifications at the epialleles

Preliminary evidence for differences in DNA methylation between the epialleles was observed (Mittelsten Scheid *et al.,* 2003), but a detailed analysis was missing. Therefore, I established an accurate epigenetic map of the epialleles, with a special focus on the promoter sequence. Applying bisulfite sequencing and chromatin immunoprecipitation, I investigated DNA cytosine methylation and histone methylation at the epiallele. The silent (S) epiallele showed high levels of cytosine methylation in every sequence context. CG methylation was the most prevalent, and with a close-up view via bisulfite sequencing the transcription factor binding sites turned out to be highly methylated in diploid and tetraploid plants. In contrast, the active epiallele (R) was almost fully unmethylated at cytosines, and I did not detect any methylated cytosine at the transcription factor binding site. In addition, histone H3 dimethylation at lysine 9 together with H3 dimethylation at lysine 27 were predictive of silenced states at the epiallele (S), while expressed states (R) correlated with H3 methylation at lysine 4. The different epigenetic patterns and expression states were stably maintained independent of their ploidy level as similar results were obtained for diploid and tetraploid lines.

Stability of epiallelic expression states

Beside the stability of epiallelic expression states over generations and upon ploidy changes, the unchanged expression levels and stable epigenetic modifications of epialleles in UV-stressed seedlings and in callus cultures are another indication for

the permanence of their states. Genotoxic stresses, like ultraviolet radiation, have been shown to cause global gene expression changes, due to reduced genome stability upon stress treatment in Arabidopsis (Ries *et al.,* 2000; Molinier *et al.,* 2005). Yet the silent epiallele was not reactivated in UV-stressed seeds or seedlings, indicating that the mechanism of silencing regulation must be very firm. Recently, several independent publications described epigenetic changes in dedifferentiated plant cells in cell suspension or prolonged callus culture (Berdasco *et al.,* 2008; Tanurdzic *et al.,* 2008; Krizova *et al.,* 2009). Changes in DNA methylation occur frequently in cultured plant tissues and are suspected to be a major cause for somaclonal variation (Kaeppler *et al.,* 2000). Plants regenerated from cell culture can have highly variable DNA methylation patterns and provide additional evidence that DNA modifications are less stable in culture than in seed-grown plants (Kaeppler *et al.,* 2000). Epialleles are known to be induced in plant cell culture leading to meiotically heritable transmission of epigenetic changes in regenerated plants (Meins and Thomas 2003). Epigenomic consequences of long-term plant cell culture include DNA hypomethylation and loss of H3K9me2 of heterochromatic DNA. Specific transposable elements reveal drastic shifts in small RNA abundance and small RNA length due to cell culture (Tanurdzic *et al.,* 2008). Changes in DNA methylation patterns and levels observed in plant cell culture and hypermethylation in genic regions due to prolonged plant cell culture might be the cause for the emergence of epialleles in regenerated plants (Tanurdzic *et al.,* 2008). Other studies describe the impact of DNA methyltransferases and 5-methylcytosine glycosylases in the establishment and maintenance of undifferentiated states in plant cells. These studies show that the specific repression of genes is a result of promoter DNA hypermethylation caused by the undifferentiated cell state (Berdasco *et al.,* 2008; Zhang *et al.,* 2009). In contrast, the epialleles studied in this thesis were not affected by callus culture or genotoxic stress, indicating an extremely high degree of stability. It is still possible that epigenetic changes due to cell culture have played a role in the initial generation of the inactive and active epialleles as polyploids were generated via protoplast cell culture from a common diploid progenitor (Mittelsten Scheid *et al.,* 2003). A diploid progenitor line containing the identical transgene always maintained high expression, while partial or complete silencing was found in several independent autotetraploid derivatives. However, other parameters such as hormone effects, tissue culture conditions or even propagation of pre-existing epigenetic states in

individual cells due to protoplast culture could be possible sources of silencing. Nevertheless, an association with polyploidy is very likely based on the potential for *trans*-acting silencing between inactive and active epialleles which is limited to tetraploid hybrids (Mittelsten Scheid *et al.,* 2003).

Polyploidy-associated transcriptional gene silencing

The observed association between polyploidy and gene silencing leads to another important issue addressed in this thesis. While the problem of TGS is widely studied, especially in the background of transgenic systems (van Blokland *et al.,* 1997; Meyer 2000; Huettel *et al.,* 2007; Matzke *et al.,* 2009), only little is known about polyploidy-associated transcriptional gene silencing (Mittelsten Scheid *et al.,* 1996; Mittelsten Scheid *et al.,* 2003). Polyploidisation is considered an important source of genetic as well as epigenetic changes (Osborn *et al.,* 2003; Adams and Wendel 2005a). Compared to freshly formed allopolyploids, gene expression changes in autotetraploids are less frequent (Wang *et al.,* 2004), but studies in autopolyploids allow one to uncouple the effects of ploidy changes from the effects caused by the combination of two genomes. There is strong evidence of ploidy effects on TGS (Mittelsten Scheid *et al.,* 1996; Mittelsten Scheid *et al.,* 2003), while another recent study shows no effect of ploidy level on transgene-induced PTGS in autopolyploids (Pignatta *et al.,* 2008). Here one has to consider (Rocha *et al.,* 2005) that the silencing system in plants is usually not exposed to man-made transgenes, suggesting an independent development of silencing mechanisms, therefore making the HPT resistance gene well suited for epigenetic studies.

3.2. Only few known epigenetic factors are involved in maintaining paTGS at epialleles

It is widely accepted that epigenetic factors play major roles in regulating gene expression. A variety of TGS-deficient mutants is already known, and previous studies have highlighted the complexity and diversity of chromatin-based epigenetic silencing mechanisms. With regard to the stable maintenance of the differently

expressed epialleles and their opposing epigenetic modifications, we addressed the potential of known epigenetic factors for maintenance of silencing by introgressing the silent epiallele into lines with mutations in epigenetic regulator genes (this thesis; Milos 2006). We could already show in a previous study that *ddm1* (an ATP-dependent SWI/SNF chromatin remodeler) possesses the potential to overcome the silencing (Mittelsten Scheid *et al.,* 2003). None of the other applied mutations led to reactivation of the silent transgene and thus resistance to hygromycin (this thesis, Milos 2006). Beside *ddm1*-effects, only *met1* (a DNA methyltransferases) showed a slight increase of *HPT* transcript level, but too low to lead to hygromycin resistance (*HPT*, Figure 14). The importance of *MET1* in maintenance of silencing is known, as maintenance of methylation and TGS in the subsequent generations in the absence of an RNA trigger was *MET1*-dependent, whereas initiation of TGS was *MET1*-independent (Jones *et al.,* 2001). Hygromycin resistance requires a specific *HPT* level, with a threshold between resistance or sensitivity dependent on the antibiotic concentration (Mittelsten Scheid *et al.,* 1996). In *met1* background this threshold was not reached, rendering the plants sensitive. The analysis of the silent epiallele in the mutant background led to another interesting observation. Duplication within the epiallele allowed a comparison of two identical regulatory elements with a distance of only 2 kb, which revealed different responses to chromatin changes in the mutants. This is an interesting observation, as some known epigenetic mutations released silencing specifically at the P2 transcript, independent of the *HPT* resistance gene (Figure 14). It seems that the duplicated regulatory element in the same genomic location reacts differently to chromatin changes, indicating that regulation of the two 35S promoters is not fully coordinated. A possible reason may be the transcription through the downstream copy, or alternatively, the differences in the neighboring sequences or chromatin packaging.

One can imagine two possible explanations for the missing effects of known epigenetic mutations. It is possible that new, non-identified epigenetic factors may regulate the silencing. This was addressed in the lab with a forward mutant screen. The only, so far identified mutants were strong alleles of the previously known genes *DDM1* and *HOG1* (Baubec 2009). Another explanation would be a tight control of the epiallele via two or more overlapping silencing networks, so that one mutation alone is not sufficient to trigger reactivation, or only a combination of two pathways (e.g.

histone modification change and DNA demethylation) could lead to a reactivation of the silent epiallele, similar to the molecular changes of the epiallele observed in *ddm1*.

From the molecular point of view, one can imagine two scenarios for regulation of the silent epiallele: (1) The mutation of an epigenetic regulatory gene does not affect the *HPT* gene. This would indicate that the mutated epigenetic factor is not involved in *HPT* regulation. (2) Epigenetic changes occur without consequences on *HPT* activity, thus questioning a tight relationship between the modification and the gene activity state.

