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Titel der Diplomarbeit

A reverse genetic approach to analyse ploidy-associated gene silencing in *Arabidopsis thaliana*

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Verfasser:	Michael Milos
Matrikel-Nummer:	0008228
Studienrichtung:	molekulare Biologie (A490)
Betreuerin:	Prof. Dr. Ortrun Mittelsten Scheid
	Gregor Mendel Institut für molekulare Pflanzenbiologie
	Österreichische Akademie der Wissenschaften
	Dr. Bohr -Gasse 3 A-1030 Wien

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1. Inhaltsangabe

Polyploidisierung, die Vervielfachung des gesamten Chromosomensatzes, ist eine von mehreren Möglichkeiten, die genetische Vielfalt zu erhöhen und kann dadurch zur Reaktion auf umweltbedingten Veränderungen beitragen. Dieser Mechanismus ist in Pflanzen weit verbreitet und hat vermutlich die Entstehung einer Vielzahl von Phänotypen ermöglicht und polyploide Pflanzen evolutionär erfolgreich gemacht. Unter anderem ist Polyploidisierung vielfach mit transkriptioneller Genstilllegung durch epigenetische Mechanismen verknüpft. Epigenetisch stillgelegte Zustände können über Generationen hinweg stabil weitervererbt werden und zeichnen sich durch DNA Methylierung, diverse Histonmodifikationen und verdichtete Chromatinstrukturen aus.

Verschiedene Pflanzengene, die epigenetische Suppression und Chromatineigenschaften regeln, sind bekannt, darunter Methyltransferasen, Histonmodifikationsenzyme, Polycombproteine, sowie Proteine, die Chromatin zusammensetzen bzw. umbauen oder an RNA Interferenz beteiligt sind. Viele Elemente dieser verschiedenen Gruppen interagieren in komplexen Netzwerken. Ob und wie sie auch in die polyploidie-assoziierten epigentischen Regulationen integriert sind, ist jedoch nicht bekannt. Ich habe einen reversen genetischen Ansatz in *Arabidopsis thaliana* verwendet, um zu testen, welche der bekannten Regulatoren an der Aufrechterhaltung der polyploidie-assoziierten Genstilllegung beteiligt sind.

Ich habe verschiedene Pflanzen mit definierten Mutationen in Genen für einzelne epigenetische Regulatoren mit Pflanzen der Linie (C2S), die ein Hygromycinresistenzgen (HPT) trägt, gekreuzt. Diese Linie ist ein diploider Abkömmling von einem tetraploiden Vorläufer, in dem das HPT Gen eine transkriptionelle Genstilllegung erfahren hat. Nach dem Kreuzen habe ich die F1 Hybride zur Erzeugung von F2 Generationen verwendet. Diese sollten Pflanzen enthalten, die homozygot für die Mutation und hemi-oder homozygot für das HPT Gen sind. Die F2 Generationen wurden durch Selektionsversuche auf hygromycin-haltigem Medium auf die Reaktivierung des stillgelegten Gens getestet. Ohne Selektion aufgezogene Geschwisterpflanzen wurden genotypisiert und die gewünschten Pflanzen

durch Selbstbefruchtung bis in die F4 Generation gebracht. Geeignetes Material wurde ebenfalls durch Selektionsversuche auf die Reaktivierung des HPT Gens getestet und mit molekularen Methoden auf HPT Expression und Methylierungsstatus des HPT Gens untersucht.

2. Abstract

Polyploidization, the multiplication of whole chromosome sets, is one of several possibilities to increase genetic diversity and therefore may help to react to environmental changes. The mechanisms is very common in plants and believed to create a variety of different phenotypes, making polyploid plants evolutionary successful. Among other consequences, polyploidization is frequently associated with transcriptional silencing of genes by means of epigenetic mechanisms. Silent epigenetic states can be stably inherited throughout generations and are manifest by methylation of DNA, diverse modifications of histones and a more condensed chromatin structure.

Several plant genes are known to regulate epigenetic transcriptional silencing and chromatin properties in general, including methyltransferases, histone modification enzymes, chromatin assembly/remodeling factors, RNAi processing components and Polycomb proteins. Many elements of these different groups are interacting in complex networks. However, if and how they contribute to polyploidy-associated gene silencing is not known. I have used a reverse genetic approach in *Arabidopsis thaliana* to test which of these regulators is also involved in maintenance of polyploidy-associated silencing.

I crossed several plants carrying defined mutations in genes for epigenetic regulators with plants of a line (C2S) carrying a hygromycin resistance gene (HPT). This line is a diploid derivative derived from a tetraploid progenitor in which the HPT gene had undergone transcriptional gene silencing (TGS). After crossing, F1 hybrids were grown into F2 populations that are expected to contain plants homozygous for the mutations and hemi- or homozygous for the HPT gene. F2 populations were assayed for reactivation of the silenced marker by selection on hygromycin. Siblings grown without selection were genotyped and appropriate plants further propagated to F4. Suitable material was screened on hygromycin for reactivation of the silenced HPT gene and analyzed with molecular methods for HPT expression and DNA methylation status at the HPT gene.

3. Introduction

3.1. Polyploidy

As plants are not able to move away from unfavourable conditions, they have to react to environmental challenges in a different way. One potential adaptation mechanism is a high genetic diversity, allowing exposing a variety of different phenotypes to the environment. One element of genetic variation in plants is the frequent formation of polyploid individuals. Polyploidy is the multiplication of the whole chromosome set, beyond the diploid state (2n) consisting of one maternal and one paternal chromosome.

Although polyploidization does occur in some animals, it is much more widespread in plants (reviewed in Comai 2005). Polyploidy does not always affect the whole organism, but can be restricted to a special tissue or individual cells.

Polyploidy can arise in different ways. There is autopolyploidy, where the organism duplicates its own genome, or allopolyploidy, when two different genomes are combined and doubled. In this case, genetic diversity is very high and allows for heteromultimer formation, functional specialization and rapid genome arrangements.

Polyploidization acts in different ways, reviewed in (Osborn *et al*. 2003; Comai 2005), The increased chromosomal number, together with genetic heterogeneity, can cause dosage effects, changing the ratio of different gene products to each other. This can change phenotypes determined by complexes composed of multiple elements. Further, the increased chromosomal number can augment the regulatory mechanism several fold, especially in allopolyploids where regulatory factors can stem from the divergent genomes. Beside the dosage and regulatory alterations, gross genetic changes like duplications, deletions or inversions can occur during polyploidization. These might result from different processes like homologous recombination, DNA rearrangements, point mutations, gene conversions or transposon activation.

In addition to dosage effects and genetic changes, polyploidization can trigger epigenetic changes of gene expression (reviewed in Osborn *et al*. 2003). These are characterized by heritable alterations of gene expression without

altering the DNA sequence. Although relatively stable, but in contrast to mutational changes, they are potentially reversible. Therefore, they represent a component of phenotypic diversity that is intermediate between transient adaptation of gene expression and true mutations and can be of great selective advantage.

3.2. Epigenetics

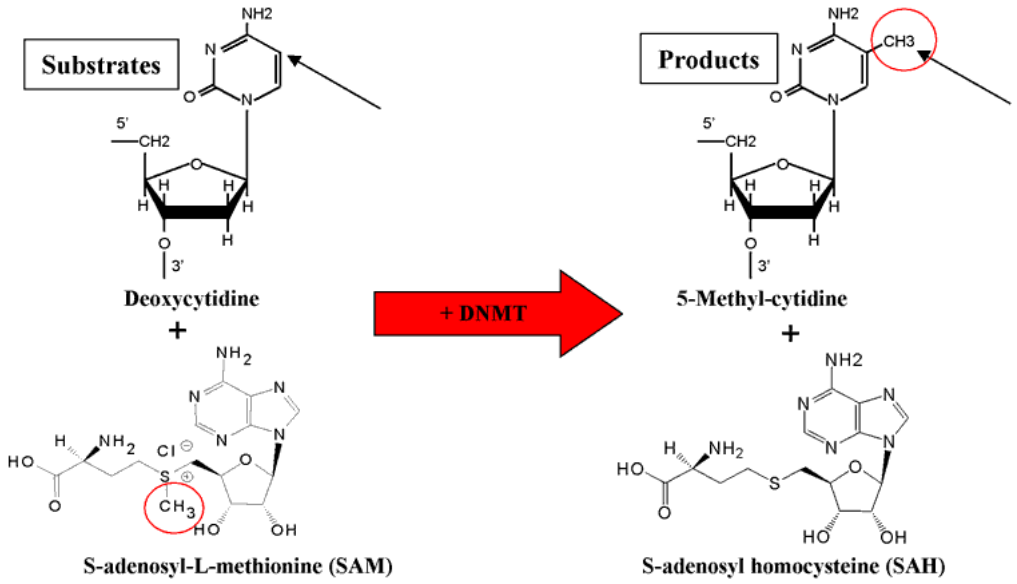
Epigenetic mechanisms regulate transcriptional activity and inactivity of DNA sequence and the stability of transcripts on longer terms. Epigenetic regulation does not alter the DNA sequence but it can chemically modify nucleotides (reviewed in Chan et al. 2005), change the density and modification of chromatin (reviewed in Tariq and Paszkowski 2004), the accessibility of the DNA strands for the transcription machinery as well as the stability of transcription products (reviewed in Mittelsten Scheid and Matzke 2006). Epigenetic mechanisms therefore change the formation of specific gene products, in connection with environmental changes, pathogen defeat or developmental switches in cell fate (Pruitt *et al*. 2003; Rapp and Wendel 2005). Epigenetic changes can be either reset between generations or stably inherited over many subsequent generations.

Several mechanisms are known to be involved in epigenetic regulation. The most prominent marker for transcriptionally silenced genes is DNA methylation, reviewed in (Chan *et al*. 2005), but there are other means like chemical modification of the DNA-associated histone proteins, reviewed in (Fuchs *et al*. 2006) that form the nucleosomes. These are the structural elements essential for wrapping the DNA threads and forming a higher order nuclear organisation, together with many other components. Although DNA methylation is an important component of epigenetic regulation in mammals and higher plants, there is some evidence that genes can be active even if they are methylated (Amedeo *et al*. 2000; Tariq *et al*. 2002), (reviewed in Vaucheret and Fagard 2001). Methylation alone therefore cannot be the only marker involved in epigenetics, and it is likely that there is a complex interplay with other elements like histone modifications or histone density (reviewed in

Wagner 2003). In addition, the regulation of RNA stability by all varieties of small RNA molecules (siRNA, miRNA, tasiRNA) and the protein factors processing it, can have a strong feedback effect on transcriptional activity of the genes serving as templates for the transcription of the progenitor RNA (reviewed in Matzke and Birchler 2005; Meins *et al*. 2005; Brodersen and Voinnet 2006). Since my thesis is dealing with epigenetic transcriptional gene silencing in plants, the following brief introductions into several classes of epigenetic regulatory elements will focus on the factors known to be involved here. I will especially introduce some mutations in genes encoding for individual epigenetic regulators since they are relevant for my experimental work described later.

3.2.1. DNA Methylation

The only DNA base which was found to be methylated in genomic plant DNA is cytidine (^{m}C). The modification is achieved by the transfer of a methyl group from the donor molecule S-adenosyl-L-methionin onto position 5 of the ring (Fig 1). While in mammalian DNA ^{m}C is found nearly exclusively at CpG (cytosine-guanine) sites, one can distinguish three different types of methylation on genomic plant DNA: symmetric methylation at CpG (cytosine-guanine) and CpNpG (cytidine-no guanine-guanine) sites and asymmetric methylation at CpNpN (cytosine-no guanine within the next two bases) (reviewed in Tariq and Paszkowski 2004). Symmetric sites allow restoration of the methylation pattern after strand separation during replication, due to the "memory" of ^{m}C at the opposite strand, while asymmetric sites do not bear this possibility. According to convention in molecular biology (http://en.wikipedia.org/wiki/Nucleotide), the letter N stands for either A, C, T or G, while the letter H represents the nucleotides C, T or A. Therefore, the distinction should correctly be made between CpG, CpHpG and CpHpH. However, since the wrong terms have been widely applied in the literature, I will follow convention and refer to CpNpG and CpNpN in the following.



www.med.ufl.edu/biochem/keithr/fig1pt1.html

Figure 1: Biochemistry of DNA methylation
The substrate Deoxycytidine is methylated via a DNA methyltransferase. The methylgroup is provided by the donor molecule SAM.

Several protein components of the plant DNA methylation machinery were identified by characterization of mutations in their coding genes.

met1-3

MET1 represents a DNA methyltransferase in plants which is related to DNMT1 in mammals. MET1 is important for maintenance of symmetrical CpG methylation. Several mutant alleles are available (Vongs *et al*. 1993; Kankel *et al*. 2003; Saze *et al*. 2003). In *met1-3*, the conserved MT motif region has been disrupted by a T-DNA insertion and represent a loss-of-function allele.

cmt3:

CMT3 is a chromomethylase. It has been identified by a suppressor screen for the hypermethylated *Arabidopsis* SUPERMAN locus and belongs to the chromodomain proteins. Several alleles have been found. CMT3 is specific for maintaining CpNpG methylation (Bartee *et al*. 2001; Lindroth *et al*. 2001). *cmt3-illa* is a point mutation with a strong effect on CpNpG

drm1,2

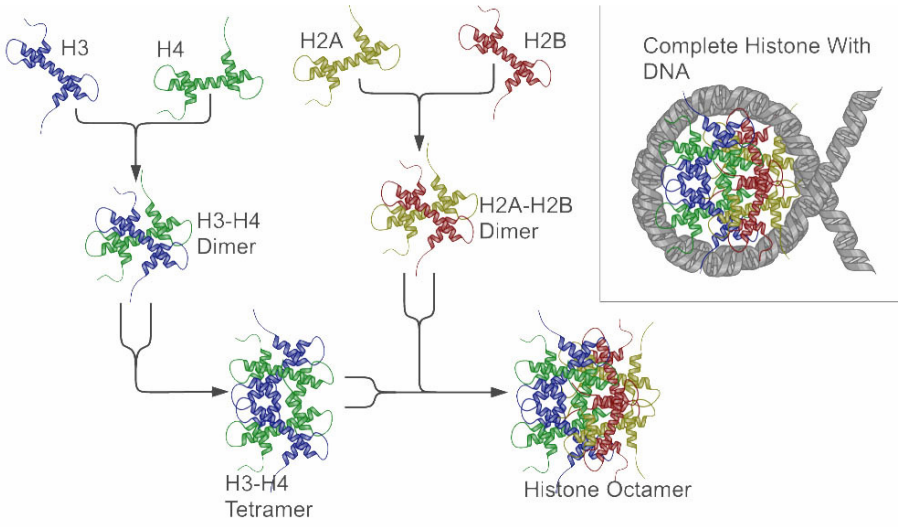
DRM1 and DRM2 (domains rearranged methylases) are needed for de novo DNA methylation. *drm1, drm2* double mutants lack de novo methylation and show some reduction in ^{m}CpNpN and a strong reduction of ^{m}CpNpG at some loci. CpG is not affected at all. DRM1 and DRM2 are acting in context with CMT3, since only triple mutants show a complete loss of asymmetrical methylation. (Cao and Jacobsen 2002).

hog1

HOG is not directly methylating or demethylating DNA. It encodes the S-adenosylhomocystein hydrolase (Rocha, Sheikh et al. 2005) and is involved in the biosynthesis of the SAM precursor providing the methylgroup. The mutation *hog1* had been generated either via X-irradiation or EMS (Ethylmethane-sulfonate) treatment (Furner *et al*. 1998).

