The nuclear pore complex: Its role in chromatin structure and RNA export

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Emilia Hristova

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auf Antrag von

Prof. Dr. Thomas Boller

Dr. Etienne Bucher

Dr. Susana Rodriguez-Navarro

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The Dean of Faculty

Prof. Dr. Jörg Schibler

To my beloved husband

Frederic

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SUMMARY

Epigenetic regulation of gene expression is a relatively new and rapidly developing research field. It studies the mechanisms of regulation of gene expression, which although heritable, occur independently of changes in the DNA sequence and certainly aid to the complexity of the this process and provide fine tuning to it.

Now, it is known that many essential processes in plants such as: development, signaling, innate immunity, symbiosis, etc. are epigenetically regulated. However, little is known about the epigenetic regulation of cell's specialization and differentiation. Here, we postulate the existence of a tissue-specific epigenetic code. This study consists primarily of a forward genetic screen, based on a tissue-specific GFP reporter line- silex, which reports adequately on the epigenetically regulated developmental gene *APUM9* in *Arabidopsis thaliana* (Chapter 2). Of the numerous mutant lines that we recovered in the mutant screen, two epigenetic regulators are presented in this thesis.

First, a new allele of the well-known histone deacetylase HDA6 was recovered and it was found that this protein has separable activities in the euchromatin and the heterochromatin (Chapter 2). The second mutant was found to be defective in AtSAC3B, a nuclear pore associated protein, which up until now hasn't been associated with epigenetic regulation of gene expression. The homologues of AtSAC3B in different model organisms are involved in nuclear-cytoplasmic export of mRNAs. By using different mutant alleles of *AtSAC3B* for studying the nuclear-cytoplasmic export, the requirement of the protein in the process in plants was validated. The assessment of the transcripts present in the different cell compartments, nucleus and cytoplasm of the mutant, revealed an export bias towards antisense RNAs (asRNAs), suggesting that the selectivity of the export process in plants is dependent on AtSAC3B (Chapter 3). This indicated that AtSAC3B is an important player in the regulation of gene expression through its' selectivity in the RNA export process.

Likewise the nuclear pore complex that is known to influence the chromatin organization, the studies on the chromatin organization and the dynamics of selected histones modifications in *atsac3b*, revealed the importance of AtSAC3B for the heterochromatin organization in plants (Chapter 4).

Chapter 1

General Introduction

The history of epigenetics

The term "epigenetic" was first introduced by Waddington, a developmental biologist, who used it to refer to "branch in biology that studies the interactions between genes and their products, which brings the genotype into being" (Waddington, 2014). From a broader perspective, Waddington's definition of epigenetics explains why despite of the identical genetic information that cells carry, they can develop into different cell types and tissues. His "epigenetic landscape" model, illustrates the process of cells specialization (Fig.1.1). In this model the pluripotent cell is represented as a marble at the top of a hill. The valley down the hill contains many paths that the marble can roll down and each of them represents different cell fates. The features of the landscape, such as: branching, steepness, etc. are determined by a network of interactions between genes from underneath the valley's surface.

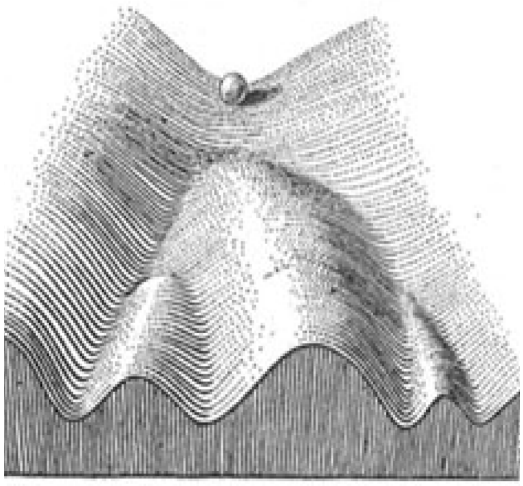


Figure 1.1 Waddington's "epigenetic landscape"

(Taken from (Goldberg et al., 2007))

The marble represents a pluripotent cell evolving in the epigenetic landscape. Its' fate is determined by the canals in which the ball is rolling.

To date there are numerous definitions of epigenetics. In this thesis the following definition will be used: Epigenetics is the study of mitotically and /or meiotically heritable changes in the gene functions without any changes in the DNA sequence (Haig D., 2004). Therefore, epigenetics describes a type of inheritance that is not in accordance with the classical Mendelian laws of heredity.

Among the earliest examples for the existence of a non-Mendelian type of inheritance was the discovery of the *paramutation* in maize (Brink et al., 1968). During studying anthocyanin genes in maize Brink demonstrated that, the epigenetic state of one allele (paramutagenic) can be transferred to another (paramutable) allele. The coexistence of the two alleles in a heterozygous state can result in changes in the expression levels of the paramutable allele due to gene silencing (Brink et al., 1968; Coe, 1968; Pilu, 2011).

Later work in Arabidopsis (*Arabidopsis thaliana*), revealed the existence of a mechanism that resembles paramutation and differs from it only by being non-allelic. Namely, in the phosphoribosylanthranilate isomerase (PAI) system composed of four genes at three unlinked loci (*PAI1*, *PAI4*, *PAI2* and *PAI3*), spontaneous deletion of *PAI1* and *PAI4* due to genome rearrangements causes activation of the other two genes, resulting in a mosaic phenotype. This indicted that *PAI1* and *PAI4* have paramutagenic control over the unlinked *PAI2* and *PAI3* loci (Bender and Fink, 1995; Martienssen, 1996).

To understand the molecular mechanism underlying paramutation, two models have been proposed. The "paring" model proposes direct interaction between two homologous chromosomal regions. During this interaction one of the regions (paramutagenic) induces modification at the other (paramutable), by transferring epigenetic marks (DNA methylation, histone modifications) and/or altering chromatin organization. The second model suggests existence of a mediator molecule (RNA) involved in the transfer of the epigenetic state from the paramutagenic to the paramutable locus, and/or changes the expression levels of the paramutable one. These two models are not exclusive and can coincide (Arnheiter, 2007; Chandler and Alleman, 2008; Chandler and Stam, 2004).

Following the discovery of paramutation in maize, a number of other epigenetic phenomena have been observed in plants. Most of them can't be classified as paramutations in *sensu stricto* (because they are not induced by other alleles and/or are not heritable through meiosis), but all of them show the significant role of silencing in the epigenetic regulation of gene expression.

One of the very first transgene silencing events was demonstrated in tobacco. In transformation experiments with T-DNA (Matzke et al., 1989), was shown that sequential

transformation of tobacco plants with transgenes causes their inactivation. In doubly transformed plants, integration of a second T-DNA construct in the plant genome leads to inactivation of the first one in *trans*. This transgene inactivation was found to be reversible; it was associated with increased levels of DNA methylation in the promoter of the inactivated transgene and the inactivation efficiency was shown to be highly dependent on the insertion site (locus) of the second T-DNA. These experiments suggested that the sequence homology between the two transgenes was triggering the silencing event.

Transgene induced gene silencing was also shown to alter the expression of endogenous loci, which share sequence homology with the transgene construct. This phenomenon was called "co-suppression" due to the silencing of both loci (endogene and transgene) (Napoli et al., 1990; van der Krol et al., 1990). In an attempt to overexpress the petals pigmentation gene *chalcone synthase* (*CHS*) in petunia, in a substantial number of plants, the pigment synthesis was blocked and instead of the expected increase of the color intensity, white flower petals were observed. Follow-up work (Van Blokland et al., 1994), showed that the DNA methylation levels in the promoters of the *chs* genes wasn't changing. This indicated that unlike the earlier described case of transgene induced silencing in tobacco plants, where the silencing coincided with increased DNA methylation levels in the transgene promoter (Matzke et al., 1989), the mechanism underlying co-suppression is rather different. The *chs* genes were shown to be transcriptionally active, yet the mRNA levels coming from the endogene and the transgene were reduced.

The mechanisms underlying gene silencing have been elucidated and two types of gene silencing are known: transcriptional gene silencing (TGS) resulting from the inactivation of the promoters (silencing of the genes at transcriptional level/DNA level) and posttranscriptional gene silencing (PTGS) where mRNAs are degraded, or the protein synthesis is impaired. While TGS can be heritable, PTGS is reset after meiosis (Stam et al., 1997).

Posttranscriptional gene silencing (PTGS)

First described as "co-supression", PTGS is known under different names in different organisms: PTGS in plants (Napoli et al., 1990; van der Krol et al., 1990), RNA interference (RNAi) in animals (Fire et al., 1998) and quelling in *Neurospora crassa* (Romano and Macino, 1992). An umbrella term for all these phenomena is RNA silencing, since all of these gene silencing phenomena occur at the posttranscriptional level (Aufsatz et al., 2002a).

PTGS utilizes sequence homology of small RNAs (sRNAs) for targeting mRNAs for degradation. These small RNAs are 20-25 nucleotides long RNA molecules, products of endogenous or foreign double stranded RNAs (Baulcombe, 2004; Carthew and Sontheimer, 2009; Castel and Martienssen, 2013; de Alba et al., 2013; Hamilton, 1999; Hamilton et al., 2002; Mello and Conte, 2004; Sijen et al., 1996; Waterhouse et al., 1998).

The class of small RNAs is diverse and the two best-studied types of small RNAs are: small interfering RNAs (siRNAs) and the micro RNAs (miRNAs). These two classes of small RNA have different origins. siRNAs are primarily derived from transgenes and viruses, and are produced from long perfectly complementary double stranded RNAs as opposed to miRNAs, which are products from endogenous double stranded RNAs with imperfect complementarity that form a stem-loop hairpin structures (Carthew and Sontheimer, 2009; Tomari and Zamore, 2005).

The first miRNAs - *lin4* and *let7* were identified in Caenorhabditis *elegans* (Lee et al., 1993; Reinhart et al., 2000). The biogenesis of the miRNAs involves several steps starting from the Pol II-dependent transcription of the *MIR* genes (so-called "pri-miRNA", caped and polyadenylated transcripts), via formation of the stem-loop intermediate (known as "pre-miRNA"). This stem-loop precursor is than cleaved into miRNA:miRNA* duplexes (miRNA is the mature, miRNA* is the traveler molecule), a step which in plants is controlled by four Dicer like (DCL) RNase III endonucleases. Unlike in animals, in plants the formation of the miRNA:miRNA* duplexes occurs in the nucleus. The duplex is exported to the cytoplasm by the plant expotin 5 homologue-HASTY, where the duplexes are unwound by a helicase, and the mature miRNA is loaded into ARGONAUTE1 (AGO1, a PAZ and PIWI domain containing protein) to form the RNA-induced silencing complex (RISC). The miRNA in the RISC complex is then used for the sequence specific selection of the silencing targets by the RISC complex. AGO proteins form an RNase H-like fold,

with a slicer endonuclease activity (the PIWI domain), and can cleave targets that are complementary to the loaded miRNA. In plants, likewise in animals, miRNA mediated gene silencing can result in RNA cleavage or inhibition of translation (Beauclair et al., 2010; Li et al., 2013; Yang et al., 2012b).

Plants miRNAs are predominately 21 nucleotides (nt) long molecules, but their length can vary from 20 to 24 nt. This length variation is a result of differences in the activities of the different DCLs, namely DCL1 gives 21 nt, DCL2 gives 22 nt and DCL3-24 nt cleavage products (Bartel, 2004; Reinhart et al., 2002; Rogers and Chen, 2013).

Defects in miRNAs biogenesis and regulatory pathways have pleotropic effects on the plant development. This is due to the fact that about 50% of their targets are transcription factors that control different processes (Zhang et al., 2006a). Moreover, miRNA are mobile molecules, and in plants the silencing signal can be spread from cell to cell via plasmodesmata, or some of them move systemically (Brosnan and Voinnet, 2011; Melnyk et al., 2011). The miRNA regulate a) plant development: leaf, root (Guo et al., 2005), shoot development as well as floral transition phase (Chen, 2004; Wu and Poethig, 2006); b) signal transduction (Paul et al., 2015; Zhang et al., 2006a) c) innate immunity (Li et al., 2012).

Transcriptional gene silencing (TGS)

TGS is a silencing mechanism that inhibits transcription. A hallmark of TGS is its' association with increased DNA methylation in the promoters of the silenced genes. These changes in the epigenetic state of silent loci can be mitotically and/or meiotically heritable (Fagard and Vaucheret, 2000; Matzke et al., 2000). One of the main functions of TGS is to protect the host genome against transposable elements and transgenes (Bucher et al., 2012; Zilberman, 2008), but it also influences the expression of endogenous genes. TGS is associated with covalent modifications at DNA (DNA methylation) and at histone residues but also with changes in chromatin organization via chromatin remodelers (Goldberg et al., 2007).

DNA methylation

The methylation of cytosine residues in DNA, in a form of 5-methylcytosine (5mC) (Ehrich and Wang, 1981) is an epigenetic modification that plays an important role in transcriptional regulation. In mammalians DNA methylation occurs predominantly at the cytosine residues in CG sequence context with an exception of the embryonic stem cells, where DNA methylation can also be found in non-CG context (Lister et al., 2009). In plants, cytosine can be methylated in CG, CHG and CHH contexts (H=A, T, C) (Suzuki and Bird, 2008). Due to their symmetric nature DNA methylation in the CG and CHG contexts can be copied to the complementary DNA strand after DNA replication by specific DNA methyltransferase enzymes. This is not the case for CHH methylation where the DNA methylation information can be lost on the newly synthesized DNA strand. Therefore, CG and CHG methylation is also referred to as "symmetric", whereas methylation in CHH context is knows as "asymmetric".

In Arabidopsis, DNA methylation plays an important role in the maintenance of the genome stability (Chan et al., 2005; Lisch, 2009; Bucher et al., 2012; Mirouze et al., 2009; Miura et al., 2001; Zilberman, 2008). Genome stability can be compromised by transgene insertions and/or transposable elements. Transposable elements (TEs) are DNA elements, which have the potential to "move" within the genome, thereby causing mutations and genome rearrangement (translocations). DNA methylation keeps TEs in a silent (inactive) state (Martinssen and Colot, 2001; Lister et al., 2008; Zhang et al., 2009).

DNA methylation was also shown to play an important role in the genetic imprinting, in both - plants and animals (Chen et al., 2009). Imprinting is an epigenetic phenomenon, which can result in differential silencing of genes, part of chromosomes or entire chromosomes, depending of their parent of origin (Finnegan et al., 2000; Garnier et al., 2008; Köhler et al., 2012; Pfeifer, 2000; Zilberman, 2008). Gene imprinting in plants occurs predominantly in the endosperm. The differential expression of the two parental alleles is associated with differences in their methylation levels, and improper regulation of this process, leads to biallelic expression that can result in improper development (Bauer and Fischer, 2011; Kinoshita et al., 1999; Köhler and Aichinger, 2010). The best-described cases of imprinted genes in Arabidopsis are the maternally expressed *FLOWERING*

WAGENINGEN (*FWA)*(Kinoshita et al., 2004), *MEDEA (MEA)*(Grossniklaus et al., 1998), *FERTILIZATION INDEPENDEN SEED 2* (*FIS2)* and the paternally expressed *PHERES* (*PHE1)* genes (Köhler et al., 2005).

Genome wide studies in Arabidopsis have revealed that the DNA methylation is not restricted to the gene promoters, but it is also present in the gene bodies, where it is associated with active transcription. DNA methylation in the gene bodies was shown to be almost exclusively associated with CG methylation as opposed to the heterochromatic regions, which also have CHG and CHH methylation (Wang et al., 2014; Zemach et al., 2013).

DNA methyltransferases

Methylation of DNA is an enzymatic reaction in which a methyl group from S-adenosyl- L-methionine (AdoMet) is transferred to cytosine residues. This transfer reaction is catalyzed by DNA methyltransferases (Fig. 1.2) (Cao et al., 2000; Junjun et al., 2010; Wada et al., 2003).

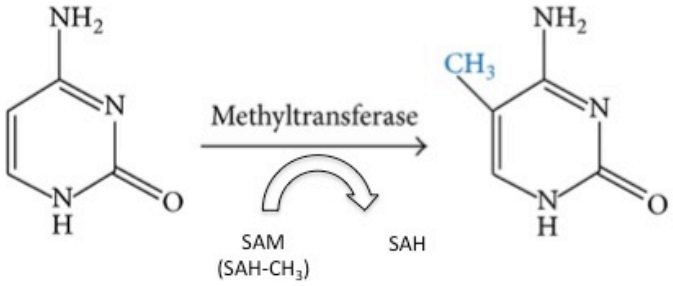


Figure 1.2 Methylation of cytosine

Methylation of cytosine base in DNA is an enzymatic process catalyzed by DNA methyltransferases

DNA methylation is established by the class of "*de novo*" DNA methyltransferases: DNA methyltransferase 3 (DNMT3) in animals and DOMAINS REARENGED METHYLTRANSFERASE 2 (DRM2) in plants. DNA methylation is maintained by the class of so-called "maintenance" DNA methyltransferases: DNA methyltransferase 1 (DNMT1) in animals and METHYLTRANSFERASE1 (MET1)

and CHROMOMETYLASE3 (CMT3) in plants (Cao and Jacobsen, 2002; Cao et al., 2000; Kim et al., 2008).

Establishment of DNA methylation in plants

The DRMs, which are plant homologues of Dnmt3a and Dnmt3b, are *de novo* methyltransferases, which deposit methyl groups to cytosine in CHH context (Law and Jacobsen, 2010). One very specific feature of DRMs is the rearrangement of the conserved catalytic motifs (I-X) compared to the rest of the eukaryotic methyltransferases (Fig.1.3). DRMs have several ubiquitin-associated (UBA) domains at the N-terminus that are involved in the recognition of DNA target sites for *de novo* methylation. This class of methyltransferases is represented with three members in Arabidopsis: DRM1, DRM2 and DRM3, with DRM2 being the most abundant one. DRM3 is a catalytically defective DNA methyltransferase, and can't compensate for DRM2 loss of function in vivo. However, it is required for establishment and maintenance of DNA methylation in the CHH sequence context.

DRM mutants don't show developmental defects, and no drastic loss of DNA methylation at a global scale, but they are defective in *de novo* methylation at specific gene loci (Cao and Jacobsen, 2002; Junjun et al., 2010).

Figure 1.3 Schematic representation of the domain organization of DRM2 in Arabidopsis (modified from Chan et al., 2005)

The ubiquitin associated domains (UBAs) target sites in DNA for de novo methylation. The methyltransferase catalytic domains are indicated with numbers I-X, are rearranged in DRMs.

Maintenance of DNA methylation by Dnmt1/MET1

The mouse Dnmt1 is the first described DNA methyltransferase and although initially described as a *de novo* DNA methyltransferase, the enzyme has higher affinity towards hemi-methylated than non-methylated DNA and is therefore known as a maintenance methyltransferase. During DNA replications, mammalian maintenance

DNA methyltransferases are primarily associated with the replication fork and are involved in restoring DNA methylation on the newly synthesized hemi-methylated DNA (Law and Jacobsen, 2010; Kim et al., 2008).

Dnmt1 is a large enzyme with several domains. In addition to the catalytic methyltransferase domain at the C-terminus, further important Dnmt1 domains are the cysteine-rich CXXC-type zinc finger domain and the two Bromo-Adjacent Homology domains (BAH1 and BAH2) at the N-terminal site that are involved in DNA binding and protein-protein interactions respectively (Fig. 1.4) (Callebaut et al., 1999; Bestor, 2000; Frauer et al., 2011).

Figure 1.4 Schematic representation of the domain organization of MET1 in Arabidopsis

The Bromo-Adjacent Homology domains (BAHs) are shown as green boxes and the catalytic DNA methyltransferase domain as a blue box (modified from Chan et al., 2005).

Loss of function of Dnmt1 is lethal in mice. It results in a global DNA demethylation, which causes pleiotropic developmental defects such as biallelic expression of some of the imprinted genes, transient activation of all X chromosomes to activation of transposable elements (Bestor, 2000).

In Arabidopsis, the class of maintenance DNA methyltransferases is represented by a gene family of four members (MET1, METIIa, METIIb and METIII), with MET1 being the best studied and characterized one (Genger et al., 1999). *METIII*, encodes a truncated protein and is not essential, whereas the METIIa and METIIb are functional proteins, that are involved in maintenance of DNA methylation, but can`t substitute MET1 loss of function (Genger et al., 1999). MET1 shares about 50% sequence identity with mouse Dnmt1, within the catalytic-methyltransferase domain (Finnegan and Kovac, 2000). A notable difference between the mouse Dnmt1 and the plant MET1 is the N-terminal cysteine rich region that is missing in plants (Finnegan and Kovac, 2000).

In Arabidopsis, mutations in MET1 result in drastic reduction of DNA methylation primarily in CG context at repetitive sequences and in the gene bodies, but also in CHG and CHH context, suggesting for a more global role for MET1 (Cao and Jacobsen, 2002). The lost methylation in *met1* can be inherited to the next generation. Restoration of CG-DNA methylation is slow and requires a functional MET1 copy (Mathieu et al., 2007).

MET1 was show to be important for the paternal imprinting of the *FWA* locus. *FWA* is a maternally expressed locus, due to the DNA hypomethylation in the female gametophyte and DNA hypermethylation in the male gametophyte. MET1 maintains the hypermethylated state of the male gametophyte, and imprinting is lost in crosses between wild type maternal plant and *met1* paternal plant. Phenotypically, *met1* exhibit delay in flowering, which is a result of hypomethylated *FWA* (a repressor of flowering) epialleles leading to its ectopic expression (Kankel et al., 2003).

Chromomethylases

Chromomethylases (CMTs) are plant-specific class of DNA methyltransferases, which predominately maintain methylation at symmetric CHG context but are also known to establish *de novo* DNA methylation in the nonsymmetrical CHH context (Dangwal et al., 2014; Kawashima and Berger, 2014; Junjun et al., 2010). Chromomethyltransferases have a bromo domain and a chromodomain which is inserted between the catalytic motifs I and IV (Fig.1.5) (Bartee et al., 2001), which are involved in the recognition and binding of histone modifications (H3K9me2 in particular) (Du et al., 2012; Platero et al., 1995; Paro and Hogness, 1991). In the Arabidopsis genome three genes are encoding for chromomethylases: CMT1 (Henikoff and Comai, 1998), CMT2 and CMT3 (McCallum et al., 2000). In some Arabidopsis accessions, the *CMT1* gene is disturbed due to transposon insertions or frame shift mutations (Papa et al., 2001; Henikoff and Comai, 1998). CMT3 is involved in maintaining CHG-DNA methylation patterns, whereas CMT2 additionally establishes *de novo* CHH-DNA methylation patterns (Stroud et al., 2013; Lindroth, 2001). On a structural base, CMT2 and CMT3 differ in the N-terminal domain and CMT2 doesn`t complement loss of function of CMT3. Mutation in CMT3 leads to strong decrease of methylation in CHG context and has a weak effect on the methylation in the CG context. Despite the lack of phenotypic abnormalities, *cmt3* mutants show substantial transcriptional activation of TEs (Lindroth, 2001).

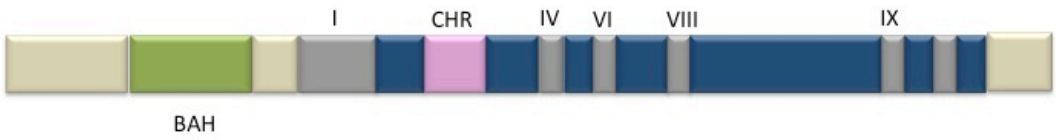


Figure 1.5 Schematic representation of the domain organization of chromomethylases in Arabidopsis (modified from Chan et al., 2005)

Numbers I-IX indicate the catalytic domains of the enzyme. Catalytic domain I is surrounded by the bromo-adjacent domain (BAH) the chromodomain (CHR) responsible for recognition and binding to H3K9me2.

DDM1

All of the aforementioned enzymes have direct DNA methylatransferase domains and activities thus directly regulate DNA methylation levels. DECREASE IN DNA METHYLATION1 (DDM1) is a chromatin remodeler (SWI2/SNF2-type), without DNA methyltransferase activity, and yet is involved in maintaining DNA methylation and in the regulation of gene expression (Jeddeloh et al., 1999). Chromatin remodelers, utilize energy derived from ATP to disturb histone-DNA interaction, which makes DNA accessible for numerous proteins among which DNA methyltransferases. Therefore the regulatory role of chromatin remodelers in DNA methylation is rather indirect (Zemach et al., 2013; Ryan and Owen-Hughes, 2011; Brzeski and Jerzmanowski, 2003).

DDM1 maintains protein coding genes and transposons transcriptionally silent by maintaining the DNA methylation in all cytosine contexts and countering the influence of the linker H1 histone, therefore creating less compact chromatin structure (Zemach et al., 2013). Loss of function in DDM1, causes strong reduction of DNA methylation at transposons and repetitive sequences, which can lead to the mobilization of transposable elements. It also leads to developmental defects, that become more severe with the inbreeding homozygous lines for several generations (Zemach et al., 2013; Jeddeloh et al., 1998; Kakutani et al., 1996; Vongs et al., 1993).

DNA demethylation

DNA methylation is a reversible process. Removal of methyl groups from cytosines in the DNA can be a passive or an active process. Passive DNA demethylation occurs when DNA methylation-maintenance machinery fails to propagate the methylation patterns after DNA replication. The active removal of methylated cytosines in DNA involves the direct removal of the methyl group from the cytosine ring, excision of the methylated cytosine and /or chemical modification of the 5mC followed by replacement (Piccolo and Fisher, 2014).

In plants active DNA demethylation is mediated by group of enzymes known as DNA glycosylases (Piccolo and Fisher, 2014; Zhu, 2009). The mechanism of action of DNA glycosylases involves direct cleavage of the bound between the 5meC and the deoxyribose. This creates gaps in the DNA helix, which are repaired by the basic excision repair pathway, by adding unmodified cytosines.

In Arabidopsis four DNA glycosylases have been identified: REPRESSOR OF SILENCING (ROS1), DEMETER (DME) and DEMETER-LIKE2 and 3 (DML2, DML3). ROS1, DML2 and DML3 are expressed in adult tissues in Arabidopsis, where they control the levels of 5mC at specific loci (DML2 and DML3) or on a broader scale (ROS1). DME has a tissue-specific expression pattern. It is active in the central cell of the female gametophyte and it`s activity is essential for a proper genomic imprinting. This tissue-specific expression and the lack of DME activity in the male gametophyte, provides the differential expression of the maternally expressed genes (*MEA*, *FIS2*, *FWA*) (Zhang and Zhu, 2013; Zhu, 2009).