Detailed molecular analysis of the silent epiallele in *ddm1* background revealed DNA demethylation in all sequence contexts, while in *cmt3* only CHG methylation was decreased. A switch from heterochromatic to euchromatic signals within the epiallele was observed in *ddm1* only, whereas other epigenetic mutants (including *kyp1*) did not change the chromatin state of the silent epiallele. The loss of a single chromatin modification does not restore transcriptional activity from the strong 35S promoter. Only *ddm1* and *met1* revealed molecular effects together with *HPT* reactivation, suggesting that CG methylation and/or H3K9me2 are the major silencing regulators. Another indication for the extremely stable silenced epiallele is the limited number of mutations leading to reactivation. It appears that both modifications cooperate to control transcriptional suppression. Therefore, double mutants and lines treated with chemicals removing epigenetic marks were screened for *HPT* expression or resistance, but none of the mutant combinations between histone methyltransferases and/or DNA methyltransferases conferred resistance. The inhibition of DNA methylation via zebularine alone or in combination with epigenetic mutations did not lead to a significant reactivation. Only the application of DZNep (SAH-hydrolyse inhibitor, (Miranda *et al.,* 2009)) reactivated *HPT* expression. Defects for SAH hydrolase activity were also reported for *hog1* (Rocha *et al.,* 2005), which was also identified in the forward-directed mutant screen (Baubec 2009). In addition, another independent study described strong gene activating effects of SAH downregulation (Mull *et al.,* 2006). Together these results suggest that in the paTGS system an interplay between two modifications is needed to stably regulate *HPT* expression, as only removing both modifications converts the silent epiallele into an active one. This mechanism may have a wider impact also for stable silencing of transposons or

endogenous genes (Lippman *et al.,* 2004; Zilberman and Henikoff 2004; Rangwala and Richards 2007; Saze and Kakutani 2007).

3.3. Structural impact on expression levels and chromatin states of epialleles

There are two ways how maintenance of TGS in the silent epiallele could occur, either *trans*-factors regulate the silencing, or the epiallele is regulated by its own sequence in *cis*. Repeatedly, effects of the sequence composition itself on TGS were reported, for example the implication of DNA structure or the involvement of small RNAs from tandem repeats involved in TGS (Chan *et al.,* 2006; Henderson and Jacobsen 2007; Henderson and Jacobsen 2008). While the previous discussion dealt with the involvement of regulatory factors outside the reporter gene, now I discuss the impact of structure and sequence of the epiallele on maintaining the silent state.

3.3.1. *Cis*-mutants

A genetic analysis in tetraploids is rather complicated, therefore the mutant screen was performed in the diploid sensitive line (Baubec 2009). Out of 21 resistant plants, six reactivated due to a locus rearrangement releasing silencing and leading to an autonomous active behavior of the *HPT* transgene (*cis*-mutants). On the one hand, the existence of six lines showing rearrangements or deletions within the gene of interest together with the appearance of three independent mutations in *ddm1* are an indication of a fully saturated mutant screen. On the other hand, the identification of those *cis*-mutants allows analysis of the importance of structural details leading to the stable TGS observed in the silent epiallele. Reactivation was coupled to major and minor deletions and/or rearrangements within the epiallele. A detailed analysis of the structure in the reactivated *cis*-mutants revealed a deletion hot spot in the 3'-region of the epiallele harboring the direct repeat of vector backbone and promoter DNA. The bias to structural changes in the 3' region appears plausible since deletions or rearrangements affecting the first promoter or the *HPT* gene would not have allowed detection of hygromycin resistance. Interestingly, tandem repeats occur in nature in

many gene families (Stam *et al.,* 2002; Kinoshita *et al.,* 2007; Sekhon and Chopra 2009). TGS is often associated with the presence of homologous sequences in the genome. Therefore, it is tempting to suggest that reactivation in the *cis*-mutants could be explained by the loss of the duplicated region. However, some *cis*-mutants revealed only a small deletion, the smallest being just 65 bp, without any involvement of the tandemly organized duplication, thus making it unlikely that the repeated structure *per se* is causing the silencing.

In TGS, blockage of transcription initiation is thought to result from reduced accessibility of transcription factors to the promoter sequence due to heterochromatinization. Indeed, a reduction of heterochromatic marks could be observed in the *cis*-mutants, correlating with the increase in *HPT* transcription. It is possible to imagine that, rather than a specific sequence, the structural rearrangements change the overall organization at this locus, leading to a loss of heterochromatin. Similar structural changes of transgenes were previously reported to affect TGS (Morino *et al.,* 2004; Yang *et al.,* 2005). Frequently, T-DNA transformations were reported to induce chromosomal rearrangements which may lead to changes in transcriptional activity (Nacry *et al.,* 1998; Parinov and Sundaresan 2000; Muller *et al.,* 2007). The positioning of the rearrangements and deletions in *cis*-mutants may be explained by the need of microhomologies between the T-DNA and target sites (Muller *et al.,* 2007). The terminator sequences (T35S) in the residing epiallele and the T-DNA used for the insertional mutagenesis (Mengiste *et al.,* 1997)) were identical, thus fulfilling the need of homology during the primary contact between the T-DNA and the target DNA. This seems to be the case for two *cis*-mutants where the deletion occurs shortly after the terminator (in 283) or exactly at the terminator (in 975). In the other *cis*-mutants, sequences close to the second copy of the 35S promoter are affected, which may be explained by a recombination hotspot in the 35S promoter sequence (Kohli *et al.,* 1999). Kohli and colleagues showed that a 19 bp palindromic sequence, including the TATA box of the 35S promoter, acted as a recombination hotspot, making it specifically involved in genomic rearrangements. Regions close to the recombination hotspot were also affected in some *cis*-mutants (279, 1146, 1341, 298). Of special interest was the reduction of DNA methylation at the transcription factor binding site in the promoter as sequence changes in *cis*-mutants were accompanied by epigenetic changes. Line

975 showed that the transcribed region harboring the transcription factor binding sites is much less methylated than the non-transcribed region upstream of the ASF-1 binding site. A demethylation of the promoter, biased towards the ASF-1 transcription factor binding site and transcribed sequences downstream of TF binding, was observed in other TGS systems using 35S promoters (Linn *et al.,* 1990; Inamdar *et al.,* 1991; van Blokland *et al.,* 1997) and in *ddm1* and *hog1* mutant background (Baubec 2009). Together these data suggest that the erasure of DNA methylation is correlated with transcription leading to a sharp drop of DNA methylation close to the transcriptional initiation site.

The deletions in the *cis*-mutants varied in size, ranging from 65 bp to up to 4750 bp. A recent publication describes the importance of a single nucleotide for the activation of a transcriptionally silent gene (Shibuya *et al.,* 2009), therefore the importance of sequence composition changes and its effects on transcription need to be considered. Shibuya *et al.* showed that DNA methylation at a specific CG within the investigated region can induce up-regulation of a gene. They speculated that the specific DNA methylation at a negative *cis*-element (silencer) interferes with the binding of its cognate transcriptional repressor, leading to derepression of transcription. Analogous mechanisms were provided for DNA methylation within, or near sequences, of a positive *cis*-element (enhancer), where DNA methylation would interfere with the binding of a related transcription factor, which in turn causes TGS (Deng *et al.,* 2001). Although it is tempting to speculate that a similar mechanism (binding of a silencer) regulates the silent epiallele, this is rather unlikely as the active and inactive epialleles are isogenic, making it impossible to distinguish them on pure sequence levels. In addition, a detailed alignment between the *cis*-mutants did not reveal a direct link to such a regulatory mechanism, as deleted regions did not overlap in all *cis*-mutants (e.g. between 298 and 1146), excluding the possibility of a single “silencer” element.

3.3.2. The role of transcripts in the silencing process

Polyadenylation of HPT transcript

Detailed analysis showed that all transcripts derived from active epialleles, including the aberrant transcripts from *HPT* and the non-coding P2 transcript driven by the second promoter, are polyadenylated. Aberrant transcription in combination with polyadenylation was already observed in epialleles in a mammalian system, suggesting an involvement of downstream transcription units leading to alternative polyadenylation (Druker *et al.,* 2004). In the mammalian system, Druker *et al.* speculated that this resembles transcriptional interference, which is defined as a suppressive influence of an active transcriptional unit on another unit linked in *cis* (Eszterhas *et al.,* 2002). Suppression of downstream expression in tandem constructs is relieved when polyadenylation and a “pause” site at the terminator separate the genes (Greger *et al.,* 1998). Thus, the read-through transcription in the system described here could be one mechanism leading to transcriptional interference. Yet, other studies document interference with expression of the downstream gene even when the upstream gene has a robust polyadenylation site (Eszterhas *et al.,* 2002). Very recently, transcriptional interference as a mechanism responsible for gene silencing was described for the first time in a plant system. An enhanced 35S promoter from a T-DNA insertion caused a strong expression extending into the genomic neighborhood of the integration site, leading to suppression of the downstream endogenous Arabidopsis gene (Hedtke and Grimm 2009). If there are similarities with these other systems, they are likely based on mechanisms other than antisense transcript or promoter function of the non-plant DNA (carrier), since there is no evidence for their involvement above detection level.