3.2.2. Histone modifications

Histones are multimeric protein complexes with a basic surface, so that they associate easily with the acidic DNA. They form specific protein complexes called nucleosomes that consist of several subunits (H2A, H2B, H3, H4). Each is represented twice so that one nucleosome consists of an octamer (reviewed in Turner 2002).



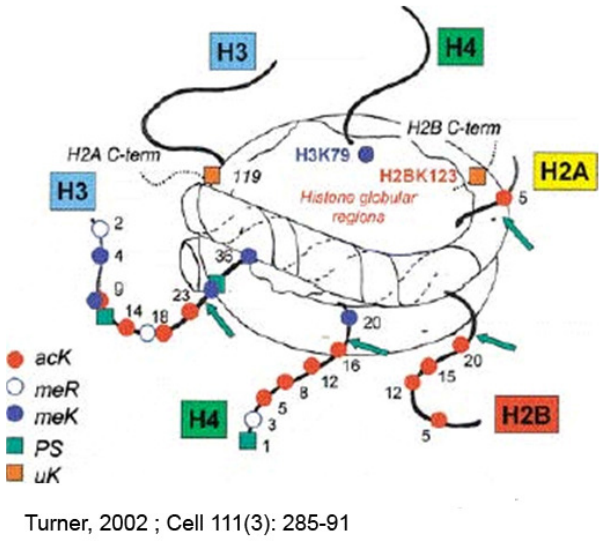
http://en.wikipedia.org/wiki/Image:Nucleosome_structure.png

Figure 2: Histone subunits and nucleosome assembly

These complexes are highly conserved throughout evolution. The histone proteins are very important for the chromosomal organization in the nucleus. Several different amino acid residues can be modified post-translationally in different ways: methylation, acetylation, phosphorylation, ubiquitination, glycosylation, ADP-ribosylation, carbonylation and/or sumoylation (reviewed in Fuchs *et al*. 2006).

These modifications are very specific for several amino acids and are believed to represent a “histone code” (Turner 2002). Methylation for example occurs mainly at lysin (K) residues. Several lysins (H3: K4, 9, 27, 36 and H4 K20) can become methylated, not only with one methyl group (mono methylation), but also with double or triple modifications (di- or trimethylation), thereby allowing many different combinations. Depending on the site and degree of histone methylation, the modifications are marking the expression and chromatin states of the associated DNA, Methylation at H3K4 and H3K36 are markers for active, accessible euchromatin. H3K9, H3K27 and H4K20 on the other hand are known to be marks for repressive heterochromatin structures. However, the situation is even more complex: while trimethylated H3K9 residues in mammals are associated with transcriptionally silent genes, the same mark is found on active chromatin in higher plants (Fuchs *et al*. 2006).

Several lysins (K) are accessible for modification by acetylation (H4: 5, 8, 12, 16 and 20; H3: 9, 14, 18 and 23). The acetylation status is important for the condensation of chromatin, and therefore for accessibility to nuclear protein complexes. An important role of histone acetylation is suggested by the presence of numerous histone acetyltransferases and histone deacetylases, representing large gene families in many organisms and indicating a highly complex and specific interplay (Wagner 2003).



Turner, 2002 ; Cell 111(3): 285-91

Figure 3: Nature and location of possible histone modifications

Since the other histone modifications are not relevant in the course of the thesis they are not described in more detail.

Several enzymes that carry out histone modifications were identified by characterization of mutations in their coding genes. On the other hand, many related elements did not show up in mutant screens, likely due to redundancy among large gene families.

axe1/rts1/sil1

RTS1 is coding for histone deacetylase 6 (HDA6). Deacetylation increases the binding strength of histones to DNA, making the DNA less prone to transcription. Several alleles were identified in different mutant screens (Murfett *et al*. 2001; Aufsatz *et al*. 2002) and revealed a role of HDA6 in transcriptional silencing (Furner *et al*. 1998; Murfett *et al*. 2001), RNA-directed DNA Methylation (RdDM) (Aufsatz *et al*. 2002) and acetylation of rDNA (Probst *et al*. 2004). The *rts1-1* allele is a point mutation leading to a loss-of-function.

kyp1/suvH4

SUVH4 belongs to the family of SET domain proteins identified in *Drosophila melanogaster.* It has a major role in methylating H3K9 in heterochromatic regions to dimethylated states and is involved in maintenance of silencing. Histone methylation of KYP/SUVH4 triggers indirectly CpNpG methylation. This is fulfilled via LHP1 which on one hand interacts with methylated histones and on the other hand with CMT3. The mutation kryptonite has been found within a screen for suppressors of DNA methylation at the SUPERMAN locus, other alleles come from reactivation of silent PAI genes (Jackson *et al*. 2002; Malagnac *et al*. 2002)

suvH2

SUVH2 is a homologue to KYP/SUVH4, but in contrast to SUVH4 (responsible for H3K9 mono – and dimethylation), H3K27 mono – and dimethylation and monomethylation on H4K20 are also reduced upon mutation of SUVH2. All these marks are significant for heterochromatin. Methylation processes induced by SUVH2 involve symmetric as well as asymmetric DNA methylation and occur independently of CMT3, but require MET1 and DDM1 (Naumann *et al*. 2005).

3.2.3. Chromatin assembly

During replication, the association between DNA and nucleosomes has to come transiently loose, and afterwards, the nucleosome arrangement must be restored or newly assembled for both strands, respectively. This is done with the involvement of several chromatin assembly factors (Figure 3). The subunits differ slightly but share significant homology between different model systems.

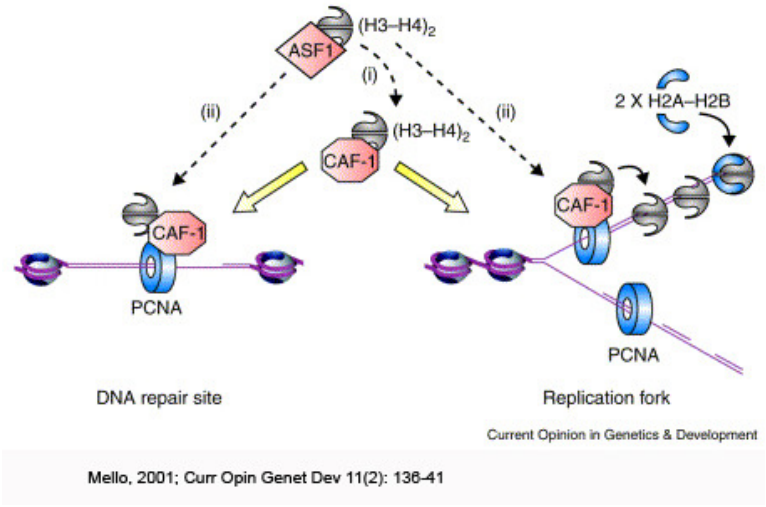


Figure 4: Nucleosome / Chromatin assembly
The process of nucleosome assembly involves histone chaperone CAF-1, which is a multimeric protein complex.

fas1 and *fas2*

FAS1 and FAS2 represent the large and the medium subunits of the *Arabidopsis* chromatin assembly factor 1 (CAF-1) (Kaya *et al*. 2001). They are required during embryogenesis and are necessary for the organization of the SAM (shoot apical meristem) and the RAM (root apical meristem), as can be seen from strong morphological abnormalities in the mutants (fas for fasciation, bundling of vascular tissue, shortening of internodes, (Kilby *et al*. 1992). FAS1 and FAS2 are further involved in stable gene silencing, since silent marker genes become reactivated in *fas* mutants (Takeda *et al*. 2004; Ono *et al*. 2006).

bru1

The phenotype of *bru1* mutations is similar to those of the *fas* mutants and to *mre11,* an element of DNA repair. *BRU1* has two conserved protein-protein interaction domains and additionally a predicted coiled coil domain and a leucin zipper for DNA interaction (Takeda *et al*. 2004). Although there is no evidence for BRU1 as a chromatin assembly factor, it is likely to be also involved in genetic and epigenetic modifications of the chromosomes since mutations cause increased sensitivity to DNA damage and reactivate silent marker genes, however without affecting DNA methylation (Takeda *et al*. 2004).

3.2.4. Chromatin remodelling

The nucleosomes can complex the DNA with different densities. In heterochromatin there are usually more nucleosomes per length of DNA, while in more active areas the density of nucleosomes is reduced. Certain proteins have the ability to shift histones along the DNA strand and are thereby remodelling chromatin (Langst and Becker 2001)(Figure 5)

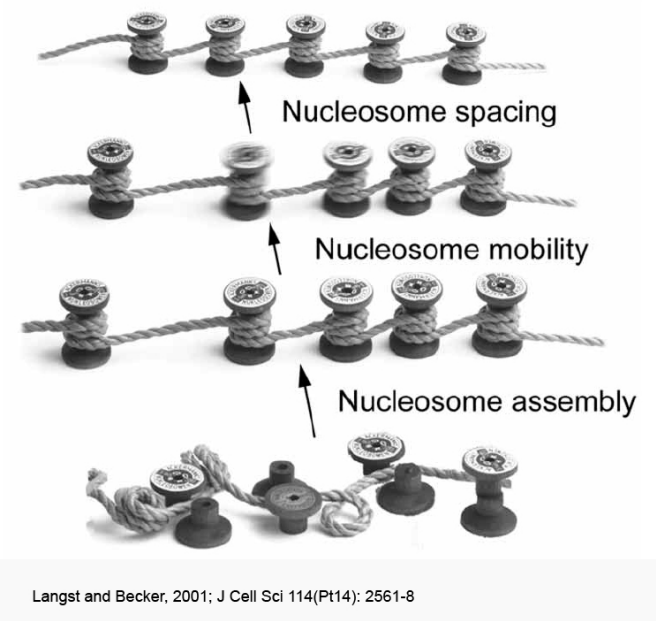


Figure 5: Chromosome remodelling involves nucleosome assembly, shifting of nucleosomes and changing the density of nucleosomes within a certain DNA segment

Also for chromatin remodelling factors, several mutants have been described and affect gene expression and accessibility.

ddm1-5

DDM1 (decrease in DNA methylation) has been originally found in a screen for mutants affected in cytosine methylation within repetitive centromeric regions (Vongs *et al.* 1993), however it turned out to belong to the SWI2/SNF2 like protein family, whose members are involved in chromatin remodelling. Remodelling activity has been shown for DDM1 *in vitro* (Brzeski and Jerzmanowski 2003). The effect of DDM1 on DNA methylation is therefore likely indirect (Zemach *et al.* 2005) and it might work as a transcriptional repressor, triggering the silencing machinery to methylate specific genomic

regions. *ddm1* mutants loose this control progressively and accumulate second-site mutations due to transposon activation (Vongs *et al*. 1993; Kato *et al*. 2004)

lhp1

LHP1 (like heterochromatin protein1) is a member of the chromodomain superfamily. This domain is highly conserved and found in various species like in polycomb proteins in *Drosophila melanogaster*. Many homologs are important components of inactive heterochromatin. Arabidopsis LHP can interact with H3K9 methylation and possibly to other proteins. Mutations in LHP1 cause changes in plant architecture, leaf development and flowering time (Gaudin *et al*. 2001; Mylne *et al*. 2006).

mom1-1 and *mom1-2*

MOM1 is another component required for maintenance of transcriptional silencing at several marker genes. Loss of the gene reactivates heavily methylated loci without reducing the methylation. A part of the gene is similar to approximately half of the ATPase region of the SWI2/SNF2 family involved in chromatin remodelling, but the functional relevance is not yet clear (Amedeo *et al*. 2000; Tariq *et al*. 2002).

drd1

DRD1 belongs to the SWI2/SNF2 like proteins, has a complete ATPase region and is important for de novo RNA directed DNA methylation. *drd1* mutations result in a significant decrease in non-CpG methylation in target genes. But this is not a global effect, since methylation in centromeric or rDNA repeats are not reduced. (Kanno *et al*. 2004; Kanno *et al*. 2005)

3.2.5. Genome stability

Beside of histones, there are other proteins attached to the DNA. Some of them are important for DNA stability, repair of DNA damage and recombination between different DNA molecules. Especially the telomeric ends require a complex maintenance to avoid unprogrammed chromosome fusion and breakage, and some proteins are specialized to provide stability and prevent telomeres from being treated as strand breaks. Interestingly, proteins involved in telomere stability are also involved in strand break repair, reviewed in (Riha *et al*. 2006).