RNA-Directed DNA methylation (RdDM) pathway

RNA-directed DNA methylation (Wassenegger et al., 1994) is a plant specific form of RNA silencing, which causes sequence-specific DNA methylation changes in the genome (Aufsatz et al., 2002a). Reminiscent to PTGS, it utilizes small RNAs to target regions in the genome (based on sequence homology) for *de novo* DNA methylation. RdDM is entailed in *de novo* DNA methylation in all cytosine contexts (CG, CHG, CHH) (Castel and Martienssen, 2013; Holoch and Moazed, 2015; Furner and Matzke, 2010; Matzke et al., 2009; Huettel et al., 2007).

RdDM plays a role in protection of the genome stability and silencing of transposable elements (Mosher et al., 2011; Ito et al., 2011; Mirouze et al., 2009) .

Histone modifications

Histone proteins are the main components of the nucleosomes - the building blocks of chromatin. The core histones H2A, H2B, H3, H4 are grouped in an octamer structure around which DNA is wrapped creating nucleosomes. The individual nucleosomes are connected with the linker histone H1(Zhang et al., 2006b; Pandey et al., 2002; Luger et al., 1997). The nucleosomes are further packed into supercoiled structures, creating the backbone of the chromatin. Two different chromatin structures can be observed: a relaxed/open, which is transcriptionally active-euchromatin and condensed/closed transcriptionally silent-heterochromatin (Grewal, 2003).

Core histones have globular structure, but their C- and N-terminal "tails" are free and can undergo posttranslational modifications. Even though most of the posttranslational modification are found at the tails, some of them are localized in the globular domain of the histone (Berger, 2007; Kouzarides, 2007). These posttranslational modifications of histone tails alter the chromatin structure by influencing the strength of the DNA-histone interaction, making the DNA more accessible (open chromatin) or less accessible (closed, compact chromatin) thereby influencing gene expression. However, not all histone modifications have a direct effect on the gene expression and some of them act via mediator molecules that recognize and bind the modification. The heredity of the histone modifications is opening discussions on whether they can be classified as *bona fide* epigenetic marks. Namely, so far only for two histone modifications - H3K27me3 and H3K9me2 have been shown to be heritable (Burgess, 2014).

The theory arguing against the histone modifications being true epigenetic marks, gives importance to the chromatin remodelers and the nucleosome occupancy. It suggest that dynamic processes that affect the nucleosomes, create histone modifications patterns, which in turn affect the physical properties of the nucleosomes and help to maintain active or silent chromatin state (Henikoff and Shilatifard, 2011; Cosgrove et al., 2004).

Opposing to this, the hypothesis of the "histone code" suggests that multiple histone modifications on one or multiple tails, acting in a combinatorial or sequential fashion,

specify unique downstream functions (la Cruz et al., 2005; Spotswood and Turner, 2002; Jenuwein and Allis, 2001; Strahl and Allis, 2000). Two recent works in *C.elegans* and Arabidopsis have shown a trans generational effect of H3K27me3 (Crevillén et al., 2014; Gaydos et al., 2014), supporting the epigenetic nature of the histone modifications.

The best-studied histone modifications include: acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation (Liu et al., 2010; Berger, 2007; Kouzarides, 2007). The overall effect of the different histone modification on gene expression is difficult to predict. This is due to the complex interplay between the different modifications. Some of the histone modifications are exclusive, whereas others can coincide. Ultimately the type of modifications, their number (amount), the position and the surrounding environment will determine the effect one modification will have on gene expression.

In Arabidopsis, acetylation can be associated with several lysine residues at different positions in histone H4: K5, K8, K12, K16 and K20 ("K" stands for lysine residue, and the number indicates the position of the lysine in the histone). Lysine residues in histone three (H3) can also be subjected to acetylation. Likewise acetylation of lysine residues in histone 3 (H3) also can occur at several positions (K9, K14, K18 and K23).

Methylation is found to be associated with lysine residues at histone H3 (K4, K9, K27, K36) and histone H4 (K5, K8, K12 and K16) (Liu et al., 2010; Zhou, 2009; Zhang et al., 2006b). Histones modifications are reversible and can be established and erased upon a stimuli in a short period of time (Kouzarides, 2007). Different classes of enzymes are involved in the establishment and removal of the different modifications (Tab.1.1).

Histone modification		Effect on transcription	"Writers" enzymes adding the mark	"Erasers" enzymes removing the mark
H3K4	me1 me2 me3	Transcriptional activation	Trithorax(trxG) (ATX1;ATX2)	Jumanji (JMJ15; JMJ16; JMJ18)
H3K9	me1 me2 me3	Transcriptional repression (repetitive sequences and transposable elements)	SUVH (SUVH4;SUVH5;SUVH6)	Jumanji (JMJ25)
H3K27	me1 me2 me3	Transcriptional repression	Polycomb (PRC2)	Jumanji (JMJ12)
H4		Transcriptional activation	HATs (GANT;p300/CREB; TAF250 and MYST)	HDACs

Table 1.1 Histone modifications in Arabidopsis, their effect on transcription, "writers" and "erasers"

Histone modifying enzymes

Histone acetyltransferases (HATs) and deacetylases (HDACs)

Histone acetylation is related to transcriptional activation. Acetylation of the lysine residues reduces the positive charge of the histone tails, which in turn reduces their affinity for DNA. This results in relaxation of the condensed chromatin, making it more accessible for transcription factors (Struhl, 1998; Vettese-Dadey et al., 1996). Using acetyl-coenzyme A as a donor the enzymes-histone acetyltransferases (HATs) mediate the transfer of an acetyl group to the lysine residues at the histone tails (Kuo and Allis, 1998).

Based on the sequence homology with the mammalian HATs, the Arabidopsis homologues can be classified into four groups: ***a)*** GNAT, ***b)*** p300/CREB (CBP), ***c)*** TAF250 and ***d)*** MYST(Servet et al., 2010; Pandey et al., 2002).

HATs regulate many processes in Arabidopsis that vary from adaptation to stress to different developmental changes (AtGCN5) (Servet et al., 2010), to flowering time (p300/CBP) (Deng et al., 2007; Han et al., 2006) and sexual reproduction (MYST) (Latrasse et al., 2007).

Histone acetylation is a reversible process. The removal of the acetyl groups is performed by histone deacetylases. They can be classified into three groups: ***a)*** RPD3/HDA1 (Reduced Potassium Dependance3/Histone Deacetylase 1), ***b)*** SIR (Silent Information Regulator 2) and ***c)*** HD2 (Histone Deacetylase 2). The last class is plant specific. In the Arabidopsis genome 18 genes encode for HDACs. Most of them (about 12) belong to the group of RPD3/HDA1. Among them the best characterized members are: HDA6, HDA19, HDA7 and HDA9 (van Zanten et al., 2014; Xuncheng et al., 2014; Pandey et al., 2002).

HDA7 is required for the female gametophyte development. Mutations in HDA7 result in unfertilized ovules and /or aborted seeds (Cigliano et al., 2013). HDA9 defective plants display pleotropic defects. First, HDA9 was reported to control flowering time by regulating the expression of two flowering genes: FLOWERING LOCUS T (FT) and the MADS-box protein-AGAMOUS-LIKE 19 (AGL19) (Kim et al., 2013; Yun et al., 2012). Recent studies have reported on the role of HDA9 in the control of seed dormancy, and showed that *hda9* plants exhibit reduced seed dormancy and enhanced germination speed (van Zanten et al., 2014). HDA19 is ubiquitously expressed and is needed for proper development of reproductive tissues. Mutations in HDA19 result in reduced fertility, and aborted seeds. HDA6 is involved in silencing of TEs, transgenes, and repetitive sequences (To et al., 2011a; Hollender and Liu, 2008; Probst et al., 2004; Murfett et al., 2001).

Histone methyltransferases (HMTs) and demethylases

Histone methylation is involved in regulation of important processes like chromatin stability, development or cellular memory. Histone methylation can occur at lysine residues (K) as mono-, di- or tri- methylation and at the arginine residues (R) as mono- and di- methylation. Methylation can be associated with activation as well as with repression of genes. This is determined by the number of methyl groups added as

well as the position of the methylated residues. Whereas the aforementioned histone acetylation reduces the positive charge of the histone tails, the methyl groups don't have this kind of effect. They are recognized by proteins (eg. heterochromatin protein 1-HP1), which then alter the chromatin structure (Bannister and Kouzarides, 2005; Zhang and Reinberg, 2001).

The addition of methyl groups to the lysine and arginine residues at the histone tails is performed by a group of enzymes called histone methyltransferases.

SET (Su (var)-E (z)-trx) - domain proteins are the best studied histone methyltransferases. They facilitate the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to lysine residues (Dillon et al., 2005). In *Drosophila melanogaster*, the 130-160 amino acid SET domain was found in:

a. Suppressor of variegation 3-9 (SU(VAR)3-9) involved in heterochromatin-dependent gene silencing by methylation of H3K9 (Schotta et al., 2002; Tschiersch et al., 1994);
b. Enhancer of zeste (E(z)) (Jones and Gelbart, 1993) - member of the polycomb group (*PcG*), involved in maintaining the repressive state of chromatin and
c. trithorax (*trx*) – maintain the activity of homeotic genes during development (Dillon et al., 2005; Baumbusch et al., 2001).

The Arabidopsis genome has 29 genes encoding SET- domain proteins that can be distributed in one of these three categories (Table 1.2). A special feature for the group of SUVH in Arabidopsis is the presence of a plant-specific domain that is common for SET and RING domain proteins. It is called SER domain (SET and RING associated) (Johnson et al., 2007; Baumbusch et al., 2001).

Arabidopsis E(z) homologues play important roles in the plant development. *MEDEA* (*MEA*) (Grossniklaus et al., 1998) is required for a proper embryo and endosperm development. This maternal gene is imprinted and loss-of function results in seed abortion (Kinoshita et al., 1999). *CURLY LEAF* (*CLF*) (Goodrich et al., 1997) is important for leaves and flower development. Direct CLF targets are genes involved in flowering such as: *FLOWERING LOCUS C* (*FLC*), *FLOWERING LOCUS T* (*FT*) and the MADS-box protein-*AGAMOUS* (*AG*). In *clf* these targets are miss expressed, and have reduced levels of H3K27me3 (Lopez-Vernaza et al., 2012). ENHANCER OF ZESTE FROM ARABIDOPSIS (EZA) or *SWINGER* (*SWN*) is a *CLF* homologue and acts in a redundant manner. Mutations in *SWN* don't cause severe developmental

phenotype. However, *clf swn* plants are severely impaired and develop callus like structures (Chanvivattana et al., 2004).

Drosophila melanogaster	***Arabidopsis thaliana***	**References**
SUV(VAR)3-9	• SUVH1-SUVH10	• Bambusch 2001
E(z)	• CURLY LEAF (CLF) • MEDEA (MEA) • SWINGER (SWN)	• Goodrich 1997 • Grossniklaus 1998 • Lindroth 2004
Tritorax	• ATX1-ATX5 • ATXR1-ATXR7	• Bambusch 2001

Table 1.2 Orthologous *D. melanogaster* histone methyltransferases that have been identified in Arabidopsis

For a long time histone methylation was considered to be a stable and irreversible modification (Bannister et al., 2002). However, the nature of this modification and its' role in regulation of gene expression requires reversibility of histone methylation. The transcriptional activity of a gene can be altered (from active to silent state and vice versa) very rapidly upon stimuli. This requires fast changes also in the gene-associated factors including chromatin modifications like histone methylation.

In plants histone methylation is actively removed from histones by two groups of proteins: KDM1/LSD1-like histone demethylases and JmjC domain containing histone demethylases (Chen et al., 2011; Liu et al., 2010; Lu et al., 2008).

Although histone modifications and their role in regulation of transcription are extensively studied fields, the many possible combination of histone modification, is an obstacle in their full understanding. The best-studied ones in Arabidopsis include:

a. Methylation of H3K4 (lysine 4 at histone 3). H3K4 exists in mono- (H3K4me1), di- (H3K4me2) and tri- (H3K4me3) methylated form. All three forms of methylated H3K4 are associated with transcriptional activation (Feng and Jacobsen, 2011; Zhang et al., 2009). Trithorax proteins deposit methylation groups at H3K4. In Arabidopsis ATX1 and ATX2 create

H3K4me3 and H3K4me2 respectively. ATX1 mutants display an early flowering phenotype.

b. JMJ18 is H3K4me2 and H3K4me3 demethylase. It was also shown that JMJ18 is promoting flowering via repressing the flowering inhibitor FLC (Yang et al., 2012a). In addition to JMJ18, JMJ15 and JMJ16 also showed H3K4 demethylation activity (Shen et al., 2013).
c. H3K9 is present as mono- (H3K9me1), di- (H3K9me2), and the less abundant tri-methylated form (H3K9me3). H3K9me1 and H3K9me2 show enrichment at repetitive sequences and transposable elements, suggesting that they play an important role in silencing of heterochromatin regions (Du et al., 2012; Feng and Jacobsen, 2011; Bernatavichute et al., 2008; Lippman et al., 2004). This type of methylation is established by SUVH4/KYP (homolog of SU(VAR)3-9), SUVH5 and SUVH6 (Feng and Jacobsen, 2011; Liu et al., 2010; Thorstensen et al., 2005).
d. Mutations in these methyltransferases do not cause developmental defects, but loss of DNA methylation is observed in CHG context. This is due to the SUVH4/KYP interactions with CMT3 (Lindroth et al., 2004). Taken together, these observations suggest a complex interplay between DNA methylation and histone modifications. INCREASE IN BONSAI METHYLATION1 (IBM1), also known as JMJ25, catalyses demethylation of H3K9. This enzyme keeps the CHG DNA methylation away from the gene bodies. Loss of function in IBM1 causes hypermethylation especially at genes with methylated bodies (Chen et al., 2011).
e. H3K27me1, H3K27me2 and H3K27me3 are the three forms of methylation of the lysine residues at position 27 in H3. H3K27me1 is found to be associated with heterochromatin, while H3K27me3 is associated with transcriptional silencing, tissue specific gene expression and regulation of developmental processes (Liu et al., 2010).

Polycomb proteins deposit methyl groups at the H3 histone tails. Although, polycomb proteins were found in the Arabidopsis genome, the histone methyltransferase activity of these proteins has not been confirmed yet. Instead they create a complex known as polycomb repressive complex 2 – *PRC2* that shows methyltransferase activity. In Arabidopsis there are three PRC2 complexes:

a. the *FIS* containing complex – controlling imprinted genes;
b. the *EMF* complex- that regulates the floral transition and expression of floral homeotic genes and
c. *VRN2* regulating vernalization(Liu et al., 2010).

RELATIVE OF EARLY FLOWERING 6 (REF6) also known as JMJ12 that was identified as a H3K27me2 and H3K27me3 demethylase (Crevillén et al., 2014; Lu et al., 2011).

Interplay between the different epigenetic marks

The epigenetic modifications described earlier create a complex network, and can't be analyzed/interpreted individually without considering the big picture. Some of them are exclusive, others have synergistic effect or some require the activity of another.

An example for this complex network is the association of H3K9 with DNA methylation. Genetic studies have shown that there is a tight correlation between the CHG-DNA methylation levels and the H3K9me2 histone mark, suggesting interplay between DNA methylation machinery and the histone methyltransferases. It is a self-reinforcing loop, in which the DNA (CHG and CHH) methylation recruits the methyltransferase-SUVH4/KYP, which deposits two methyl groups at H3K9 (H3K9me2). H3K9me2 is then recognized by CMT3, which in turn methylates the targeted locus. SUVH4/KYP can also be guided to the target sequences by siRNAs produced by the RdDM pathway. The DNA methyltransferase CMT2 deposits methyl groups at cytosines in all sequence contexts. Both, CMT2 and CMT3 utilize their chromo domain and the bromo domain for dual recognition and binding to H3K9me2 (Greenberg et al., 2013; Zemach et al., 2013; Saze and Kakutani, 2011).

Another regulatory network is the one between DNA methylation and histone acetylation levels. It was shown that there is direct interaction between the histone deacetylase-HDA6 and the CG-maintenance DNA methylatransferase-MET1, which makes them act coordinately in silencing TEs (Liu et al., 2012; To et al., 2011b).

The Nucleus

The compartmentalization in eukaryotes led to the development of highly specialized organelles, assigned to very specific processes and functions. Despite the spatial and functional separation, the cell functions as a unit and absolutely isolating border between the different compartments cannot be set. The nucleus is separated from the cytoplasm with the nuclear membrane, which thereby guards the genetic information stored in the nucleus. The nuclear membrane is a double layer envelope that consists of inner nuclear membrane (INM) and the outer one (ONM), separated with perinuclear space. The outer nuclear membrane is fused with the endoplasmic reticulum and the inner membrane in metazoans is connected with the nuclear lamina (Meier, 2007). The two membranes (outer and inner) fuse at several points, making "holes" in the membrane. Nuclear pore complexes are embedded into these fusions. The nuclear membrane separates the genome from the rest of the cell, and the nuclear pores regulate the traffic of molecules (import and export of proteins and RNAs) between the nucleus and the cytoplasm (Güttinger et al., 2009).

Nuclear pore complexes (NPCs) and nucleoporins (Nups)

Nuclear pore complexes are large (40-60MDa) multicomponent structures, which facilitate exchange of molecules between the nucleus and the cytoplasm (Grossman et al., 2012; Capelson et al., 2010; Cook et al., 2007). The size of the NPCs differs among yeast, plants and vertebrates, with yeast having the smallest and metazoans the largest complexes (Roberts and Nortcote, 1970). Nucleoporins (Nups) are the building blocks of the NPCs. Approximately 30 Nups have been identified in Arabidopsis (Tamura et al., 2010). NPCs share eight-fold symmetry, meaning that each of the NUPs is presented with at least eight copies, creating funnel-like large complexes. A large part of the Nups is embedded into the nuclear envelope, creating the central transport channel of the NPC surrounded by central spoke ring and two outer rings - cytoplasmic and nuclear. Eight filaments are attached to each of the rings (at the nuclear and the cytoplasmic site). On the nuclear site, these filaments are organized in a structure "nuclear basket", that is not present at the cytoplasmic side (Fig.1.6) (Grossman et al., 2012; Strambio-De-Castillia et al., 2010; D'Angelo and Hetzer, 2008).

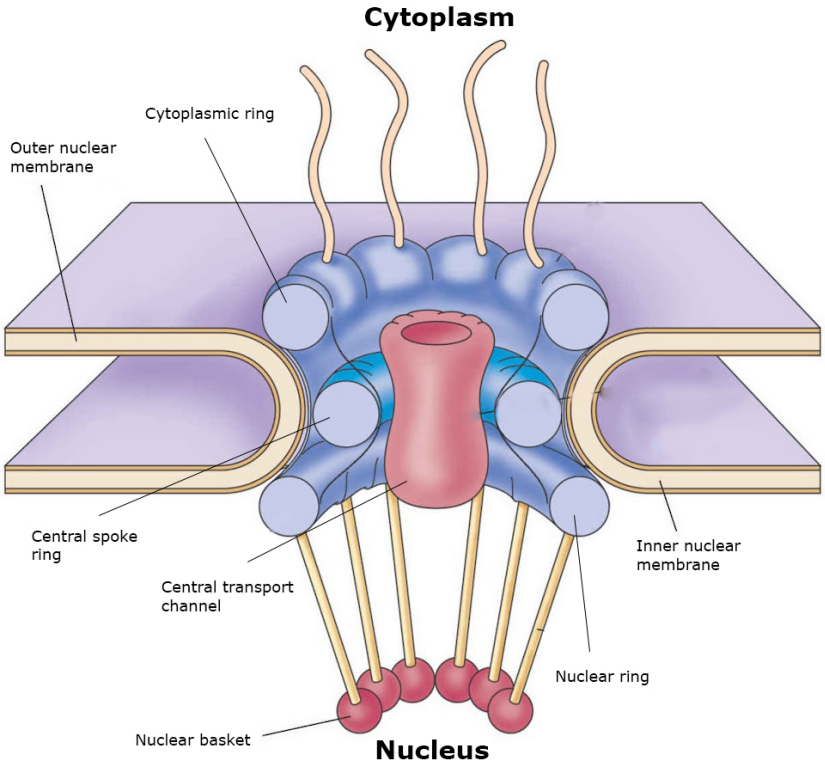


Figure 1.6 Organization of the nuclear pore complex

(modified from Strambio-De-Castillia et al., 2010**)**

The central transport channel represents the core part of the nuclear pore complex and it is the main route for the transport between the nucleus and the cytoplasm. The central spoke ring and the nuclear and the cytoplasmic rings stabilize the central transport channel. The filamentous Nups on the nuclear site are organized in a nuclear basket.

Five motifs can be identified in the Nups, all of them involved in establishing or maintenance of protein-protein interactions, therefore mediating the transport processes. Among them are: alpha solenoids, beta propellers, phenylalanine- glycine (FG) repeats, coiled coil and transmembrane motifs (D'Angelo and Hetzer, 2008; Devos et al., 2006).

Based on the position in the NPC and the motifs present, Nups can be divided into: **a)** transmembrane, **b)** scaffold (Nups at the central spoke ring, the outer rings, and the linker Nups), and **c)** barrier Nups (FG Nups from the central channel, cytoplasmic and nuclear FG Nups) (Grossman et al., 2012). Transmembrane Nups are anchoring the

NPCs to the nuclear membrane, the scaffold Nups stabilize the complex via connecting the transmembrane and barrier Nups, whereas the barrier Nups provide the selectivity of the transport through the membrane (Grossman et al., 2012; Tamura et al., 2010; Patel et al., 2007).

The entire traffic of molecules between the nucleus and the cytoplasm occurs via NPCs. NPCs are permeable for small molecules and ions, but molecules with molecular mass lager than 40kDa have to be actively transported through the NPCs (Stewart, 2010).

The active transport requires nuclear transport factors (NFTs), as well as short amino acid transport signals at the molecules subjects to transport, in a form of nuclear localization sequences (NLSs) or nuclear export sequences (NESs) that are recognized and bound by the NTFs (karyiopherin-β and importin-β) (Grünwald et al., 2011; Strambio-De-Castillia et al., 2010).

Unique features of the transport through the nuclear membrane are selectivity and directionality and several models have emerged trying to explain them. The "virtual gate model" (Rout et al., 2000), "selective phase model" (Ribbeck and Görlich, 2001), "spaghetti oil model" (Macara, 2001), and the "two dimensional model" (Peters, 2005), even though different, all of them attribute the selectivity of the nuclear pore transport to the FG domains containing Nups (D'Angelo and Hetzer, 2008; Terry et al., 2007; Fried and Kutay, 2003).

The directionality of the nuclear pore transport is achieved with the GTPase Ran and the asymmetric distribution of its' two forms (RanGTP and RanGDP) in the nucleus and in the cytoplasm. RanGTP has higher affinity for binding cargo. It prevails in the nucleus, where the affinity of Ran for GTP hydrolysis is very low. In the cytoplasm the presence of factors such as RanGAP promotes the hydrolysis of GTP by Ran to GDP and dissociation of the cargo (Fried and Kutay, 2003).

Additional roles of NPCs

NPC regulates gene expression in transport-dependent and transport-independent manner (Raices and D'Angelo, 2012; Capelson and Hetzer, 2009). The first one is related to the association of the RNA surveillance machinery with the the nuclear basket and the NPC. The transport-independent regulation of gene expression on the other hand is related to the nuclear organization, which reveals the role of the nuclear

pore in the organization of the chromatin (Ptak et al., 2014; Strambio-De-Castillia et al., 2010; Qureshi and Mehler, 2010; Capelson and Hetzer, 2009).

The NPCs influence the chromatin structure in several aspects:

a) In yeast the nuclear periphery is associated with patches of heavily condensed heterochromatin. The regularity of condensed chromatin along the nuclear rim is disrupted by the NPCs (nuclear basket in particular) where the chromatin is open (relaxed) (Ptak et al., 2014; Raices and D'Angelo, 2012). Studies in yeast have shown that the position of a certain gene within the nucleus can greatly influence its transcription and that transcriptionally active genes are associated with the nuclear pores. The inducible genes *INO1* and *HXK1* in *Saccharomyces cerevisiae*, are an example for this phenomenon. In favorable conditions, these genes get activated and translocated to the nuclear basket, and this translocation was shown to be independent of active transcription, indicating that translocation happens prior to transcription initiation (Taddei et al., 2006; Brickner and Walter, 2004). These observations are supporting the theory of "gene gating" (Blobel, 1985), according to which the tethering of genes to the NPC leads to transcriptional activation.

b) In addition to the role of the nuclear pores in gene activation via the process of gene gating, the nuclear pores play a role also in supporting the stability of the replication fork and ultimately support the chromosome stability. During replication in S phase a topological stress is created, which is especially profound when the replication fork clashes with transcription units. The positive supercoiling can cause chromosomal breaking and fork reversal, whereas the negative supercoiling can cause formation of R-loops (Skourti-Stathaki and Proudfoot, 2014). R loops are DNA/RNA hybrids formed between the replication forks and the newly synthesized RNA from the transcription machinery. With the gating process chromosomes get attached to the nuclear pore at several points, creating barriers that disperse the topological pressure. The association of the NPC with chromosome stability is supported by the fact that gene-gating mutants exhibit formation of R-loops (Gonzalez-Aguilera, 2008).

c) The epigenetic state of a certain sequence can influence the epigenetic state of neighboring regions. The so-called "boundary elements" can block the

communication between active and silent chromatin. Boundary activity was shown for Nup2 in yeast (Burgess-Beusse et al., 2002).

d) NPCs and Nups were associated with epigenetic transcriptional memory (Ptak et al., 2014; Light et al., 2013;Van de Vosse et al., 2010). Transcriptional memory explains the faster activation of genes that have recently been active but were repressed compared to genes that were silenced for long time (Light et al., 2013; Ptak et al., 2014; Brickner et al., 2007). In yeast, DNA loops created upon gene tethering to the pore, stay associated with it even in repressive conditions, thus allowing faster Pol II association in activation conditions (Tan-Wong et al., 2009). This mechanism is highly conserved from yeast to humans. In both systems transcriptional memory is a multistep process that involves interactions of the pore members Nup98 and Nup100p with the promoters of the activated genes, which leads to accumulation of H3K4me2 in the promoter region of the activated genes and faster reactivation (Light et al., 2013).