Small RNAs

Aberrant transcripts are often involved in TGS, and in some cases they produce dsRNA and lead to the activation of RNAi, followed by RdDM and TGS (Mette *et al.,* 1999; Mette *et al.,* 2000). The presence of homologous sequences led me to speculate whether the TGS is based on RNAi-mechanisms. Repetitive sequences

can be a source of endogenous siRNAs and are preferred targets for RdDM. Small RNA species can direct changes in the chromatin structure to DNA regions with which they share sequence identity, mediating TGS if they have homology to the promoter sequence (Matzke and Matzke 2004). Small RNAs in plants lead to methylation mainly of cytosines in non-CG context, providing a primary mark for the formation of transcriptionally silent heterochromatin (Mathieu and Bender 2004). Plant-specific RdRPs (Pol IV and Pol V) play a role in siRNA-directed DNA methylation and gene silencing. Pol IV is required for siRNA production (Pikaard *et al.,* 2008) and Pol V for generating non-coding transcripts at (silent) target loci (Wierzbicki *et al.,* 2008). The epiallelic sequence was scanned for homology with the endogenous siRNAs reported by ((Lu *et al.,* 2005), http://mpss.udel.edu/at) and by ((Gustafson *et al.,* 2005), http://asrp.cgrb.oregonstate.edu/) but no significant homology with any sequence in those databases could be found. Northern blot analysis did not reveal the existence of small RNAs related to the epiallelic sequence but it could still be that small RNAs are produced only at a special time point in plant development or at levels below northern blot detection limit. Additionally, introgression of the silent epiallele into RNAi mutant background did not restore hygromycin resistance. Furthermore, crossing the stably silenced epiallele with other 35S promoter-driven transgenes did not show any *trans*-silencing effect of the silent epiallele on marker genes (Pecinka A. and Mittelsten Scheid O., unpublished results). Although RNA gene silencing signals are graft-transmissible, grafting experiments with silent and active epialleles did not give any indication for a role of a similar mobile signal in epiallelic TGS (Hödl 2007). All these independent attempts did not provide evidence that small RNAs are involved in maintaining the transcriptionally silent state. It has previously been observed that TGS at a marker gene was inherited independently of RNA triggers (Jones *et al.,* 2001). The inactive *HPT* epiallele may not need RNAi for silencing maintenance, though an involvement in establishment of the silent epiallele still needs to be elucidated.

Cryptic promoter functions and anti-sense transcripts

Frequently, cryptic promoters drive aberrant transcripts causing transcriptional interference and thus silencing of related genes (Eszterhas *et al.,* 2002; Hedtke and

Grimm 2009). There are studies, where rearrangements were accompanied by loss of aberrant transcripts followed by reactivation of silent genes (Morino *et al.,* 2004). In my case, an initial level of aberrant transcripts, provoked by the truncated terminator and the observed read-through activity, could have induced a low level of DNA methylation via RNA-dependent RNA polymerase (RDRP)-dependent dsRNA production and RdDM, followed by recruitment of chromatin factors, and creating into a situation where 35S promoter is no longer active. A similar mechanism, involving read-through transcripts, for a system driven by a *nos* promoter was suggested, showing that cryptic promoters at the transgene insertion site transcribe the *nos* promoter sequence and therefore lead to differences in expression of transgenes (Eike *et al.,* 2005). Gene silencing in combination with a duplication was also observed in a 35S-driven transgenic system in rice (Yang *et al.,* 2005). The authors state that it is not the duplication *per se* but rather the organization of the transgene which leads to aberrant promoter transcripts. Another study showed evidence that tandem repeats may rather be a consequence than a cause of epigenetic control, as not all Arabidopsis ecotypes use tandem repeats for *FWA* silencing (Fujimoto *et al.,* 2008). In the case of mutant line 279, silencing happens even in the absence of the tandemly repeated promoter copies, as 279 is lacking the duplication, promoting the idea of repeat-independent silencing.

Nevertheless, other possibilities to regulate silencing remain. A recent study in *S. cerevisiae* shows that cryptic unstable transcripts synthesized by RNA polymerase II can lead to *trans*-silencing despite the absence of the entire RNAi pathway in this organism (Berretta *et al.,* 2008). This is not the only example where cryptic unstable transcripts derived from pervasive transcription regulate transcription. In human cells the accumulation of a new class of short, polyadenylated and highly unstable transcripts was reported, suggesting a potential role for non-coding RNA transcription in DNA methylation and hence in transcriptional repression (Preker *et al.,* 2008). Another mechanism through which cryptic transcription affects gene expression is by influencing the epigenetic state of chromatin. Two recent publications show the impact of non-coding transcription on repressive histone modifications at genes that need to be switched on or off rapidly on metabolic change, emphasizing the significance of this type of regulation in the response to changes in nutrient conditions (Houseley *et al.,* 2008; Pinskaya *et al.,* 2009). The process of transcription

per se also has a very important role in the modulation of gene expression, mainly by changing the state of chromatin (Berretta and Morillon 2009). However, the precise role of nascent transcription in targeting or regulating chromatin factors remains to be elucidated (Berretta and Morillon 2009). Additionally, one has to take into account that, once the silent state is established, another mechanism may be involved in regulation, thus it is important to distinguish establishment from maintenance of TGS.

3.3.3. Stability of transcript reactivation

Diploid reactivated *cis*-mutants gave some insight into the structural impact on silencing. To find out whether polyploidization *per se* can induce re-silencing, *cis*-mutants were polyploidized. Three out of four tetraploid *cis*-mutants stably maintained the active state acquired in the diploid progenitor. Only one line (279) progressively silenced over subsequent diploid generations and upon polyploidization. Similar silencing over generations was already previously observed in other diploid systems (Kilby *et al.,* 1992; Fischer *et al.,* 2008). In these cases, the silencing increased progressively in later generations, but a detailed epigenetic analysis of the progressive silencing is still missing. The fourth, selfed generation of the *cis*-mutant 279 (S4) showed an increase in active histone modification while it still maintained a relatively high level of H3K9 dimethylation, a mark for inactive chromatin. I could show a correlation between a decrease of hygromycin resistance and decrease of the active chromatin marks over generations (Figure 34). This indicates that the re-silencing in 279 was not due to polyploidization but rather to the co-existence of heterochromatic and euchromatic marks. It is possible that the presence of such bivalent, co-existing modifications are prone to induce silencing, making other silent signals join the cluster and leading to progressive silencing. Fischer and colleagues (2008) suggested a similar hypothesis, stating that repetitive sequences are themselves subjected to silencing mechanisms and might be particularly associated with proteins involved in gene inactivation, such as DNA methyltransferases, histone deacetylases or chromatin remodeling factors. Such an increased local concentration might allow further implementation of transcriptional inactivation and DNA methylation in reaction to sequence-specific RNA signals provided by the silencer transgene. Alternatively, they suggest that activators might

be attracted by expressed genes. In this view, transcriptional activity of each promoter would be the net sum of antagonistic processes that can be shifted in both directions depending on kinetics and stochiometry.

3.3.4. Activation and DNA repair

Reactivation in *cis*-mutants could not be connected to loss of a defined, single region within the epiallele, and beside deletions, a sequence rearrangement in 975 also released silencing. The sequence composition of 975 suggests that T-DNA mutagenesis caused a double strand break, which in the special case of 975 allowed likely followed by DSB repair by homologous recombination due to the duplication of the 35S promoter. The result was that in 975 the sequence upstream of promoter 1 is copied upstream of promoter 2. Therefore, a possible role of the DNA repair pathway in the *cis*-mutant activation process needs to be discussed. Complete or aborted integration events during T-DNA mutagenesis may lead to DSBs, and the duplication present in the epiallele might trigger a repair attempt via homologous recombination. Evidence for this comes from the rearranged sequence in 975 where the duplicated P35S may have led to the insertion of an upstream plant DNA sequence into the epiallele. While detailed studies of illegitimate recombination on the DNA level have been performed (Kohli *et al.,* 1999), little is known about epigenetic changes underlying DNA repair. Additional studies are needed to investigate whether a DSB can cause reactivation of silent epialleles. To do so, one could use an *in vivo* system, where a rare-cutting endonuclease induces targeted DSBs in inactive genes (Puchta *et al.,* 1993). Other studies have revealed several factors (e.g. FAS1 and FAS2, BRU1, RPA2) involved in replication, repair and TGS reactivation in *Arabidopsis*, suggesting a relation of repair and transcriptional control (Kaya *et al.,* 2001; Takeda *et al.,* 2004; Elmayan *et al.,* 2005; Kapoor *et al.,* 2005). Recent studies have shown that DNA repair, similar to transcription, is facilitated by histone tail modification and ATP-dependent chromatin remodeling (Pandita and Richardson 2009). Chromatin remodeling plays a key role in the regulation of gene expression, but is also important for other chromatin-based processes such as DNA repair (Osley and Shen 2006; Bao and Shen 2007). With regard to the reactivation effect of *ddm1* (a chromatin remodeler) on the silent epiallele, and in combination with the *cis*-mutants,

where deletions and possibly their repair process lead to reactivation, it is tempting to speculate about an impact of nucleosome dynamics in maintaining silencing. The questions of “how nucleosomes are organized during transcription” and “how nucleosomal organization is reestablished after repair of double strand breaks” are possibly connected, as both involve regulation via chromatin remodelers. It will be interesting to find out how DNA sequence and chromatin remodeling complexes influence nucleosome positioning and transcription.