Some mutants affecting genome stability will be described in the following.

ku70 and *ku80*

KU70 and KU80 are proteins involved in the stabilization of the telomeric regions of chromosomes and in the non-homologous end joining (NHEJ) repair process of double strand breaks. In NHEJ, they act as subunits of a multimeric protein complex. Each protein forms a ring and builds a heterodimeric protein complex containing one monomer each. KU70 and KU80 are important for the genome stability, since lack of them in mutants causes telomere shortening, incomplete chromosomal pairing during mitosis and double strand breaks, ending in cell cycle arrests. (Riha *et al*. 2002; Tamura *et al*. 2002)

mim

MIM is a member of the SMC (structure maintenance of chromosomes) protein family, which is involved in DNA replication and repair. In contrast to KU70/KU80 it is involved in homologous repair (HR) of double strand breaks, acting under genotoxic stress. (Mengiste *et al*. 1999; Hanin *et al*. 2000)

mre11

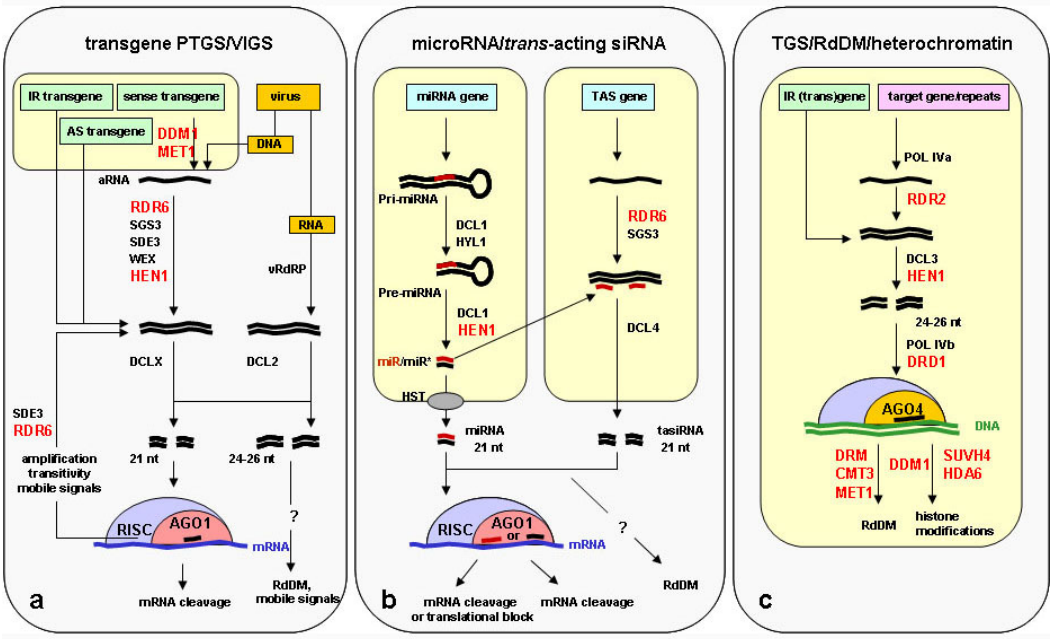
MRE11 is also involved in DNA repair. It has domains for single strand nuclease activity as well as for double strand nuclease activity. MRE11 is involved in HR and NHEJ. (Puizina, Siroky et al. 2004)

ter

TER is the reverse transcriptase subunit of the telomerase, important for maintenance of telomere length. Telomere shortening limits cell proliferation because uncapped telomeres are recognized as double strand breaks. Attempts to repair them lead to chromosome fusion-breakage cycles and the cell cycle is arrested. (Riha *et al*. 2001)

3.2.6. RNAi pathway

RNAi stands for RNA interference and was first encountered in *Petunia* and later in *C.elegans*. It is characterized by a well-defined processing of RNA, triggering the specific degradation of homologous RNA and a resulting reduction of expression levels of affected genes, reviewed in (Mittelsten Scheid and Matzke 2006). At the beginning, RNA becomes double-stranded (dsRNA), either by the action of RNA-dependent RNA polymerases (RDRP) or due to the ability of RNA with inverted repeats to form a loop and fold back. This dsRNA is cut to a length of 21-26 nt by a protein called dicer. The small dsRNA fragments become then separated into both strands and are integrated into a multiprotein complex (RISC). The RISC complex has the ability to find matching mRNA's and to degrade them. Altrenatively, the binding of small RNA can lead to translational arrest, or they can induce methylation of homologous DNA templates and transcriptionally silence corresponding regions. The RNAi machinery is involved in many pathways, defending the organism against foreign nucleic acids of transposons and viruses, as well as contributing to developmental and metabolic regulation. However, there might be specificity and redundancy of components at the same time, as apparent from slight differences between the pathways (Figure 6). This can be exemplified with the different size of the small RNA fragments. For post-transcriptional silencing through RDR's or folding of the RNA, the RNA is cut into fragments of 21 nt. The fragments connected with virus-induced gene silencing are larger (24 to 26 nt). Here I will concentrate on the elements that are involved in feedback silencing at the DNA level (Figure 6c) and describe the mutants available in this pathway.



Matzke M, Mittelsten Scheid O (2006) Epigenetic regulation in plants. In: Epigenetics (eds. Allis CD, Jenuwein T, Reinberg D), Cold Spring Harbor Laboratory Press (in press)

Figure 6: A schematic overview on RNAi pathways

ago4

AGO4 is involved in RNA-mediated posttranscriptional silencing coupled with RNA-dependent heterochromatin formation, with DNA methylation and histone modifications. It seems that AGO4 is positioned upstream of KYP, influencing H3K9 methylation and further downstream being involved in DNA methylation via CMT3 interacting with KYP and DRM. AGO4 has an N-terminal PAZ and C-terminal PIWI domains that is characteristic of the argonaute gene family (Zilberman *et al.* 2003; Meins *et al.* 2005)

dcl3

While in animals one dicer protein does all the processing of dsRNA, Arabidopsis has a dicer protein family with four members and different functions. In my context DCL3 is the most important, generating those endogenous siRNA with a length of 24-26 nt that are involved in the heterochromatin formation pathway of RNAi silencing (Xie *et al.* 2004).

hen

The exact length of small RNAs after processing and strand separation is very important. HEN1 is a methyltransferase and methylates the most 3' nucleotide of the RNA. This protects the RNAs from uridinilation and degradation (Boutet *et al*. 2003; Li *et al*. 2005)

rdr2 and rdr6

RDR2 and RDR6 are RNA-dependent RNA polymerases which can copy ssRNA into dsRNA (Dalmay, Hamilton et al. 2000; Mourrain, Beclin et al. 2000; Peragine, Yoshikawa et al. 2004; Xie, Johansen et al. 2004). As can be seen in Figure 6, the two polymerases are acting in different pathways. RDR6 is acting in PTGS for sense-transgene mediated silencing and silencing of DNA viruses but not on inverted repeat-mediated silencing. RDR2 is involved in heterochromatin formation and may process specifically PolIV-derived RNA templates. It is further an important factor of miRNA regulation in Arabidopsis (Lu *et al.* 2006).

3.2.7. Polycomb Proteins

Polycomb group proteins (PcG) are the antagonistic proteins to the Trithorax group proteins (TrxG) (Ringrose and Paro 2004). Both were originally described in *Drosophila melanogaster* but later shown to be common and involved in developmental differentiation of many organisms, including plants (Calonje and Sung 2006). Functionally, they are involved mainly in stabilization of epigenetic states after switches in gene activity and chromatin remodelling. TrxG are activating, PcG are repressing transcription. They modify histone tails or alter the nucleosomal conformation. PcG proteins can methylate H3K9 and H3K27, also they have the ability to ubiquitinylate H2AK119. While in *Drosophila* several binding motifs guiding the PcG and TrxG to their targets are known, there is little information about binding motifs in plants. However, there are several mutants of PcG-like proteins available.

clf and *swn*

CLF (curly leaf) and SWN (swinger) are homologues of ENHANCER OF ZESTE (E(Z)) from *Drosophila.* Both genes show similar expression pattern, and overlapping functions, but are not completely redundant. Both proteins have a SET domain, like the histone methyltransferases SuvH4 and SuvH2. CLF determines leaf and flower development, as the *clf* mutation causes delayed flowering and the characteristic leaf curling (Goodrich *et al*. 1997; Chanvivattana *et al*. 2004; Schubert *et al*. 2005)

emf

EMF (embryonic flower) is a homologue of SUPPRESSOR OF ZESTE found in *Drosophila.* EMF is a Zinc finger protein, directly interacting with DNA. It is found in vegetative shoot meristems and lateral organ primordia. It has direct interaction with CLF. *emf* causes severe defects in shoot establishment, resulting in extremely early flowering (Yoshida *et al*. 2001; Chanvivattana *et al*. 2004)

msi1

MSI (homologue of multicopy suppressor of IRA) can interact with several protein complexes controlling chromatin dynamics. Targets for MSI1 are histones, in combination with B-type acetyltransferases for H4K5 and H4K12 of newly synthesized histones, the CAF1 Complex, and also E(Z) like proteins. MSI is directly involved in gametophyte and early seed development. No direct catalytic function is known (Hennig *et al*. 2003; Hennig *et al*. 2005)

3.2.8. Miscellaneous

A few mutants that I included in my study are difficult to assign to a certain functional group and are therefore listed here.

hmgb1

HMG (high mobility group proteins) are small, abundant, non-histone proteins also associated with chromatin and seem to play a role in maintaining nuclear architecture. I have chosen a member of the B subgroup with a characteristic DNA binding motif. They do not recognize specific DNA sequences but rather DNA structures (like four way junctions, DNA minicircles, etc.) and A/T rich promoter sequences. HMGB has bending activity and can enhance the structural flexibility of DNA and therefore make it more accessible (Grasser 2003).

pkl

PKL (Pickle) is a CHD protein (chromatin remodelling factors, involved in repression of transcription) with a chromodomain, an SNF2-related and a DNA binding domain. It is necessary for repression of LEC1, which is a critical factor for embryo development (Ogas *et al*. 1999). Pkl seems to be involved in a gibberellin-modulated pathway (gibberellin is a growth regulator). Gibberellin promotes germination. *pkl* is defective in repressing embryonic identity characteristics after germination (like genes for seed storage).

ttg2-9/2/5 and *ttg2-12/4/4*

Two allelic mutations in the Transparent testa glabra gene 2 were only recently found to affect transcriptional gene silencing (Hofmann 2004). TTG2 is a member of a large (72 members) superfamily of transcriptional regulators. Charakteristic for these proteins is the N-terminal amino acid sequence. This WRKY domain spans about 60 amino acids and is highly conserved. The domain enables specific DNA binding at the recognition site (T)(T)TGAC(C/T).

The list of potentially important elements of epigenetic regulation is constantly growing, and I am aware that some published factors are missing in the previous description. However, the ones mentioned represent diverse entry points into a complex network of interaction and dependency and have therefore been chosen for the reverse genetic approach described in the following.

3.3. Experimental and genetic approach

Most of the epigenetic regulators described before were identified either by their role in maintenance of transcriptional silencing (mutant screens), by morphological consequences of gene defects, or by deducing their role in plants from that in other model organisms. None was connected with the epigenetic responses to polyploidy that were described in paragraph 4.1. Therefore, I decided to investigate systematically whether any of the known elements of transcriptional gene silencing was involved in polyploidy-associated transcriptional gene silencing (PA-TGS).

As a reporter technique to score for the effects on PA-TGS, I have chosen the following assay system. Previous work in the lab (Mittelsten Scheid *et al*. 2003) had produced a diploid Arabidopsis line transgenic for a hygromycin resistance gene (hygromycin phosphotransferase, HPT) under control of a constitutive CaMV (Cauliflover Mosaic Virus) 35S promoter. The transgene (called C-locus) inserted in the middle of chromosome 3 bottom arm (Figure 7). The HPT gene was stably expressed and the plants resistant. From this diploid progenitor line, several tetraploid plants were generated through a

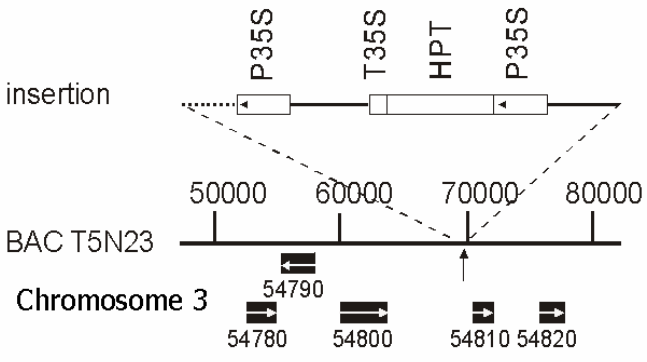


Figure 7: Location of the C-locus
The HPT gene is under control of a 35S Promoter, further downstream is a second copy of the promoter, but in sense.

protoplast culture and subsequent regeneration of several independent plants. Surprisingly, some of these plants lost the hygromycin resistance (termed C4S, for C-locus, tetraploid, sensitive), while others maintained the resistant state (C4R, C-locus, tetraploid, resistant). The gene in C4S became highly methylated and was not transcribed any longer, representing a gene silencing occurring in conjunction with the polyploidization. After bringing the silent line back to a diploid state by backcrossing, the sensitivity for hygromycin remained. This state is inherited very stably (C2S), as is a diploid derivative from the resistant tetraploids (C2R). Therefore, four lines, all from the same progenitor and therefore supposedly genetically identical, differ in degree of ploidy (diploid or tetraploid) and expression state of the HPT gene (resistant or sensitive) (Figure 8).

