THE ROLE OF EPIGENETICS IN THE MAINTENANCE OF PLANT GENOME STABILITY

ANDRIY BILICHAK
Bachelor of Science, Precarpathian University, Ukraine, 2008

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Dedicated
To my ever supportive parents Yurij and Lybov Bilichak
And to my beloved wife Nina

Abstract

Significant alterations in the environmental conditions can have pronounced effects on plant genome stability. Recent evidence argues for a global involvement of the components of epigenetic modules in the regulation of genome homeostasis both immediately after stress exposure and long after environmental cues were acquired. The last observation is of particular interest as the memory of imposing stress can be maintained at the molecular level throughout plant ontogenesis and may be faithfully propagated into the following generation. Our study provides evidence that epigenetic repercussions exerted by stress exposure of parental plants manifest themselves in untreated progeny at all three levels of the epigenetic module: DNA methylation, histone posttranslational modifications and small RNA metabolism. Additionally, the results of our study shed new light on the engagement of the epigenetic machinery in the maintenance of plant genome integrity by counteracting the activity of invading nucleic acids.

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List of Abbreviations

ABA – abscisic acid
ACD11 – ACCELERATED CELL DEATH11 gene
AGO4 – ARGONAUTE4
ARF1 – AUXIN RESPONSE FACTOR1
ARP6 – ACTIN-RELATED PROTEIN6
ATGB2 – Rab2-like small GTP-binding protein gene
ATP – adenosine triphosphate
BAR – BIALOPHOS RESISTANCE gene
BER – DNA base excision repair pathway
braAGO1 – Brassica rapa AGO1 gene
CAF-1 – CHROMATIN ASSEMBLY FACTOR1
CaMV – cauliflower mosaic virus
C. elegans – Caenorhabditis elegans
CBC – cap-binding complex
cDNA – complementary DNA
CDS – coding sequence
ChIP – chromatin immunoprecipitation
CMT3 – CHROMOMETHYLASE3
COBRA – combined bisulphite restriction analysis
CpG – cytosine and guanine separated by a phosphate
CPM – counts per minute
CSD1 and 2 – CU-ZN SUPEROXIDE DISMUTASE1 and 2 enzymes, respectively
CT - control
DCL – Dicer-like protein
ddc – triple mutant deficient in non-CpG methylation (*drm1/drm2/cmt3*)
DIG – digoxygenin
DME – DNA demethylase DEMETER
DML – DEMETER-LIKE
DMS3 –DEFECTIVE IN MERISTEM SILENCING 3
DNA – deoxyribonucleic acid
dpg – day post germination
DRB4 – DICER RNA binding factor4
DRD1 – DEFECTIVE IN RNA-DIRECTED DNA METHYLATION1
DRM1 and 2 – DOMAINS REARRANGED METHYLASE 1 and 2, respectively
ds – double stranded
DSB – double strand break
FDR – false discovery rate
GO – gene ontology
GSR – general stress response
GT – gene targeting
GUS – β–glucuronidase gene (uidA)
HAB1 – HYPERSENSITIVE TO ABA1
HAD – histone deacetylase
HAT – histone acetyltransferase

hc- or ra-siRNAs – heterochromatic or repeat-associated siRNAs, respectively
HEN1 – the methyltransferase HUA ENHANCER1
HOS15 – HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES
HR – homologous recombination
HS – heat shock
HSR – heat stress response
HST – exportin-5 homolog HASTY
nt – nucleotides
HUB1 – HISTONE MONOUBIQUITINATION1
HYL1 – dsRNA-binding protein HYPONASTIC LEAVES1
H3K9me2 and H3K9ac – dimethylation and acetylation of histone 3 lysine 9, respectively
LB medium – Luria-Bertani broth medium
LINE – long interspersed nucleotide element
LTR – long tandem repeat
LUC – luciferase gene
meC – methylated cytosine
MES – 2-(N-morpholino)ethanesulfonic acid
MET1 – METHYLTRANSFERASE1
miRNA – micro RNA
MMS – methyl methane sulfonate
MS medium – Murashige and Skoog medium
MSH6 – mismatch repair protein
nat-siRNA – natural-antisense transcript-derived siRNA
NHEJ – non-homologous end joining
NtGPDL – tobacco *GLYCEROPHOSPHODIESTERASE-LIKE* gene
OD_{600} – the optical density of bacterial cell culture at λ=600 nm
PAGE – *polyacrylamide gel* electrophoresis
PAT – PHOSPHINOTRICIN ACETYL TRANSFERASE
PcG – polycomb group protein
PCR – polymerase chain reaction
Pol II – polymerase II
pol IVb – RNA Polymerase IVb
PIE1 – PHOTOPERIOD-INDEPENDENT EARLY FLOWERING1 gene
PPRL – PENTATRICOPEPTIDE REPEATS (*PPR*) protein-like gene
PP1 – PROTEIN PHOSPHATASE1
PTM – posttranslational modification
pri-miRNA – primary miRNA transcript
P5CDH – DELTA-1-PYRROLINE-5-CARBOXYLATE DEHYDROGENASE
qPCR – quantitative PCR
RAT-gene – *RESISTANT TO AGROBACTERIUM TRANSFORMATION* gene
R-gene – resistance gene
RC – rolling-circle transposon
RdDM pathway – RNA-dependent DNA methylation pathway
RDM1 – RNA-DIRECTED DNA METHYLATION1
RDR – RNA-dependent RNA polymerase