3.4. Paramutation-like behavior of epialleles in tetraploids

The *HPT* epialleles in tetraploid plants (but not in diploids) interact *in trans* and lead to heritable gene silencing, persisting even after segregation from the inactive epiallele. This mechanism, resembling paramutation, leads to the establishment of paTGS on a previously active epiallele (Mittelsten Scheid *et al.,* 2003). Thus, the combination of the epialleles can be used to study effects connected with establishment of polyploidy-associated TGS.

The detailed analysis of epialleles in this thesis allows a thorough investigation of the mechanism behind the *trans*-silencing involved in the paramutation-like interaction. Our system is one of the few models where ploidy influences the occurrence of silencing. Ploidy-mediated paramutation was also reported at the tomato *sulf* locus, which maps close to heterochromatin and is enriched in repetitive sequences (Hagemann 1993). How ploidy influences paramutation is still unclear. One can speculate that expression levels and epigenetic states of genes are affected upon polyploidization, leading to a new balance between different chromosomes (Chen 2007). What makes a gene responsive to dosage effects in polyploids remains to be investigated. It might be that the repetitive nature of epialleles plays a role in dosage effects. Repeated sequences contribute to the formation of silenced chromatin, and repeats turned out to be important in other cases of paramutation (Stam *et al.,* 2002). To investigate the sequence requirements in our system, structurally rearranged tetraploid *cis*-mutants were crossed with the paramutagenic silent line (SSSS) and other control lines. I could show that the paramutation-like phenomenon between SSSS and the tetraploid *cis*-mutants was independent of the tandem repeat, as three

cis-mutants were paramutable. Only one line was resistant to the *trans*-silencing effect, suggesting that the sequence composition may play a role in paramutation, as previously reported for other paramutation phenomena (Stam *et al.,* 2002).

SSSS and RRRR are isogenic, thus the epialleles could not be distinguished on the genetic level. In contrast, the sequence differences of the *cis*-mutants allow genotyping of the epialleles, I could show that both epialleles are present in the hybrids, ruling out allelic exclusion which might have eliminated the active epiallele and kept only the silent version in the genome. Paramutation was shown to be associated with changes in DNA methylation and chromatin structure (Sidorenko and Peterson 2001; Stam *et al.,* 2002) but their exact role is not yet elucidated. I could demonstrate that epialleles involved in paramutation-like behavior carry different DNA methylation and histone modifications. Methylation analysis revealed that in the F1 generation, the epialleles are still in their parental epigenetic modification, indicating (in accordance with the hygromycin resistance data) that the *trans*-inactivation event happens later. Genotyping of F2 plants from crosses between the different lines (1146 4n/Wt4n, 1146 4n/SSSS) allowed the investigation of homozygotes and/or heterozygotes based on their genotype. A comparison of the epialleles reveals changes in DNA methylation patterns in F2 generation, which is also the point when the *trans*-silencing is apparent for the first time. I was able to confirm that the previously active *cis*-mutant (1146 4n) increases in DNA methylation after being in contact with the silent epiallele (SSSS). In the future, the data presented here will permit queries as to whether the previously active and paramutable allele acquires paramutagenic features (secondary paramutation).

Currently, there are three models which might explain paramutation: by RdRP, by physical interaction, or a combination of the two (Figure 42). It was recently reported that the RNAi pathway plays a major role in regulating paramutation in maize. Several groups have identified RNAi factors involved in maize gene silencing (Alleman *et al.,* 2006; Nobuta *et al.,* 2008; Sidorenko and Chandler 2008; Erhard *et al.,* 2009). Certainly, *MOP1* is a major determinant of establishing and maintenance of paramutation in maize, although the role of the small RNA there awaits further investigation, since small RNAs are also present in plant material expressing the gene (M. Arteaga-Vasquez and V.L. Chandler, personal communication; (Stam

2009)). Nevertheless, the Arabidopsis ortholog of MOP1 (the RDR2 homologue) did not reactivate the silent *HPT* epiallele, congruent with the lack of small RNAs. It would be possible that small RNAs may regulate *trans*-silencing only at a certain developmental time point, or that they are present at a level below detection limit. This problem could be addressed by future experiments in which establishment of silencing, in the absence of factors shown to be involved in paramutation in maize, would be investigated. Analysis of the small RNA pool via deep sequencing may also help to identify potential regulators of the *trans*-silencing. Paramutation is known to violate Mendel's law, describing that alleles segregate unchanged from each other during meiosis. Considering that in the Arabidopsis system a change in expression was only observed in F2 generation, the epialleles might have to go through meiosis together for the *trans*-silencing to occur. This suggests a role for physical pairing between alleles that is limited or enforced especially in the tetraploid state. In maize paramutation, a tissue-specific interaction mediated by sequence-specific transcription factors upregulated only in a certain tissue was suggested (Louwers *et al.,* 2009). Other support for the role of meiosis-based interaction comes from the increase of *trans*-silencing at higher temperatures, as previous studies have shown an interdependence of duration of meiosis, chromosome dynamics, ploidy level, and temperature (Bennett 1977). To address whether the *in trans* interactions play a role in the Arabidopsis system, FISH (fluorescence in situ hybridization) or 3C (chromosome conformation capture) techniques would be potential tools. However, temperature is also involved in control of siRNA genesis (Szittya *et al.,* 2003), and regulatory RNAs may be present only during meiosis in tetraploids. This would provide another not mutually exclusive explanation for the limitation of the observed effect to F2 generation.

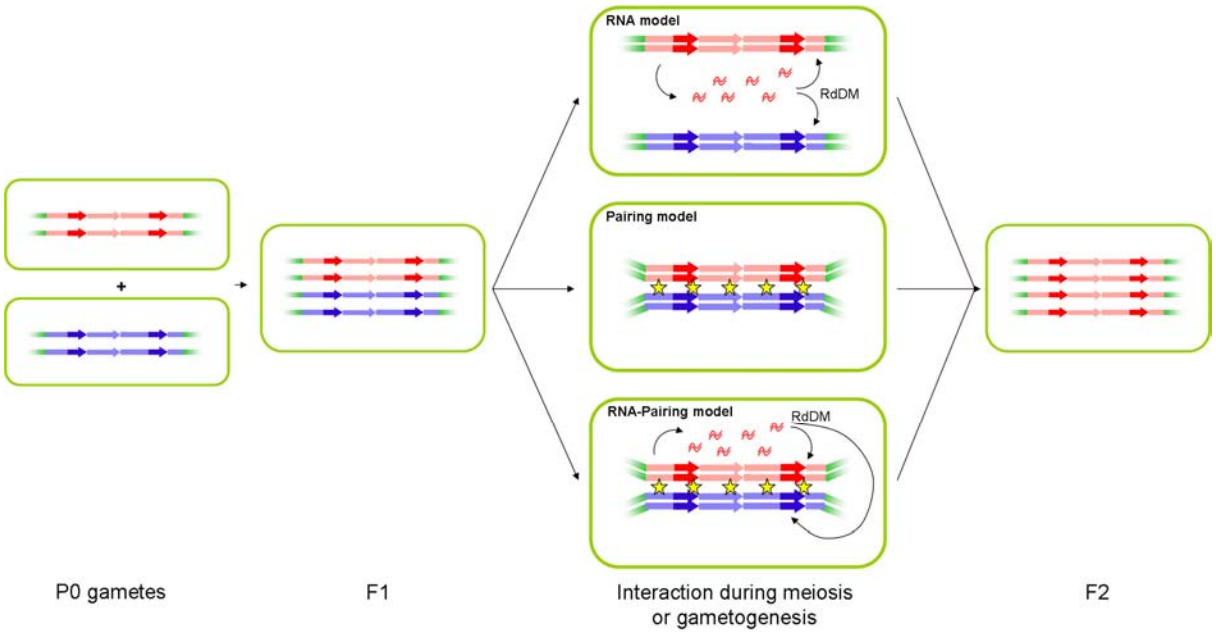


Figure 42. Paramutation models in tetraploids. Two gametes from homozygous parents form a new tetraploid F1 plant. As the *trans*-silencing effect is only apparent in F2 generation, interaction and exchange of silencing information takes place between F1 and F2 generation, most probably during meiosis or gametogenesis. Three interaction models are illustrated. *The RNA model*: small RNAs are produced from the tetraploid silent epiallele and *trans*-silencing happens by RNA-dependent DNA methylation (RdDM) and chromatin silencing. *The Pairing model*: the silent and active epialleles physically interact, leading to a transfer of epigenetic modifications from the silent to the active epiallele. *The RNA-Pairing model*: both RNA and physical interaction are required for the *trans*-inactivation, as only a combined action is leading to silencing.