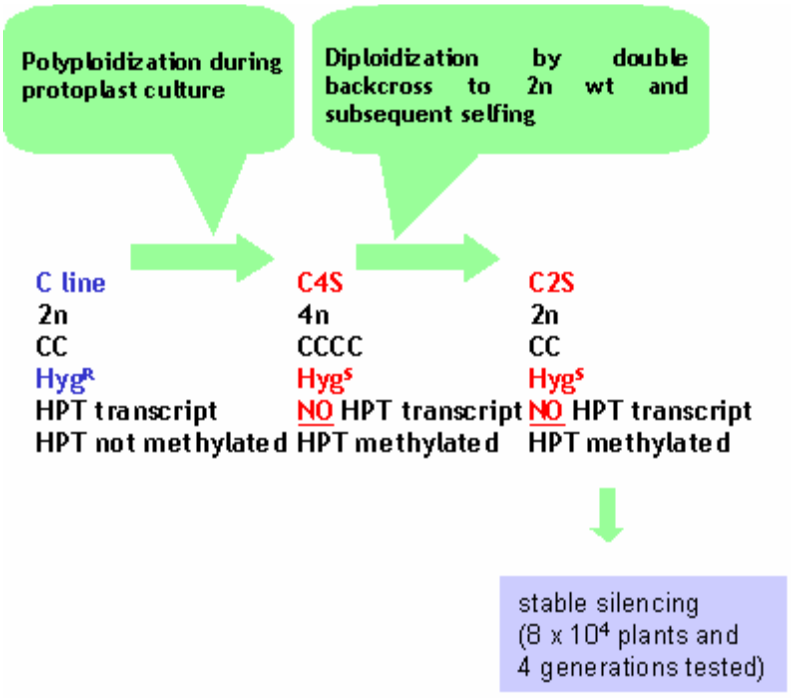


Figure 8: Generation of the C2S line

Genetic experiments and mutational techniques with tetraploid lines are much more complex than with diploids, but the stability of the silent state in diploid derivatives allowed me to apply a reverse genetic approach, to challenge the role of genes known to be involved in epigenetic silencing. Therefore, I chose well-defined mutants out of every group I described before, to perform crosses with C2S and inbred the plants. The rationale was that the hybrids would

segregate different genotypes, among them some in F2 that would be homozygous for the mutation and contain one or two copies of the C-locus (Figure 9). If the function of the defined components would be required to maintain the silencing of the HPT gene, these genotypes would be expected to restore HPT activity and thereby become resistant. Although recessive mutations should reveal their effect in F2, being homozygous for the first time after the outcross, I decided to continue inbreeding till to the F4 generation, to allow for delayed effects described for some mutations (e.g. (Vongs *et al.* 1993). I performed a selection experiment with seeds of the F2 and F4 generations. In addition, I investigated different molecular features, like transcription, DNA integrity and methylation state within the C-locus in the mutant backgrounds.

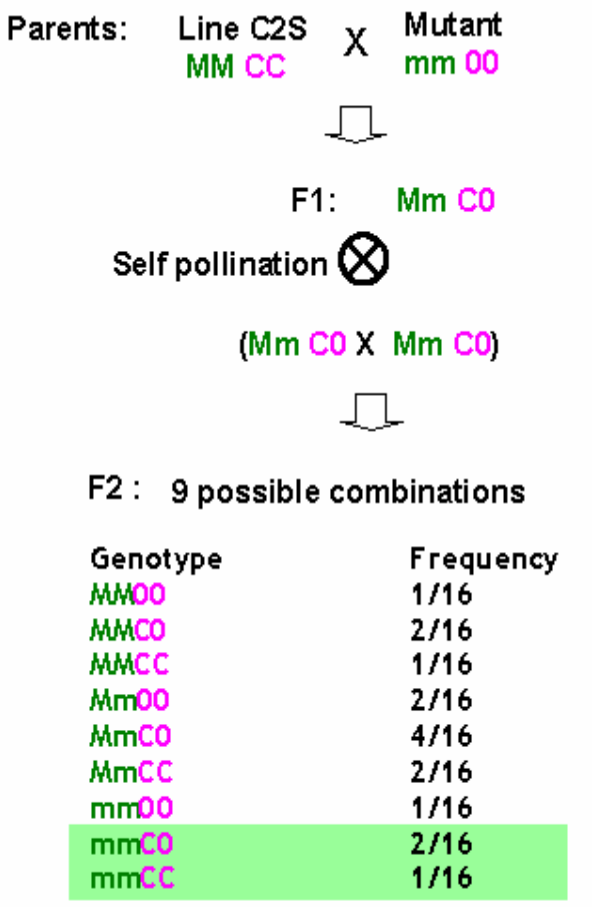


Figure 9:
Scheme of the crosses, possible genotypes and expected segregation. Highlighted in green are the genotypes of interest.

4. Materials and methods

4.1. Plant material

4.1.1. Plant lines

I used mutant lines affecting several epigenetic mechanisms and in different ecotype backgrounds. All lines are described within the introduction, and technical data are collected in Table 1 in the Appendix. The crossing partner line C2S, diploid, hygromycin-sensitive and with a silent HPT gene, and the control line C2R (also diploid, but hygromycin-resistant and with an expressed HPT gene) were generated as described (Mittelsten Scheid *et al*. 2003) (Figure 8) and are in the background of ecotype Zürich (Zh).

4.1.2. Crossing

For crossing *Arabidopsis thaliana* it is important to avoid self-pollination of the plants. Therefore, I emasculated flowers before maturity of the anthers. I fixed the whole inflorescences under a binocular and removed older flowers (where white petals were visible) and the youngest buds that were too small for emasculation. The remaining buds, around six with a size of around 2-3 mm, were opened carefully with surface-sterilized fine forceps and all anthers were removed. I let the plants grow for 2 more days so that the stigmata were fully developed. Then I pollinated the flowers with pollen of the designated paternal line and marked the flowers properly. I was able to harvest the mature siliques around three weeks later. Seeds were collected in small paper bags, allowed to desiccate completely and stratified for 1 week at 4°C.

4.1.3. Seed sterilization

For the hygromycin selection assay, I needed to surface-sterilize the seeds. I prepared a 5% sodium hypochlorite solution with some drops of Tween 20 (to minimize the surface tension). I incubated the seeds for 6 minutes with additional shaking. Under a laminar hood I washed the seeds properly with sterile water through filter paper and let them dry. The dry seeds could be stored under sterile conditions for a long period.

4.2. Media

4.2.1. Stock solutions:

- macro (1 litre): KNO_3 – 19 g, NH_4NO_3 - 16.5 g, $CaCl_2$ x H_2O - 4.4 g, $MgSO_4$ x 7 H_2O - 3.7 g, KH_2PO_4 - 1.7 g; one component dissolved before adding the next one, autoclaved
- B5 micro (100 ml): $MnSO_4$ x H_2O – 1 g, H_3BO_3 – 300 mg, $ZnSO_4$ x 7 H_2O – 200 mg, KJ – 75 mg, Na_2MoO_4 x 2 H_2O – 25 mg, $CuSO_4$ x 5 H_2O - 2.5 mg, $CoCl_2$ x 6 H_2O - 2.5 mg; one component dissolved before adding the next one, filter-sterilized
- MS vitamin (500 ml): m-inositol – 5 g, glycine – 100 mg, thiamine – 50 mg, pyridoxine – 25 mg, nicotinic acid – 25 mg; filter-sterilized
- Ferric citrate (500 ml): ammonium iron citrate – 5 g; filter-sterilized
- MES (100 ml): MES – 14 g; dissolved in ca. 80 ml H_2O, pH 6 adjusted with 2 M NaOH, filled up to 100 ml and filter-sterilized

4.2.2. GM (germination medium):

Solution 1:MS macro – 50 ml, B5 micro – 1 ml, ferric citrate – 5 ml, sucrose – 10 g; filled up with H_2O to 100 ml, pH 5.6 and filter-sterilized

Solution 2: Merck agar – 4 g, MES – 2,5 ml; filled up with H_2O to 450 ml and autoclaved.

50 ml of solution 1 and 450 ml of solution 2 were finally mixed.

4.2.3. GMH10 (selective germination medium):

Hygromycin B (Calbiochem) stock solution (filter-sterilized) was added to GM at a final concentration of 10 mg/l.

4.3. Genotyping

4.3.1. Preparation of FTA squashes

For many genotyping PCRs, filter-bound DNA prepared by squashing leaf tissue onto FTA filter paper (Whatman) was sufficient. The squashes were dried over night, and discs of ∅ 2 mm were punched out and incubated twice for 5 min with FTA reagent (Whatman), followed by two washes with TE buffer for another 5 min. Finally, I dried the discs for 10 min at 56°C. These discs were added directly to the PCR reaction tubes as templates.

4.3.2. DNA isolation

Clean genomic DNA for more demanding PCR conditions was prepared with the Nucleon Phytopure plant DNA extraction kit (Amersham Biosciences), as described in the manual.

4.3.3. Overview on mutant alleles and genotyping protocols

Table 1 in Appendix: Description of mutant alleles, ecotype background, morphological consequences, primer sequences and PCR-based polymorphisms for mutants involved in the investigation

4.3.4. PCR

PCR reactions were done using a standardized protocol, adjusted to the primers' melting temperatures. The only variable parameter was the length of the elongation step, which was adjusted according to the expected fragment length (ca. 30 sec per 400 bp). The water volume of the blank control was reduced according to the volume of the added DNA template solution.

General PCR reaction	
	µl
H_2O	17.4
10 X PCR Buffer	2.5
dNTPs (2.5 mM each)	2.5
Primer A (10 µM)	1.25
Primer B (10 µM)	1.25
Taq Polymerase (Eppendorf)	0.1

PCR Cycle Protocol		
Temperature	Time	
94°C	5 min	
94°C	30 sec	30 cycles
55°C	30 sec	
72°C	30 sec -3 min, depending on expected fragment length	

4.3.5. Restriction digest

Restriction digests of PCR fragments or genomic DNA were all done under conditions recommended by the enzyme suppliers. Fermentas produced most restriction enzymes used.

4.3.6. Agarose gel electrophoresis

In general, I prepared 1.5 – 2 % agarose gels in TAE buffer. For separations of small or very similar restriction fragments, I used gels up to 4 %. For DNA gels I used the 100 bp marker (Fermentas), for RNA gels the size of the rRNA served as standard.

For the documentation, I used ethidium bromide and UV light to visualize the DNA bands. RNA gels were stained with RR (Radiant Red from BioRad) and also inspected under UV light.

4.4. Gene expression analysis

4.4.1. RNA isolation

For RNA isolation, I applied a method based on the phenol-containing reagent Trizol (MRC-Molecular Research Center inc.). First, I prepared 1.5 ml Eppendorf tubes with 6-8 small glass beads (∅1,7-2mm, Roth). Then I filled the tubes to 50 % with leaf material and froze it immediately in liquid nitrogen. For homogenization, I used a bead mill (Retsch). The disrupter barrels were pre-cooled in liquid nitrogen. I homogenized the tissue by milling for 4 min at maximum speed. The result was a fine green powder. Immediately after homogenization, I added 1 ml of Trizol. I vortexed the mix and incubated it for 10 min at room temperature (RT). Then I added 0,2 ml chloroform and vortexed it vigorously for 15 sec, followed by 3 min incubation at RT. This is followed by a centrifugation step for phase separation for 15 min at 4°C with 12000x g. I transferred the upper aqueous phase and precipitated the RNA by adding 0,5 ml of isopropanol. This mixture was incubated at RT for 10 min and centrifuged for 10 min at 4°C with 12000x g. After removing the supernatant, the obtained RNA pellets were washed by adding 1 ml of 75 % EtOH. I vortexed it once and centrifuged it again for 5 min at 4°C with 7500x g. The EtOH was removed and the pellets were dried. Then I dissolved the pellets in DEPC (diethylpyrocarbonate)-treated water (treatment to eliminate RNases). Then I stored the RNA at -80°C.

4.4.2. Blotting

4.4.2.1. Northern Blot

For the northern blots, I ran RNA gels based on denaturation with glyoxal, which is less toxic than other common protocols.

I dried 5-10 µg of RNA per sample in a speed vac. Meanwhile I prepared a master mix consisting of 6 µl H2O, 3 µl glyoxal, 1 µl 20 x gel buffer (50 x GB ph7: 577ml of Na_2HPO_4 500mM + 423 ml of NaH_2PO_4 500mM) and 10 µl DMSO per sample. The RNA pellets were dissolved in 20 µl of the master mix. I incubated the solution 1 h at 50°C. In the meantime, I prepared a 1,5 %

agarose gel in 1xGB. Before loading the probes onto the gel, I cooled the probes to RT and added 4 µl sample buffer (SB: glycerol 50% + bromophenolblue 0,4% in 1 x GB) to each probe. The gel was run at 80 V for about 1,5 h. The gels were stained by incubation in RR (Radiant Red) for 30 min. The integrity of the rRNA bands was checked on a UV transilluminator and the gel cut to the appropriate size for blotting.

For blotting, I prepared two pieces of Whatman 3MM paper and one layer of Hybond N membrane (Amersham) according to the size of the gel. Before rinsing the membrane, I marked one edge with pencil to remember the orientation. Whatman paper and Hybond N were soaked in 2 x SSC (20 x SSC: 175,3g NACl, 88,2g sodium citrate – $2H_20$ in $1lH_2O$, pH7). A plastic tray was filled with 20 x SSC. A sheet of Whatman 3MM was cut into shape to form a bridge, soaked in 20 x SSC and used to cover a glass plate so that the ends of the paper were immersed in the SSC solution. Some ml of 20 x SSC were pipetted onto the bridge and the RNA gel transferred carefully onto it. If necessary, the surface of the gel was rinsed with 20 x SSC before covering it with the membrane. The membrane was covered by the two pre-soaked Whatman filter papers 3MM. It was very important to avoid any air bubbles trapped between the bridge paper, the gel and the membrane. The gel was surrounded by stripes of parafilm to avoid shortcut flow of the transfer solution. A stack of paper towels or other soaking paper covered the sandwich. Finally, the whole stack was covered by a second glass plate with a weight of about 1 kg placed on top, located centrally to ensure equal distribution of the weight. The blotting procedure was allowed to proceed over night. After disassembling the stack, the membrane was washed with 2 x SSC for a minute with shaking. Then it was drained, dried and cross-linked through UV exposure (Stratagene crosslinker) with 2500 kJ. Subsequently, the membrane was washed with 0,1 % SDS to remove the glyoxal. Now the membrane was ready for hybridization.