Role of the Nups in developmental processes and tissue specificity

Different developmental programs in an organism are based on different transcriptional activities. Hence, regulators of transcription can influence the development of the organism. Since the NPCs are involved in regulation of transcription, their importance in the control of development doesn't come as a surprise. The diversity of the NPCs functions is displayed via pleotropic developmental defects in the different Nup mutants.

In humans the role of NUP98 in leukemia was reported (Lam and Aplan, 2001), whereas NUP133 is required for neural differentiation and embryonic development in mice (Lupu et al., 2008).

In Arabidopsis NUPS are predominately associated with control of flowering time, but were shown also to be involved in processes like fungal and rhizobia symbiosis, innate immunity, or hormone signaling (Table 1.3).

Nucleoporins in *Arabidopsis thaliana*		
NUP160	Flowering time and cold tolerance	(Dong et al., 2006)
NUP96/MOS3	Hormone signaling and flowering time	(Zhang and Li, 2005)
NUA/AtTPR	Flowering time, fertility, growth	(Xu et al., 2007)
NUP85	Symbiosis	(Saito et al., 2007)
NUP88/MOS7	Innate immunity	(Cheng et al., 2009)
SARE	Hormone signaling	(Parry et al., 2006)

Table 1.3 Best-studied nucleoporins in Arabidopsis and their physiological roles

Some of the NUPs have tissue-specific expression patterns. In mammals NUP155 shows heart specific expression patters, and is essential for its development. Lack of function in NUP155 causes cardiac disease (Zhang et al., 2008). The tissue specificity makes them essential for the establishment of specific developmental programs. Therefore, developmental defects in NUP mutants can be partially assigned to the tissue-specificity of their expression.

NUPs can also show selectivity towards the transport export factors (specific NUPs will bind only certain subset of transport export factors). Their higher affinity towards some of the transport export factors will favor the export of their cargoes. This can in great extend have an influence on the development of the organism.

Nuclear-cytoplasmic export of RNAs

Messenger RNAs are exported form the nucleus as large ribonucleoprotein complexes (RNPs). Therefore, the translocation through the nuclear pores requires the assistance of transport factors (NTFs). Until now four NPC mediated mRNA export pathways have been identified in higher eukaryotes - three of them are CRM1 dependent (Chromosomal maintenance 1, known as Xpo1 in yeast) and one is NXF1/NXT1 dependent pathway (also known as TAP/p15; Mex67/Mtr2 in yeast) (Natalizio and Wente, 2013).

CRM1 (Xpo1) encodes for β- kariopherin, and since kariopherins require Ran-GTP for functioning the entire pathway is a Ran-GTP dependent process, and the release of

the cargo (primarily rRNAs, snRNAs) requires hydrolysis of Ran-GTP to GDP (Natalizio and Wente, 2013; Rodriguez et al., 2012; Hutten and Kehlenbach, 2007).
Conversely, the NXF1/NXT1 (Mex67/Mtr2) pathway is a Ran-GTP independent pathway with strict RNA quality control and is primarily utilized by the bulk mRNAs. (Natalizio and Wente, 2013; Reed and Hurt, 2002; Clouse et al., 2001; Hurt, 2000; Strässer et al., 2000; Segref et al., 1997).
Whereas some of the RNAs (tRNAs) create secondary structures that can be recognized by transport (export) factors, direct recognition of mRNAs doesn't occur due to their great diversity in size and sequence. Therefore, several transport adaptor proteins are linked to transcription. From the point of transcription to the point of export, maturation of mRNAs occurs (5'capping, 3'polyadenylation and splicing). Maturation of mRNA is a co-transcriptional process, and the factors involved in these steps, associate with the C-terminal domain of Pol II. Association of some factors involved in the maturation steps, promotes the binding of export adaptor proteins, creating the transcription - export (TREX) complex (Katahira, 2012; Dieppois and Stutz, 2010; Kelly and Corbett, 2009; Köhler and Hurt, 2007; Vinciguerra and Stutz, 2004).

TREX-THO complex

The TREX complex is involved in coupling transcription and export and escorts nascent RNAs on their way to the nuclear pore. In yeast the complex consists of export adaptor factors – Yra1, Sub2 and Tex1 and a sub complex THO (Tho2, Hpr1, Mft1 and Thp2) (Katahira, 2012; Stewart, 2010; Köhler and Hurt, 2007; Sträßer et al., 2002; Chavez et al., 2001). Mutations in TREX-THO members cause nuclear retention of mRNAs (Sträßer et al., 2002).
The mechanism of TREX assembly in yeast is associated with transcription and it is splicing independent, due to fact that only 5% (mostly highly expressed genes) of yeast genes contain introns (Parenteau et al., 2008; Sträßer et al., 2002; Zenklusen et al., 2002; Strässer and Hurt, 2000;). Assembly of the TREX complex and it's loading to the transcripts is coupled with transcription elongation and 3' end processing (Kelly and Corbett, 2009; Gwizdek et al., 2006). Consistent with this model are the reports on mRNA nuclear retention in mutants of the 3' end processing machinery,

and hyperpolyadenylation of mRNAs in *mex67* and *yra1* mutants (Hammell et al., 2002; Jensen et al., 2001).

Yeast THO is known to be required for transcription elongation. Mutations in its members affect in particular the expression of CG - rich and repeat-containing genes, causing stalling of the transcription complex (Voynov et al., 2006; Chavez et al., 2001). Impaired transcriptional elongation can result in R-loops formation, which was observed in *tho/trex* mutants (Katahira, 2012; Jimeno et al., 2002).

Metazoan genes are rich on introns hence a different, cap and splicing - dependent model of TREX assembly exists (Müller-McNicoll and Neugebauer, 2013; Stewart, 2010; Köhler and Hurt, 2007; Cheng et al., 2006; Masuda et al., 2005). In metazoans the TREX complex consists of: the transport adaptors UAP56 (homolog of Sub2), Aly (homolog of Yra1) and Tex1, and the THO sub complex (Cheng et al., 2006).

In plants the mRNA export pathway is poorly understood. Paralogs of the TREX/THO members have been identified in Arabidopsis, indicating that the complex is evolutionary conserved. The Arabidopsis TREX/THO complex consists of: THO1-THO7 and UAP56 (Meier, 2012). In addition to regulation of the mRNAs export, the complex also regulates the production of siRNAs. Loss of function in THO1, THO6 and THO3 (also known as TEX1) result in reduced levels of siRNAs originating from inverted repeats and transgenes. While TEX1 regulates the processes of siRNA production, THO2 is more involved in the production of miRNAs. No direct interaction of the TREX/THO complex with any of the small RNAs synthesis pathways has been identified so far, suggesting an indirect role of the complex in these pathways. However, the complex is required for the translocation of the small RNAs' precursors to the processing sites (Francisco-Mangilet et al., 2015; Furumizu et al., 2010; Jauvion et al., 2010; Yelina et al., 2010).

The TREX-2 complex

The transcription and export complex 2 (TREX2, consisting of Sac3, Thp1, Cdc31, Sus1) was initially identified in yeast as an important component of nuclear-cytoplasmic export of mRNAs (Fischer et al., 2002). Even though TREX-2 homologues have been identified in plants (Lu et al., 2009), fruit fly (Kopytova et al., 2010) and in humans (Jani et al., 2012), most studies were carried out in yeast.

Mutations in TREX-2 members exhibit several phenotypes. One of them is impairment of mRNA export. Yeast TREX-2 represents a bridge at the nuclear pore,

connecting the export machinery Mex67p-Mtr2p and the nucleporins Nup1, and Nup60, thereby targeting the newly synthesized transcripts to the pore channels (Fischer et al., 2002). Deletions of yeast TREX-2 members (eg. Sac3, Thp1) results in accumulation of mRNAs in the nucleus, a phenotype which can be also observed in TREX/THO mutant backgrounds (Gallardo et al., 2003; Lei, 2002), indicating that both complexes are involved in the mRNAs nuclear-cytoplasmic export.

Sacharomyces cerevisiae	***Mus musculus***	***Drosophila melanogaster***	***Arabidopsis thaliana***
Sac3	GANP/SHD1	Xmas-2	Sac3A, Sac3B, Sac3C
Thp1	PCID		Thp1
Sus1	ENY2	E(y)2	Sus1
Cdc31	CEN2		Cen1, Cen2
Sem1	DSS1	DSS1	Dss1

Table 1.4 Members of the TREX-2 complex in yeast and their homologues in different model organisms (García-Oliver et al., 2012)

TREX-2 was also shown to be required for transcription of long transcripts and transcripts with high CG content, and a general down regulation of these transcripts is observed in TREX-2 mutants (*sac3* and *thp1*)(Santos-Pereira et al., 2014). Therefore, mutants in TREX-2 components exhibit defects in transcription elongation and genome stability. This phenotype is consistent with the phenotype of THO/TREX mutants, which supports the notion that a coordinated activity of the two complexes takes place and for their important role in transcription and export (Santos-Pereira et al., 2014; Faza et al., 2009; Gonzalez-Aguilera, 2008).

In yeast TREX-2 consists of several subunits: Sac3, Thp1, Cdc31, Sus1, and the small protein Sem, which stabilizes the complex (Faza et al., 2009; Köhler and Hurt, 2007; Fischer et al., 2002). The homologues of these yeast proteins in higher eukaryotes are given in table 1.4 (Wickramasinghe et al., 2010; Kurshakova et al., 2007).

Sac3 (Suppressor of actin 3)

Sac3 is the core protein in the TREX-2 complex around which the other subunits are organized (Ellisdon et al., 2012; Jani et al., 2009; Fischer et al., 2002). The central-

CID region of the protein interacts with two Sus1 (Sus1A and Sus1B) and one Cdc31 proteins, which work synergistically in providing the connection of TREX-2 to the NPC, and stabilizing the long Sac3-CID alpha helix (Jani et al., 2009). The protein has a conserved SAC3/ GANP domain, which together with the N-terminal domain binds to Thp1, and the Mex67-Mtr2p export complex (Jani et al., 2012).

Deletion analyses of the Sac-CID domain and its N-terminal domain result in mRNA export and growth defects in yeast, suggesting that both domains are responsible for the mRNA export functions of Sac3 (Ellisdon et al., 2012; Jani et al., 2012).

Thesis outline

In recent years, research on epigenetic regulation of gene expression in Arabidopsis has been very intensive, especially in the field of RNA-directed DNA methylation. Even though numerous detailed mechanisms have now been revealed, surprisingly little is known about how epigenetic mechanisms influence plant development. More specifically, little is so far known about epigenetic regulation of tissue specific gene expression, which should play a central role in development and adaptation.

The initial aim of this thesis was to uncover novel epigenetic regulators in Arabidopsis that regulate tissue-specific gene expression. The basis of this thesis was a forward genetic mutant screen that was previously performed in the lab that was designed to discover tissue-specific epigenetic regulators.

In Chapter 2, I present data describing the transgenic reporter line that was used in the forward genetic mutant screen. I first confirm via forward genetics that the reporter line behaves as expected and then describe a novel mutant allele (*epic1*) of the histone deacetylase HDA6 that was recovered in the mutant screen.

In Chapter 3 I then mapped the causal mutation in *epic3*. This mutant is defective in a central component of the nuclear pore complex, thus resulting in defects in the nuclear-cytoplasmic export of polyadenylated RNAs. Using transcriptomics I show that *epic3* plants behave like heat-stressed plants and I show that heat stress inhibits polyA RNA export in Arabidopsis. Based on transcriptomes carried out separately on nuclear and cytoplasmic RNA I show the very surprising finding that EPIC3 plays a role in the export of antisense transcripts.

In Chapter 4 I then focus on the role of EPIC3 and the NPC in regulating the chromatin organization. I show that EPIC3 is required to maintain proper heterochromatin structure and that it is required to global levels of repressive histone marks.

A general discussion is then presented in Chapter 5, where I put all my findings into the general context.

Chapter 2

The forward genetic mutant screen and characterization of epic1

Modified version of this chapter was published in the journal "*Plant Physiology*" (April, 2015 pp.00177.2015) under the title:
"HDA6 controls gene expression patterning and DNA methylation-independent euchromatic silencing"
Authors: Emilija Hristova, Kateryna Fal, Laurin Klemme, David Windels and Etienne Bucher

Abstract

Cellular differentiation is a process, determined by the implementation of specific transcriptional programs. Up until now, the epigenetic aspect of gene expression patterning and the link between the epigenetic regulators and cell differentiation was missing. We addressed this question by using a novel epigenetically controlled and highly tissue-specific GFP based reporter line, which is reporting on the epigenetically regulated *APUM9* in Arabidopsis (*Arabidopsis thaliana*). A forward genetic screen on this line led to the identification of several mutants (*epic1*- *epic7*) that activated the transcription of the transgene in different tissues, indicating on different epigenetic regulators being involved in silencing of the transgene in different tissues. Among the recovered mutants is a novel HDA6 allele (*epic1*, *hda6-8*). This allele differs from the previously reported alleles, as it did not affect DNA methylation and only had a very modest effect on the release of transposable elements and other heterochromatic transcripts. Overall the data shows that HDA6 has at least two clearly separable activities in different genomic regions.

Introduction

Background

All of the cells in a given multicellular organism have a common "ancestor" cell, therefore carry the same genetic information, and have potential to develop/specialize in any cell type. One of the most fundamental biological questions is "what is determining cell's fate"? Understanding the molecular mechanisms behind this process and identification of the factors involved in it is a still ongoing process. Even though the role of transcription factors in the establishment of tissue specific (transcription) programs has been elucidated (Odom et al., 2007; Naya et al., 1995; Maniatis et al., 1987), little is known about the epigenetic aspect of these processes.

Genome wide studies of plants defective in different epigenetic pathways have provided valuable information about the localization of different chromatin marks and their general effect on transcription (e.g. (Reinders et al., 2008; Zhang et al., 2007)). While there are some reports proposing a tissue-specific distribution of these marks in plants (Caro et al., 2007; Costa and Shaw, 2006), majority of the studies have focused on germ cells (She et al., 2013; Hsieh et al., 2009; Slotkin et al., 2009). Currently, little is known about how different epigenetic marks influence tissue-specific gene expression patterns in sporophytic tissues.

To investigate the role of different epigenetic regulators and pathways in the establishment and maintenance of tissue-specific gene expression programs, we selected a gene that displays a complex epigenetic regulation and tissue specific gene expression in Arabidopsis for our genetic studies.

The Arabidopsis *APUM9* gene

APUM9 is a member of the Pumilio/PUF protein family in Arabidopsis (Abbasi et al., 2011). These proteins are known to be regulators of embryogenesis, development and differentiation in mammals (Quenault et al., 2011).

Although being generally known as translational repressors, PUF proteins have versatile mechanisms of action. They control gene expression primarily by influencing mRNA stability (Wharton and Aggarwal, 2006). The PUF family members contain the characteristic PUM-HD domain (composed of eight 36 amino-acid long repeats), that is involved in recognition and binding to a specific sequence at

the 3' end of mRNAs, thereby controlling mRNAs stability. The mRNA recognition is base specific (e.g. an adenine base in the RNA is bound by specific cysteine and glutamine residues of the PUF protein) (Filipovska et al., 2011; Quenault et al., 2011; Francischini and Quaggio, 2009; Miller et al., 2008). PUF proteins were also shown to influence the subcellular localization of the mRNAs by targeting them to specific cellular compartments, which can result in both, transcriptional repression or activation of a certain gene (Quenault et al., 2011; Gu et al., 2004).

In Arabidopsis the APUM family comprises 25 PUF proteins, 12 of which share between 50% and 75% sequence homology with the PUF proteins in Drosophila. Their function is also conserved. Notably, six members of the family (APUM1-APUM6) were shown to regulate developmental genes like: *WUSCHEL*, *CLAVATA* and *FASCIATA* (Abbasi et al., 2011; Francischini and Quaggio, 2009). APUM5 was shown to have a protective role against virus infection (Un Huh and Paek, 2013), which shows the diverse roles of these proteins in plants.

The *APUM9* gene (*AT1G35730*) is located on chromosome one in Arabidopsis. The region is of high interest, due to the presence of a *copia*-like retrotransposon (*ROMANIAT5*) in close proximity of the *APUM9* transcriptional start site (772bp upstream of the transcriptional stat site) (Fig. 2.1A). Transposable elements (TEs) are under tight epigenetic repression in order to prevent their mobilization. The TEs themselves and their epigenetic state can highly influence the fate of their neighboring genes (Slotkin and Martienssen, 2007; Girard and Freeling, 1999). The question arising is if the epigenetic state from *ROMANIAT5* can spread to *APUM9*, and if it is the case, to what extend is it influencing the expression of *APUM9*. A previous study from (Yokthongwattana et al., 2010) reported on the coordinated transcriptional regulation of *APUM9* and the *ROMANIAT5*. First it was shown that transcriptional activation of *APUM9* corresponds with transcriptional activation of *ROMANIAT5*, and that both of them are under similar epigenetic regulation being targets for several epigenetic regulators (*MOM1*, *DRM2, Pol V*).

In order to monitor APUM9 expression, we obtained a GFP based reporter line that reports on APUM9 expression and used this line in forward and reverse genetic studies. We performed a forward genetic mutant screen on that reporter line and recovered several lines (*epic1-7*; for epigenetic control), mutated in distinct epigenetic regulators. All of these mutations caused activation of the silenced transgene in different tissues, providing evidence for the potential existence of the tissue-specific

epigenetic programs, and allowing assessment of how the different epigenetic regulators contribute to the gene expression patterning.

Among the recovered mutants from the genetic screen is *epic1*, a novel allele of the histone deacetylase HDA6, which is well known epigenetic regulator, involved in silencing TEs, rRNA genes, transgenes and developmental processes (Earley et al., 2010; Probst et al., 2004; Aufsatz et al., 2002b) via regulating the dynamics of the acetylation levels in histones and DNA methylation (Liu et al., 2012; Vaillant et al., 2006; Aufsatz et al., 2002b).

In this chapter I further characterize the novel HDA6 allele and found that it affects histone acetylation, but not DNA methylation. The detailed analysis of this novel allele revealed that HDA6 has at least two independent euchromatic and heterochromatic functions. This allowed the postulation of a new regulatory mechanism in which HDA6 may inhibit *de novo* DNA methylation in the CG context.

Results

The silex reporter line

In a mutant screen for modifiers of *MOM* (Morpheus' molecule) (Amedeo et al., 2000), a genetic interaction between MOM and Pol V was revealed. These two TGS regulators were shown to have a synergistic effect in transcriptional activation at selected loci in the Arabidopsis genome (Yokthongwattana et al., 2010). Yet, this synergistic effect was suggested to be highly dependent on the target loci and their chromosomal location. The *APUM9* gene in Arabidopsis is one such locus that is regulated by MOM1 and Pol V. Loss of function in *mom1* plants causes transcriptional activation of *APUM9* that is even more pronounced in *mom1nrpe1* (Yokthongwattana et al., 2010). This suggests that MOM1 and Pol V independently silence *APUM9* creating a double lock on its transcription.

We used a transgenic reporter line to investigate the regulation of *APUM9* in more details. The reporter line was retrieved from the collection of GFP reporter lines of low expressing genes in Arabidopsis (Xiao et al., 2010). The transgene construct includes the promoter of *APUM9* and 1.5 kb of the *ROMANIAT5* that lies upstream (Fig. 2.1B). Arabidopsis plants transformed with the reporter construct showed no visible GFP expression at the juvenile stage, and tissue-specific expression in adult phase, where GFP was observed in the fruits (siliques), hence the name of the reporter line "silex" (silique expression) (Fig.2.1C). Segregation analyses showed that the construct was inserted as a single copy at chromosome three, in a gene (*AT3G07640*) of unknown function.

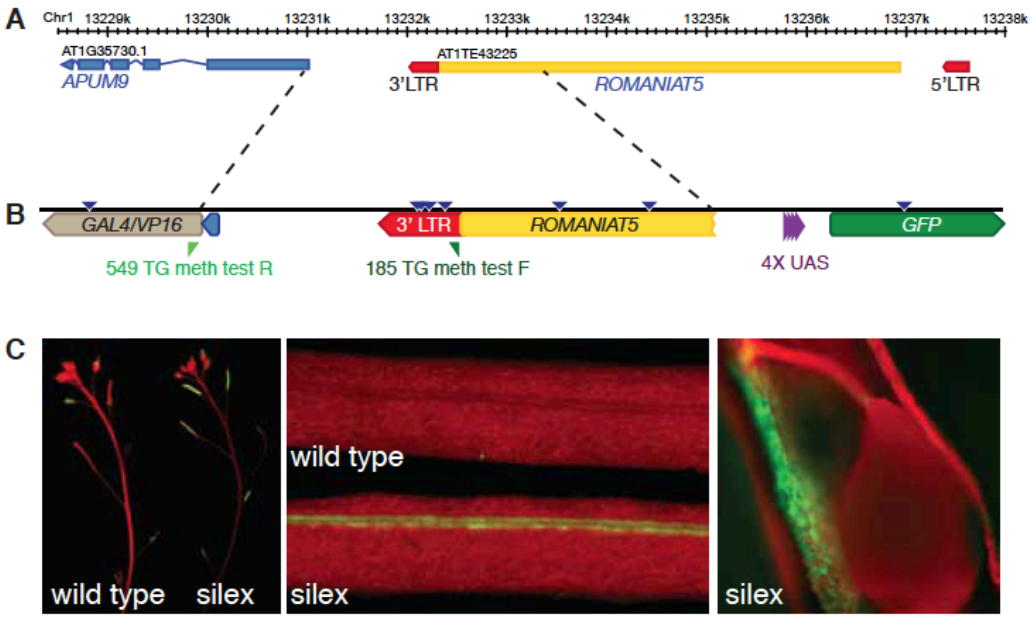


Figure 2.1 The silex GFP reporter line

(A) Schematic representation of the endogenous *APUM9* locus on chromosome 1 of Arabidopsis. The exons of *APUM9* are indicated by blue boxes. The yellow box upstream of *APUM9* marks the *ROMANIAT5* retrotransposon and the red boxes represent its LTRs. (B) Representation of the transgene in the silex line. It contains 2395 bp of the DNA sequence upstream of the CDS including 75 bases of the *APUM9* CDS. It was cloned in front of *GAL4/VP16*, which in turn will recognise the 4X UAS sequence in front of *GFP* to drive GFP expression. (C) Fluorescence images of the silex reporter line. GFP expression is green and chloroplast autofluorescence is red. Left panel shows GFP expression in siliques, GFP was detected in the valve margin of siliques (central panels). A dissected silique is shown on the right panel depicting a seed and the green fluorescent valve margin. Wild type non-transgenic plants are shown as controls.

The *silex* reporter transgene is epigenetically repressed

The transgene construct and endogenous *APUM9* were found to be under similar epigenetic regulation. Notably, introgressions of *mom1* (*mom1-2* (Habu et al., 2006)), *nrpe1* (*nrpe1-2* (Pontier et al., 2005)) and *mom1nrpe1* into the silex line resulted in GFP expression that was visible on the abaxial side of the leaves in the case of *mom1nrpe1*, as opposed to *mom1* and *nrpe1*, where no GFP signal could be detected visually (Fig. 2.2 A). Quantification of *GFP* mRNA levels by real-time PCR corresponded well with the observed increase in GFP fluorescence. *GFP* transcript accumulation was the highest in *mom1nrpe1* and showed weak activation of the transgene in *nrpe1* (two fold increase) and activation in *mom1* plants (Fig. 2.2 B).

However, activation in *mom1* plants was insufficient to produce microscopically detectable GFP fluorescence (Fig. 2.2).

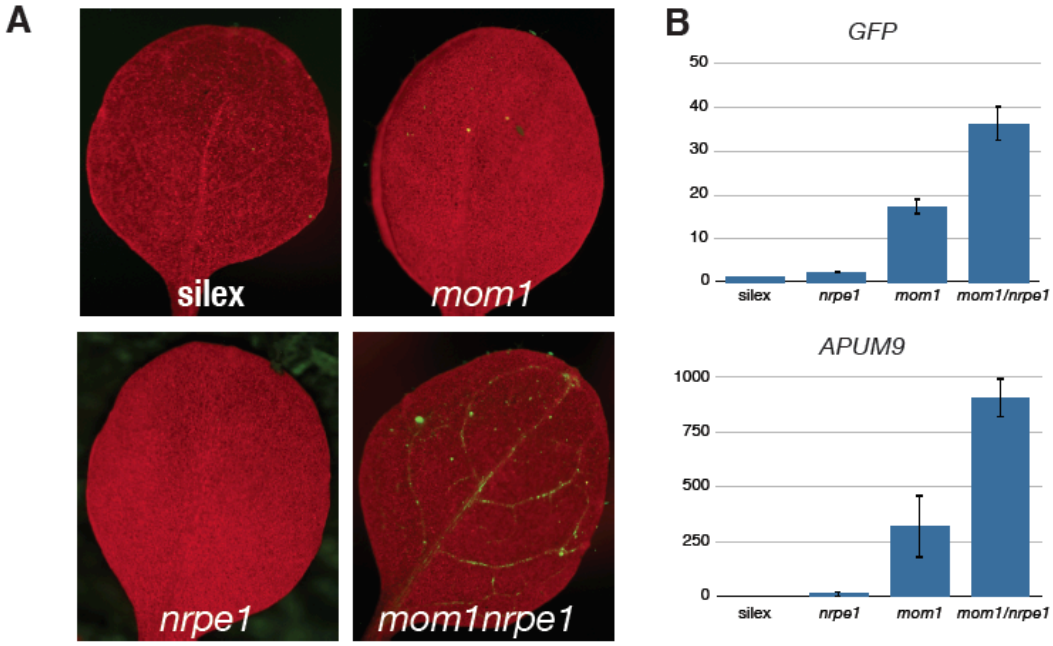


Figure 2.2 The silex transgene is repressed by the synergistic activity of MOM1 and NRPE1.
(A) Fluorescence imaging of the abaxial side of leaves. *mom1, nrpe1* and the combination of both mutations were introgressed into the silex reporter line. Only the double mutant resulted in visible release of GFP expression in veins. (B) Real-time PCR based measurement of *GFP* and *APUM9* transcript levels. The error bars indicate s.e.m. of three biological replicates.