3.5. Conclusion and perspectives

In this thesis I studied epialleles in diploid and tetraploid Arabidopsis, demonstrating that silencing at the inactive epiallele is stable over many generations and even upon dedifferentiation in callus culture. The expression states of the epialleles correlate with their epigenetic modification, where the inactive line reveals high levels of DNA methylation and H3 lysine 9 dimethylation, while the active line has high H3 lysine 4 trimethylation and lacks DNA methylation. Regulation of nucleosome density, histone and DNA methylation seems to be crucial for silencing, as a mutation in *DDM1* (a chromatin remodeler) led to reactivation of the silent epiallele. Genomic rearrangements that reactivated the silent *HPT* gene suggest a structural role in regulating silencing and suggest an involvement of the repair process in epigenetic

regulation. *Trans*-silencing of the active epiallele by the inactive homolog resembles paramutation. This paramutation-like interaction is limited to tetraploid epialleles, indicating the importance of polyploidy in gene regulation.

Polyploidy is known to have a broad impact on adaptation and evolution, based on modified gene expression, genomic rearrangements and epigenetic effects. The Arabidopsis system described here is suitable for a genetic approach to address different questions since epigenetic changes are easy to investigate. It is expected that endogenous sequences underlie a similar regulation, as there are numerous examples of epigenetic modifications in polyploids. paTGS can apparently generate very tightly controlled epialleles with an extremely low frequency of reversion and with the potential to be propagated and even spread among plant populations. It should be considered to be an important source of epigenetic diversity with an evolutionary impact.

Endogenous targets regulated in a similar fashion can be identified by follow-up experiments including microarray-based comparison of gene expression and DNA methylation patterns between tetraploids lines and the mutant derivatives. The detailed knowledge of the *HPT* epialleles will assist in analyzing possible endogenous targets in the future. Additionally, the structurally different *cis*-mutants are a strong tool for further investigation of the paramutation-like interaction. In order to fully understand the mechanism behind the *trans*-silencing, further experiments are needed. Those should include deep sequencing of small RNAs (the most sensitive method to date to screen for their involvement). In addition, further experiments should be performed to elucidate the role of RNA-mediated silencing factors, meiosis and physical pairing in *trans*-silencing.

4. Material and methods

4.1. Plant material, growth conditions and resistance assay

In this study *Arabidopsis thaliana* plants in the background of ecotypes Zürich (Zh), Columbia (Col), Wassilewskija (Ws), and Landsberg erecta (Ler) were used. Line C, carrying a single copy *HPT* transgene (Mittelsten Scheid *et al.,* 1996) is derived from ecotype Zh. The mutants *ago4-1* (Zilberman *et al.,* 2003) and *cmt3* (Lindroth *et al.,* 2001) are in Ler/Col and Ws background, respectively. The mutant *ddm1-5* is derived from ecotype Zürich (Mittelsten Scheid *et al.,* 1998; Jeddeloh *et al.,* 1999). The mutant *met1-3 (Saze et al., 2003)* and *nrpd1-7* (Smith *et al.,* 2007) are in Col background. The mutants *kyp1*, *mom1-2*, *suvH2*, *nrpd1-4*, *nrpe1*, and *nrpd2* are T-DNA insertion lines derived from the SALK collection in Col background (http://signal.salk.edu/cgi-bin/tdnaexpress) (Alonso *et al.,* 2003; Yamada *et al.,* 2003). Genotyping information can be found in Table 2.
Cold-treated seeds were grown on soil under long day conditions (16 h light, 8 h dark) or surface-sterilized (5 % sodium hypochlorite and 0.05 % Tween-80), washed and air-dried to be plated on solid germination medium (GM, MS agar base).
Hygromycin resistance was determined by growing a minimum of 50 seeds per line on GM containing hygromycin B (10 µg/ml) and evaluating resistance 3 to 4 weeks after germination, as the percentage of plants with green, expanded leaves and well developed roots.

4.2. Callus induction

Callus samples were obtained by cutting cotyledons from 2 week-old seedlings (C-line, Zh) grown on standard GM plates. Explants were transferred onto GM callus-inducing medium, consisting of GM base supplemented with auxin (α-naphtalene acetic acid, NAA, 2 µg/ml and 2,4-dichlorophenoxyacetic acid, 2,4-D, 0.05 µg/ml), cytokinin (6-benzyl aminopurin, BAP, 0.15 µg/ml and kinetin, 0.15 µg/ml), 3 % (w/v) sucrose, and 4 % (w/v) glucose. Four weeks later, callus samples were transferred to

new plates, propagated every two weeks and collected at different time points for analysis.

4.3. Cell suspension culture

Cell culture was initiated from about 500 sterile seeds (Col) germinated in liquid MS medium supplemented with 3 % (w/v) sucrose, 2 x Gamborg B5 vitamins (Duchefa), 0.5 µg/ml 2,4-dichloro-phenoxyacetic acid, 2 µg/ml 6-(γ,γ-methylallylamino)-purine riboside. Suspension culture was propagated every week by pipetting 5 ml of suspension culture into a new sterile flask containing 20 ml fresh medium. Cultivation was done at 25°C and shaking at 130 rpm in the dark.

4.4. Generation of polyploid plants and counting of ploidy

Tetraploid *A. thaliana cis*-mutants were generated as described previously (Henry *et al.,* 2005). In short, two week-old seedlings grown on GM plates were submerged for 2 h in 0.1 % (w/v) colchicine, washed extensively with water and transferred to soil. Seeds were harvested from individual plants, large seeds were cold-treated and propagated into the next generation.

Nuclear suspensions from leaf tissue of these plants was stained with DAPI (4',6-diamidino-2-phenylindole, 1 µg/ml) and nuclear DNA content was estimated by flow cytometry (Partec Flow Cytometer). In addition, chromosome counting on mitotic divisions was performed after spreading of flower buds as follows. Inflorescences were rinsed in sterile water and citric buffer (10 mM sodium citrate, pH 4.6), and incubated in 0.3 % (w/v) pectolyase, cellulose and cytohelicase in citric buffer at 37°C for 2-3 h. Individual flower buds were detached and macerated in 60% acetic acid on microscopic slides. The slides were placed on a hot plate (45°C), and the drop was gently stirred with a needle for 30-60 seconds. Subsequently, 200 µl of ethanol/acetic acid (3:1) were added and then the slide was dried with a hair drier and stored at 4°C until use. Visualization of DNA was done with DAPI (1 µg/ml (Vector Laboratories)), and chromosomes in mitotic division were analyzed using a Zeiss Axioplan 2 epifluorescence microscope. Images were acquired with MetaVue (Universal Imaging) and processed with Adobe Photoshop (Adobe).

4.5. Cloning techniques

Cloning of long PCR constructs (> 3 kb) was performed with the TOPO XL PCR cloning kit (Invitrogen, K4700-20) according to manufacturer's instructions, using TOP10 chemically competent E. coli cells for transformation. Other constructs including amplicons after bisulfite conversion (see below) were cloned into pGEM-T easy vector (Promega, A1360) according to manufacturer's instructions and transformed into DH5α chemically competent E. coli cells.
For transient expression assays to test promoter activity of the carrier (part of the *HPT* transgenic locus), the plasmid pCBK04 was used. It contains a GUS gene under control of promoter P35S and a kanamycin resistance gene. The 35S promoter was replaced with the carrier sequence via *BamHI* and *PstI* restriction sites. The construct was transformed into electrocompetent cells of *Agrobacterium tumefaciens* strain (AGL) containing rifampicin and carbenicillin resistance genes.