4.4.2.2. Southern blot

The Southern protocol differed from that of the northern mainly in the preparation of the gel after electrophoresis. Genomic DNA digests were run in specially cleaned electrophoresis chambers, starting the run with 30 V to let the probes enter the gel without aggregating. Then voltage was increased up to 150 V. After checking and documenting the gel under UV light, it was trimmed to the right size and marked on one edge.

Before blotting, the gel was incubated in HCl (250 mM = 25 ml of 37 % HCl/l) for 10 min at RT on a shaker (the bromophenolblue of the loading buffer should turn yellow). This was followed by a denaturation step with incubation in a denaturation solution (500 mM NaOH, 1,5 M NaCl) for 30 min (the indicator should turn blue again). Finally, the gel was neutralized by shaking the membrane in neutralization buffer (500 mM Tris, 1,5M NaCl, 1 mM EDTA, pH 7,2) twice for 15 min, changing the solution once. The final pH should be lower than 8,5.

The following procedure was identical with that of the northern blot, just omitting the wash with 0,1 % SDS.

4.4.3. Preparation of the hybridization probes

As probe I used 25 ng EtOH-precipitated probe DNA (produced by PCR reactions with appropriate primers) diluted in 45 µl of TE buffer. After boiling for 5 min at 100°C for complete denaturation, I cooled the probe on ice for 5 min. I added the sample to a REDIPRIME reaction tube (Amersham Biosciences) (no mixing at this point). In the next step 5 µl of ^{32}P dCTP were added and mixed by pipetting several times. After an incubation step of at least 10 min at 37°C, the reaction was stopped by adding 5 µl of 0,2M EDTA.

For separation of the non-incorporated nucleotides, spin columns (Amersham probequant G50) were equilibrated first. For this, the columns were vortexed, placed on top of a 1,5 ml screw cap tube (the cap needs to be open a ¼ turn). This was followed by a centrifugation step at 735 g for 1 min. After placing the column in a new tube, I applied the probe to the centre of the column, paying attention not to disturb the matrix. The following spinning step for 2 min at 735 g removed the spare nucleotides, which are retained in the column, while the

probe was in the screw cap tube. After boiling for 5 min at 100°C and chilling on ice for another 5 min the probe was ready for hybridization.

4.4.4. Probes

I used 4 different probes:

- HPT ORF (northern, C-locus expression analysis): PCR fragment containing the HPT coding region
- TSI (northern, repeat expression analysis): PCR fragment representing a common region of TSI templates (pericentromeric repeats, not expressed in wt)
- HPTtotal, (Southern, C-locus methylation analysis): PCR fragment including CaMV 35S promoter region and HPT coding region
- 180 bp repeats (Southern, repeat methylation analysis): PCR fragment representing one unit of the centromeric repeats

4.4.5. Hybridization for Southern and northern blots

The hybridization solutions were mixed freshly for every experiment out of stock solutions (500 mM EDTA, 20 % SDS, NAPI: 89 g Na_2HPO_4 x $2H_2O$ in 996 ml H_2O + 4 ml H_3PO_4). The blots were pre-incubated with about half of the hybridization solution (25 ml NAPI, 7,5 ml H_2O, 17,5 ml SDS and 100 µl EDTA) for about 30 min at 65°C. Then the solution was discarded and replaced by the rest of the hybridization solution, the denatured probe was added and incubated in a rotating hybridization oven overnight at 65°C. After hybridization, the membranes were washed with washing solution (10 ml NAPI, 85 ml H2O, 5 ml 20 % SDS) as follows. The first washing took 10 min at 65°C, followed by two further washing steps at 60°C for 20 min each.
Afterwards, the blots were exposed to a phosphor-imager screen or X-ray film.

5. Results

5.1. Crosses

In order to investigate whether the non-expressed epiallele, stably silenced in a tetraploid line and its diploid derivative, would be reactivated upon lack of any of the known components of transcriptional gene silencing, I have introgressed the silent HPT gene by genetic crosses into the background of several loss-of-function mutants.

Crosses were performed between the diploid C2S line and mutant plants. In most cases C2S was used as the paternal line. Reciprocal crosses were also performed additionally. The C2S line contains a hygromycin phosphotransferase gene (HPT) in a stably silenced and hypermethylated state. The mutants, consisting of the alleles *ago4-1*, *bru1-1, cmt3-illa, dcl3-1, drd1-6*, *ddm1-5, drm1,2, emf2-3, fas1-1, fas2-1, hen1-5*, *hmgb1 (SAIL261-B02)*, *hog1-1, ku70, ku80, kyp (SALK041474), lhp1, met1-3, mim1-1, mom1-1), mom1-2, mre11-3, pkl1-1, rdr2-1, rdr6 (sgs2-1), rts1-1, suvH2 (SALK079574), swn-3, ter, ttg2-9/2/5, ttg2-12/4/4,* have been shown or are assumed to be involved in epigenetic regulation, determining DNA methylation, chromatin modification, chromatin assembly or remodelling, recombination and repair. I assorted the mutants into different groups according to their functionality (Table 2).

The C2S line is in the background of ecotype Zürich, while most mutants are in different accessions. Inter-ecotype crosses therefore could potentially lead to changes in epigenetic states that are not due to mutations. Since crosses in all combinations would have exceeded the range of the experiments here, I have chosen to perform control crosses of C2S with wild type plants of ecotype Columbia (Co), representing the background of the majority of mutants. No effect on the expression state of the HPT gene was seen in these controls.

5.2. Generation of F2 populations

After the crosses I collected the mature siliques with hybrid seeds and grew a small number of F1 plants on soil. Since C2S plants are homozygous for the silent HPT locus and most mutant plants used for crossing were homozygous for the mutation, I expected to obtain hetero- and hemizygous F1 plants in all cases. I nevertheless genotyped these plants and confirmed the presence of the mutation and the C-locus. This was especially important for the few cases of heterozygous mutant parents, but proved to be generally helpful to ensure the correct genotype for further propagation (Table 2). Since some of the mutant lines were only viable as heterozygotes and some parental lines turned out not to have contained the mutant locus (e.g. *hog*1), some F1 plants obtained from these crosses could be discarded early. The other F1 plants of suitable genotypes were grown and allowed to self-pollinate to obtain F2 populations (Table 2). These were expected to segregate for different genotypes and were either used to score for hygromycin resistance (see below) or grown again on soil, to allow genotyping for selection of further generations. Twenty plants per combination were genotyped. This sample size was chosen because I expected 18.75%, corresponding to 3-4 plants, with the required genotype. This number was calculated based on the expectation to find 25% F2 plants homozygous for the mutant locus (genotype mm) and 75% of these having at least one (hemizygous, genotype C0, 50%) or both (homozygous, genotype CC, 25%) HPT alleles. This was successful for most but not all of the mutants (Table 2).

In addition to the homozygous mutant plants, segregating siblings containing the HPT gene, but the wild type alleles for the mutations, were also collected. These plants could be used as control material to exclude any changes in HPT expression due to different ecotype background. However, due to the results described below, these controls were not yet further analysed.

5.3. Genotyping

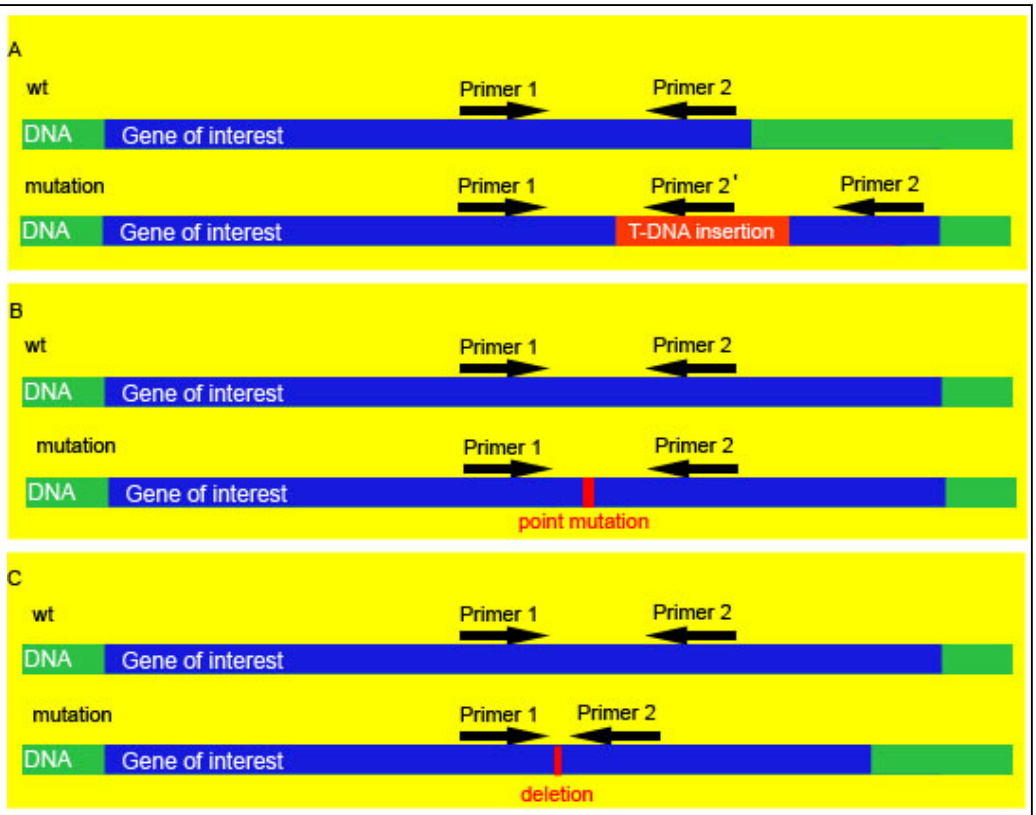


Figure 10: Principles of genotyping.
A: mutations caused by insertions of T-DNA were identified by differential PCR with primers located outside of the insertion site (wt) or outside and within the insert (mutation). A similar principle was applied to genotype for the HPT-insert. B: mutations caused by single base exchanges were identified by PCR with primers around the site and then identified by either restriction fragment polymorphism or by sequencing of PCR products. C: mutations caused by insertion or deletions were identified by PCR with primers around the site and comparison of fragment length.

Most mutants, especially those established by T-DNA insertions, were genotyped with four separate PCR reactions per plant (Figure 10). One PCR with primers in the insertion and in the flanking DNA addressed the presence of the mutant allele, another with primers in both flanking sequences the absence of the wt allele (negative result) as a confirmation that the plant is homozygous for the mutant allele. One reaction with primers in the HPT gene challenged the presence of the transgene insertion and finally one (negative) PCR with primers in the regions flanking the insert confirmed the homozygosity of the HPT gene (Figure 10). The template at the insertion site is too long for a standard PCR reaction. For smaller rearrangements and point mutations, the strategy was modified as follows. Primers were designed

around the mutation site and used to amplify the corresponding region. A small insertion, like in the case of *ddm1*-5, was visible as a shift in band size of the PCR product, either for both alleles (homozygous), for one fragment (heterozygous) or not at all (wild type) (Figure 10). Some point mutations had created a restriction polymorphism (e.g. *ago4-1*). In these cases, I digested the PCR product with the corresponding restriction enzyme (see Table 1). Complete digestion indicated a homozygous, polymorphic digestion a heterozygous, and no digestion the wild type genotype (Figure 11).

However, some mutations were not detectable by such assays at all. In the case of the *fas* mutants, the obvious mutant phenotype could be exploited to identify homozygous genoptypes (Figure 11). For the remaining mutants, like *hog1*, the PCR product was subjected to DNA sequence analysis. An overview on the desired genotypes obtained is given in Table 2.

Mutations	F1		F2				F4		
	Seeds obtained	Required genotype	Seeds obtained	Required mutant genotype	HPT	Screen	Seeds obtained	Required mutant genotype	Screen
DNA methylation mutants									
cmt3	+	+	+	+	+	-	+	mmCC	-
drm1,2	+	+	+	not yet	not yet	-			
hog1	+	missing							
met1	+	+	+	Mm	+	-	+	MmCC	-
Histone modification mutants									
axe1/rts1/sil1	+	missing							
kyp1/suvH4	+	+	+	+	+	-	+	mmCC	-
suvH2	+	+	+	+	+	-	+	mmCC	-
Chromatin assembly mutants									
bru1	+	+	+	+	+	-	+	mmCC	-
fas1	+	+	+	+	+	-	+	mmC0	-
fas2	+	+	+	+	+	-	+	mmCC	
Chromatin remodelling									
ddm1	+	+	+	+	+	-	+	mmCC	partial
drd1	+	+	+	not yet	not yet	-			
lhp1	+	+	+	not yet	not yet	-			
mom1-1	+	+	+	+	+	-	+	mmCC	-
mom1-2	+	+	+	+	+	-	+	mmCC	-
Genome stability									
ku70	+	primer probl	+						
ku80	+	+	+	+	+	-	+	mmCC	-
mim	+	+	+	+	+	-	+	mmCC	-
mre11	+	+	+	not yet	not yet	-			
ter	+	+	+	not yet	not yet	-			
Mutants in RNAi pathways									
ago4	+	+	+	+	+	-	+	mmCC	-
dcl3	+	+	+	not yet	not yet	-			
hen	+	repeat cross							
rdr2	+	+	+	not yet	not yet	-			
rdr6	+	+	+	not yet	not yet	-			
Polycomb group mutants									
clf	+								
emf	not crossed								
msi1	not crossed								
swn	not crossed								
Miscellaneous									
hmgb1	+	+	+	not yet	not yet	-			
pkl	+	+	+	+	+	-	+	mmCC	-
ttg925	+	+	+	+	+	-	+	mmCC	-
ttg1244	+	+	+	+	+	-	+	mmCC	-
wtCo	+	+	+	not yet	not yet	-			

Table 2: Overview about the advance and the results for the different crossing.
The table lists the material available from crosses between different mutants and the C2S line, the information obtained by genotyping and the results from the growth assays under selective conditions. “Required genotype” stands for heterozygous mutant alleles and hemizygous HPT gene (F1), or for homozygous mutant alleles (F2).