We further analyzed the role of DNA methylation in repressing the transgene. For this purpose silex seedlings were grown on a MS medium supplied with 5-aza-2'-deoxycytidine (AzaC), a drug known to cause global DNA methylation reduction (Doerfler, 1983). In treated seedlings we observed stochastic release of GFP silencing in cotyledons (Fig. 2.3 A). Drug-induced release of *GFP* transcription was also confirmed at the transcriptional level (Fig. 2.3 B). Methylation sensitive analyses in the transgene promoter additionally confirmed the synergistic *mom1nrpe1* interaction. Whereas no loss of DNA methylation was identified in *mom1* (*MOM1* causes transcriptional activation without changes in DNA methylation), there is significant change of DNA methylation in *nrpe1*, and this effect is even more pronounced in *mom1nrpe1* (Fig. 2.3 C).

Thus DNA hypomethylation at the transgene promoter to a certain extend contributes to GFP activation in *mom1nrpe1*.

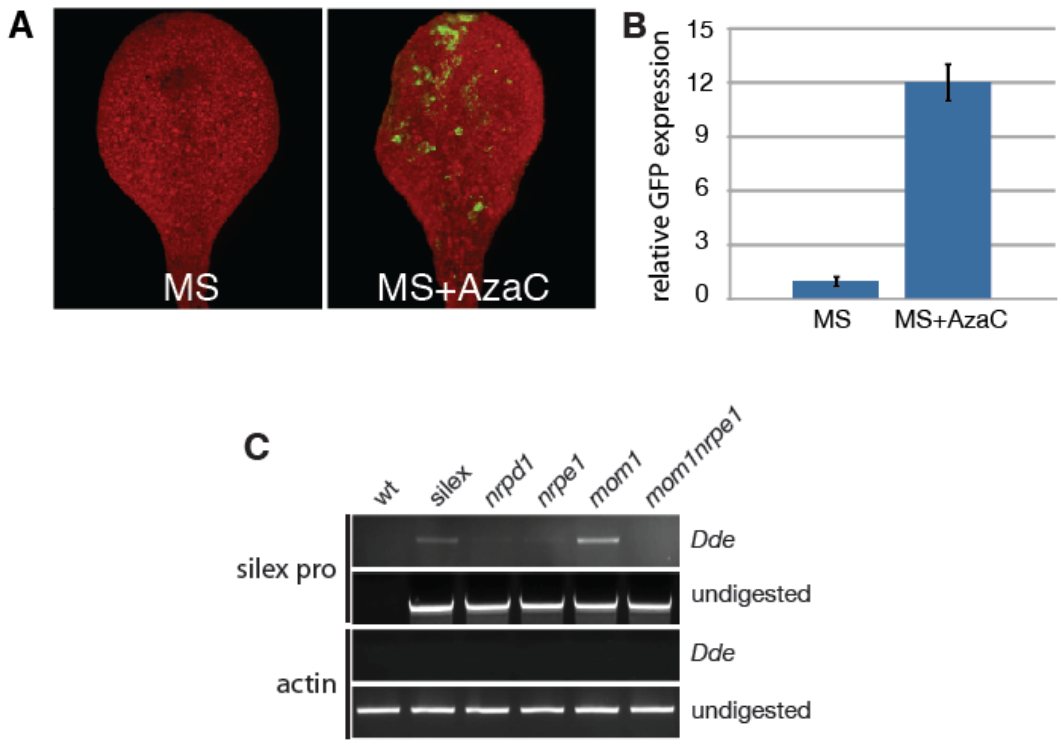


Figure 2.3 The transgene is methylated in *silex*
(A) Fluorescence imaging of cotyledons of silex seedlings grown on MS medium supplied with AzaC shows stochastic GFP activation. Non-treated seedlings are shown as a control. (B) Real-time PCR measurements of the GFP transcripts in AzaC treated and non-treated plants. (C) Methylation sensitive restriction combined with PCR analyses of the CG methylation at the transgene promoter shows the synergistic effect of *mom1nrpe1* on the transgene. *ACT2* is shown as a digestion control.

Taken together these data show that the transgene, likewise the endogene, is regulated by at least two epigenetic regulators, MOM1 and NRPE1, which in combination have a stronger, synergistic effect. Our GFP reporter line is adequately reporting on the epigenetic state of the endogenous *APUM9*. The observation of changes in the GFP expression pattern in different epigenetic regulators backgrounds, prompted us to seek novel epigenetic regulators. For that purpose, a forward genetic screen was performed.

Mutagenesis and mutant screen

Seeds from the silex were subjected to EMS (ethyl methanesulfonate) mutagenesis. EMS is an alkylating agent that causes randomly distributed mutations in the genome. It creates single nucleotide polymorphism (SNPs) resulting in changes of the bases G:C->A:T. The EMS mutation mechanism is based on modification of the nucleotides bases, which reduces their affinity towards their complementary nucleotides and causes miss paring. The miss paring is recognized by the DNA repair machinery, which exchanges the bases with new, complementary ones (Greene et al., 2003).

The seedlings from the progeny (M2) of the mutagenized silex seeds were screened for activation of GFP expression. The screen resulted in several lines, which activated the reporter transgene in different tissues (eg. shoot apex, hydatodes and vasculature, leaf edge) (Fig 2.4). Assuming that our mutants may be affected in epigenetic control of gene expression we named them "*epic*" mutants.

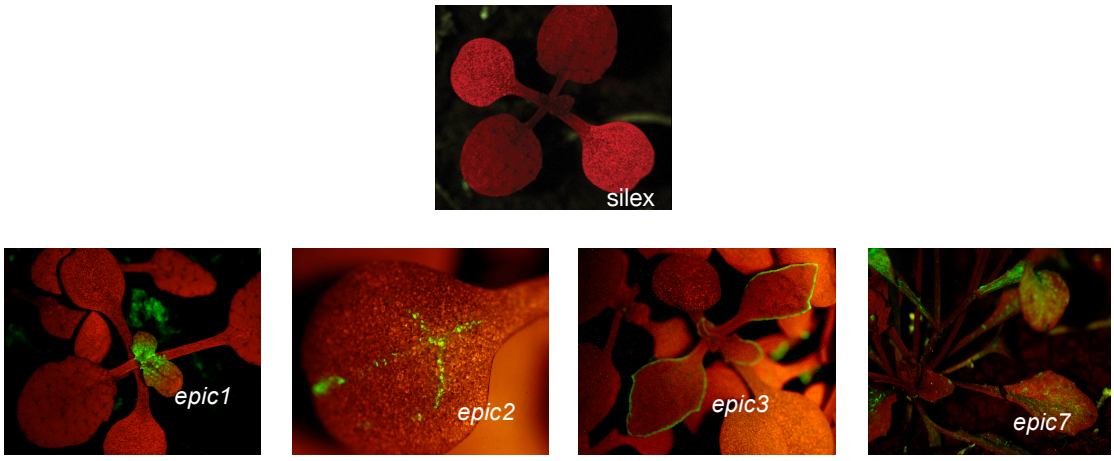


Figure 2.4 Tissue-specific transgene activation

Fluorescence imagining of silex and the "epic" lines (*epic1*, *epic2*, *epic3*, *epic7*) recovered upon EMS mutagenesis shows activation of the transgene in numerous different tissues.

Characterization and identification of the *epic1* mutation

The first line we identified in our mutant screen was *epic1* (epigenetic control of gene expression-1). It activates the transgene in the shoot apex (Fig. 2.5 A). The *epic1* mutation was recessive and was backcrossed to the parental silex line twice prior to further analysis in all following experiments. Real-time PCR measurements of *APUM9* and *GFP* transcript levels in *epic1* seedlings confirmed that these targets were de-repressed (Fig.2.5 B). Steady-state transcript levels of these two targets were also assessed in dissected young leaves where the GFP fluorescence is present in

epic1 and compared to fully developed leaves and the corresponding tissue in silex (Fig. 2.5 C).

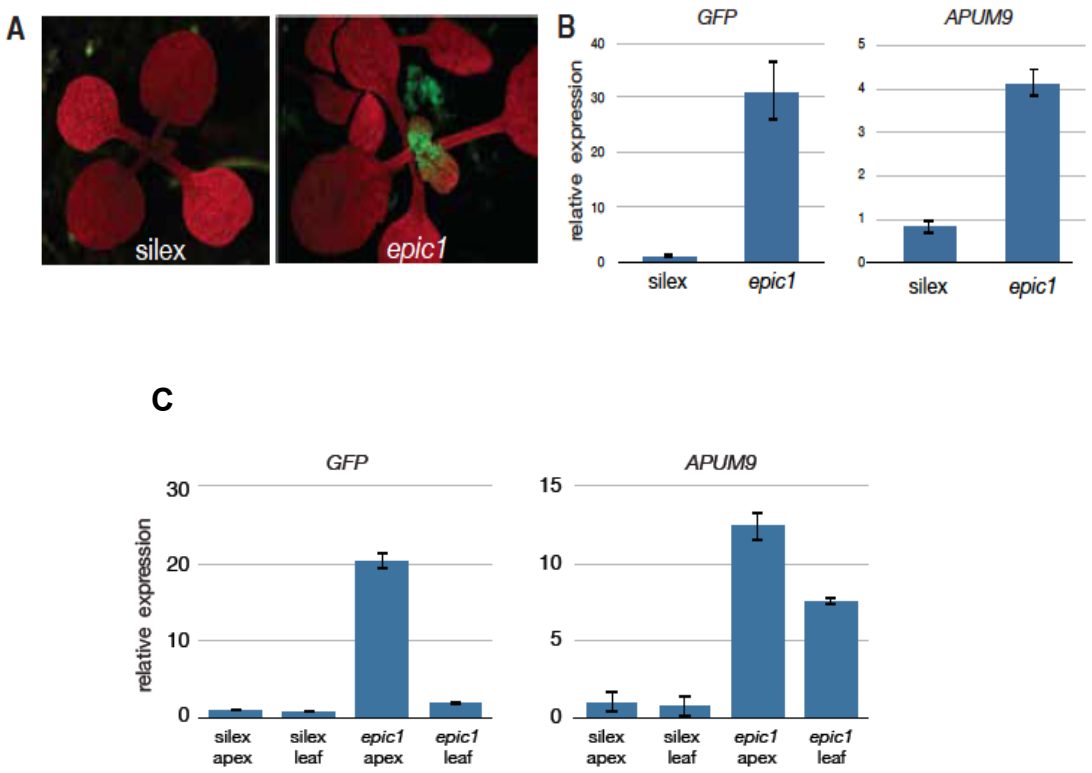


Figure 2.5 *epic1*, a novel mutant allele of HDA6, releases GFP expression in the shoot apex

(A) Fluorescence images of the silex and the epic1 mutant that released GFP silencing in young leaves (B) Real-time PCR based quantification of the release of GFP and APUM9 silencing in epic1 (error bars show s.e.m. of three biological replicates) (C) Real-time PCR measurements of APUM9 and GFP transcript levels in dissected plant parts to compare expression between leaves and the shoot apex. Error bars show s.e.m. of three biological repeats.

In order to identify the causal mutation in *epic1* we performed whole-genome re-sequencing on a pool of DNA extracted from 10 GFP positive F2 plants resulting from a backcross of *epic1* into the silex line. This allowed us to map the mutation to *HISTONE DEACETYLASE 6 (HDA6*, Fig 2.6).

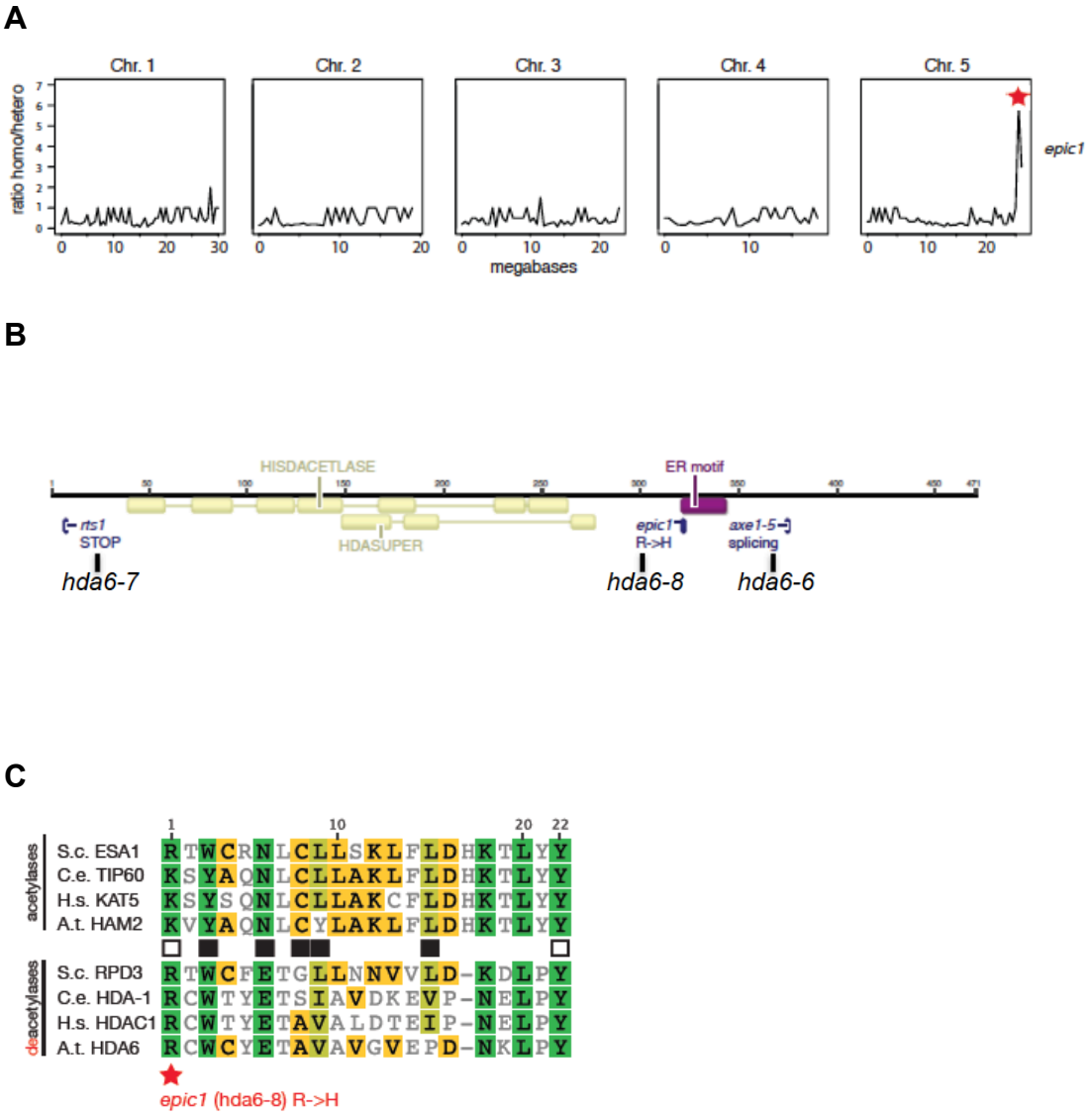


Figure 2.6 *hda6-8* carries a mutation in conserved domain of HDA6

(A) Homozygous versus heterozygous EMS mutation counts in 500 kb windows plotted along the five Arabidopsis chromosomes. DNA of a pool of 10 *epic1* plants coming from a backcross to the parental silex line was subjected to whole genome sequencing. This was sufficient to detect strong linkage disequilibrium at the lower arm of Chr. 5 (B) The conserved domains of HDA6 and the mutant alleles used in this study (C) *hda6-8* is mutated in a highly conserved E-R domain of HDA6 that is shared between histone deacetylases and histone acetylases. The colors indicate similarity levels (green for high and yellow for low). The *hda6-8* mutation was mapped to the first amino acid of this motif and resulted in a substitution of an arginine to histidine (indicated by the red star). The filled boxes indicate amino acids that have been found to be required for the enzymatic activity of the histone acetylases and histone deacetylases in yeast. Empty boxes indicate highly conserved amino acids that were shown to be dispensable for the enzymatic activity of both types of enzymes.

To confirm that a mutation in HDA6 could release GFP expression in young leaves we introgressed the *hda6-6* (*axe1-5*) allele of HDA6 (Murfett et al., 2001) into silex. This resulted in the same GFP expression pattern as we had previously observed in *epic1* (Fig. 2.7). We then rescued *epic1* by transformation with *HDA6* under its native promoter and thereby confirmed that the causal mutation was indeed located in *HDA6* (Fig. 2.7). In order to identify in which tissues HDA6 was expressed, we created an *HDA6::GUS* reporter line. GUS staining was observed in young developing leaves corresponding well with the release of GFP expression we had observed in *hda6-8* (Fig. 2.7).

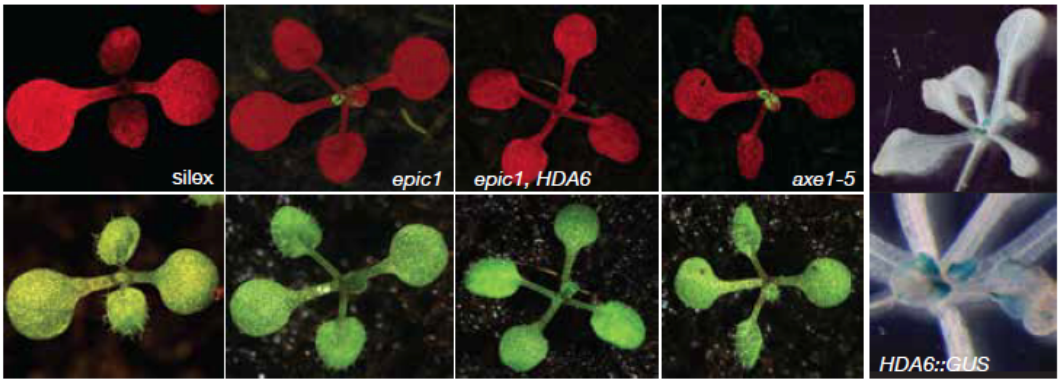


Figure 2.7 Confirmation of the mapping of the epic1 mutation to HDA6

From left to right: The *epic1* mutation was rescued with full-length HDA6 under its endogenous promoter (*epic1*, HDA6). Introgression of *hda6-6* (*axe1-5*) into the silex reporter line resulted in the *epic1* phenotype. GUS staining of the *HDA6pro:GUS* reporter line shows the expression pattern of HDA6.

Since we validated *epic1* to be mutated in *HDA6* we termed this allele *hda6-8* (Fig. 2.6 B shows the HDA6 alleles used in this study and their position in the protein). Predictions of the secondary structure of HDA6 suggested that the *hda6-8* mutation did not cause a change in the secondary structure of the protein (Rice et al., 2000). However, we found that the *hda6-8* allele was of interest because it was mutated in a highly conserved amino acid of the previously described E-R (Esa1-Rpd3) motif. Notably, this motif is present in both, histone acetylases and histone deacetylases (Adachi, 2002) (Fig. 2.6 C). Phenotypically, *hda6-8* did not differ form *hda6-6* or *hda6-7* (*rts1*) and also showed delayed flowering as was previously reported for *hda6-6* (Yu et al., 2011).

hda6-8 affects histone acetylation but not DNA methylation

Because HDA6 is required for histone deacetylation we tested if the chromatin at the promoters of the *APUM9* endogene and the silex transgene were hyperacetylated in *hda6-8* plants. Indeed we observed a strong increase in H4-tetra-acetylations in the promoter region of these loci (Fig. 2.8 A).

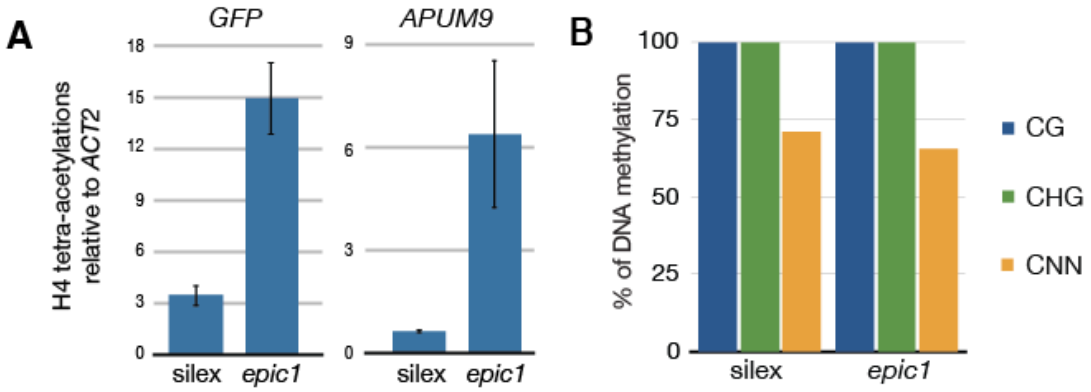


Figure 2.8 *hda6-8* affects histone acetylation but not DNA methylation at the transgene promoter

(A) Chromatin immunoprecipitation of acetylated histones (H4 tetra-acetylations) and quantification by real time PCR at the transgene and endogene *APUM9* promoters (error bars show s.e.m. of three biological replicates) (B) bisulfite sequencing of the *ROMANIAT5 LTR* present in the transgene.

Since HDA6 has been reported to play a role in the maintenance of DNA methylation (Probst et al., 2004) we assessed its' levels at the transgene locus using bisulfite sequencing. However, we did not detect significant changes in DNA methylation in any of the sequence contexts in *hda6-8* (Fig. 2.8 B).

We then compared transcription of known target genes controlled by HDA6 in *hda6-8* and the well-characterized *hda6-7* (*rts1-1*) null mutant allele of HDA6 (Aufsatz et al., 2002b; Pontvianne et al., 2012). We found that silencing of targets controlled by RNA-directed DNA methylation (RdDM) such as *solo LTR* (Huettel et al., 2006) and *AT4G04293* (*AtIS112A*) (Numa et al., 2009; Yokthongwattana et al., 2010) were released in both *hda6-7* and *hda6-8*, however *hda6-8* tended to have a weaker effect (Fig. 2.9 A). A clear difference between *hda6-8* and *hda6-7* was observed when we assessed transcription of the HDA6 targets *AT5G41660* and *AT3G4470* (Fig. 2.9 A)

(To et al., 2011a). In contrast to *hda6-7*, *hda6-8* showed no release of transcriptional suppression of these two targets.

To test if histone acetylation levels at these HDA6 targets were affected in *hda6-8* we performed chromatin immunoprecipitation (ChIP) (Jaskiewicz et al., 2011) of histone H4 lysine residues associated with tetra-acetylation (K5, K8, K12, and K16 on H4). Confirming previous reports (To et al., 2011a), we observed strong increase of H4 tetra-acetylation in the *hda6-7* null mutant at all tested targets (Fig. 2.9 B). Compared to the silex reporter line *hda6-8* also showed significantly increased H4 tetra-acetylation levels at *solo LTR* and *AT4G04293*. In the case of *AT5G41660* and *AT3G44070* we only observed a very modest increase of H4 tetra-acetylation in *hda6-8*. It has been reported that release of silencing of certain targets in HDA6 defective plants was coinciding with loss of DNA methylation in the CG context (Earley et al., 2010; To et al., 2011a). One such target that has previously been described is *AT5G41660*. Confirming these previous reports, we found loss of CG methylation at *AT5G41660* in *hda6-7*, but we did not observe loss of DNA methylation in *hda6-8* (Fig. 2.9C). Other tested HDA6 targets did not show loss of DNA methylation in either *hda6-7* or in *hda6-8* (Fig. 2.9 C).

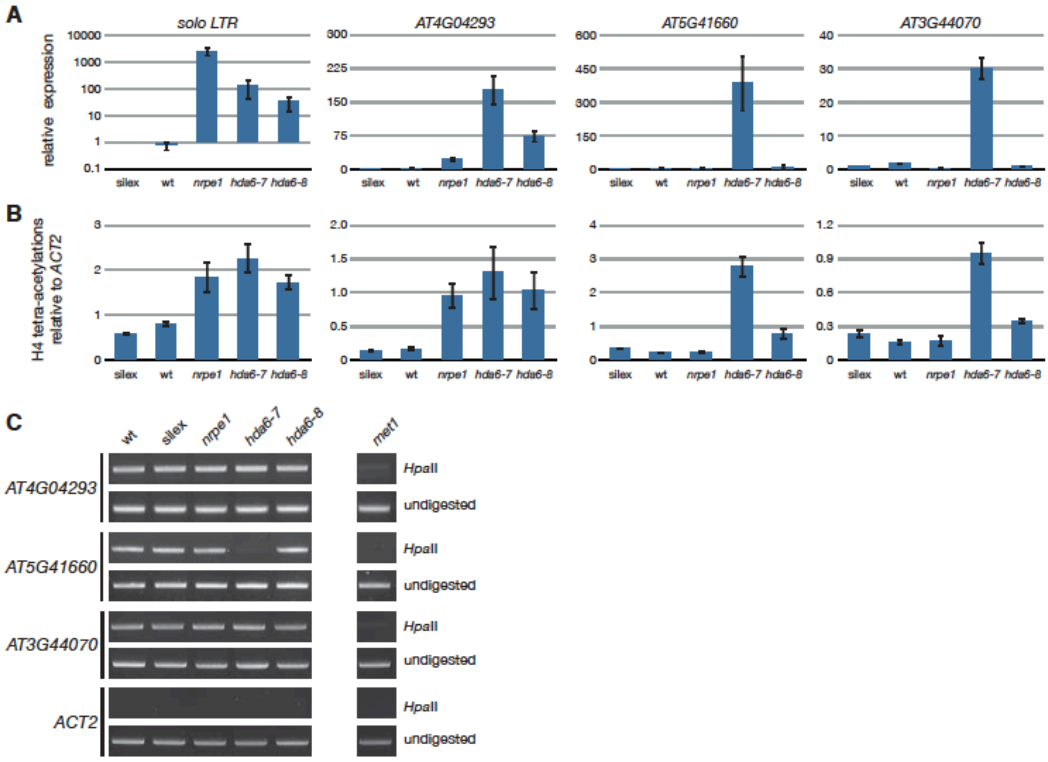


Figure 2.9 *hda6-8* influences HDA6 target specificity

(A) Comparison of the release of transcription of HDA6 targets in the *hda6-7* null mutant and *hda6-8*. (B) H4 tetra-acetylation levels at the HDA6 targets assesses by ChIP followed by real-time PCR. Error bars show s.e.m. of three biological replicates(C) DNA methylation levels in the CG context of HDA6 targets. Genomic DNA was digested by the methylation sensitive *HpaII* restriction enzyme reporting on CG and CHG methylation and then PCR amplified with target specific primers.