4.6. *Agrobacterium tumefaciens*-mediated transformation of cell suspension culture

Agrobacterium cultures were initiated by inoculation of 25 ml of LB medium supplemented with Rifampicin (40 µg/ml), Carbenicillin (100 µg/ml) and Kanamycin (50 µg/ml) to select for cells containing the plasmids. The cultures were grown to an OD_{600} of 0.9 to 1.0, collected by centrifugation and resuspended in $1/10^{th}$ of the original volume in cell suspension media. Before starting Agrobacterium infiltration, cell suspension was treated with 0.5 mM acetosyringon to facilitate the transformation procedure. Agrobacteria containing the respective plasmids and cell suspension were co-cultivated for 3 days at 25°C shaking in the dark at 130 rpm. After transformation, the cell suspension was washed several times with suspension medium to remove bacteria, followed by fixation and/or GUS staining.

4.7. GUS detection

In vitro GUS activity of cell suspension culture was performed with or without fixation (0.3 % formaldehyde, 0.3 M mannitol). Cell suspension was washed in 0.1 M sodium phosphate buffer pH 7.0, and GUS staining was performed over night at 37°C in the

dark in 0.1 M sodium phosphate buffer pH 7.0, 10 mM EDTA, 0.1 % Triton X-100, 100 µg/ml chloramphenicol, 2mM potassium ferrocyanide, 2 mM potassium ferricyanide and 0.5 mg/ml X-glucuronide. The reaction was stopped by washing with 0.1 M sodium phosphate buffer and cells were fixed with 70 % ethanol.

In situ GUS activity was detected after incubation in GUS staining solution including 15 min (seedlings) to 30 min (leaf tissue) vacuum infiltration and overnight staining at 37°C in the dark. Subsequent washes with 70 % ethanol at 37°C were performed in order to remove residual chlorophyll.

All samples were analyzed using a Leica MZ16FA binocular microscope with a Leica DFC300FX CCD camera. Images were acquired with Leica Application Suite and processed with Adobe Photoshop (Adobe).

4.8. Immuno-labeling detection

Nuclei were prepared from young leaves rinsed and vacuum infiltrated with 10 mM Tris buffer pH 7.5. Leaves were then chopped in 600 µl chromosome isolation (CI) buffer (15 mM Tris, 2 mM EDTA, 0.5 mM spermin, 80 mM KCl, 20 mM NaCl, 15 mM beta-mercaptoethanol, 0.1 % Triton X-100, pH 7.5) and filtered through a 32 µM nylon mesh. Eighty µl of nuclei suspension and 250 µl of CI buffer were transferred onto microscope slides using Cytospin (2500 rpm, 5 min). After centrifugation, slides were shortly rinsed in ice-cold 1x PBS, transferred into 50 % glycerol and stored at -20°C until use.

Immunodetection of histone modifications was performed as previously described (Jasencakova *et al.,* 2000). Slides were washed for 2 h in ice-cold 1x PBS and postfixed in 4 % paraformaldehyde/PBS for 20 min, followed by blocking (3 % BSA, 10 % horse serum, 1x PBS) for 30 min at 37°C in a humid chamber. The primary antibody (H3K9me2 from the lab of Thomas Jenuwein, IMP Vienna) was diluted 1:500 (1 % BSA, 10 % horse serum, 0.1 % Tween 80, 1x PBS), and slides were incubated at room temperature for 1 h. Secondary antibody (goat-anti-rabbit-AF488, 1:500) was applied for 1 h at 37°C and slides were dehydrated in an ethanol series. The slides were counterstained with DAPI (1 µg/ml) and analyzed using a Zeiss Axioplan 2 epifluorescence microscope. Images were acquired with MetaVue (Universal Imaging) and processed with Adobe Photoshop (Adobe).

4.9. Application of UV-C stress

UV-C irradiation (254 nm) was applied in doses of either 3600 J/m² or 6000 J/m² to germinating seeds on GM plates for a total of 3 days every 24 h using a UV crosslinker (Stratalinker 2400). Twelve day-old seedlings grown on GM plates were exposed twice to UV-C dosages equivalent to 2000 J/m² in an interval of 24 h.

4.10. Nucleic acid isolation and gel blot analysis

Genomic DNA was isolated from 3 week-old seedlings using either DNeasy Plant Mini Kit (Qiagen) or Phytopure (Amersham), following the manufacturers' protocols, except that genomic DNA was eluted in sterile water. Total RNA extraction from 3 week-old seedlings was performed with RNeasy Plant Mini Kit (Qiagen) including an on-column DNase I digest (Qiagen). Small RNA was isolated from either pooled inflorescences or seedlings (21 days old) using *mir*Vana miRNA Isolation Kit (Ambion). MicroPoly(A)Purist Kit (Ambion) was used to isolate poly(A) RNA from total RNA obtained with Trizol (Invitrogen) RNA isolation. All RNA kits were following the manufacturers' protocols, except that RNA was eluted in RNase-free water.
For Southern blot analysis, 10 µg of genomic DNA were digested overnight with 2 U restriction enzymes. For methylation-specific Southern blot analysis, the methylation-sensitive restriction enzymes (*Hpa*II, blocked by mCG, and *Msp*I, blocked by mCHG) were used. Digested samples were electrophoretically separated on 1.2 % TAE agarose gels, depurinated for 10 min in 250 mM HCl, denaturated for 30 min in denaturation solution containing 0.5 M NaOH and 1.5 M NaCl and neutralized twice in 0.5 M Tris, 1.5 M NaCl and 1 mM EDTA at pH7.2 for 15 min. For northern blot analysis of total and poly(A) RNA, 5 µg of RNA was denatured with 15 % glyoxal and DMSO for 1 h at 50°C and separated using 1.5 % agarose gels in 10 mM sodium phosphate buffer pH7 in a Sea2000 circular flow electrophoresis chamber (Elchrom Scientific). DNA and RNA gels were blotted onto Hybond N+ (Amersham) membranes over night with 20x SSC, washed and UV-crosslinked using a Stratalinker (Stratagene). Hybridization was performed as described by Church and Gilbert (1984). Radioactively labelled sequence-specific probes were synthesized from 25 ng of DNA using the Rediprime labeling kit (Amersham) and 50 µCi dCTP-α-

^{32}P (Amersham or Hartmann Analytic) and purified on G50 Probequant (Amersham) columns. Signals were detected with Phosphoimager Screens (Bio-Rad) and scanned with a Molecular Imager FX (Bio-Rad).

Small RNAs were denatured in 96 % deionized formamide and 20 mM EDTA at 95°C and were analysed by separation on 15 % polyacrylamide-7 M urea gel. Small RNA gels were electroblotted (Bio-Rad Semi Dry Electro Blotter) on Hybond N+ Membrane (Amersham) with 0.5 x TBE at 10 V for 1 h. Hybridization was performed in UltraHyb Oligo hybridization buffer (Ambion #8663) at low stringency at 42°C. Using T7 or SP6 RNA polymerase (Fermentas) and 50 µCi dCTP-α-^{32}P (Hartmann Analytic), I generated internally labelled *in vitro* transcripts in both antisense and sense orientation. The template DNA for riboprobe preparation was removed from the reaction by treatment with RNase-free DNase I (Fermentas). Oligo-probes (< 40nt, Metabion) were 5' end labelled with 50 µCi ATP-γ-^{32}P (Hartmann Analytic) with T4 polynucleotidekinase (10 U/µl, Roche). Washes were carried out twice in 2 x SSC, 0.5 % SDS at 42°C for 30 min.

4.11. Genotyping of *Arabidopsis* mutants

Young leaf tissue was ground to fine powder (Retsch machine) and vortexed in extraction buffer (0.2 M Tris-HCl pH7.5, 0.25 M NaCl, 25 mM EDTA, 0.5%SDS). After a short centrifugation the supernatant containing the DNA was precipitated with an equal volume of isopropanol. The pellet was washed in 70% ethanol and resuspended in sterile water. 1 µl extracted DNA was added to 20 µl of a standard PCR mix (5Prime).

4.12. Rapid amplification of cDNA 3' ends

3'-RACE was performed according to the instructions of SMART RACE cDNA Amplification Kit (Clontech). Total RNA (700 ng) was treated with DNaseI (Fermentas), then reverse-transcribed with RevertAidRT (Fermentas) with 3-RACE A primer (5–AAGCAGTGGTATCAACGCAGAGTAC(T)30V N–3) in a 20 µl reaction. Two µl of cDNA reaction was used as a template in 3'-RACE PCR. For RACE PCR reactions, Advantage 2 PCR Kit (Clontech) was used according to manufacturer's

instructions. A control primer (Actin, Act2F primer: 5-GCCATCCAAGCTGTTCTCTC-3) and gene-specific primers were used in combination with UniA_45 (5–CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT–3).