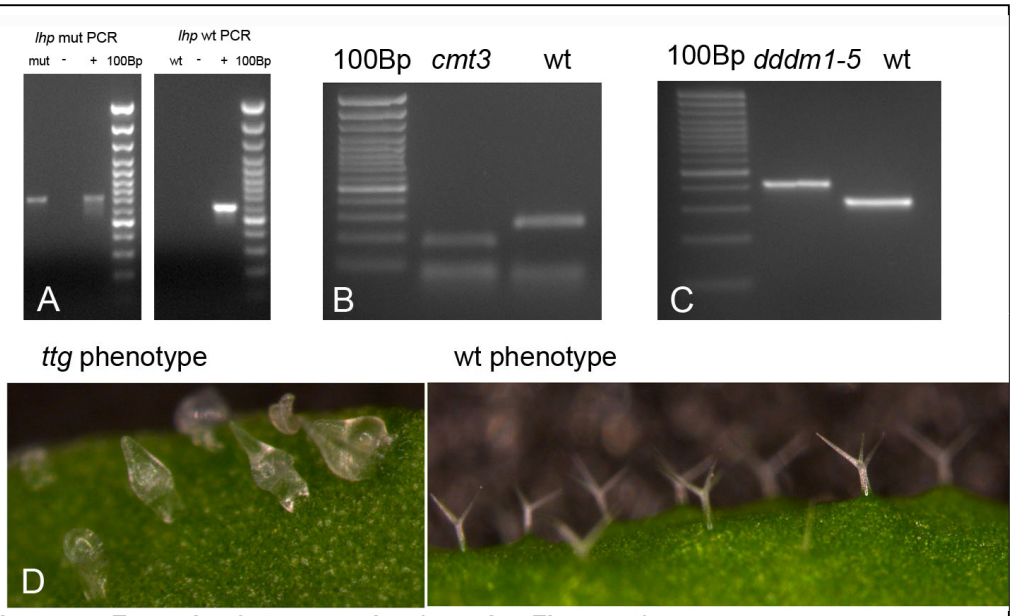


Figure 11: Examples for genotyping (see also Figure 10).
A: Detection of insertional homozygous mutant *lhp* by differential PCR (see also Table1) mut: existence of mutant allele; wt: no insertion; –: negative control; +: positive control of PCR reaction. B: Detection of the *cmt3-illa* allele by restriction fragment polymorphisms after PCR amplification (see also Table 1). C: Detection of insertion in the *ddm1-5* allele by PCR amplification polymorphism (see also Table 1). D: Detection of trichome abnormalities caused by the ttg2-9/2/5 allele.

In some mutants it was difficult to obtain the required genotype. Especially the evaluation of weak phenotypes and the under-representation of homozygous plants in the case of the *fas* mutants impaired to obtain the required plants. Therefore, some genotypes still need to be verified in later generations. For two mutants (*hog1, rts1-1*), it was not possible to recover the correct F1 genotype since the parental plants had not contained the mutant alleles. These crosses need to be repeated with plants from confirmed original seed batches.

5.4. Generation of F4 populations

Plants with the wanted genotypes from the segregating F2 populations were propagated further by two subsequent self-pollinations into generation F4. Again, this was achieved for most mutants (Table 2); however, some of the mutants showed reduced fertility and did not yield sufficient seed numbers for the hygromycin resistance screen or the molecular analysis, or they had germination problems. This was true for *fas1-1*, *met1-3* and *mim1-1*. Others were strongly affected by their mutational defect in F4. It was difficult to gain material for the molecular analysis, especially when there is a combination of reduced germination and reduced plant size, like in *ddm1-5* and *ttg2* mutants. However, material lacking key components like CMT3, KYP, SUVH2, MOM1 and AGO4 was successfully established as families homozygous for the mutation and homozygous for the HPT gene.

5.5. Hygromycin resistance assays

The screen for reactivation of the silent HPT gene in the mutant background was performed for the mutant material as available in the generation F2 and F4. F2 populations are expected to contain the HPT gene in the first generation of plants homozygous for the mutations after outcrossing, while genotyped and selected F4 populations would contain the HPT gene after transmission through two homozygous generations. I could not observe restoration of the hygromycin resistance in any of the F2 populations. This was true also for all crosses which were bred into F4, with the exception of *ddm1-5* where I found some weakly resistant plants (Figure 12). However, the phenotype of these plantlets, in comparison to fully resistant control plants, indicated only a partial reactivation: the plants are able to develop further than fully sensitive plants of the controls or other F4 populations, but are not able to survive the hygromycin selection on long terms (Figure 12). The limited reactivation was also demonstrated with molecular analysis (see below). The partially resistant plants were transferred first to non-selective *in vitro* conditions and after 2 weeks to soil, but did not recover and died after 2 more weeks.

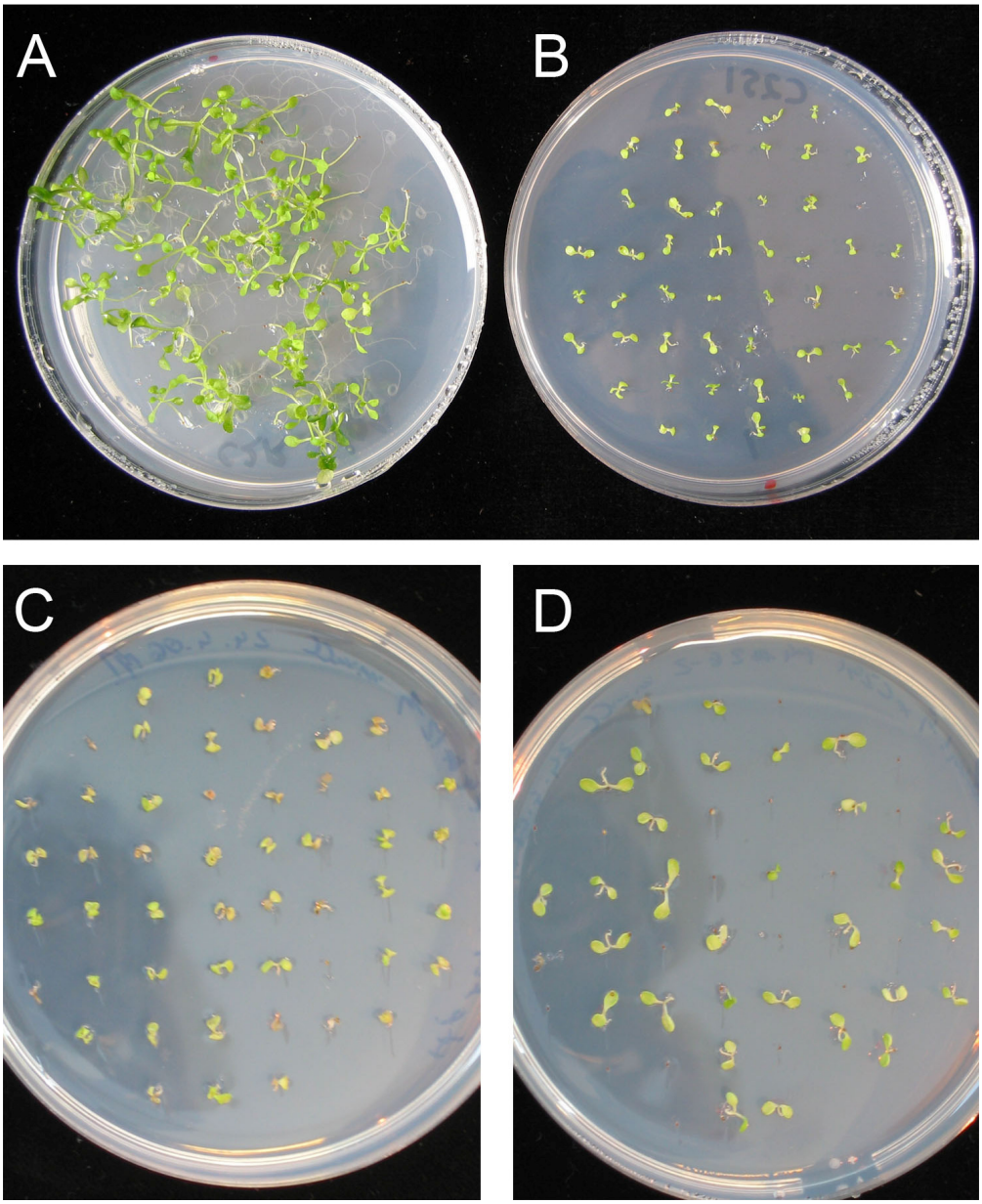


Figure 12: Resistance assays on selective medium (GMH10 containing 10 mg/l hygromycin), asking for activity of the HPT gene.
A: Control line C2R, full expression of the HPT gene. B: Line C2S, carrying the silent epiallele and used as parental line in the crosses. C: F4 population of the cross *ttg2-9/2/5* x C2S with genotype mmCC, showing no hygromycin resistance. D: F4 population of the cross *ddm1-5* x C2S with genotype mmCC, showing partial hygromycin resistance.

5.6. Molecular analysis of HPT gene expression

5.6.1. Northern Blot

Among all mutants where suitable material was available and tested, only *ddm1-5* restored hygromycin resistance partially and with a delay of two generations. *ddm1-5* is one of the most drastic silencing mutants and causes DNA hypomethylation as well as significant changes in chromatin architecture (Vongs *et al*. 1993; Probst *et al*. 2003). The other mutants did not show any regain of hygromycin resistance at all. Therefore, I decided to investigate the expression level of the HPT gene in my crosses at the molecular level, to see if the mutations had any effect on the C-locus at all. The results of the molecular analysis showed a more distinct picture of the effects in the different mutations. Analyzing the transcriptional level of the HPT gene by northern blot (Figure 13) revealed a relatively strong reactivation in the *ddm1-5* background. Though significant, the amount of HPT mRNA is not as high as in the fully resistant line C2R, and thereby correlates with the limited resistance found in the selection assay. I observed weak HPT expression also in heterozygous *met1-3* (MmCC) plants and very faint bands in *bru1-1*, *mom1-1*, *ttg2-9/2/5* and *ttg2-12/4/4* plants. The northern blot was rehybridized with a TSI probe (Figure 13). TSI stands for Transcriptionally Silent Information and the probe represents a family of repetitive DNA sequences, which are positioned near to the centromeric region of all chromosomes. These repeats are normally methylated and not transcribed in wild type plants, but hypomethylated and expressed in several silencing mutants (Steimer *et al*. 2000). With the TSI probe I found some reactivation in *bru1-1, cmt3-illa, ddm1-5, met1-3, mom1-1, mom1-2, ttg2-9/2/5 and ttg2-12/4/4*. The spectrum of HPT reactivation is therefore overlapping, but not identical with that of TSI reactivation.

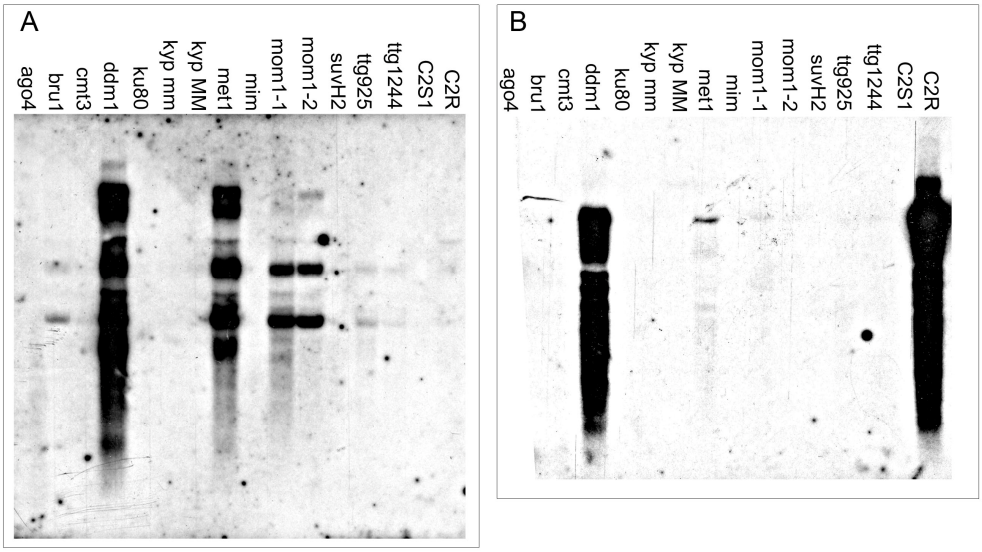


Figure 13: HPT and TSI expression analysis by northern blot.
Total RNA preparations from homozygous mutant plants (met1: heterozygous) containing the HPT gene in F4 generations was hybridized to a probe representing the transcriptionally silent pericentromeric repeats TSI (A) or the HPT open reading frame (B).

5.6.2. Reverse transcription-PCR

HPT expression was further monitored by reverse transcription. In comparison to the northern blot, reverse transcription assays are more sensitive and yield specific information about the region of the transcript under investigation, but they cannot reveal the length of a transcript. Unfortunately, the analysis was hampered by an erratic source of contamination in the PCR reactions in the whole lab. This problem could not be solved in the time frame of my thesis and the data therefore are not conclusive in this part.

5.6.3. Southern blot

Some mutants are expected to act on DNA methylation, like *cmt3, ddm1 and met1.* Therefore, I performed Southern blot analysis with methylation-sensitive enzymes for the HPT gene in the background of these mutations, including that of *ago4, mom1,* C2S and C2R as controls. The experiment was done by digesting genomic DNA with the methylation-sensitive restriction enzymes *Hpa*II (not cutting $^{m}C^{m}CGG$ or $C^{m}CGG$) and *Msp*I (not cutting $^{m}CCGG$ but cutting $C^{m}CGG$). The digested DNA was run on a gel and then blotted to a membrane. The probes I used for subsequent hybridization were specific for the C-locus and the 180bp repeats. The latter are found in the centromeric region and are normally highly methylated at CG and CNG sites and transcriptionally inactive. They have been shown to be demethylated at cytosines in all sequence context in the background of different silencing mutants and were used to control the general effect of the mutations. Indeed, the methylation patterns of the repeats in the different mutants were as described before (Vongs *et al.* 1993; Jeddeloh *et al*. 1999; Bartee *et al.* 2001; Lindroth *et al*. 2001; Kankel *et al*. 2003; Saze *et al*. 2003; Zemach *et al*. 2005):

- *met1* is important for the maintenance of methylation patterns at both types of sequences with a preference for ^{m}CG. Here I expected a more pronounced loss for ^{m}CG methylation.
- *cmt3* is a key determinant for the CNG methylation. I expected a loss in ^{m}CNG, but not in ^{m}CG methylation.
- *ddm1-5* is important for CG as well as CNG methylation, therefore I expected a loss of ^{m}C at both sites.