Transcriptional release of heterochromatic regions differ in *hda6-6* and *hda6-8*

In order to globally compare the genomic regions that were transcriptionally activated in *hda6-6* and *hda6-8* we generated a transcriptome of *hda6-8* using tiling arrays and compared it to the previously reported *hda6-6* transcriptome (To et al., 2011a). It was shown earlier that the *hda6-6* and *hda6-7* mutant alleles efficiently release transcription of TEs (Blevins et al., 2014; Probst et al., 2004; To et al., 2011a). In order to obtain an overview of the chromosomal distribution of the up-regulated transcripts in *hda6-6* and *hda6-8* they were plotted onto the five Arabidopsis chromosomes (Fig. 2.10 A). Notably on the chromosomes 4 and 5 a high number of up-regulated transcripts can be observed in heterochromatic regions (represented as TE-rich regions here) in *hda6-6* while it is not the case in *hda6-8*. In accordance with

this observation *hda6-8* released transcription of very few TEs compared to *hda6-6* (Fig. 2.10 B).

A highly conserved amino acid in HDA6 prevents *de novo* CG methylation

We then wanted to assess if HDA6 could play a role in *de novo* DNA methylation by testing if DNA methylation that was lost in *hda6-7* could be recovered by restoring HDA6 activity. *hda6-7* plants were transformed with wild-type HDA6 or with the mutant *hda6-8* allele (*hda6-7,HDA6* and *hda6-7,hda6-8* respectively). Transformation of *hda6-7* with *HDA6*, did not restore CG methylation at *AT5G41660*, similarly to the *ETR7* gene that was previously described (Blevins et al., 2014). However, we observed acquisition of *de novo* DNA methylation at *AT5G41660* in *hda6-7* plants rescued with *hda6-8* (Fig. 2.10 C). Same results were obtained with two independent transformants for each line presented here. All plants were genotyped for the presence of the transgene and the *hda6-7* mutation. This data suggests that the highly conserved E-R motif of HDA6 is involved in repressing *de novo* DNA methylation activity at certain targets. As an additional control we included *AT2G34655* (*ETR15* in (Blevins et al., 2014)), which is up regulated in *hda6* mutants but where CG methylation is not lost (Fig. 2.10 C).

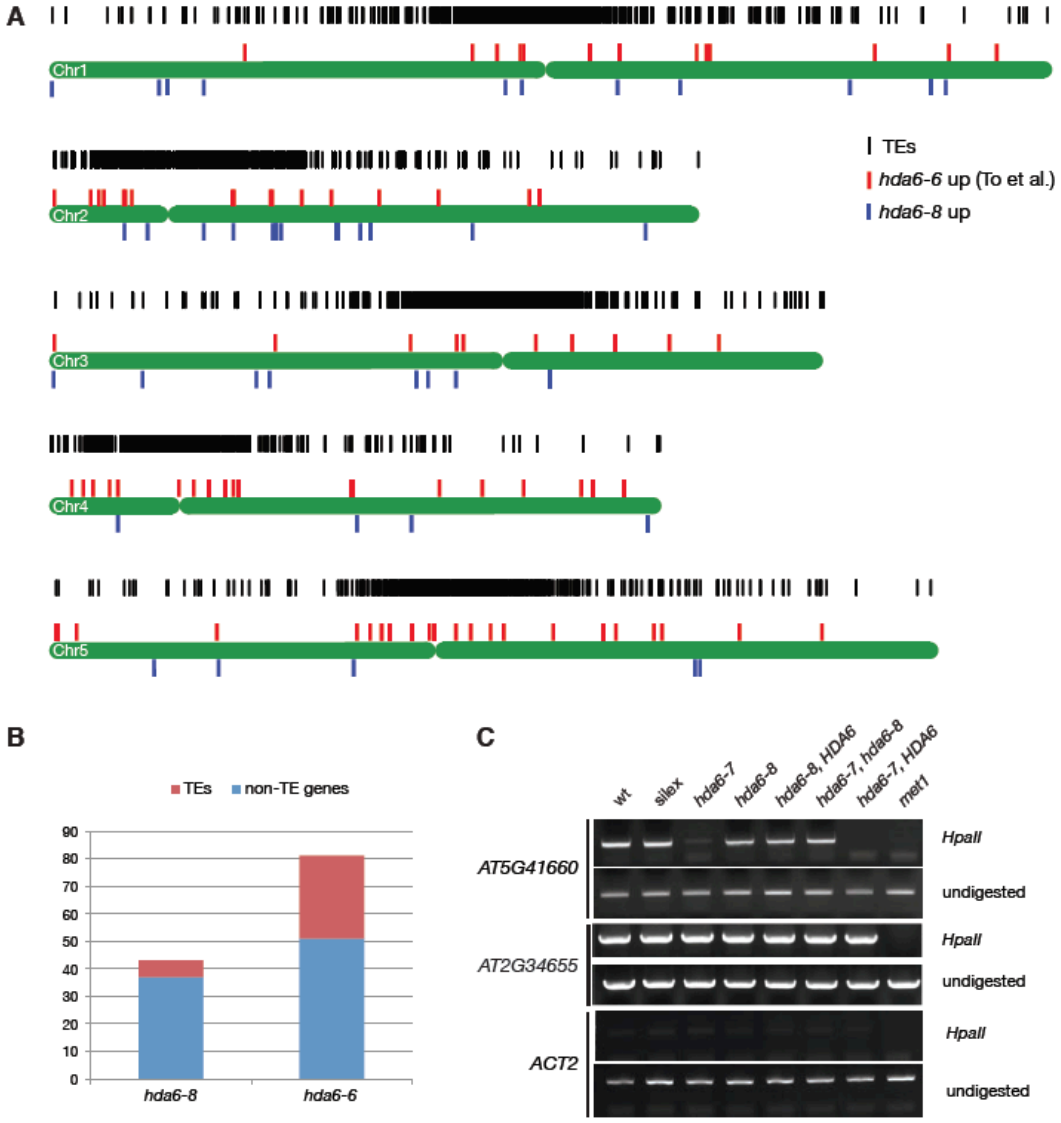


Figure 2.10 *hda6-8* regulates euchromatic silencing and may play a role in *de novo* DNA methylation

(A) Graphic representation of the 5 Arabidopsis chromosomes (green), distribution of TEs (black), distribution of *hda6-6* up-regulated annotated transcripts (in red, from (To et al., 2011)) and *hda6-8* up-regulated genes (blue). (B) Number of up-regulated TEs and protein coding genes in *hda6-8* and *hda6-6*. (C) Chop-PCR based analysis of the CG methylation state of *AT5G41660* and *AT2G34655* in the different HDA6 alleles and in the *hda6-7* lines that were rescued with wild type HDA6 or the *hda6-7* allele. *ACT2* is shown as a digestion control.

Discussion

Numerous factors repressing transcription of silenced chromatin in *Arabidopsis* have been described (see (Eun et al., 2012; Furner and Matzke, 2010; Matzke and Mosher, 2014) for recent reviews). Intriguingly, many of these factors only affect certain subsets of silenced TEs and genes (Slotkin, 2010; Vaillant et al., 2006; Yokthongwattana et al., 2010) suggesting that each factor has a distinct target specificity. However, how these factors contribute to gene expression patterning has not been investigated in detail so far.

In order to explore this systematically we used the epigenetically controlled and highly tissue specific silex reporter line.

Epigenetic regulation of gene expression patterning

The silex reporter line showed GFP expression in the valve margin of siliques. This was unexpected as transcription of the *APUM9* gene is under tight epigenetic repression implemented by MOM1 and NRPE1 (Yokthongwattana et al., 2010). This suggests that both MOM1 and NRPE1 may be inactive in this tissue. Interestingly, this observation fits well with the developmental relaxation of TE silencing that has been proposed (Martínez and Slotkin, 2012). In *Arabidopsis* this has been extensively studied in reproductive and gametophytic tissues but little is known concerning sporophytic tissues. The presented GFP reporter line now allows addressing this tissue as well.

Introgression of the individual *mom1* and *nrpe1* mutations into the silex line showed no visible release of GFP expression, nevertheless we could detect some GFP mRNAs in *mom1* plants. Interestingly, the *mom1 nrpe1* double mutant released GFP expression at the veins, which was only visible on the abaxial side of the leaves. Taking into consideration previous reports on the global effect of *mom1* in transgene activation in the 6b5 reporter line, this phenotype was unexpected (Vaucheret and Fagard, 2001). The vein specific activation of the transgene in *mom1 nrpe1* plants, and the stochastic activation upon AzaC treatment, indicated that several epigenetic regulators might influence the expression pattern of the silex transgene. The forward genetic screen confirmed these observations because we recovered *epic1*, a mutant

releasing GFP expression in the shoot apex of seedlings. This mutant depicted a distinct GFP expression pattern compared to *mom1nrpe1* (Fig. 2.2 and 2.5).

We mapped the *epic1* mutation to HDA6 (and we thus renamed this allele *hda6-8*), a well-characterized histone deacetylase that has been recovered in several mutant screens designed to identify factors involved in transcriptional gene silencing (TGS) (Aufsatz et al., 2002b; Murfett et al., 2001; Probst et al., 2004). The GFP expression pattern corresponded well with the specific transcription of *HDA6* in the shoot apex (Fig. 2.7, (Winter et al., 2007)) and to the pattern of release of GUS silencing initially described in the *HDA6* mutant *axe1-3* (Murfett et al., 2001). This suggests that the main activity of HDA6 takes place in the shoot apex, where it repressed the GFP transgene of the silex line. This is well in line with a recent report that showed that expression of meristem-specific silencing factors is important to repress TEs (Baubec et al., 2014; Yadav et al., 2009). Notably, as leaves expand and mature GFP fluorescence is lost. It is thus possible that other epigenetic regulators, such as the histone-deacetylase HDA19 that is more ubiquitously expressed in seedlings (Zhou et al., 2005), take over the role of silencing the transgene in these tissues. Another possibility that cannot be excluded is post-transcriptional repression of the transgene that might occur as the leaf ages.

A highly conserved domain in *HDA6* is required for its target specificity

Since *hda6-8* solely carried a single amino acid substitution we wanted to compare its effect on the release of known HDA6 targets to an *HDA6* null mutant (*hda6-7*, *rts1-1* (Aufsatz et al., 2002b)). We found that *hda6-8* strongly released RdDM-dependent HDA6 loci (*solo LTR* and *AT4G04293*) but not the RdDM-independent HDA6 targets (*AT5G41660* and *AT3G44070*). The effect on transcription was also reflected by changes in epigenetic marks since the RdDM-independent targets did not gain histone H4 acetylation in *hda6-8* (Fig. 2.9 B). Interestingly, we also found that at *AT5G41660* DNA methylation was lost in the CG context in *hda6-7* but not in *hda6-8* (Fig. 2.9 C). The finding that CG methylation was not reduced in *hda6-8* indicates that recruitment of MET1 by HDA6 was not affected in *hda6-8* plants (Liu et al., 2012; To et al., 2011b). Detailed analysis revealed that the mutation in *hda6-8* was located in the highly conserved ER motif that is present in histone acetylases and deacetylases (Adachi, 2002) (Fig. 2.6 C). Combined with our observation that only a subset of

HDA6 targets were affected in *hda6-8* suggests that the mutated amino acid in the ER motif may play an important role in targeting HDA6 to specific loci. More specifically, our data supports observations made in yeast that the first amino acid of the ER motif is not required for the enzymatic activity of histone deacetylases (Adachi, 2002), since *hda6-8* retained its histone deacetylase activity at the RdDM-independent targets. The ER motif may therefore be involved in recognition of specific DNA sequence contexts and/or chromatin modifications and thereby defining the target specificity of HDA6. Alternatively, the mutation in the ER motif may influence protein-protein interactions that are required for proper targeting of HDA6 to specific regions in the genome. In yeast, RPD3 the ortholog of Arabidopsis HDA6, has been shown to be part of large protein complex that includes transcriptional repressors such as SIN3 and UME1 (Grzenda et al., 2009). It is thus possible that the *hda6-8* mutation in the ER motif affects the interaction of HDA6 with such transcriptional repressors.

HDA6 has distinct activities in euchromatin and heterochromatin

The strong mutant alleles of HDA6 have been shown to release silencing of heterochromatic transcripts (Blevins et al., 2014; Liu et al., 2012; Probst, 2004; To et al., 2011b). A striking difference we observed in the *hda6-8* transcriptome was that it had only very little effect on TEs and heterochromatic transcripts (Fig. 2.10 A and B). This suggests that the amino acid change in the highly conserved ER domain only plays a role in silencing euchromatic genes and that it has little to no role in heterochromatin silencing, probably because this mutation does not affect the interaction of HDA6 with MET1 (Liu et al., 2012; To et al., 2011b). This observation is intriguing because it suggests that depending on the chromosomal location HDA6 may interact with different proteins to silence genes or conversely, that interacting proteins define HDA6 target specificity.

It has been documented that epigenetic changes caused by defects in *met1-3* and *ddm1* can cumulate over generations of inbreeding (Kakutani et al., 1996; Mathieu et al., 2007). We analyzed *hda6-8* directly after the second backcross, thus excluding potential inbreeding effects as they might have accumulated in *hda6-6* and *hda6-7*. These mutants have been discovered more than a decade ago and likely have been inbred over multiple generations since their initial discovery (Aufsatz et al., 2002b;

Murfett et al., 2001). We thus cannot exclude that some of the differences between the mutant alleles that we observe may also be caused by such inbreeding effects.
Interestingly, *hda6-8* efficiently released the GFP transgene in silex, even thought it had acquired heterochromatic properties. This observation might be due to the insertion of the transgene in euchromatin and is therefore under less repressive state then *bona fide* heterochromatic regions.

The HDA6 ER motif represses *de novo* DNA methylation

HDA6 is implicated in silencing diverse endogenous targets, either via RdDM (Aufsatz et al., 2002b) and in interaction with MET1(Liu et al., 2012; To et al., 2011b). An interesting group of targets has been described previously (Blevins et al., 2014). The described group E targets lose DNA methylation in *hda6-6* however this methylation is not recovered in *hda6-6* plants rescued with HDA6, showing that HDA6 is required to maintain DNA methylation. We found the same to be true at the *AT5G41660* locus. However when we complemented *hda6-7* with the *hda6-8* allele of HDA6 we reproducibly found that CG methylation could be restored. This observation is in line with experiments that were carried out in animal cells where inhibition of histone deacetylases lead to an increase in CG methylation at certain targets (Jia et al., 2015). It suggests that even thought DNA methylation is lost in *hda6-7* plants and not restored in the complementation assay, an epigenetic memory, either in the form of histone modifications or small RNAs is still present allowing DNA methylation to come back under certain circumstances. Notably the mutation in *hda6-8* is located within the C-terminal region of HDA6 that has been shown to interact with MET1 (Liu et al., 2012). It is therefore possible that the ER motif is involved in regulating MET1 activity. It is currently unclear how *hda6-8* may target *de novo* methylation and it will be interesting to investigate this activity in more detail in the near future.

Conclusions

The processes of cell specialization and differentiation depend on a complex network of highly controlled expression of gene sets in space and time. Chromatin modifications and epigenetic regulation of gene expression play important roles in the regulation of cell fate. However, our understanding about the mechanisms involved in these processes is currently limited. The results presented here open the door to tissue and cell-type specific analysis of epigenetic regulation of gene expression. It will be very interesting to further investigate these aspects since it may answer one of the most fundamental questions in molecular biology: How do genes know when and where to be expressed?

Materials and methods

Plant material, mutagenesis and mapping

All plants used in this study come from the Columbia accession. The silex reporter line was obtained from a collection created by The Institute for Genomic Research (J. Craig Venter Institute, line AGRAC-60-1-1) (Xiao et al., 2010). Mutants used in this work were: *mom1-2* (Habu et al., 2006), *nrpe1-2* (formerly *nrpd1b-2*; (Pontier et al., 2005), *met1-3* (Saze et al., 2003), *axe1-5* (Murfett et al., 2001) and *rts1*(Aufsatz et al., 2002b). EMS mutagenesis was carried out as described previously (Weigel and Glazebrook, 2002) and plants were grown in Sanyo MLR-350 chambers at 24°C with 16 hours light.

Causal EMS mutations were mapped by whole genome sequencing combined with classical mapping by crossing the mutants with the Wassilewskija accession (WS). Reads were mapped against the reference genome and SNPs called in Geneious (Biomatters Ltd.). Using R SNPs were filtered for EMS mutations (G:C->A:T) and zygosity called based on the variant frequency provided by Geneious (>=80% homozygous mutation, >=45% and <=55% heterozygous mutation). Plots were then created by calculating the ratio of the number of homozygous and heterozygous and mutations in a 500 kb window.

Transgenic lines

Promoter and rescue constructs were all cloned into the pCAMBIA1304 plasmid. The *HDA6* promoter (including 1057 bp upstream of the transcription start site) and the full length *HDA6* gene including the promoter (4044 bp) were PCR amplified from genomic DNA of wild-type (rescue and promoter constructs) or *hda6-8* plants (rescue constructs). The obtained PCR products cloned into pGEM-T easy (Promega), sequenced and then cloned into pCAMBIA1304.

DNA methylation analysis

For methylation sensitive PCR, genomic DNA from fresh leaf tissue was isolated using the DNeasy Plant Mini Kit (Qiagen). 50 ng of DNA was then digested with *Dde*I and *Hpa*II restriction endonucleases (NEB) overnight. It was then PCR-amplified using specific primers for the promoter regions of target genes.

Bisulfite analyses were carried out as previously described (Yokthongwattana et al., 2010) with the following modification: to be able to differentiate between the endogenous and

transgenic *ROMANIAT5* LTR, genomic DNA was digested with *Ssp*I (NEB) and re-ligated prior to bisulfite treatment. This resulted in an inverse PCR-like approach allowing specific amplification of the LTR present in the transgene.

Real-time PCR and transcriptome analysis

Total RNA from 100 mg of fresh leaf tissue of Arabidopsis plants was isolated with innuPREP Plant RNA Kit (Analytik Jena). 500 ng of RNA were used for cDNA synthesis (iScript cDNA synthesis kit, Bio-Rad). Expression of target genes was measured by quantitative PCR (qPCR) in a Light-Cycler 480 (Roche), using SYBR Green I Master Mix. Steady state mRNA levels were calculated with the Light-Cycler 480 software (Roche) using *ACT2* for normalization.

Transcription profiling on the silex reporter line and *epic1* was carried out on RNA extracted from leaves of 17 days old plants as described previously on one biological replicate (Yokthongwattana et al., 2010). The raw data has been submitted to the GEO repository as study GSE65640.

Chromatin Immunoprecipitation

ChIP experiments were performed on chromatin extracted from leaves of three weeks old plants as described by (Jaskiewicz et al., 2011) using anti-H4 tetra-acetylation antibody (06-866) from Milipore (To et al., 2011a). Relative histone acetylation were calculated using the comparative C_T method (Schmittgen and Livak, 2008) by normalizing against input and *ACT2*.

Chapter 3

epic3: Uncovering a novel role of the nuclear pore complex in RNA export

Abstract

The nuclear-cytoplasmic export of mRNAs plays an essential role in the regulation of gene activity in eukaryotes. Eukaryotes have evolved specialized RNA export pathways that mediate the export of the different RNAs: tRNAs, micro RNAs (miRNAs), small nuclear RNAs (snRNAs), messenger RNAs (mRNAs) and ribosomal RNAs (rRNAs). Each type of RNA associates with specific proteins in ribonucleoproteins complexes, which as such are exported to the cytoplasm. While numerous studies have been carried out in order to elucidate the export mechanisms of the aforementioned RNAs, little is currently known about the export of antisense RNAs (asRNAs). Here we show that AtSAC3B, a component of the nuclear-pore associated complex TREX-2 complex, is required for efficient poly(A) RNA export. At the transcriptional level we found that *atsac3b* mutant plants resembled heat stressed plants. We found that heat stress efficiently represses poly(A) RNA export in Arabidopsis. Using transcriptomics on nuclear and cytoplasmic RNAs we identified the nature the RNAs retained in the nucleus and found that AtSAC3B is mediating the export of antisense RNAs (asRNAs), while heat stress did not display such a bias. Our data shows that AtSAC3B is required for the export of asRNAs suggesting that these RNAs must play important functions in eukaryotic cells.

Introduction

Nuclear-cytoplasmic export of messenger RNAs (mRNAs) is an indispensable step of the regulation of gene activity, ensuring occurrence of the final step in gene expression, namely protein synthesis. mRNAs are exported to the cytoplasm as ribonucleoprotein particles (RNPs), small complexes composed of the nascent mRNAs and RNA-binding proteins (Erkmann, 2004). On their way to the nuclear membrane the RNPs are additionally accompanied by general export factors and the transcription-export (TREX) complex (Sträßer et al., 2002). The subunits of the nuclear-pore complexes (NPCs) recognize and bind to the general export factors. This process is not random, but affinity based and the NPC subunits display higher affinity towards some of the export factors compared to others. Thereby the NPCs not only facilitate the export of mRNAs but also contribute to selectivity of the export process. In addition to all these factors that determine or influence the mRNA export process, the environment, especially stress conditions such as high temperatures and ethanol were shown to have an effect on the process (Názer et al., 2012; Saavedra et al., 1997).

The nuclear-cytoplasmic export via the NPC is an intensively studied process, and the number of factors involved in it is constantly increasing. Among them is the transcription and export complex-2 (TREX-2). Initially identified in yeast (Fischer et al., 2002), the complex also known as Thp1-Sac3-Cdc31-Sus1 was shown to be associated with the nuclear pore complex (NPC), and its members to be involved in transcriptional elongation, thereby coupling transcription and export of mRNAs into the cytoplasm. Mutations in the TREX-2 complex members Sac3 and Thp1 in yeast result in impaired cytoplasmic mRNA export and drastic, microscopically detectable accumulation of polyadenylated (poly(A)) RNAs in the nucleus (Faza et al., 2009; Jani et al., 2009; Fischer et al., 2002). The Arabidopsis TREX-2 homologue has been identified recently. It consists of: AtSAC3B, AtSAC3A, AtTHP1, AtCEN1 and AtCEN2 (Lu et al., 2009).

Whereas the export pathways of different RNAs, such as: t-RNAs, miRNAs and mRNAs have been intensively studied, little is known about the export of the antisense RNAs (asRNAs). This is due to the fact that for a long time they have been considered as transcriptional noise, and their regulatory potential was underestimated. Recent studies however, have shown that more than 30% of the annotated human transcripts have antisense transcripts (Pelechano and Steinmetz, 2013). This high abundance of antisense transcripts is only an indicator of the potential that these transcripts have in the regulation

of the gene expression. The asRNAs originate from independent, bidirectional or cryptic promoters and they can regulate gene expression at the transcriptional, and posttranscriptional level (Sigova et al., 2013; Xu et al., 2011). In plants the best-studied asRNA regulated gene is the FLOWERING LOCUS C (FLC), where the transcription of the FLC is suppressed on a transcriptional initiation level, by changes in the chromatin organization induced by the asRNA-COOLAIR (COLD-ASSISTED INTRONIC NON CODING-RNA)(Swiezewski et al., 2009). Most of the asRNAs localize in the nucleus, but some of them were reported to go to the cytoplasm where they regulate gene expression on posttranscriptional level (Derrien et al., 2012). In yeast asRNAs utilize the general export factors (such as Mex67) that are also used by the mRNAs for export. How are asRNAs exported in plants is not known yet.

In the forward genetic screen for epigenetic regulators introduced in Chapter 2, we recovered new alleles of AtSAC3B, a component of the TREX-2 complex. We confirmed that AtSAC3B is required for poly(A)s RNA export and found that plants deficient in AtSAC3B resemble heat stressed plants at the transcriptional level. Finally, we show TREX-2 to be required for the export of antisense RNAs, which has implications on transcriptional regulation and genome stability.

Results

Mapping and characterization of *epic3*

The GFP-based reporter line-silex, introduced in Chapter 2, allowed us monitoring of the epigenetically controlled *APUM9* expression. In the mutant screen for regulators of silex we recovered couple of epigenetic regulators that are influencing the tissue localization of the reporter GFP. In Chapter 2 I introduced *epic1* (*epigenetic control 1*), a mutant deficient in the histone deacetylase *HDA6* leading to a release of GFP expression in young leaves (Chapter 2, Fig. 2.4). Another mutant that was recovered in the mutant screen was *epic3* (*epigenetic control 3*). This mutant carries a recessive mutation, which activates the transgene in the leaf margins (Fig.3.1 A).

In order to map the causal mutation in *epic3*, whole genome sequencing and classical genetic mapping were employed as previously described ((Hristova et al., 2015) and Chapter 2). The mapping data showed that the *epic3* mutation was located on chromosome 3, in *AT3G06290* encoding for the *SUPRESSOR FOR ACTIN 3-B* (*AtSAC3B*) gene. The

mutation in *AtSAC3B* was confirmed with the identification of two additional alleles: *epic3-2* and *epic3-3* (Fig. 3.1A) that were recovered in complementation tests (crosses). Since we validated the causal *epic3* mutation to be located in *AtSAC3B,* the recovered *epic3-1*, *epic3-2* and *epic3-3* alleles were renamed into: *atsac3b-3*, *atsac3b-4* and *atsac3b-5*, respectively. In two of the alleles (*atsac3b-3* and *atsac3b-5*) the mutation is located in the conserved SAC/GANP domain (Fig. 3.1B). For further characterization the *atsac3b-3* allele was used, after it was backcrossed twice to the parental line (silex).

The tissue specific transcriptional activation of the transgene registered in *atsac3b-3* was analyzed with QT-PCR. When the levels of the transgene transcripts in *atsac3b-3* were compared to the parental silex line, we observed weak but significant difference that can explain the observed phenotype. However, no significant difference was detected in the levels of the endogenous *APUM9* transcripts in *atsac3b-3* (Fig. 3.2 A).