4.13. Reverse transcription and real time PCR

Two µg of total RNA were treated with DNase I (RNase free, Fermentas) for 30 min at 37°C in a total volume of 20 µl. The reaction was inactivated in the presence of EDTA and with an additional incubation at 65°C for 10 min. Half of the DNase I treated total RNA was used for reverse transcription. In a total volume of 12 µl RNase-free water, 1 µg RNA and 0.2 µg random hexamer primer (Fermentas) were incubated at 70°C for 5 min and chilled on ice. The following components (Fermentas) were added: 4 µl 5X reaction buffer, 1 µl RiboLock Ribonuclease inhibitor (20 U/µl) and 2 µl 10 mM dNTP mix. The whole set was incubated at 25°C for 5 min and 20 U RevertAid H M-MuLV reverse transcriptase (Fermentas) were added, with subsequent incubation at 25°C for 10 min and at 42°C for 1 h. The reaction was stopped by heating the reaction up to 70°C for 10 min and chilling on ice.

Real time PCR on cDNA was performed with 2x SensiMix Plus SYBR & Fluorescein Kit (Quantece) in a total volume of 15 µl according to the manufacturer's instructions. Quantification was done with an iQ5 Real-Time-PCR System (Bio-Rad). The obtained Ct values were analyzed with the iQ5 Optical System Software Version 2.0 (Bio-Rad), applying the mathematical model for relative quantification in Excel (Microsoft) described by (Pfaffl 2001).

4.14. Bisulfite sequencing

Two µg of RNase A-treated genomic DNA were used for bisulfite conversion. Bisulfite modification and desulfonation of genomic DNA were performed using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturers' instructions, with the following modification: the bisulfite conversion step was extended by an extra denaturation step and by additional 2 h incubation at 60°C. Prior to amplification of the sequence under investigation, the conversion was tested for completeness using distinct primer

sets for either unconverted or converted DNA strands, amplifying a region previously shown to be always unmethylated (At5g66750) and already described in (Hetzl *et al.,* 2007). PCR amplification was performed with TrueStart Taq DNA polymerase (Fermentas, #EP0619) for 30 cycles with annealing at 50°C and elongation at 72°C. The PCR products were inserted into vector pGEM-T easy (Promega). At least five clones for each line from each amplicon were selected for plasmid isolation, incubation with the BigDye terminator cycle sequencing kit (Applied Biosystems), and sequencing with an ABI Prism 3100 capillary sequencer (VBC Biotech). Obtained sequences were aligned in MegAlign (Lasergene, DNAStar), processed in ClustalX 1.83 (http://www.ebi.ac.uk) into FASTA format and analyzed for their methylation status using CyMATE (Cytosine Methylation Analysis Tool for Everyone, (Hetzl *et al.,* 2007), www.cymate.org).

4.15. Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed on 1.5 g of 3 week-old seedlings as described in http://www.epigenome-noe.net/researchtools/protocol.php?protid=13. For crosslinking of callus samples, the time under vacuum was doubled compared to seedlings; otherwise, ChIP on callus samples was performed as described for seedlings. The chromatin was immunoprecipitated with antibodies to histone H3 dimethyl K9 (prepared in the lab of Thomas Jenuwein, IMP) and other histone antibodies:

H3K4me3	#07-473	Upstate
H3K27me2	#07-452	Upstate
H4K20me1	#07-748	Upstate
H4K5,8,12,16ac	#06-598	Upstate
H3S10ph	#06-570	Upstate

Immunoprecipitated DNA was purified using the Qiagen PCR Purification Kit and eluted in 50 µl buffer EB. The control PCR was carried out in a total reaction volume of 25 µl and PCR conditions were: 96°C, 3 min; 30 cycles of 94°C, 30 s; 51°C, 30 s; 68°C, 1 min; followed by 68°C, 6 min. The primer sets for control PCR are listed in (Huettel *et al.,* 2006) and are located in either typically euchromatic (At4g0404, phosphofructokinase beta subunit) or heterochromatic (At4g03770, cinful-like retrotransposon) regions in the Arabidopsis genome.

PCR for semiquantitative analysis was performed with Taq polymerase (5Prime) and amplification intensity was measured on 1.2 % agarose gels with GelDoc XR (Quantity One 4.6.1, Bio-Rad). Quantitative real time PCR ChIP data were obtained using the 2x SensiMix Plus SYBR & Fluorescein Kit (Quantace) in a 20 µl quantitative PCR reaction according to the manufacturer's instructions. The samples were amplified using an iQ5 Real-Time-PCR System (Bio-Rad). QPCR data were analyzed according to the % of input method described by (Haring *et al.,* 2007).

4.16. Cation-exchange high pressure liquid chromatography

Total cytosine methylation was determined as described by (Rozhon *et al.,* 2008). In summary, 5 µg genomic DNA were RNase A-treated and digested at 37°C overnight in a total volume of 50 µl with 2.5 U/ml nuclease P1 and 500 U/ml DNase I in 20 mM acetic acid, 20 mM glycine, 5 mM $MgCl_2$, 0.5 mM $ZnCl_2$, and 0.2 mM $CaCl_2$ at pH 5.3. After the mixture had been incubated at 37°C overnight, 5 µl of 100 mM NaOH and 1 µl of calf intestine alkaline phosphatase (1 U/µl) were added. The mixture was incubated at 37°C for a further 24 h. Samples were diluted with 44 µl of 12 mM HCl and injected into the HPLC system (Dionex). Chromatograms were analyzed with Chromeleon 7 (Dionex) and Excel (Microsoft).

Allele	Gene identifier	Eco-type	Type of mutation	Primer pair (wildtype)	Fragment size (bp)	Primer pair (mutant)	Fragment size (bp)	Restriction enzyme
ago4-1	At2g27040	Ler/Col	Point mutation	AGO4-F AGO4-R	450+550	AGO4-F AGO4-R	1000	*Ava*II
cmt3	At1g69770	Ws	Point mutation	CMT3-F CMT3-R	240 (2 sites)	CMT3-F CMT3-R	240 (3 sites)	*Mse*I
ddm1-5	At5g66750	Zh	82 bp insertion	DDM+ DDM-	332	DDM+ DDM-	414	/
drd1-6	At2g16390	Col	Point mutation	DRD1-F DRD1-R	636	DRD1-F DRD1-R	413+223	*Bcl*I
drm1,2	At5g15380 At5g14620	Ws	T-DNA Wisconsin	DRM1or2-F DRM1or2-R	1: 2160 2: 2110	TL2 DRM1or2-R	1: 1600 2: 590	/
kyp1 *(suvH4)*	At5g13960	Col	T-DNA SALK 041474	KYP1 KYP2	430	KYP1 LBb1	643	/
met1-3	At5g49160	Col	T-DNA Hohn lab	MEF-1 MER-1	1000	MEF-1 barbiG	1200	/
mom1-2	At1g08060	Col	T-DNA SAIL_610_G01	301T36 301SP1	600	301T36 LB3	500	/
nrpd1-4	At1g63020	Col	T-DNA SALK 083051	NRPD1A4F NRPD1A4R	1300	NRPD1A4F LBb1.3	420	/
nrpd1-7	At1g63020	Col	Point mutation Baulcombe lab	SDE41005F SDE41294R	251+42	SDE41005F SDE41294R	293	*Bse*GI
nrpe1 *(nrpd1b)*	At2g40030	Col	T-DNA SALK 017795	NRPD1BF NRPD1BR	733	NRPD1BF LBb1.3	1038	/
nrpd/e2-1 *(nrpd2a)*	At3g23780	Col	T-DNA SALK 095689	NRPD2A1F NRPD2A1R	724	LBb1.3 NRPD2A1R	883	/
rdr2	At4g11130	Col	T-DNA Garlic 1277	RDR2F RDR2R	458	LB3 RDR2R	~350	/
suvH2	At2g33290	Col	T-DNA SALK 079574	SALK574F SALK574R	524	LB-b1 SALK574R	~650	/

Table 2. Summary of mutant alleles and genotyping information.