While these effects can be seen with the 180 bp probe (Fig.14), other tested mutants (*ago*4-1, *mom*1-1, *mom*1-2) do not show any effect on methylation at the repeats as expected based on previous work (Amedeo *et al*. 2000; Tariq *et al*. 2002) and their pattern resembles that of the two test lines C2S and C2R and the wild type (data not shown). Slight differences in the size of the large fragments with *Hpa*II are due to different efficiency of restriction digestion, as could be seen on the ethidium bromide-stained gels (data not shown).

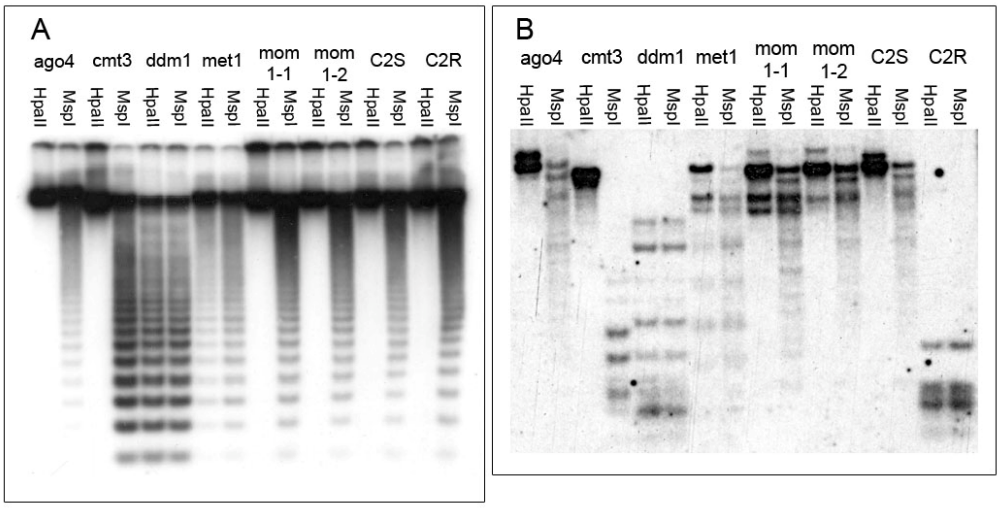


Figure 14: Analysis of DNA methylation by Southern blot.
Genomic DNA from homozygous mutant plants (met1: heterozygous) containing the HPT gene in F4 generations was digested with restriction enzymes HpaII and MspI, and hybridized to a probe representing the highly methylated centromeric 180 bp repeats (A) or the promoter and the HPT open reading frame of the C-locus (B).

The HPT probe reveals the following finding. While the HPT gene in genomic DNA of C2R (containing the expressed version) is cut into fragments smaller than 1 kb by both enzymes, thereby indicating no DNA methylation at CG or CNG, the inactive HPT in line C2S is not well digested by either enzyme, rendering fragments of larger than 5 kb (Figure 14). The HPT gene in the background of *ago*4-1, *mom*1-1 and *mom*1-2 remains hypermethylated as in the parental C2S line. Smaller fragments indicating hypomethylation due to the mutations are found only for *MspI* digests in *cmt*3, and for *Hpa*II and *Msp*I digests in *ddm*1-5 and *met*1-3. Since no HPT transcript was found in the *cmt*3 progeny, the loss of CNG methylation is obviously not sufficient to reactivate the gene, and the different level of demethylation at CG between *ddm*1-5 and *met*1-3 corresponds well with the different level of reactivation described earlier.

6. Discussion

6.1. Conclusions

In the course of the work presented here, I have applied a reverse genetic approach to investigate which *trans*-acting factors are required for maintenance of polyploidy-associated transcriptional gene silencing at a single copy transgene in *Arabidopsis*. Although the silent epiallele was first observed in tetraploid plants, it was stable also in diploid derivatives, and this allowed to introgress the silent locus into the background of numerous of recessive mutants, asking for continuity or release of the silencing upon the lack of defined epigenetic regulators.

I have chosen mutants affected in DNA modification, DNA repair or chromatin features. I was successful in generating the expected hybrids for most of these mutants. I also achieved to generate most F2 and many F4 populations with the required genotypes. The reactivation and demethylation of the *HPT* gene and the partial resistance in F4 *ddm*1-5 mutant background confirm earlier results and can be taken as a proof that the experimental concept was correct. However, it also revealed that, in spite of the relatively strong reactivation of the *HPT* gene in *ddm*1-5, the amount of transcript was lower than that provided by the active epiallele and not sufficient for a comparable resistance level. DDM1, a SWI2/SNF2 chromatin remodelling factor, is not directly acting on methylation. The loss of methylation and the resulting morphological aberrations are increasing over subsequent generations. The Southern blot data show that methylation is not completely lost in the F4 generation, and the transcriptional level achieved so far may not be high enough to confer complete resistance. Delayed demethylation, in comparison to that of repetitive sequences, has been observed also for other single copy loci in a *ddm* mutant background (Vongs *et al*. 1993). Therefore, as the silent HPT gene is a single copy insert, more generations without functional DDM1 are probably needed to restore complete resistance.

All other mutants so far analyzed in F2 or F4 did not restore hygromycin resistance. However, some of them caused a limited reactivation of *HPT* transcription detectable on Northern blots. The *met*1 mutant yielded a

detectable *HPT* signal in the Northern blot which corresponds to approximately 3 % compared to the fully active C2R line. The signal was much weaker than that of the repetitive TSI sequences used as a control. This occurred in plants that were heterozygous for the mutants. However, although a loss-of-function mutation is expected to be recessive, this result is plausible based on the role of MET1 during gametogenesis (Saze *et al*. 2003). In animals, gametes are fusing directly after or even before the end of meiosis, whereas in higher plants the haploid products of meiosis undergo further mitotic divisions before zygote formation. In female gametogenesis, the haploid gametocytes undergo three further mitotic cell divisions, producing an embryonic sac containing eight nuclei. The two male sperm nuclei are formed by two postmeiotic divisions. During these replications, DNA methylation can get passively lost if the mutated allele is inherited within the haploid cells . The *HPT* gene that is transmitted together with the mutant allele through the haploid gametophytes would thereby lose DNA methylation at CG as well as at CNG. If both DNA strands have lost their DNA modification, the methylation pattern is not regained immediately in the heterozygous progeny. However, the loss of methylation is not complete and correlated with only a minimal amount of *HPT* transcription, not sufficient to confer resistance. It will be interesting to see if this represents a final limit of demethylation upon the lack of MET1 or if there is a cumulative hypomethylation over subsequent generations.

DDM1 and MET1 are both affecting DNA methylation. Therefore, other mutants that modify the regulation of DNA methylation were especially interesting in their effect on the silent epiallele. While for the crosses with *drm*1 and 2 the required double mutant genotype is not yet available, the progeny of *cmt*3 could be included in my analysis. As expected (Bartee *et al*. 2001; Lindroth *et al*. 2001), the *HPT* gene shows significant loss of CNG methylation, but RNA analysis revealed no *HPT* transcription. This leads to the conclusion, that either loss of methylation including that at CG is required to restore *HPT* transcription, CNG methylation does not influence gene expression in this case, the lack of CMT3 does not completely remove CNG methylation at the *HPT* gene, or that CMT3 acts in silencing together with other components. Future experiments will challenge these alternatives.

Although the correlation between transcription/hypomethylation and silencing/hypermethylation is strong in general and also supported by the data discussed so far, there are some exceptions of this rule. (Amedeo *et al*. 2000) have shown in the *mom*1 mutation, that loss of methylation is not always obligatory for reactivation of transcriptionally silent genes. Their evidence was generated based on a repetitive transgenic insert of the same *HPT* gene as in the single copy insert of my analysis. Interestingly, I found a faint *HPT* expression in the background of both *mom*1 alleles, again much weaker than the TSI expression. However, the *HPT* signal is one order of magnitude lower than the already limited transcription in *met*1. Since I used leaf material to generate the total RNA preparations in this study, and MOM1 has a role in silencing throughout most leaf cells the very limited *HPT* transcript level is likely to reflect a generally low level of reactivation, and not a significant reactivation restricted to just a few cells.

I found also some faint reactivation in *ttg2-9/2/5* and *ttg2-12/4/4*. These mutations are allelic supressor mutations for TGS (Hofmann 2004). In the course of the *arabidopsis genome initiative 2000* (http://www.arabidopsis.org) this locus has been identified as coding for a WRKY-type DNA binding protein. This is a plant-specific superfamily of transcription regulators with at least 72 members in *Arabidopsis.* Interestingly, the mutations affect a member with a tissue-specific transcription regulation. It is expressed in developing seed covers, in root tips and in all stages of trichome development. Therefore, reactivation of the HPT upon lack of TTG2 might be limited to only a subset of cells, explaining the very faint reactivation signals. Additionally, TTG2 is not known to be involved in DNA methylation regulation, so this gene could be involved in other regulatory pathways (Hofmann 2004).

In all the other mutants where the required material was available and tested, no *HPT* transcription could be detected. Although the mutants expected to modify epigenetic regulation at the chromatin level are involved in different, complex networks, none of them seems to have an effect on transcriptional silencing at the *HPT* gene at all. One possible explanation would be, that this locus is controlled by two overlapping silencing networks, so that one mutation alone is not sufficient to trigger reactivation. It might be, that loss of chromatin modifications or improper chromatin assembly is not

suffcient if not accompanied by a certain amount of DNA demethylation, allowing access of transcription factors. Alternatively, silencing at the *HPT* gene might be under control of regulators different from those identified to be involved in spontaneous silencing of mainly repetitive targets independent from polyploidy of the genome.

The latter explanation provides an optimistic view on the parallel approach ongoing in the lab, to identify *trans*-acting regulators of polyploidy-associated gene silencing in a forward screen, by selecting for hygromycin-resistant plants in the M2 generation after mutagenesis of line C. Several of such plants were found and are likely to be affected in genes others than those tested in my analysis, thereby hopefully allowing to identify previously unknown genes or even new pathways of epigenetic regulation.

6.2. Outlook

Although I obtained a lot of F4 generations with the wanted combination of homozygous mutations and *HPT* gene, some gaps need to be filled. The material for this is available in different preceeding generations. These require genotyping, further propagation and subsequent analysis in resistance assays and by molecular techniques, as Southern blot, northern blot and RT-PCR analysis. The most time- and labor-intensive work is the genotyping, especially in the segregating F2 generations. In many cases, it requires 4 independent PCR reactions per plant, for mutant and wild-type allele and *HPT* gene and empty insertion site respectively. I started with 20 plants per cross in F2 and, for many combinations, ended up with approximately 5 plants having the wanted homozygous mutant genotype and at least one copy of the HPT gene. The perfect genotype, homozygosity for the mutant allele as well as for the C-locus, was represented by a maximum of one or two plants. Some mutations (*bru1-1, dcl3-1, hmgb1 and rdr6*) are located on the same chromosome as the C-locus, and even those are distant enough to expect sufficient recombination in between. Based on the classical Mendelian rules of independent segregation, I expected 18.75% (3/16) for hemizygous C-locus and 6.25% (1/16) for homozygous C-locus mutant plants. Therefore, the values for most crosses fit with the expectation. Those cases were the mutant

genotype was underrepresented might be caused by the damage provoked by the mutation. For these I have to go back one or two generations and grow larger populations. Even if short in comparison to other plants, the generation time of Arabidopsis is a limiting step, and it was therefore not possible to fill all gaps in the time frame of the thesis. From the missing mutants, most interesting to complete is the progeny from the cross with *drm*1,2. Since it is involved in establishing as well as in maintaining methylation (Cao and Jacobsen 2002), and *ddm*1 and *met*1 have some limited effect while *cmt*3 does not. This material might give more clues about the role of DNA methylation. However, also the effect of mutants involved in RNA-based silencing is interesting, although there is no hint so far about a role of RNA in PA-TGS. Also the mutations in polycomb protein genes are interesting candidates, although they have been associated so far with the regulation of rather specific endogenous target genes, reviewed by (Ringrose and Paro 2004).

Beside of the limited role of DDM1 in maintaining silencing at the *HPT* gene, none of the tested mutants had a significant effect, and therefore, we still do not know which *trans*-acting regulators are involved in PA-TGS. However, the material I created will be an interesting basis for further studies about the role of the diverse factors on this specific locus under investigation. For example, the lack of the histone methyltransferases SuvH2 and SuvH4 have been shown to modify histones at different genomic locations and were identified by reactivating different silent genes (Cao and Jacobsen 2002; Jackson *et al.* 2002; Malagnac *et al*. 2002; Naumann *et al*. 2005). Since they do not seem to act on the single copy *HPT*, there are two alternatives. Either their lack does not affect histones associated with the *HPT* locus, or it changes histone modification without an effect on gene activity. The first case would indicate some locus- or target-specificity of the histone modifiers, while the second case would question a tight relationship between the modification and state of gene activity. Both alternatives would present valuable information about the molecular nature of epigenetic information. Experiments to analyze the chromatin state at the *HPT* epialleles are underway, and plants with the *HPT* gene introgressed into the mutant backgrounds will promote this analysis. Similarly, a detailed investigation of the DNA methylation status is in

progress, and the *HPT* gene in the methylation mutant backgrounds will be included.