In order to understand the mechanism underlying the transcriptional activation of the transgene in *atsac3b-3*, we analyzed some of the histone marks at transgene promoter. Using ChIP, we measured the levels of H3K27me3 (associated with transcriptional repression), H3K9me2 (transcriptional repression of transposable elements) and H4-tetra-acetylation (associated with transcriptional activation). We measured reduction in the H3K27me3 and H4 tetra-acetylation, but no significant change in the H3K9me2 levels (Fig. 3.2 B).

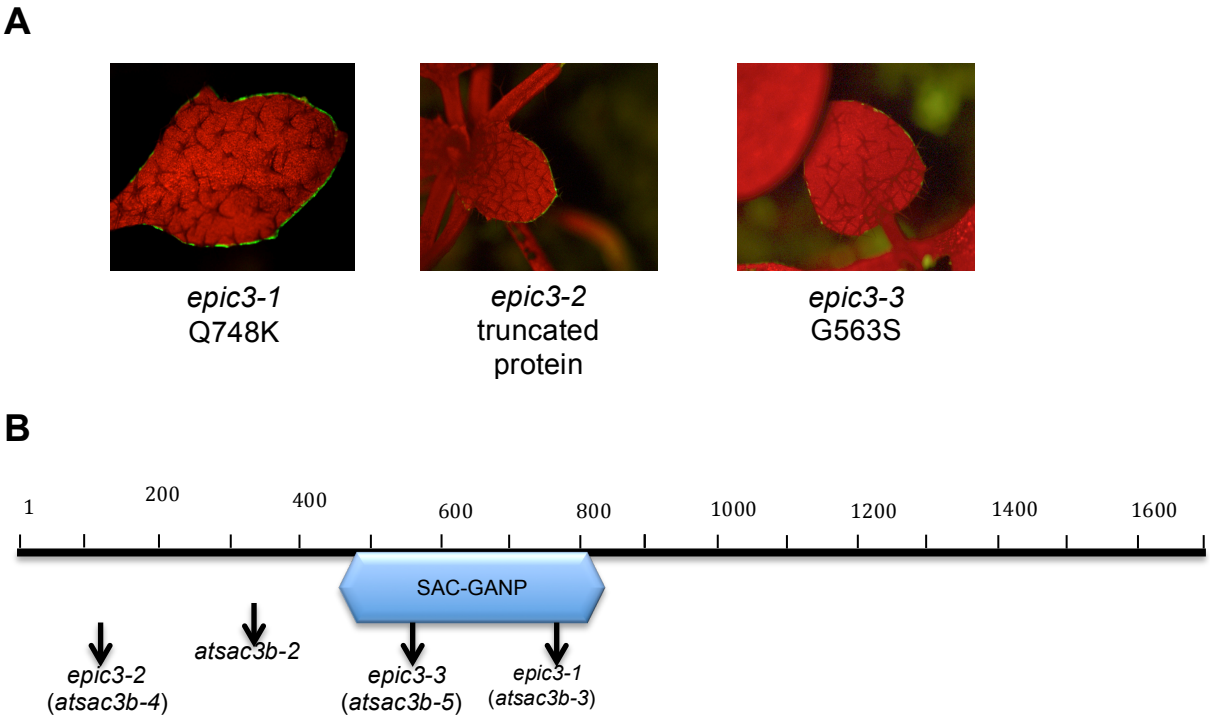


Figure 3.1 The mutation in *epic3*

(A) Fluorescence images of the different *epic3* alleles and the type of mutations that they carry (B) Schematic representation of the AtSAC3B protein and the location of the mutations discussed in this chapter.

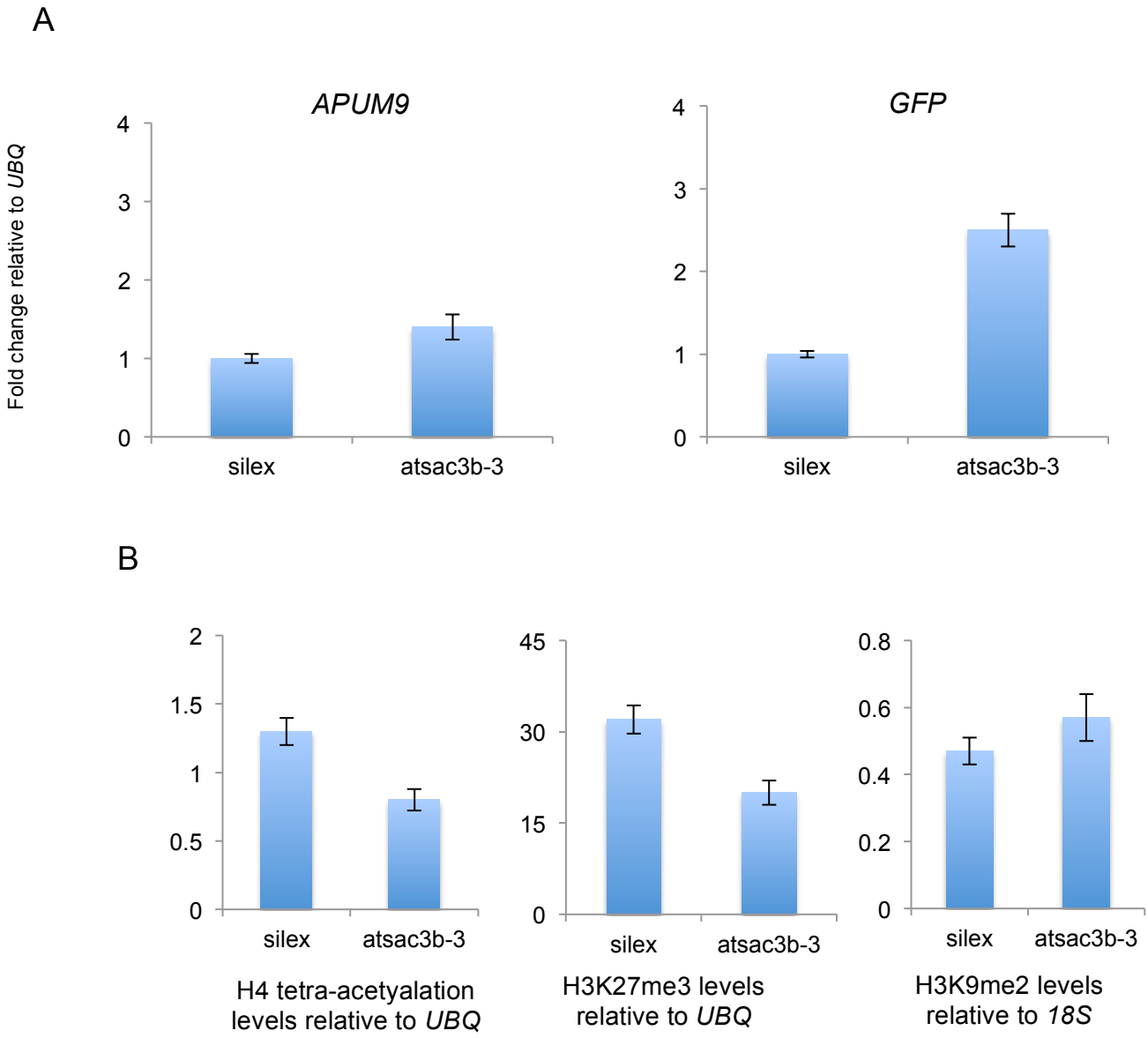


Figure 3.2 Transcriptional activation of the GFP based transgene in *epic3* is associated with changes in the histone modification at the transgene promoter
(A) Steady-state levels of GFP and APUM9 transcripts show that the mutation in *atsac3b-3* causes transcriptional activation of the GFP based transgene and has no effect on the endogene. (B) ChIP analyses of the silex transgene promoter detected significant changes in H4tetra-acetylation and H3K27me3 levels but not for H3K9me2. The error bars represent s.e.m of three biological replicates.

AtSAC3B is required for proper nuclear-cytoplasmic poly(A) RNA export in Arabidopsis

Since AtSAC3B is a component of the TREX-2 mRNA export complex, we performed mRNA whole-mount *in situ* hybridization for localization of poly(A) RNAs in the different *AtSAC3B* mutant alleles. For the purpose leaves from two weeks old plants were fixed and

hybridized with a fluorescent cyanine 3 (cy3) labeled oligo dT($_{50}$) probe, which recognizes and binds to the poly(A) tails of the mRNAs. To identify the cells nuclei, the samples were counterstained with DAPI. In all three recovered alleles, strong cy3 fluorescence signal was detectable that colocalized with the DAPI signal, indicating that the bulk mRNAs were kept in the cells nuclei. In contrast to this in the parental silex line, the cy3 signal was dispersed through the entire cells (Fig. 3.3). We also tested the previously reported *atsac3b-2* allele (Lu et al., 2009) and we obtained similar results as for our EMS alleles, albeit the intensity of the detected signal was weaker. This data establishes that AtSAC3B is required for proper nuclear-cytoplasmic poly(A) RNA export in Arabidopsis.

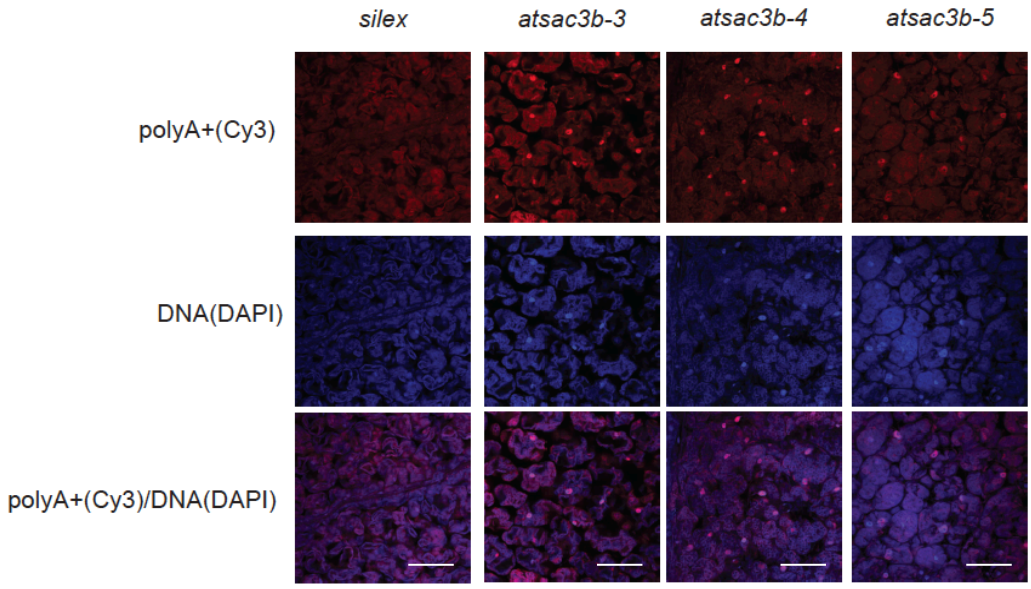


Figure 3.3 Cytoplasmic mRNA export in Arabidopsis requires functional AtSAC3B

Whole-mount mRNA *in situ* localization of the mRNAs in silex and *atsac3b* alleles. The cy3 flurofore is reporting on the localization of the mRNAs in the cells. The strong cy3 signal in *atsac3b* alleles indicated on dense subcellular mRNAs accumulation. DAPI represents a nuclear marker, binding specifically to the DNA in the nucleus. The colocalization of the cy3 signal and the signal from DAPI in *atsac3b* alleles indicates that the mRNAs accumulate in the nucleus of the cells. The fluorescent microscopy pictures are taken with confocal system. Scale bares= 40µm.

Transcription profiling

In order to identify the genes whose steady-state transcript levels are affected in *atsac3b*, we performed RNA-seq based transcription profiling using RNA extracted from areal parts of young seedlings (silex and *atsac3b-4*). Compared to silex, 486 protein-coding genes were up-regulated and 604 down-regulated in *atsac3b-4*. Gene ontology analyses (MAPMAN) allowed us to group the miss-expressed genes into distinct classes. The main affected categories belonged to RNA metabolism, regulation of transcription, protein degradation and heat stress (Table 3.1). Notably, 23 of the up-regulated genes belonged to the "abiotic heat stress" category. Because the overall transcriptome resembled that of a heat stressed plant, we decided to investigate the effect of heat stress on poly(A) RNA export.

GeneID	MapMan GO	Symbol	Expression (log2)	P-value
AT2G26150	20.2.1 stress.abiotic.heat	HSFA2	7.96	2.2E-14
AT5G12030	20.2.1 stress.abiotic.heat	HSP17.6	5.51	1.2E-107
AT1G07400	20.2.1 stress.abiotic.heat		3.51	7.4E-87
AT5G51440	20.2.1 stress.abiotic.heat		3.30	9.3E-108
AT2G19980	20.2.99 stress.abiotic.unspecified		3.20	4.0E-05
AT5G12020	20.2.1 stress.abiotic.heat	HSP17.6II	3.01	3.7E-48
AT1G53540	20.2.1 stress.abiotic.heat		2.91	2.8E-18
AT5G52640	20.2.1 stress.abiotic.heat	ATHSP90.1	2.72	1.1E-174
AT3G12580	20.2.1 stress.abiotic.heat	HSP70	2.55	8.5E-189
AT1G56300	20.2.1 stress.abiotic.heat		1.99	4.8E-08
AT3G46230	20.2.1 stress.abiotic.heat	ATHSP17.4	1.99	1.2E-27
AT4G25200	20.2.1 stress.abiotic.heat	ATHSP23.6-MITO	1.94	3.44E-04
AT1G74310	20.2.1 stress.abiotic.heat	ATHSP101, HOT1	1.84	1.7E-84
AT2G20560	20.2.1 stress.abiotic.heat		1.76	6.4E-52
AT5G37670	20.2.1 stress.abiotic.heat		1.66	7.7E-14
AT2G40330	20.2.99 stress.abiotic.unspecified	PYL6	1.54	2.3E-11
AT1G59860	20.2.1 stress.abiotic.heat		1.45	1.4E-11
AT3G14200	20.2.1 stress.abiotic.heat		1.40	3.2E-45
AT1G11360	20.2.99 stress.abiotic.unspecified		1.39	5.01E-04
AT4G19590	20.2.1 stress.abiotic.heat		1.33	8.3E-06
AT4G18880	20.2.1 stress.abiotic.heat	HSFA4A	1.26	1.5E-27

AT3G09440	20.2.1 stress.abiotic.heat		1.15	7.3E-13
AT4G11660	20.2.1 stress.abiotic.heat	HSFB2B	1.05	6.9E-22

Table 3.1 Transcripts related to heat stress that are up regulated in *atsac3b-4*

Heat stress causes a block of mRNAs export in wild-type plants

The compromising effect of high environmental temperatures on mRNA export has already been reported in *Saccharomyces cerevisiae* (Saavedra et al., 1997; Saavedra et al., 1996) and in *Trypanosoma cruzi* (Názer et al., 2012). Because the *atsac3b-3* transcriptome indicated that heat stress might affect mRNA export in plants we analyzed if mRNA export in wild type plants can be affected by heat stress. For this purpose wild type (Col-0) and silex seedlings were heat stressed at 37 °C for 24 hours. Immediately after heat stress *in situ* detection of poly(A) RNAs was performed using the aforementioned whole-mount method. In parallel whole-mount poly(A) RNA *in situ* localization was performed with wild type and silex seedlings grown at 24 °C (Fig. 3.4 A and B). In heats stressed wild type and silex seedlings we observed strong Cy3 signal in the nuclei of the cells. This nucleus specific cy3 signal was not present in the non-stressed control seedlings. This data indicates that heat stress compromised the export of the bulk of the poly(A) RNAs in wild-type plants.

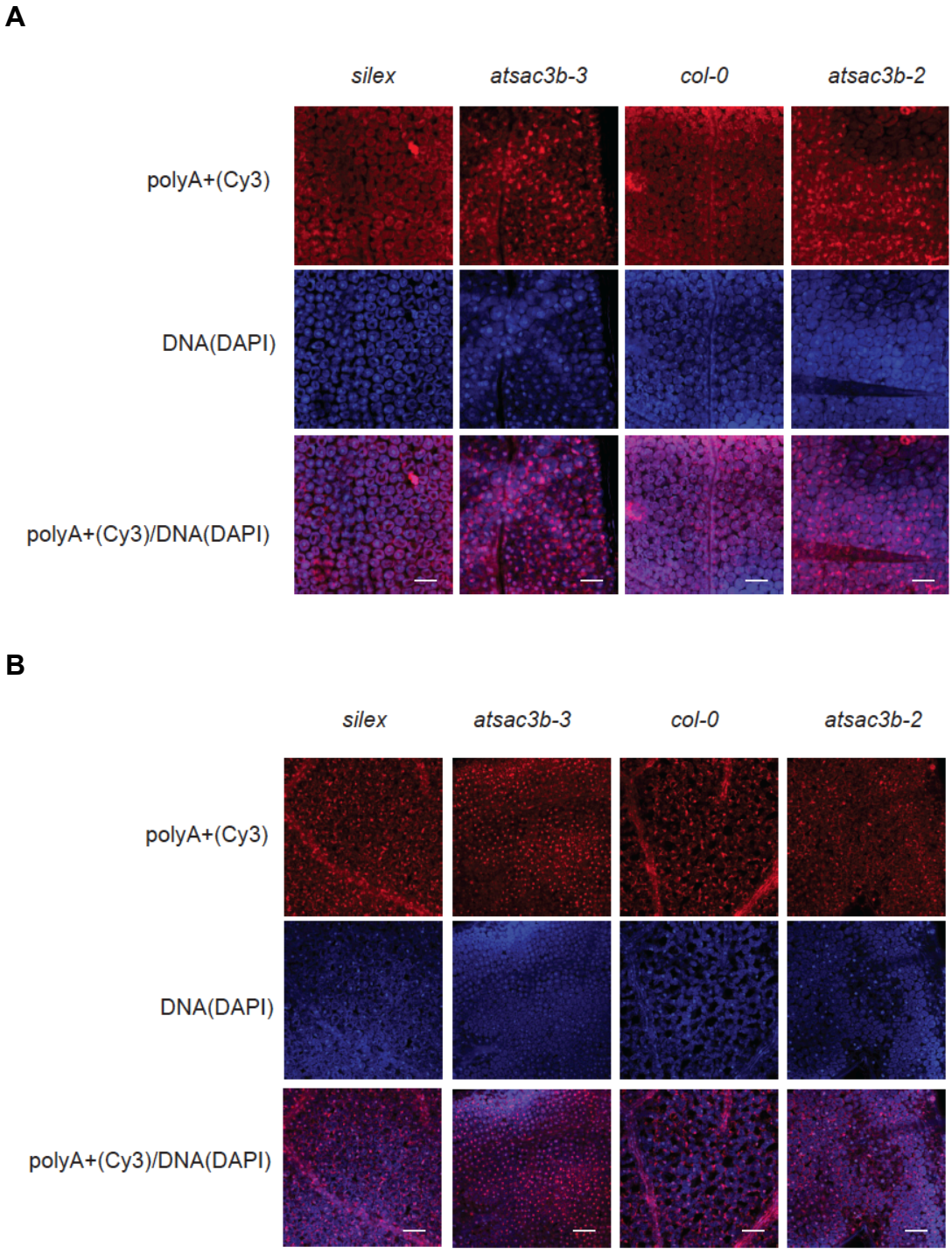


Figure 3.4 Heat stress leads to the accumulation of poly(A) RNAs in the nuclei of wild type Arabidopsis plants.

Whole mount poly (A) RNAs *in situ* localization in control plants grown at 24 °C (A) and in plants grown at 37 °C for 24 hours shows the compromising effects of high temperatures in the poly (A) RNAs export in wild type plants (B). DAPI staining was used for the nuclei localization. Scale bares= 40µm.

AtSAC3B is required for selective nuclear poly(A) RNA export

The selective poly(A) RNA export we observed in heat stressed wild-type plants (Col-0) and heat stressed silex plants as well as in *atsac3b* raised the question about the nature/type of the transcripts that are kept in the nucleus or exported into the cytoplasm. To address this, we performed nuclear-cytoplasmic RNA fractioning of three weeks old plants followed by strand-specific microarray based transcription profiling of the two fractions using three biological replicates for each sample. The purity of the created fractions was confirmed by Western blots of the proteins extracted from the very same fractions that were used for the RNA isolation. Antibodies against a nuclear protein (tetra-acetylated histone H4) and a cytoplasmic protein (UGPase) were used for the detection of the proteins in the different fractions. The results showed little to no contamination present in the separate fractions, indicating that we were able to obtain highly enriched fractions (Fig. 3.5).

The nuclear transcriptomes confirmed our previous results that we obtained with non-strand specific total RNA sequencing. Indeed, almost 30% of all up-regulated nuclear protein-coding sense transcripts were shared between *atsac3b-3* and heat stressed silex (Fig. 3.6 A, top). We observed very similar activation of sense and antisense transcripts in the nucleus (Fig. 3.6 B, top). In the cytoplasmic transcriptome we did not observe an overlap between *atsac3b-3* and heat-stressed silex plants (Fig. 3.6 A, bottom). A very surprising result however was obtained when we analyzed the ratio between sense and antisense transcripts in the cytoplasm. While heat stressed plants did not display any bias for one or the other, in *atsac3b-3* the vast majority of down-regulated transcripts were of the antisense orientation and the up-regulated transcripts were almost exclusively sense transcripts (Fig. 3.6 B, bottom).

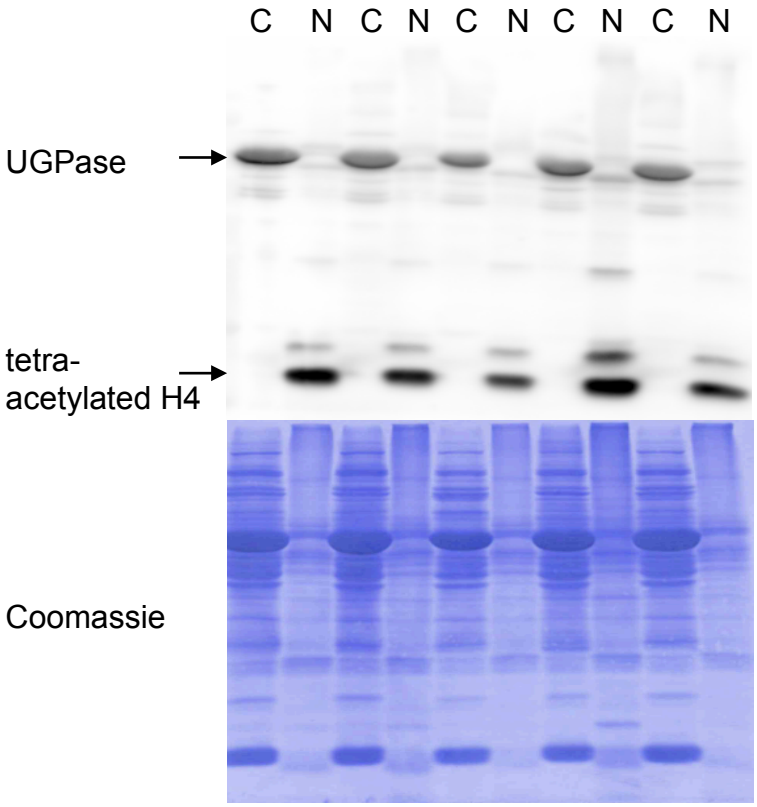


Figure 3.5 Enrichment of the nuclear and the cytoplasmic fractions

Western blot with cytoplasmic (UGPase) and nuclear (tetra-acetylated histone H4) markers, show high enrichment and no contamination of the cytoplasmic (C) and the nuclear (N) fractions respectively. Coomassie staining of the membrane is shown as a loading control.

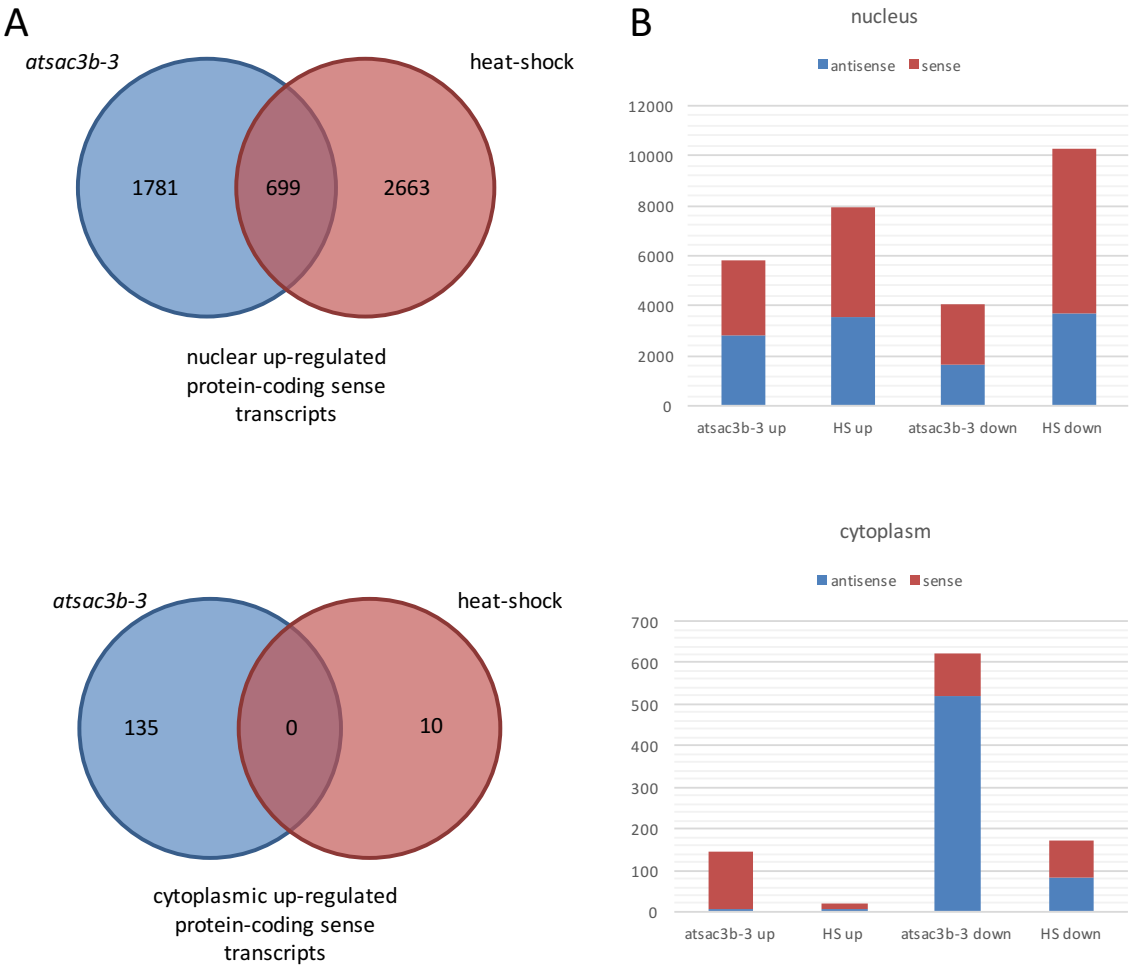


Figure 3.6 Graphical representation of the nuclear (top) and cytoplasmic (bottom) transcriptomes .