Primer Name	Primer Sequence (5' to 3')	used for
301 SP1	GCA ACT GTA GCA CAT GCA TCC AGC	genotyping
301 T36	GCA TAC CTG CAG GCA ATG AT	genotyping
AGO4-F	TGA CTG ACA GCT GAA AAT GGG ATG TGG AT	genotyping
AGO4-R	GCC ACT CCC TAG AAC TCA CCA CCT AAG TT	genotyping
B8 F	GCC ACG AAA ACC AAA CAG AC	ChIP control
B8 R	CCG GAA TTT CGA TCA ATC CT	ChIP control
barbiG	GGT TCT TAT AGG GTT TCG CTC	genotyping
BME3rtF	TCT CTT CCA ACA CCA ACT CTG AT	RT-PCR (BME3)
BME3rtR	CTC CTG AAT TGG CTA TGA GAA GA	RT-PCR (BME3)
BS control 1F	CGT CTG GTG ATT CAC CCA CTT CTG TTC TCA ACG	Bisulfite Sequencing
BS control 2F	TGT TTG GTG ATT TAT TTA TTT TTG TTT TTA ATG	Bisulfite Sequencing
BS control R	CTC TCA CTT TCT ATC CCA TTC TA	Bisulfite Sequencing
BSA-F	AAT TGA GAT TTT TTA ATA AAG GGT AAT AT	Bisulfite Sequencing
BSA-X1	ATC CCC CAA AAT CCC CAA ATA	Bisulfite Sequencing
BSA-X2	ATA AAA ACC CAC CAC CTC TAC	Bisulfite Sequencing

BSREP-F	TAT AAT AAT GTG TGA GTA TAA A	Bisulfite Sequencing
BSREP-R3	TAA TAC RRT TAT CCA CAR AAT CA	Bisulfite Sequencing
C seq 2	ACT GAC CTA CAG GGC AGC CAC	RT-PCR (P2 transcript), qPCR ChIP (P2)
C seq 2	ACT GAC CTA CAG GGC AGC CAC	PCR ChIP 6
CF5	GCG GGC TTG GAT GGT TCC AG	antisense northern, RT-PCR (P2 transcript)
CF9	ATA CCG CTC GCC GCA GCC GAA C	antisense northern
CFconfirm	GAA GTA ATG TTA GAT GTT CAA G	PCR ChIP 7
CFrevF	ACA TGA CTC CAG TCT GTA TCT	PCR ChIP 7
CMT3-F	GTT CTG CGT CAG TTA ATT GTT GAG	genotyping
CMT3-R	GCG GTT GTG ACC ACT GAT TCC TTG CG	genotyping
Cosmo	GGT AAC ATG TAT TTG GAA AAA GTC	PCR ChIP 2
CprobeF	GAT TAC GAA TTC CCA TGG AGT CA	Southern blot (P35S+HPT probe)
CprobeR	TCT AGA GGA TCC CGG ACG AGT	Southern blot (P35S+HPT probe, HPT probe)
DDM-	AAA GGA CCC ATT TAC AGA ACA C	genotyping
DDM+	CGC TCT CGA AAT CGC TCG CTG TTC	genotyping
DRD1-F	GAT GAG CTT CCT GGA CTT GCT G	genotyping
DRD1-R	CTT CCT CAG GTG ATG ACC CAG C	genotyping
DRM1-F	TGC GAT TGA CAA TTT CCA ATT TTC TCC AT	genotyping
DRM1-R	CTT GGT GTC TCA GTG TAT GTT CG	genotyping
DRM2-F	CCT CCT CCA GTA AAC TGA CGA CGA TAC AA	genotyping
DRM2-R	GGT AGA CGA ATC GGC TCG TCA TC	genotyping
FLank F	ACC GTC GCG AAC TAT ACA TCA	PCR ChIP 8
FLank R	ACG TTG AAT TGA ACT CTC CAC A	PCR ChIP 8
FRank F	CGA CAA ACA CTG ATT CAT CAT CT	PCR ChIP 1
FRank R	AGT CAC CAC CGC ACA CAT TGT	PCR ChIP 1
H3 F	CTC GAT GTC GTA TTC GCT GA	ChIP control
H3 R	GCA ACC TAT CAA CGC TTC GT	ChIP control
HM-	GTG TAT TGA CCG ATT CCT TGC GG	PCR ChIP 4
HM3F	TCC CAA TAC GAG GTC GCC AAC	antisense northern
HPT RTF	GAT CCC CAT GTG TAT CAC TGG	RT-PCR (HPT), ChIP 5
HPT RTR	TAT CGG CGA GTA CTT CTA CAC	RT-PCR (HPT), qPCR ChIP (HPT), ChIP 5
HPT start	GAT CCC GGG GGA CAA TGA GAT ATG	PCR ChIP 4, Southern blot (HPT probe)
Hugo	ATA TCT CAT TGT CCC CCG GGA	qPCR ChIP (P1), ChIP 3
KYP-1	CCT GTT CAA TTG ATT TCC ATG TGG T	genotyping
KYP-2	TCT ACA AGG AAT ATC ACC TGC C	genotyping
LB3	GCA TCT GAA TTT CAT AAC CAA TCT CG	genotyping
LBb1	GCG TGG ACC GCT TGC TGC AAC T	genotyping
LB-b1	GCG TGG ACC GCT TGC TGC AAC T	genotyping
LBb1.3	ATT TTG CCG ATT TCG GAA C	genotyping
MEF-1	GAT TGT GTC TCT ACT ACA GAG GC	genotyping
MER-1	GTT AAG CTC ATT CAT AGC CTT GC	genotyping
NRPD1A4F	GGG TTC GAA TAC GGG TCA CTT GA	genotyping
NRPD1A4R	TGT TAC ATA CTG AGA AGC ATG CT	genotyping
NRPD1BF	GCA GTG GAA TTC CTA GTC GAG	genotyping
NRPD1BR	AGA TCG GGA TCG GTG GCA TTG	genotyping
NRPD2A1F	CTT GAG TCC TGA TTC ATT ACC	genotyping
NRPD2A1R	GCA ACA CTT ACG GGT TAG TTC	genotyping
pALR	TGG ACT TTG GCT ACA CCA TG	Southern blot (180 bp probe)
pALU	AGT CTT TGG CTT TGT GTC TT	Southern blot (180 bp probe)
P35SF	CAG TCT CAG AAG ACC AAA GGG	qPCR ChIP (P1, P2), ChIP 3, ChIP 6
P35S-F	GTG ATA TCT CCA CTG ACG TAA GGG	antisense northern
Politan	ATA ATG GGA AGG TGA AAT GGC A	PCR ChIP 2
RDR2-F	TCC GGT TCT TAG AAC TCC ACC	genotyping

RDR2-R	CAT CAA TCT CAG AAG CGT CAC	genotyping
SALK574F	GTA CAT TGT TAC CAT TTC CTG AC	genotyping
SALK574R	AAG TAC ATG ATT CTT CAT ACT CTC C	genotyping
SDE4-1005F	GCA GGT TTA TGC TCT GTT ATT AG	genotyping
SDE4-1294R	GTT TCC CTC AAA GCC GAC TAG TT	genotyping
siRNA02	GTT GAC CAG TCC GCC AGC CGA T	small RNA northern
siRNA1003	ATG CCA AGT TTG GCC TCA CGG TCT	small RNA northern
TL-2	TGG ACG TGA ATG TAG ACA CGT CG	genotyping
U6 probe	TCA TCC TTG CGC AGG GGC CA	Poly(A) northern

Table 3. Primer Sequences.

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6. List of abbreviations

5mdC	5-methyldeoxycytosine
bp	basepair
CaMV	Cauliflower Mosaic Virus
cDNA	complementary DNA
Col	*Arabidopsis thaliana*, ecotype Columbia
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
F1, …	filial generation
TS-GUS	transcriptionally-silent beta-glucuronidase
HDGS	homology-dependent gene silencing
HMT	histone methyltransferase
HPLC	high pressure liquid chromatography
HPT	hygromycin phosphotransferase resistance gene
kb	kilobase
Ler	*Arabidopsis thaliana*, ecotype Landsberg erecta
M1, …	generation obtained after mutagenesis of progenitor
nt	nucleotide
paTGS	polyploidy-associated transcriptional gene silencing
PTGS	posttranscriptional gene silencing
RdDM	RNA-dependent DNA methylation
RdRP	RNA-dependent RNA polymerase
S1, …	generation obtained by selfing of progenitor
siRNA	small interfering RNA
TGS	transcriptional gene silencing
Ws	*Arabidopsis thaliana*, ecotype Wassilewskija
Zh	*Arabidopsis thaliana*, ecotype Zürich

Mutant abbreviations are explained in the text.

7. Curriculum vitae

PERSONAL DATA

Name	Andrea Maria Förster-Sümecz
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EDUCATION

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25.10.2005 — Magister rerum naturalis
Master's Degree of Science (with honours),
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01/2005 – 09/2005 — Diploma thesis with Frédérique Magdinier, PhD, in the research group of Prof. Eric Gilson, Ecole Normale Supérieure de Lyon, France.
Title: *Molecular characterization of the subtelomeric region of 4q35 involved in the facioscapulohumeral muscular dystrophy*

10/2000 – 10/2005 — Undergraduate studies at the University of Vienna
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PUBLICATIONS

Foerster AM and Mittelsten Scheid O. *Analysis of DNA Methylation in Plants by Bisulfite Sequencing.* Methods Mol Biol, **2009**, in press

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