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8. Appendix

Table 1: Summary of mutant alleles and genotyping protocol

Type of mutation	Ecotype	Morpho-logical phenotype	Allele	Primer name	Primer sequence	Fragment length (bp)	Restriction enzyme
AGO4 AT2G27040							
point mutation	Ler/Col	no	wt	ago4-F ago4-R	TGA CTG ACA GCT GAA AAT GGG ATG TGG AT GCC ACT CCC TAG AAC TCA CCA CCT AAG TT	450 + 550	*AvaII*
			ago4-1	ago4-F ago4-R	TGA CTG ACA GCT GAA AAT GGG ATG TGG AT GCC ACT CCC TAG AAC TCA CCA CCT AAG TT	no cut	
BRU1 AT3G18730							
30 bp insertion	Ws	yes, fasciation	wt	bru-1 bru-2	GAG TTA GTT GGT TAT GCA TAG GGA CAC CAT ACA TCT GGC TTG ATG A	130	no
			bru1-1	bru-1 bru-2	GAG TTA GTT GGT TAT GCA TAG GGA CAC CAT ACA TCT GGC TTG ATG A	100	
CLF AT2G23380							
complete deletion	Ws	yes, leaf curling	wt	clf50-F clf50R	GGT CTT CTA TGG AAC GAG GAG G GCC TCC TCT TCA CTA TCA CTG C	456	no
			clf50	clf50-F clf50-R	GGT CTT CTA TGG AAC GAG GAG G GCC TCC TCT TCA CTA TCA CTG C	no fragment	
CMT3 AT1G69770							
point mutation	Ws	no	wt	CMT3 F mod CMT3 R mod	CTT GAA CAG TAA TTG CCG GGG T CAG CAT GTC AAC GAC GTT TTC C	240 2 sites	*Mse1*
			cmt3 illa	CMT3 F mod CMT3 R mod	CTT GAA CAG TAA TTG CCG GGG T CAG CAT GTC AAC GAC GTT TTC C	240 3 sites	
DCL3 AT3G43920							
T-DNA Salk 005512	Col	no	wt	dcl3-F dcl3-R	CTG AAG AGC GTG AAG GAG TGG CTC CAA GCA GAC TTC AGC CGC	522	no
			dcl3-1	dcl3-F LBc1	CTG AAG AGC GTG AAG GAG TGG TGG ACC GCT TGC TGC AAC TCT	~650	
DDM1 AT5G66750							
82 bp insertion	Zh	no	wt	ddm- ddm+	AAA GGA CCC ATT TAC AGA ACA C CGC TCT CGA AAT CGC TCG CTG TTC	332	no
			ddm 1-5	ddm- ddm+	AAA GGA CCC ATT TAC AGA ACA C CGC TCT CGA AAT CGC TCG CTG TTC	414	
DRD1 AT2G16390							
point mutation	Col	no	wt	drd1-F drd1-R	GAT GAG CTT CCT GGA CTT GCT G CTT CCT CAG GTG ATG ACC CAG C	636	*BclI*
			drd1-6	drd1-F drd1-R	GAT GAG CTT CCT GGA CTT GCT G CTT CCT CAG GTG ATG ACC CAG C	413 + 223	
DRM1,2 AT5G15380, AT5G14620							
T-DNA Wisconsin	Ws	no	wt	drm1-F drm2-F	TGC GAT TGA CAA TTT CCA ATT TTC TCC AT CCT CCT CCA GTA AAC TGA CGA CGA TAC AA	2160	no
				drm1-R drm2-R	CTT GGT GTC TCA GTG TAT GTT CG GGT AGA CGA ATC GGC TCG TCA TC	2110	
			drm1	TL2 TL2	TGG ACG TGA ATG TAG ACA CGT CG TGG ACG TGA ATG TAG ACA CGT CG	1600	
			drm2	drm1-R drm2-R	CTT GGT GTC TCA GTG TAT GTT CG GGT AGA CGA ATC GGC TCG TCA TC	590	
EMF AT5G51230.1							
35 bp deletion	Ws	no	wt	emf2-F emf2-R	GAC GAA GTG TGT CCG TTT GTA CTC G TGC TAA TAC TCT GCA AAG GGT C	400	no
			emf2	emf2-F emf2-R	GAC GAA GTG TGT CCG TTT GTA CTC G TGC TAA TAC TCT GCA AAG GGT C	365	

Type of mutation	Ecotype	Morpho logical phenotype	Allele	Primer name	Primer sequence	Fragment length (bp)	Restriction enzyme
FAS1 AT1G65470							
point mutation	Enk	yes, fasciation	*fas1-1*	no PCR			no
FAS2 AT5G64630							
point mutation	Ler	yes, fasciation	*fas2-1*	no PCR			no
HEN1 AT4G20910							
T-DNA Salk 049197	Col	no	wt	hen1-F hen1-R	GAG GTA GGG ACT GGA TCC ATG GTA ATT TAA ACA TGG ACG TTC TGT G	682	no
			hen1-5	hen1-F LBc1	GAG GTA GGG ACT GGA TCC ATG TGG ACC GCT TGC TGC AAC TCT	~400	
HMGB1 AT3G51880							
T-DNA Sail 261 B02	Col	no	wt	hmgb1-F hmgb1-R	GCC TAC TAA ACG AGA GAC TCG CAG CTT TCT CTT CAT ATG GAG C	497	no
			hmgb1	hmgb1-F LB3 short	GCC TAC TAA ACG AGA GAC TCG GCA TCT GAA TTT CAT AAC CAA TCT CG	~350	
HOG1 AT4G13940							
point mutation	Ler	no	wt	hog F hog R	AAG CCA CAG ACT GAC AGG TG GGA GGC TTG TAT GGT CCC TC	328	no
			hog1	hog F hog R	AAG CCA CAG ACT GAC AGG TG GGA GGC TTG TAT GGT CCC TC	328	
KU70 AT1G16970							
T-DNA Wisconsin	Ws	no	wt	ku70-2 ku70-11	TAC TAC ACC AGA CAA AGC CGT GAT GGT T ACT TGG GAT AGC TCT TCC ACA GTA	939	no
			ku70	ku70-11 LB-CD6	ACT TGG GAT AGC TCT TCC ACA GTA GAA CAT CGG TCT CAA TGC A	639	
KU80 AT1G48050							
T-DNA ABRC	Ws	no	wt	ku80-1 ku80-2	GAG GAA GTA CTA AAG CCT GAT C CCG CAA CAA TCT CTT AGA AGC CT	402	no
			ku80	ku80-2 LBku80	CCG CAA CAA TCT CTT AGA AGC CT GAT TCT TTT TAT GCA TAG ATG CAC	>230	
KYP1 AT5G13960							
T-DNA Salk 041474	Col	no	wt	kyp-1 kyp-2	CCT GTT CAA TTG ATT TCC ATG TGG T TCT ACA AGG AAT ATC ACC TGC C	430	no
			kyp1	kyp-2 LB-b1	TCT ACA AGG AAT ATC ACC TGC C GCG TGG ACC GCT TGC TGC AAC T	643	
LHP1 AT5G17690							
T-DNA Salk 011762	Ws	yes, early flowering	wt	LHP-F LHP-R	GTA TTT GTA GGG CAA GGT TCA G TCC CAA CAC TCC CAC TGT TGG	650	no
			lhp1	LHP-R LBc1	TCC CAA CAC TCC CAC TGT TGG TGG ACC GCT TGC TGC AAC TCT	~700	
MET1 AT5G49160							
T-DNA Hohn lab	Col	no	wt	MEF-1 MER-1	GAT TGT GTC TCT ACT ACA GAG GC GTT AAG CTC ATT CAT AGC CTT GC	1000	no
			met1-3	MEF-1 barbiG	GAT TGT GTC TCT ACT ACA GAG GC GGT TCT TAT AGG GTT TCG CTC	~1200	
MIM AT5G61460							
T-DNA Versailles	Ws	yes, root length	*mim 1-1*	no PCR			no
MOM1 AT1G08060							
T-DNA Paszkowski lab	Zh	no	wt	pro3- pro4+	CAC TTT CCG ATT TCG ATT CTC G CAT GAC TCC CCC AGC CAG TAG	260	no
			mom 1-1	pro5+ barbiE	GTG GTT ACT GAT CAA GTC TCG GTG AAG GGC AAT CAG CTG TTG	600	
T-DNA SALK	Col	no	wt	301 T36 301 SP1	GCA TAC CTG CAG GCA ATG AT GCA ACT GTA GCA CAT GCA TCC AGC	600	no
			mom 1-2	301 T36 LB-3	GCA TAC CTG CAG GCA ATG AT C	500	

Type of mutation	Ecotype	Morpho logical phenotype	Allele	Primer name	Primer sequence	Fragment length (bp)	Restriction enzyme
MRE11 AT5G54260							
T-DNA Salk 054418	Col	variable	wt	mre11-1 mre11-3	CCA ATG GAT GAG GCC TGA AGT T GTC TGC CAC CAC CAT AAC AT	700	no
			mre11-3	mre11-3 LBc1	GTC TGC CAC CAC CAT AAC AT TGG ACC GCT TGC TGC AAC TCT	477	
MSI1 AT5G58230							
over-expressing and downregulating		no	wt	P35S-F msi1-oe	GTG ATA TCT CCA CTG ACG TAA GGG GCT CTC GGA GGT ATG GGT ACC G		no
			msi1-oe	P35S-F msi1-oe	GTG ATA TCT CCA CTG ACG TAA GGG GCT CTC GGA GGT ATG GGT ACC G		
PKL1 AT2G25170							
point mutation	Col	yes, short plants	*pkl1-1*	no PCR			no
RDR2 AT4G11130							
T-DNA Garlic 1277	Col	no	wt	rdr2-F rdr2-R	TCC GGT TCT TAG AAC TCC ACC CAT CAA TCT CAG AAG CGT CAC	458	no
			rdr2-1	rdr2-R rdr2 mut	CAT CAA TCT CAG AAG CGT CAC C	~350	
RDR6 AT3G49500							
point mutation	Col	no	wt	rdr6-F rdr6-R	GCA GGG ATA CTT GAA CAT GGC C CAA ACA TTT GTG ACC CCA TGC C	850 + 250 + 150	*MvaI*
			rdr6	rdr6-F rdr6-R	GCA GGG ATA CTT GAA CAT GGC C CAA ACA TTT GTG ACC CCA TGC C	850 + 400	
RTS1 AT5G63110							
deletion	Col	no	wt	rts1-1 F rts1-1 R	GAT TCT GAG TGA GAG ACG GAG AGC CAT ACG GAT CCG GTG AGG	165	no
			rts1-1	rts1-1 F rts1-1 R	GAT TCT GAG TGA GAG ACG GAG AGC CAT ACG GAT CCG GTG AGG	~130	
SUVH2 AT2G33290							
T-DNA Salk 079574	Col	no	wt	salk574F salk574R	GTA CAT TGT TAC CAT TTC CTG AC AAG TAC ATG ATT CTT CAT ACT CTC C	524	no
			suvH2	salk574R LB-b1	AAG TAC ATG ATT CTT CAT ACT CTC C GCG TGG ACC GCT TGC TGC AAC T	~650	
SWN3 AT4G02020.1							
T-DNA Salk 050195	Ws	no	wt	swn3-F swn3-R	CTG ATG TTG CTG GAT GGG GTG C GGG TTT AGC TGA GTG ATT GGC	382	no
			swn3-1	swn3-R LBc1	GGG TTT AGC TGA GTG ATT GGC TGG ACC GCT TGC TGC AAC TCT	~300	
TER AT5G16850							
T-DNA Wisconsin	Ws	no	wt	tert-6 tert-7	CTA GGA CAT ATC CAT CAA GGG C GGA AGC TGT ATT GCA CGA ACG	458	no
			ter	tert-6 TL-2	CTA GGA CAT ATC CAT CAA GGG C TGG ACG TGA ATG TAG ACA CGT CG	~600	
TTG925 / TTG1244 AT2G37260							
point mutation	Col	yes, modified trichomes	*ttg2-9/2/5*	no PCR			no
			ttg2-12/4/4	no PCR			no
C-locus/HPT insert (close to AT3G54810)							
transgenic insertion	Zh	no	wt	CFconfirm CFinsR	GAA GTA ATG TTA GAT GTT CAA G AGT TAG GTG TGA GAA ACA CTT C	912	no
			C-locus	HPTstart HM-	GAT CCC GGG GGC AAT GAG ATA TG GTG TAT TGA CCG ATT CCT TGC GG	450	

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Curriculum Vitae

Personal data

Name:	Michael Milos
Date of birth:	1. August 1979
Place of birth:	Echirolles / France
Nationality:	Austria

Education

1985 - 1989	Elementary School Notre Dame de Sion, Vienna, Austria
1989 - 1995	Albertus Magnus Secondary School, Vienna, Austria
1995 - 1999	Marianum secondary school, Vienna, Austria
22. June 1999	Matura final exam
Since 2000	Studies of molecular biology at the University of Vienna

Professional experience

Oct. 1999 - Dec. 1999	Institute for Applied Genetics, University of Natural Resources and Applied Life Sciences, Vienna Prof. Dr. Marie-Theres Hauser
Jul. 2001 - Aug. 2001	Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Stuttgart, Germany Dr. Franz-Josef Johannes
Jul. 2002 - Aug. 2002	Department of Pediatrics, Division of General Pediatrics, Medical University of Vienna, Vienna, Austria. Prof. Dr. Zsolt Szepfalusi
Nov. 2003 - Dec. 2003	Center for Brain Research Medical University of Vienna
Jul. 2004 - Sept. 2004	Gregor Mendel Institute of Molecular Plant Biology Austrian Academy of Sciences, Vienna Dr. Ortrun Mittelsten Scheid
Apr. 2005 - May 2005	Center for Brain Research Medical University of Vienna Univ.-Doz. Dipl.-Biol. Dr. Monika Bradl
Jul. 2005 - Jul. 2006	Gregor Mendel Institute of Molecular Plant Biology Austrian Academy of Sciences, Vienna Dr. Ortrun Mittelsten Scheid

Extracurricular activities

Jan 2000 - Aug 2000	Military service at Maria-Theresien Kaserne Vienna