(A) Venn diagrams indicating the number of up-regulated protein-coding sense transcripts compared to wild type untreated plants. (B) Stacked bar plots indicating the number of up or down-regulated transcripts and their orientation (antisense in blue, sense in red).

Discussion and conclusion

Recently, we have reported on a tissue-specific epigenetically regulated GFP reporter line (silex), suitable for studying different epigenetic regulators and tissue-specific epigenetic regulation of gene expression (Hristova et al., 2015). In the forward genetic mutant screen for novel epigenetic regulators that was performed on this line we recovered *epic3* (*atsac3b-3*) that caused transcriptional activation of GFP expression. The microscopically detectable GFP signal was restricted to the edges of the leaves, indicating a highly tissue-specific activation pattern of the transgene.

In order to elucidate the mechanism underlying the transcriptional activation of the transgene we analyzed the histone marks occupancy at the transgene promoter. The data showed a reduction in the levels of the repressive histone mark H3K27me3. This can be the cause for the transcriptional activation of the transgene in *atsac3b-3* (*epic3*). The possibility that other chromatin modifications that were not tested here also contribute to the transgene's transcriptional activation can't be excluded. Another possibility may be an indirect regulation of the transgene by AtSAC3B, where AtSAC3B regulates an upstream factor that influences transgene expression. However, we have data that indicates that AtSAC3B could be involved in regulation of the dynamics of the histone marks on a global nuclear scale (discussed in Chapter 4).

In a previous study (Lu et al., 2009), three possible Sac3 homologues in Arabidopsis were identified-AtSAC3A, AtSAC3B and AtSAC3C, but none of the respective mutants displayed the *sac3*Δ specific nuclear poly(A) RNA export defect that was reported in yeast, indicating that in plants these proteins might have evolved to fulfill different functions. However, using a more sensitive approach based on confocal microscopy, we were able to confirm that *atsac3b-2* (the allele used in the aforementioned study), displays a poly(A) RNA export defect thus validating that AtSAC3B is required for proper RNA export in Arabidopsis. Furthermore, we found *atsac3b-2* to be a weak allele compared to our EMS alleles. This may be due to the fact that *atsac3b-2* is a SALK line carrying the T-DNA in an intron, thus potentially still producing functional SAC3B protein. Our findings are well in accordance with the reports on the Sac3 homologues in Drosophila (Kurshakova et al., 2007) and humans (Wickramasinghe et al., 2010) where the importance of the respective homologues in the mRNA export process are shown.

Taking into consideration the involvement of AtSAC3B in the export of the poly(A) RNAs, the finding that *atsac3b-2* causes up-regulation of heat-stress related genes, raised the question of whether heat stress may have an impact on poly(A) RNA export. We show here that poly(A) RNAs export is compromised in wild type plants following heat stress. To our knowledge this has not been shown in plants before, but it is a well known phenomenon in yeast (Saavedra et al., 1996). Since the *in situ* hybridization method used was not quantitative, we couldn't identify if additional heat stress on *atsac3b-2* and *atsac3b-3* intensifies the severity of mRNA export defect.

The genome-wide analyses of the nuclear and cytoplasmic RNAs originating from heat-stressed silex plants, showed that high temperatures cause strong transcriptional activation of heat stress related genes, whose transcripts are predominantly kept in the nucleus and only small portion of those transcripts appears to be exported. Upon heat stress, transcriptional reprogramming occurs in the cells, including fast activation of heat responsive genes. However our data suggest that most of these newly synthesized transcripts remain in the nucleus.

We also measured increased levels of heat-stress related mRNAs in the nuclear fraction of *astac3b-3*, but the activation was moderate compared to the heat stressed wild type plants. This together with the fact that we found that 700 of the up-regulated genes (out of 1781 for *atsac3b-3* and 2663 for silex-hs) were in common for the nuclear fractions of *atsac3b-3* and silex-hs indicated a substantial but not complete overlap of the two conditions. Therefore heat stress does most likely not simply result in a complete inhibition or inactivation of SAC3B. Also, whereas in silex-hs the heat stress genes are activated transiently as a response to the new conditions, in *atsac3b-3* these genes seem to be activated in a long term manner, without any external stimuli.

The nuclear and cytoplasmic compartment transcriptomes revealed a striking difference in the amount of antisense transcripts that exported between *atsac3b-3* and silex-hs (Fig.3.6). The impaired export of the antisense transcripts in *atsac3b-3* implied that there might be a specialized antisense transcript export pathway in Arabidopsis. While the specificity for antisense transcripts may be surprising, the specificity of TREX-2 for the export of certain classes of RNAs is not. It has been reported previously that the human homologue of Sac3-GANP is required for the export of a specific subset of mRNAs, therefore playing a role in selective export of transcripts (Wickramasinghe et al., 2014).

The nuclear pore complex and the complexes that are associated to it (eg. TREX-2) are clearly very important component in the gene expression regulatory network. We show

here that the regulatory potential of the nuclear pore is exquisite and still not completely understood.

Materials and methods

Plant material, mutagenesis and mapping

All plants used in this study are in Col-0 background. The plants were grown in growth chambers Sanyo MLR-350 at 24°C with 16 hours light. The silex reporter line was obtained from a collection created by The Institute for Genomic Research (J. Craig Venter Institute, line AGRAC-60-1-1) (Xiao et al., 2010).

Causal EMS mutations were mapped by whole genome sequencing combined with classical mapping by crossing the mutants with the Wassilewskija accession (WS). Reads were mapped against the reference genome and SNPs called in Geneious (Biomatters Ltd.). Using R SNPs were filtered for EMS mutations (G:C->A:T) and zygosity called based on the variant frequency provided by Geneious (>=80% homozygous mutation, >=45% and <=55% heterozygous mutation). Plots were then created by calculating the ratio of the number of homozygous and heterozygous and mutations in a 500 kb window.

Real time PCR and transcriptome analysis

Total RNA from 100 mg of fresh leaf tissue of Arabidopsis plants was isolated with innuPREP Plant RNA Kit (Analytik Jena). 500 ng of RNA were used for cDNA synthesis (iScript cDNA synthesis kit, Bio-Rad). Expression of target genes was measured by quantitative PCR (qPCR) in a Light-Cycler 480 (Roche), using SYBR Green I Master Mix. Steady state mRNA levels were calculated with the Light-Cycler 480 software (Roche) using *ACT2* for normalization.

Transcription profiling on the silex reporter line and *atsac3b-4* was carried out on RNA extracted from leaves of 17 days old plants as described previously on three biological replicates (Yokthongwattana et al., 2010).

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation was performed as previously described by Jaskiewicz et al., 2011. In our study we used one-gram roseate leaves from three weeks old plants as a starting material for nuclei preparation. For the pull downs two antibodies were used: anti-H4 tetra-acetylation antibody (06-866) from Milipore and anti H3K27me3 (A299-001) from Diagenode. Relative histone acetylation and histone H3K27 trimethylation levers were calculated using the comparative C_T method (Schmittgen and Livak, 2008) by normalizing against input and *ACT2*. The experiment was performed in three biological replicates.

Whole mount mRNA *in situ* hybridization

The whole mount mRNA *in situ* hybridization was performed as previously described by (Germain et al., 2010) with slight modifications.

Leaves from two weeks old plans, (where indicated seedlings were used) were harvested in small petri dishes and immersed with 5ml fixation cocktail (50% Fixation buffer: 120 mM NaCl, 7mM Na_2HPO_4, 3 mM NaH_2PO_4, 2.7 mM KCl, 0.1% Tween-20, 80 mM EGTA, 5% formaldehyde and 10% DMSO, and 50% of the fixation cocktail consists of heptane). The leaves (seedlings) were vacuum infiltrated for 10 min and room temperature, and than gently agitated at room temperature for 30min. The samples were than dehydrated twice for 5 min each in 100% methanol, and three times, each five minutes in 100% ethanol, after which they were incubated for 30 min in ethanol:xylene (1:1) with agitation at room temperature. The samples were washed twice with 100% ethanol; each of the washes is for 5 min, and two times with 100% methanol (5 min each). After a 5 min incubation in methanol:fixation buffer without formaldehyde (1:1) the samples were post fixed in fixation buffer with formaldehyde for 30 min at room temperature. Samples were rinsed twice (5 min each) in fixation buffer without formaldehyde, and pre hybridized in 1ml PerfectHyb Plus (Sigma) at 50°C for 1 hour, with a gentle agitation. After this the PerfectHyb Plus buffer was exchanged with fresh 1ml and 5 pmol 5' end-labeled cy3 oligo $dT(_{50})$ (Microsynth)was added to the samples and incubated at 50°C in dark overnight. The samples were washed in 2X SSC and 0.1% SDS at 50°C in dark for one hour, and 0.2X SSC and 0.1% SDS for another 20 min.

Before the DAPI staining the samples were mounted in 1X PBS for 5 min. Than DAPI staining solution was applied in concentration, and the samples were incubated in dark and room temperature for 5 min. The DAPI staining solution was washed away with 3 washes in 1X PBS (5 min each). The fluorescence was observed with confocal microscope Zeiss LSM700.

Nuclear-cytoplasmic fractioning

The nuclear-cytoplasmic fractioning was performed as previously described by (Wang et al., 2011) with slight modifications. Roseate leaves (approximately 3 gr.) from three weeks old plants were harvested, grinded to fine powder in liquid nitrogen and mixed with 2ml/g lysis buffer (20 mM Tris-HCl, pH 7.5, 20 mM KCl, 2mM EDTA, 2.5 mM $MgCl_2$, 25% glycerol, 250 mM Suc and 5 mM DTT) supplemented with protease inhibitor cocktail

(Roche) in a final concentration of 1X. The homogenate was incubated on an overall shaker at 4°C for 15min. The homogenate was than filtered through a double layer of Miracloth. The flow-through was spun at 1500g for 10 min, and the supernatant, which contains the cytoplasmic fraction, was collected, and centrifuged at 12,000g for 15 min at 4°C. The supernatant was collected, and used as a cytoplasmic fraction. The pellet that was obtained after the centrifugation at 1500 g, was washed four times with 5ml of nuclear resuspension buffer-NRBT (20 mM Tris-HCl, pH 7.4, 25%glycerol, 2.5 mM $MgCl_2$ and 0.2%Triton X-100), supplemented with protease inhibitor cocktail (Roche) in a final concentration of 1X. After the last wash, the pellet was resuspended in 500µl of buffer NRB2 (20 mM Tris-HCl, pH 7.5, 0.25M Suc, 10mM $MgCl_2$, 0.5%Triton X-100 and 5mM β-merkaptoethanol) supplemented with protease inhibitor cocktail (Roche) in a final concentration of 1X. The suspension was carefully overplayed on top of 500 µl NRB3 buffer (20 mM Tris-HCl, pH 7.5, 1.7 M Suc, 10 mM $MgCl_2$, 0.5% Triton X-100 and 5mM β-merkaptoethanol) supplemented with protease inhibitor cocktail (Roche) in a final concentration of 1X. The tubes were centrifuged at 16,000g for 45 min. at 4°C. The final nuclear pellet was resuspended in 400 µl lysis buffer.

For the RNA extraction TRI Reagent (Sigma) was used, following the instructions for the manufacturer. After the RNA extraction the rest of the homogenate was kept and used for protein extraction again following the instructions from the manufacturer.

Western Blots

The concentration of the proteins from the nuclear and the cytoplasmic fractions was determined using Bradford assay, and the amount of proteins used for the nuclear and the cytoplasmic fractions were adjusted accordingly. The proteins were loaded on 12% SDS-polyacrylamide gel. Antibodies against UGPase and H4-tetra-acetylation ((06-866) from Milipore) were used for detection of the proteins.

Chapter 4

The role of TREX-2 in maintaining the chromatin structure

Abstract

In addition to the canonical functions in providing a barrier between the cytoplasm and the nucleus and regulation of the transport between the two compartments, the nuclear pore complexes-NPCs also play a pivotal role in the regulation of the chromatin organization. Whereas the role of nucleoporins, components of NPCs, in chromatin organization has been extensively studied, little is currently known about the exact role of the TREX-2, a complex associated with NPC in this process. TREX-2 is a highly conserved protein complex that has been found from yeast to humans and plants. In order to better understand the role of TREX-2 in regulating chromatin organization we studied Arabidopsis plants that are defective in AtSAC3B, a component of the TREX-2 complex. For this purpose we assessed the global heterochromatin levels and the levels of several specific histone modifications in *atsac3b* and compared them with wild type plants. We found that AtSAC3B is involved in the maintenance of the heterochromatin levels, and has a general effect on the maintenance of repressive histone modifications. Our results show that it may also influence euchromatic histone marks. The data presented here further confirm the very close links between transcription, RNA export and chromatin structure.

Introduction

Chromatin is a higher organizational level of the genome in which the genomic DNA interacts with histone proteins, creating highly compact structures that allow the accommodation of the long genomic DNA into the space-limited nucleus. The level of chromatin condensation is not ubiquitous within the nucleus and two major structurally and functionally distinguishable territories can be observed: euchromatin and heterochromatin (Heitz, 1928). The euchromatin is less compact, transcriptionally active and enriched in protein coding genes, whereas the (constitutive) heterochromatin is condensed, transcriptionally inactive and rich in repetitive sequences (satellite sequences and transposable elements) and pseudo genes.

Euchromatin can undergo organizational changes in a developmental stage-dependent manner when it becomes more compact. This type of chromatin is termed "facultative" heterochromatin and is defined as a cytological manifestation of epigenetic events that cause changes in gene expression (Gilbert et al., 2003). The distribution of the euchromatin and the heterochromatin within the nucleus is precisely organized. Along the chromosomes, constitutive heterochromatin is predominantly confined to the pericentomeric regions, at the so called "chromocenters" (CCs), but also at the nucleolus organizer regions (NORs) (Dvořáčková et al., 2015; Huisinga et al., 2006; Lermontova et al., 2015; Schoeftner and Blasco, 2009; Schueler and Sullivan, 2006; Slotkin and Martienssen, 2007).

Euchromatin and heterochromatin are also distinguishable by the different histone modifications for which they are enriched in. Higher eukaryotes' euchromatin is enriched in acetylated histones (H3ac and H4ac), as well as methylation of lysine residues (H3K4me, H3K36 and H3K79) (Martin and Zhang, 2005; Schübeler et al., 2004), which relax the chromatin structure and make it accessible for different transcriptional factors and RNA polymerase II. Heterochromatin on the other hand is enriched in methylated histone three (H3K9me) and the heterochromatin protein 1 (HP1) (Gilbert et al., 2003; Ho et al., 2014; Lachner et al., 2001).

From a nuclei-spatial perspective, heterochromatin tends to accumulate at the nuclear periphery (Bühler and Gasser 2009; Dillon, 2008). This was confirmed in gene translocation studies in yeast and animals, which showed that some genes can undergo transcriptional silencing when they are brought into close proximity to the nuclear envelope (Kosak et al., 2002). This repressive effect of the nuclear envelope was considered to be locus-specific, since it wasn't observed at some inducible yeast genes (such as INO1,

GAL1, GAL2, SUC2, FIG2, HXK1, HSP104). Thorough studies of these genes, showed that they undergo translocation upon transcriptional activation and get anchored to the NPCs (Van de Vosse et al., 2010; Casolari et al., 2004; Mendjan et al., 2006; Taddei, 2007). This suggests that despite the preferential association of heterochromatin with the nuclear periphery, there are regions (at the NPCs) free of heterochromatin that stimulate transcription.

While the spatial distribution of the genes and the role of the nuclear envelope and the nuclear pore complexes received attention in yeast and higher eukaryotes, in plants little research has been conducted on this topic. In the previous chapter, we showed that the *atsac3b-3* caused transcriptional activation of the GFP based reporter construct. Concomitant to the release of silencing we observed reduced levels of the repressive H3K27me3 and H3K9me2 marks at the transgene promoter. AtSAC3B is a member of TREX-2 (Transcription Export Complex 2), a complex associated to the nuclear pore, whose role in chromatin organization is well known in yeast and animals (Brown and Silver, 2007; Ptak et al., 2014; Sood and Brickner, 2014; Taddei, 2007; Taddei et al., 2006). Since we observed changes in the chromatin constitution at specific targets in *atsac3b* we wanted to asses if AtSAC3B also played a more global and genome-wide role in maintaining these chromatin marks.

Using whole mount immune assays we revealed here the role of AtSAC3B in heterochromatin organization, and present data that shows that this protein influences the levels of repressive histone modifications such as H3K27me3 and H3K9me1.

Results

Heterochromatin levels are reduced in *atsac3b-3*

In order to study the role of the AtSAC3B in the chromatin organization we first assessed the state of the heterochromatin in the mutant. For this purpose, we carried out quantitative analyses of global heterochromatin levels in *atsac3b* (*atsac3b-3* and *atsac3b-2*). We measured the relative heterochromatin fraction (RHF), an indicator for chromatin compaction (van Zanten et al., 2012) in 10 days old seedlings stained with propidium iodide PI (whole-mount DNA staining). High-resolution images, acquired with a confocal laser scanning microscope were used for the assessment of the RHF, which was determined by the area and fluorescence intensity of all chromocenters (CCs) in relation to the area and the fluorescence intensity of the entire nucleus (She et al., 2013; Soppe et al., 2002; Tessadori et al., 2007). Interestingly we detected a significant reduction in RHF levels in both *atsac3b-2* and *atsac3b-3* compared to the respective wild type plants (Fig. 4.1), suggesting that defects in AtSAC3B caused heterochromatin decondensation.

CCs are discrete nuclear domains of mainly pericentromeric heterochromatin. They are the main representatives of heterochromatin, and are rich in repetitive sequences, methylated DNA and dimethylated histone H3K9 (Fransz et al., 2006; Soppe et al., 2002). To exclude the possibility that the observed reductions of the RHF values in the mutant were not a result of a reduced number of chomocenters we also assessed the numbers of chromocenters (CC) in the mutant and wild type cells. A tendency for a slight increase of the number of CCs was observed in the *atsac3b-3*, that wasn't present in the *atsac3b-2* (Fig. 4.1). Since *atsac3b-3* causes transcriptional activation of the transgene in the edges of the leaves, we wanted to identify if the mutation had a stronger effect in those cells. We measured the heterochromatin content and the number of CCs in the cells at the marginal cell layer. The data we obtained showed the same tendency as we had observed in the central mesophyll cells: reduction of the heterochromatin content, and slight increase in the number of the chromocenters (Fig.4.1).

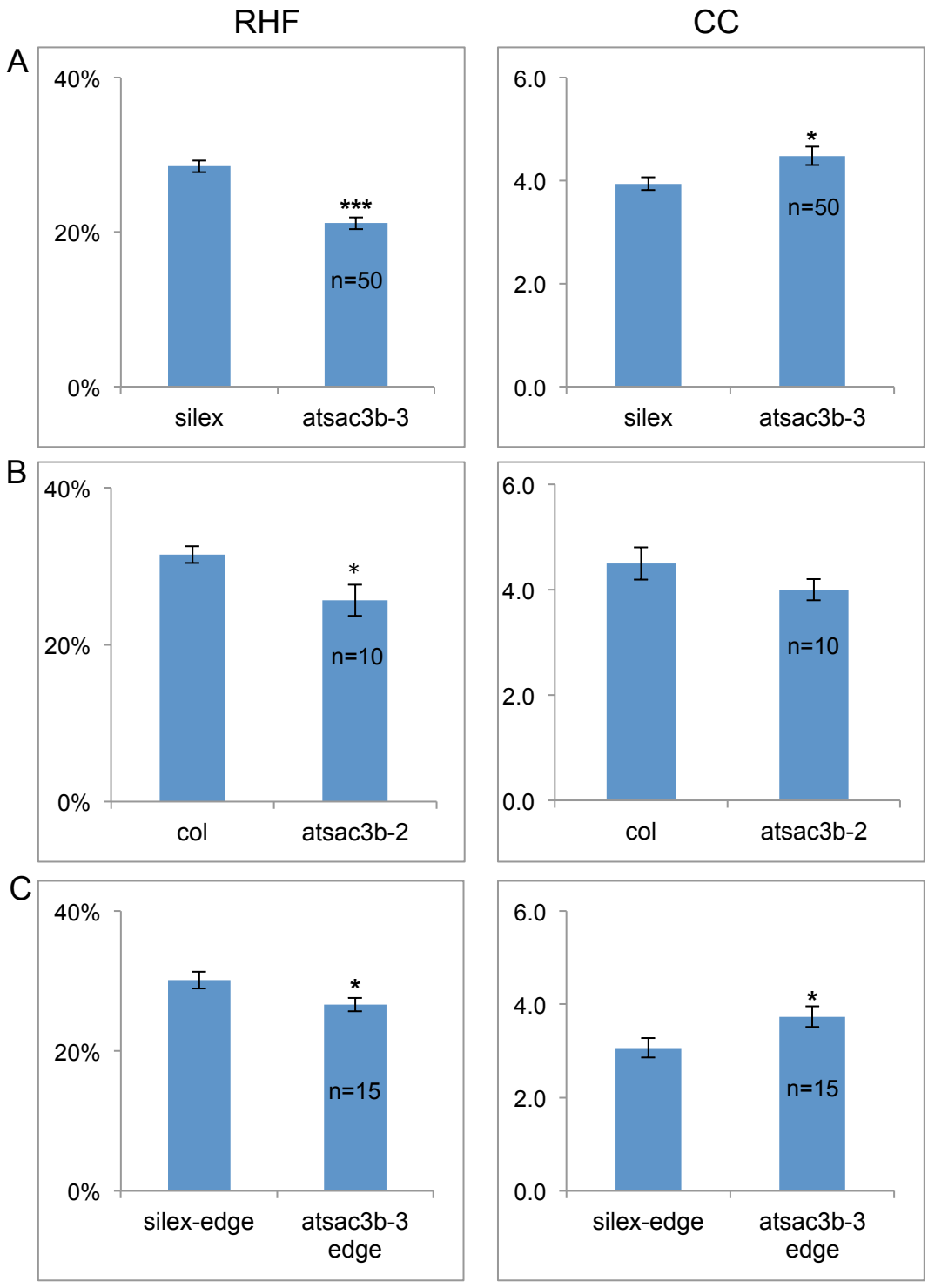


Figure 4.1 Quantification of the heterochromatin levels in *atsac3b*

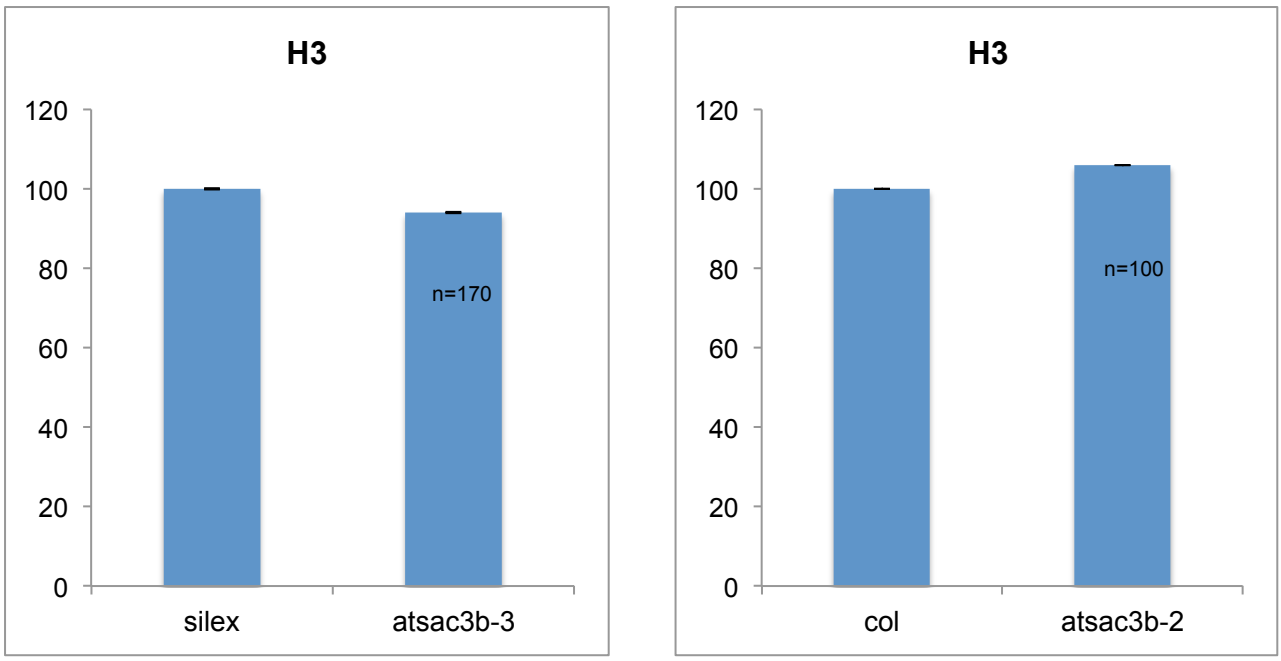
(A) Graphical representation of the heterochromatin levels (relative heterochromatin fractions-RHF, left panels) and number of chromocenters (CC, right panels) in *atsc3b-3* and *atsac3b-2* (B) quantified with PI staining. (C) Heterochromatin levels and number of chromocenters in the leaves edges of *atsac3b-3* and silex. The error bars represent s.e.m of 50 and 10 individual cells, respectively. Differences between the wild type and the mutant plants were assessed using a two-tailed Welch's test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

atsac3b influences the levels of the histone marks in plants

The finding that *atsac3b* displayed decondensation of the heterochromatin, prompted us to investigate if the mutation is also influencing the global levels of two repressive histone marks: H3K9me1 and H3K27me3. We addressed this question by performing cytological staining (whole mount immunostaining) in 10 days old seedlings. The seedlings were fixed and labeled with fluorescent antibodies against the aforementioned histone modifications. As a control to the experiment, a hybridization with antibody against histone three (H3) was carried out in order to exclude the possibility that defects in AtSAC3B may affect the global level of H3.

In order to visualize the DNA, the cells were counterstained with propidium iodide (PI) and the signal was detected with confocal laser scanning microscope. The acquired high-resolution serial images were reconstructed in 3D, and the borders of the objects of interest were defined. The fluorescence from both channels (Alexa 488-for the antibodies against the histone modifications and the PI) was measured as sum of pixel intensities in each object. The antibody fluorescence signals were normalized against the respective PI signal and these values were used for statistical analyses.

We did not detect significant differences in the amount of H3 in the two tested alleles compared to the wild type plants (*atsac3b-3* compared to silex and the *atsac3b-2* compared to Col-0) (Fig. 4.2). This wasn't the case with the histone modifications we assessed. We detected significant reduction in the levels of H3K27me3 and H3K9me1 in both tested alleles (Fig. 4.3).



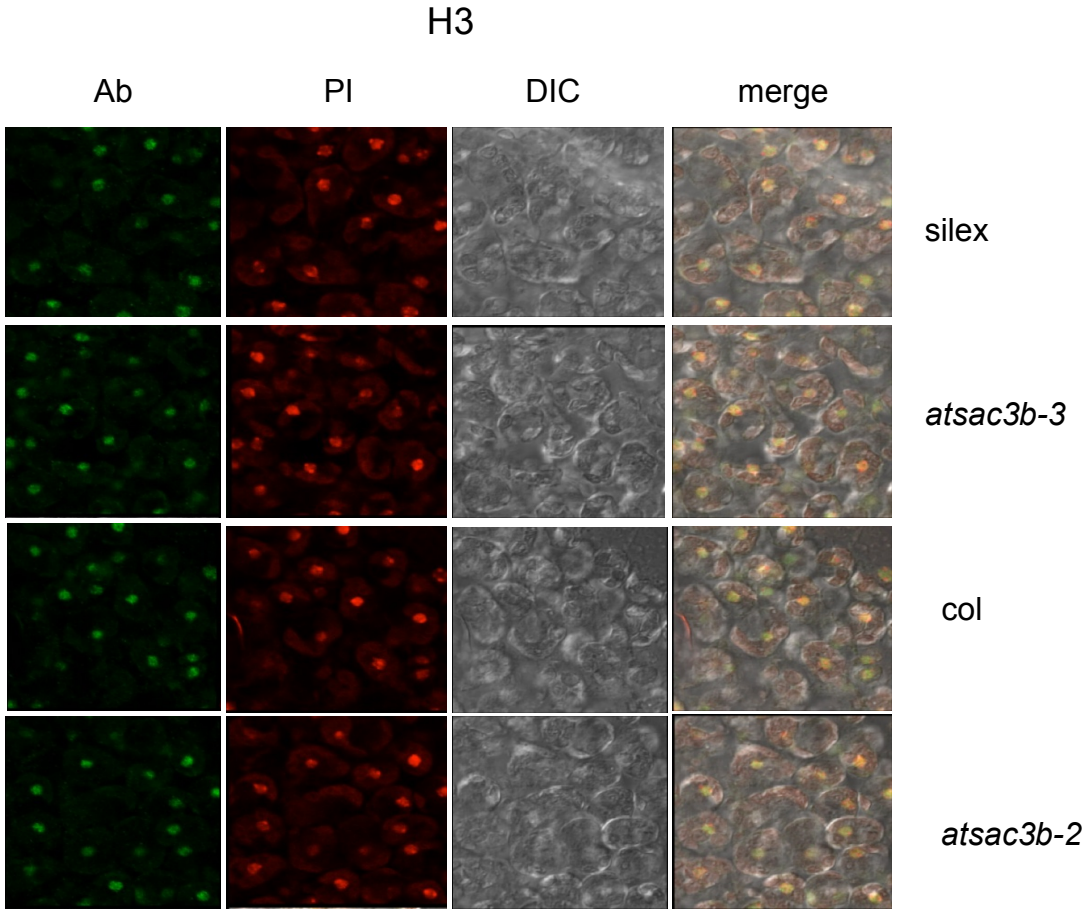


Figure 4.2. H3 levels are not affected in atsac3b

(A) H3 levels quantified with whole-mount immunostaining in *atsac3b* and wild type plants. The error bars represent s.e.m of 170 cells. (B) Microscopy pictures of the H3 antibody channel (Ab- Alexa 488), the propidium iodide (PI), and the bight field channel (DIC). Objective 63x.

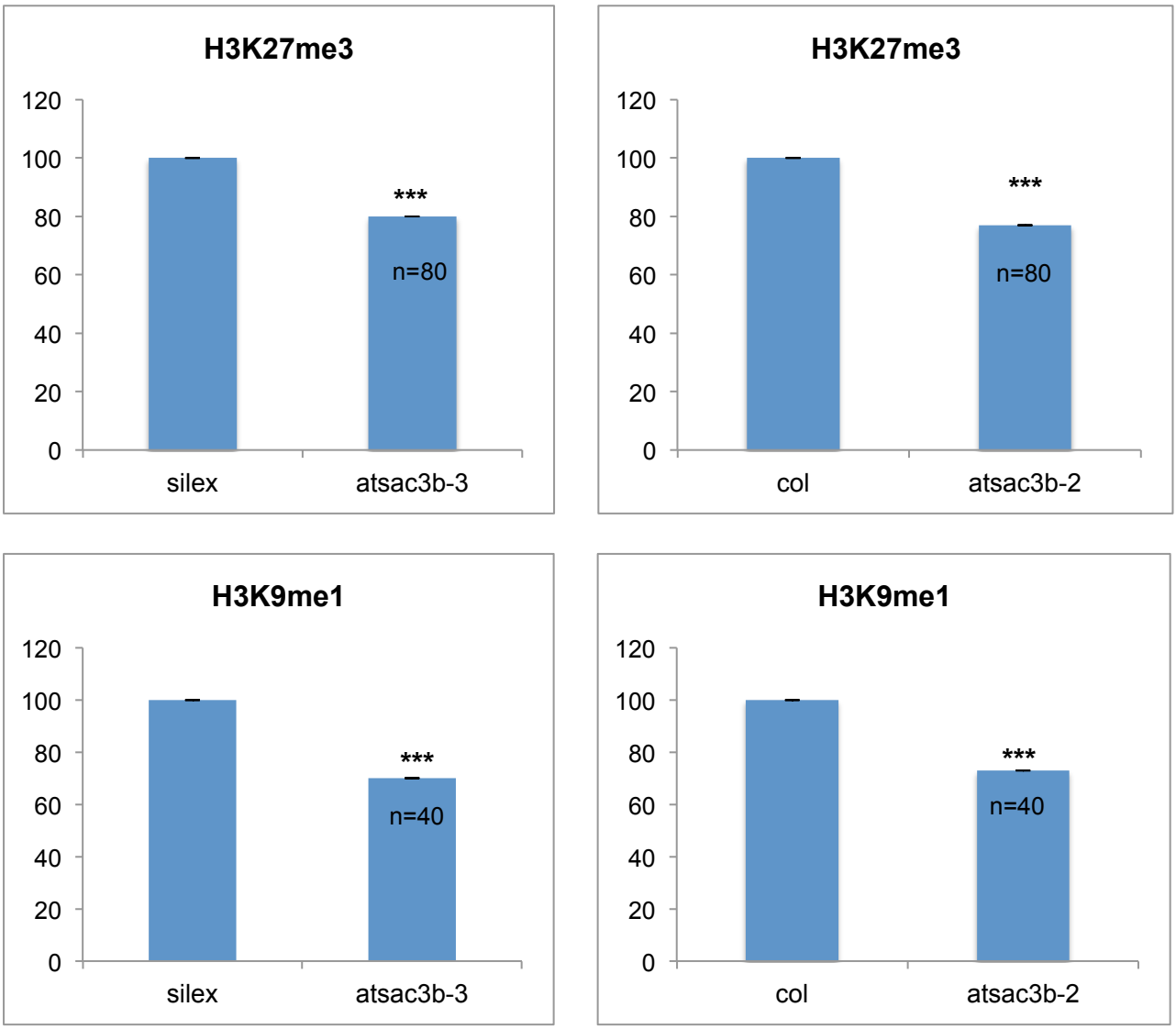


Fig.4.3 atsac3b causes global reduction in the H3K27me3 and H3K9me1 levels in Arabidopsis

H3K27me3 and H3K9me1 levels in two mutant alleles were quantified with whole mount immunostaining and compared it to the respective wily type. The error bars represent s.e.m of 80 and 40 cells respectively.

Discussion and conclusion

In the work presented here we investigated the role of AtSAC3B, a TREX-2 member in chromatin organization in plants. Using two independent alleles (*atsac3b-3* carrying a SNP, *atsac3b-2* containing a T-DNA insertion), we were able to show that loss of function of AtSAC3B led to a significant global reduction of the RHF index. Since RHF represents an indicator for the degree of condensation of the heterochromatin (van Zanten et al., 2012), and we didn't detect change in the number of the CCs, this finding implied that the mutation in AtSAC3B is causing relaxation of heterochromatin, which eventually leads to global transcriptional activation that we also observed with the transcriptome of *atsac3b*-2 (discussed in chapter 3). The thorough analyses of the heterochromatin levels in both, the central mesophyll cells and the most outer single cell layer of the leaf margin showed that AtSAC3B doesn't influence heterochromatin levels in a spatial manner.

We also measured significant reduction in H3K9me1 and H3K27me3 levels (Fig. 4.3), but observed no changes in the levels of the H3 histone (Fig.4.2), indicating that the mutation in AtSAC3B directly influences specific histone modifications, and has no effect on the global nucleosome incorporation. AtSAC3B is not known to have an enzymatic activity; therefore, the observed reduced levels of H3K9me1 and H3K27me3 are not a result of disturbed equilibrium in the placement and/or the removal of the specific modifications, but rather an indirect effect. One possibility could be that the AtSAC3B has an effect on the establishment on the so-called "chromatin boundary activities". Notably, when put in proximity heterochromatin and euchromatin can influence each other and assign properties of the oppose chromatin state to one another. Boundary activates are assigned to proteins that can prevent the spreading of the influence between the heterochromatin and euchromatin (Capelson and Corces, 2012; Kellum and Elgin, 1998).

Boundary activities were reported to the several export factors in yeast (Mex67, Cse1p, etc.), which were shown to be blocking the spreading of heterochromatin by interactions with the nucleoporin Nup2 (reviewed in (Gerasimova and Corces, 2001)). The TREX-2 complex in Arabidopsis is anchored to the NPC via the Nup2 (Lu et al., 2009). It is possible therefore that AtSAC3B has a similar mechanism of action.

Materials and methods

Plant material

All plants used in this chapter are in Col-0 background. The mutants used in the study are the *atsac3b-2*, a SALK line that caries a T-DNA insertion (Lu et al., 2009), and *atsac3b-3*, an EMS mutant, with a point mutation in the conserved SAC/GANP domain. The seeds were surface sterilized with sterilization buffer (70%EtOH supplied with 0.05% Triton X-100), for 25 min on an overall rotator. The tubes were centrifuged for 5min at 5000 rpm and room temperature. The sterilization buffer was exchanged with 1ml absolute ethanol, and the tubes with the seeds were incubated for another 20 min in an overall rotator. The seeds were dried in sterile conditions. The dried seed were sown on plates with ½ Murashige & Skoog (MS) basal media. The plates were kept in dark at 4°C for 24 hours for stratification of the seeds and afterwards germinated in long day (16 hours light and 8 hours dark) conditions for 10 days.

Tissue fixation, embedding and permeabilization

These procedures were performed as previously described by (She et al., 2013).

Briefly, 3-4 leaves from the 10 days old seedlings grown on MS media were harvested on ice in 2ml tubes containing freshly prepared fixative BVO (1xPBS, 2mM EGTA, 1% formaldehyde, 10% DMSO, 0.1% Tween-20). The leaves were fixed for 30min at room temperature with a gentle agitation. The tubes were centrifuged for 1 min at 400g. The fixative was replaced with 1ml fresh PBT (1x PBS, 0.1% Tween-20) and the tubes were kept on ice until the embedding step.

The fixed leaves were transferred on Superfrost slides (Thermo Scientific cat. No. J1800AMNZ). Using fresh razor blade, the leaves were fine-sliced without disturbing the leaf integrity. This step increased the cells' permeability and led to better PI staining. After finishing the dissection the remaining PBT was removed with fine paper and 100 µL - embedding mixture (5% acrylamide mix, prepared in 1xPBS, 20% APS and 20% NaS) was applied on the leaves, and carefully covered with 20 mm x 20 mm cover slips, avoiding the formation of bubbles. The glass slides were incubated at room temperature until the acrylamide polymerizes (45min-60min). The cover slips were removed and the embedded leaves were transferred into coupling jars in order to achieve clarification and post-fixation of the tissue (5 min in methanol, 5 min in absolute ethanol, 30 min in absolute

ethanol:xylene, (1:1), 5 min in absolute ethanol, 5 min in methanol, 15 min in PBT:2.5% formaldehyde (1:1) and final washing for 5 min in PBT).

The glass slides were dried with fine paper and a digestion mix (0.5% cellulose, 1% driselase, 0.5% pectolyase, all dissolved in 1xPBS) was applied over the leaves for degradation of the cell wall. The glass slides were incubated in moist chamber at 37°C for one hour. Two-steps wash (5 min each) with PBT was performed, and the glass slides were dried before an RNase A solution (100 µg/ml RNase A in 1xPBS, supplemented with 1%Tween-20) was applied to the fixed leaves, followed by incubation in moist chamber at 37°C for one hour. Glass slides were again washed in PBT (two times, 5 min each), before the final post-fixation for 20 min in PBT-F (PBT with 2.5% formaldehyde) and the two hours permeabilization at 4°C in 1xPBS supplemented with 2% Tween-20. Before proceeding to immunostaining the glass slides were washed twice (for 5 min) in PBT.

Immunostaining

Dilutions 1:1000 for H3K4me3 (Upstate-ab32356) and 1:200 for H3 (Abcam-ab1791), H3K9me1 (Upsate-07-450) and H3K27me3 (Upstate)) of the primary antibodies were prepared in PBS supplemented with 0.2% Tween20, and each glass slide was incubated in wet chamber with 100 µL primary antibodies for 24 hrs at 4°C. The primary antibodies were washed with PBT for 2-4 hrs at room temperature with a gentle agitation, and a secondary antibody was applied (1:200 dilution in PBS supplemented with 0.2% Tween20) for 24 hrs in wet chambers at 4°C. The secondary antibody was washed with PBT for 2 hours at room temperature.

Propidium Iodide (PI) staining

The aforementioned procedure of fixation, embedding, clearing and permebealization of the tissue was also followed when PI staining was performed for the determination of the heterochromatin content.

After the immunostaining the cells were counterstained with PI. In both cases-immunostaining and heterochromatin quantification, the PI was applied in a concentration of 10µg/mL in PBS, for 15 min at room temperature. The slides were washed with PBS for 15min. The staining and the washing were performed in dark.

Image acquisition and processing

Confocal laser scanning microscope system Leica SP5 was used for the detection of the fluorescence signal. Parameters like: laser intensity, gain, pinhole, voxel size and zoom factor were kept constant through the entire experiment. Serial, three-dimensional images (Z-stacks) were acquired with two times oversampling, following the Nyquist's rule of oversampling. The images were processed with the Imaris software (Bitplane).

Chapter 5

General discussion and outlook

Epigenetic regulation of gene expression is a fast developing research field, and even though many aspects of it are extensively studied, the complexity of the field makes it attractive research topic. The main aim of this thesis was to identify novel players and mechanisms involved in the tissue-specific epigenetic regulation of gene expression in Arabidopsis. We established a forward genetic screen that not only led to the recovery of already known epigenetic players, it also provided interesting novel insights. One chapter in the thesis discusses a new allele of *hda6* that we recovered in the screen. The core of the thesis evolves on the mutant *epic3* that I have identified and that lead to the discovery of yet unknown mechanisms influencing transcription and epigenetic marks.

State of the art and concept of the project

In the last decade numerous mechanisms involved in the epigenetic regulation of gene expression have been discovered. The most fruitful approaches that led to the discovery of proteins playing important roles in transcriptional gene silencing (TGS) were forward genetic mutant screens. The approaches taken in order to identify TGS factors evolved over the decades. One of first mutant screens that led to the identification of novel factor required for the maintenance of DNA methylation was done by Southern blots on individual plants (Vongs et al., 1993). A few years later, the discovery of silenced transgenes provided a much more effective tool for mutant screens. For instance, transcriptionally silenced antibiotic resistance genes were ideal for such screens. These plants contained the transgene in a methylated state thus repressing its expression resulting in antibiotic susceptible plants. Such plants were mutagenized and then grown on plates containing the respective antibiotic. Plants deficient in TGS release transgene expression rendering them resistant to the antibiotic. In such a way several mutants could be identified (Amedeo et al., 2000). Such screens have limitations because the transgene needs to be strongly activated in all tissues to allow plants to survive on media containing the antibiotic thereby rendering them not very sensitive. Aiming to identify subtler epigenetic regulators, possibly acting only in specific tissues, we moved to a different system based on a very sensitive

epigenetically controlled GFP reporter line (silex). The silex reporter line is based on *APUM9*, an essential developmental gene that is under complex epigenetic regulation by at least two independent silencing pathways (MOM1 and NRPE1) (Yokthongwattana et al., 2010). Introgressions of *mom1* and *nrpe1* into silex showed that similar to the endogene GFP transgene expression was epigenetically controlled, thereby showing that the reporter transgene adequately reported on *APUM9* and was suitable for further studies on it's regulation mechanisms.

The mutant screen

The forward genetic mutant screen that was performed on silex resulted in mutants that released GFP expression in numerous different tissues, a result that was quite intriguing.

In this thesis two independent lines are presented. *epic1* and *epic3* were found to be defective in HISTONE DEACETYLASE6 (HDA6) and SUPRESSOR FOR ACTIN 3B (AtSAC3B) respectively. Already at this point there was a peculiar observation: while *mom1 nrpe1* plants released GFP expression only in veins on the abaxial side of the leaf, *hda6-8* (*epic1*) released it in young emerging leaves, and *atsac3b-3* (*epic3*) in the leaf margin. That suggested that each of these proteins (MOM1, NRPE1, HDA6 and AtSAC3B) affected the reporter transgene expression in a different tissue. Why that is the case currently remains unclear but we could confirm that *HDA6* was expressed specifically in the young emerging leaves, the same tissue where we observed release of GFP expression. That might suggest that in the case studied here, it is not epigenetic marks that guide tissue specific gene expression. It rather seems to be the tissue specific expression of the epigenetic regulators that results in developmentally regulated patterning of chromatin marks.

RNA export and heat stress

In the mutant screen, I identified that a defect in the AtSAC3B protein in Arabidopsis, lead to transcriptional activation of the GFP reporter transgene and local release of GFP expression limited only to the leaf margin. AtSAC3B is a member of the TREX-2 (transcription and export complex 2), a complex that is associated with the nuclear pore complex via the nucleoporin Nup2 and which couples the transcription and export process (Fischer et al., 2002; Jani et al., 2014; Köhler and Hurt, 2007; Lu et al., 2009).

Even though the mutation appeared to have different effects on the transcriptional activities of the transgene and the endogenous gene, the fact that AtSAC3B is associated with the nuclear pore complex, a complex that is a powerful regulator not only for the nuclear-cytoplasmic

trafficking, but also the nuclear organization, prompted me to investigate it into more details. In this thesis I showed the versatility of AtSAC3B and it's potential to regulate gene expression on several levels.

First, I demonstrate here that AtSAC3B is required for the export of poly(A) RNAs in Arabidopsis. The involvement of the yeast AtSAC3B homologue in the poly(A) RNAs export process was well know (Fischer et al., 2002), however in plants this is the first study showing the role of the AtSAC3B in the nuclear-cytoplasmic export of poly(A) RNAs. With this I showed that this function of the protein is conserved across kingdoms and that AtSAC3B has the potential to directly regulate gene expression by controlling the export of transcripts. There are several possibilities how this regulation is achieved. In this context, structural studies in yeast have shown that the export phenotype in TREX-2 mutants is primarily caused by the disassociation of the TREX-2 from the nuclear pore (Jani et al., 2014; 2009). However, recent work of (Schneider et al., 2015), showed that in yeast the role of TREX-2 into regulation of gene expression is even more direct. Namely, they showed that Sac3 could directly interact with the transcription machinery and, together with other factors control transcription initiation and thereby regulate the balance of the Pol II CTD phosphorylation. It remains unknown which mechanism is utilized by the plant TREX-2 complex.

Surprisingly, the transcriptomic data of plants defective in AtSAC3B showed transcriptional activation of heat-responsive genes. This prompted me to study the poly(A) RNAs export in wild type plants under heat stress conditions. I showed that likewise in yeast, exposure to high temperatures compromises the export process in wild type plants. The accumulation of poly(A) RNAs in yeast under heat stress conditions was associated to transcriptional reprograming that occurs upon the heat stress, which results in fast production of heat stress related transcripts that are exported and help the cells to better and faster cope with the new conditions, whereas the export of all the other transcripts in the cells is paused (Saavedra et al., 1997). Another study on the nature of the accumulated transcripts went further and suggested that this preferential export of heat stress related transcripts in yeast under heat stress conditions was achieved through the activity of the RNA surveillance system. Namely, they have observed that the majority of the accumulated transcripts in the nucleus are improperly processed and have hyperpolyadenylated tails (Jensen et al., 2001). These findings imply on the existence of a general poly(A) RNAs export pathway and suggest that the selectivity of the export process is determined by the RNA processing machinery and surveillance system.

Export of antisense RNAs

Assuming that the TREX-2 export pathway would only be involved in general poly(A) RNA export pathways, then one would expect mutations in this complex to have strong developmental defects. However, we observed only late flowering in *atsac3b* indicating that only the export of some of the poly (A) RNAs is affected.

Assuming that the TREX-2 displays a certain level of selectivity in poly(A) RNA export, we studied the nature of the exported transcripts and those retained in the nucleus in *atsac3b*. The results presented here suggest the existence of several poly(A) RNA export pathways, which are specialized in export of specific subsets of poly(A) RNAs. The assessment of transcript accumulation in the different compartments (nucleus and cytoplasm) showed that in the population of transcripts showing export defects in the mutant, there was a bias towards antisense transcripts, implying that AtSAC3B is required for the export of antisense transcripts. Therefore, we assume that AtSAC3B plays a selective role in the poly(A) RNA export process. A certain selectivity of TREX-2 was already reported in humans, where it was attributed to the GANP, a homologue of Sac3 (Wickramasinghe et al., 2014). In the same direction was also the reported interaction of the TREX-2 complex with the chromatin modifying complex SAGA. The SAGA complex acts as a transcriptional co-activator of inducible and stress related genes in yeast. It was shown that a member of the SAGA complex-Sus1 interacts with the Sac3 in yeast and thereby couples transcription to export. Considering this, it can be speculated that TREX-2 favors export of the stress-related transcripts (García-Oliver et al., 2012).

AtSAC3B and chromatin organization

Even though the AtSAC3B homologues have been well studied and much is know about their role in the poly(A) RNA export process, up until now the protein wasn't put into a connection with epigenetic regulation of gene expression. Here for the first time it was shown that AtSAC3B is a general epigenetic factor that regulates the organization of the heterochromatin in plants and to certain extend the dynamics of the heterochromatin associated chromatin marks. The mechanism of action remains unknown and further work is required to be shown if this feature of the protein is unique for plants.

Conclusions and outlook

In this thesis I showed that a nuclear pore associated protein-AtSAC3B regulates gene expression by regulation of the export of poly (A) RNAs and is required for the export of antisense transcripts, therefore it contributes to the selectivity of the export process. Up until now, a connection between the TREX-2 complex and the export of the antisense transcripts hasn't been made. To my knowledge this is the first report on a protein that is most likely involved in the specific export of antisense transcripts. In yeast TREX-2 defective cells were shown to have increased genome instability, that is associated with increased numbers of R-loops (Aguilera and García-Muse, 2012; Bhatia et al., 2014; Santos-Pereira et al., 2014). On the other hand genome wide analyses in yeast have shown that the distribution of the R-loops across the yeast genome is limited to some transposons, telomeric regions, and subsets of ORF, which often have high CG content and/or are associated with antisense transcripts (Chan et al., 2014; Faghihi and Wahlestedt, 2009). Furthermore, a regulatory role for R-loops in the expression of antisense RNAs (asRNAs) was reported in mouse (Powell et al., 2013) but also in plants. In plants it was shown that the expression of the antisense transcript COOLAIR, which regulates the expression of the *FLC* flowering gene, is regulated by an R-loop which is formed at the promoter of the COOLAIR thereby preventing its' transcription (Sun et al., 2013).

Based of these findings, the connection between the TREX-2 and asRNAs that we made is consistent. We suggest that in *atsac3b*, the export of the asRNAs is affected. We expect *atsac3* to have more R-Loops at regions where the asRNA is not exported anymore. This in turn can affect the chromatin structure and sense gene expression. Indeed, it was proposed that asRNAs act as scaffold molecules for different histone-modifying enzymes. Many of these enzymes don't have specific DNA-binding domains, and asRNAs facilitate their interaction with DNA and chromatin in a locus specific manner (Magistri et al., 2012). This can explain the changes in the heterochromatin organization and in the dynamics of the heterochromatin modifications that we observed in *atsacb3* in chapter 4, even though AtSAC3B presumably has no enzymatic activity.

Taken together the results presented here emphasizes once again the complexity of the epigenetic regulation of gene expression and draws the attention to the nuclear pore complex as a powerful epigenetic regulator, which so far was neglected in the plant epigenetic research field. I show here that the different epigenetic regulatory pathways can be better understood when the problem is looked upon from a broader perspective.

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