RISC – RNA-induced silencing complex
RITS – RNA-induced transcriptional silencing complex
RNA – ribonucleic acid
RNAi – RNA interference pathway
ROPS – random oligonucleotide primed synthesis
ROS – reactive oxygen species
ROS1 – REPRESSOR OF SILENCING1
RPM - *Revolutions per minute*
SA – salicylic acid
SAR – systemic acquired resistance
SD – standard deviation
SDG8 – histone lysine methyltransferase SET (Su(var)3-9, E(z) and the Trithorax-conserved) DOMAIN GROUP 8
SE – SERRATE
SGS3 – SUPRESSOR OF GENE SILENCING3
SINE – short interspersed nucleotide element
siRNA – small interfering RNA
smRNA – small RNA
SQN – the cyclophylin protein SQUINT
SR – simple repeat transposon
SRO5 – SIMILAR TO RCD ONE5
SYD – ATPase SPLAYED
TALEN – transcription activator-like effector nucleases
ta-siRNAs – trans-acting siRNAs
T-DNA – transferred DNA
TE – transposable element
Ti-plasmid – tumor-inducing plasmid
TR – treated
tRF – tRNA-derived RNA fragment
TRV – *Tobacco rattle virus*
TSS – transcription-start site
T0 – primary transformant
T1 – first generation transgenic plant
UBP1b – OLIGOURIDYLATE BINDING PROTEIN 1b
v – software version
VIGS – virus-induced gene silencing
vir – virulence genes
XRCC4 – X-Ray Cross Complementation Protein4
YFP – yellow fluorescent protein
ZFN – zinc-finger nuclease

1. INTRODUCTION

All living beings throughout their lifespan are in a constant interaction with environmental cues which can either benefit or jeopardize their homeostasis, depending on the intensity of abiotic and biotic factors encountered. If environmental stimuli deviate substantially from the organism's optimal range, the organism initiates specific and/or non-specific stress responses. In order to reduce the influence of stress, diverse strategies can be implemented i.e., tolerance, resistance and avoidance. Unlike most animals, plants are restricted in their mobility. Hence, either the natural resistance mechanisms or the acquired tolerance mechanisms have to be initiated to withstand the consequences of stress and rapidly adapt to adverse abiotic and biotic cues without relocating themselves to a more beneficial environment (Tsaftaris, Polidoros et al. 2008, Gutzat and Mittelsten Scheid 2012). It is thought that for this reason, plants evolved the complex physiological mechanisms to cope with imposed stress conditions (Boyko and Kovalchuk 2011, Gutzat and Mittelsten Scheid 2012). At the same time, it still remains an enigma how exogenous environmental stimuli are integrated with endogenous developmental programs in plants.

A single genome influenced by different factors can give rise to multiple distinguishable transcriptomes leading to a variety of proteomes and eventually culminating in the altered phenotypic appearance of an organism. Recent evidence argues for the epigenetic nature of this phenomenon. Superimposed on the DNA sequence, epigenetic components have the potential to provide dexterity and plasticity in terms of modulating gene expression and responses to the environmental cues. At the molecular level, epigenetic factors manifest themselves through the structural adaptation of

chromosomal regions that is not accompanied by any changes in DNA sequence (Bird 2007, Kovalchuk and Kovalchuk 2012).

Curiously, our lab and other labs previously demonstrated that the memory of imposed stress can be maintained throughout plant ontogenesis and faithfully propagated into the following generation, this phenomenon known as transgenerational epigenetic inheritance (Boyko, Blevins et al. 2010, Bilichak, Ilnystkyy et al. 2012, Luna, Bruce et al. 2012, Rasmann, De Vos et al. 2012). Nonetheless, the exact factors and mechanisms that participate in the aforementioned observation remain speculative. **We hypothesize that alterations in DNA methylation, posttranslational histone modification and small RNA metabolism are among the primary causes of transgenerational epigenetic inheritance.**

Apart from regulating genome stability by suppressing the activity of transposable elements and manipulating gene activity (Chinnusamy and Zhu 2009) the epigenetic machinery also plays a critical role in the plant immune system. Both posttranscriptional and transcriptional gene silencing pathways were extensively studied as the immune factors that counteract viral replication in plant cells (Voinnet 2001, Blevins, Rajeswaran et al. 2006, Raja, Sanville et al. 2008). At the same time, the involvement of epigenetic modules in the protection of plant genome integrity against the bacterial pathogen *Agrobacterium tumefaciens*, which is widely used in biotechnology and has an ability to deliver a portion of its DNA into a plant cell, has largely remained unexplored. Based on the available reports, **we hypothesize that plants utilize similar epigenetic mechanisms to maintain their genome integrity regardless of origin of exogenous invading DNA/RNA.**

The primary goal of our work was to reveal the contribution of epigenetic factors to plant genome stability following exposure to abiotic and biotic stresses. To reach this goal, three different experiments were established with the following objectives:

1. A transgenerational salt stress experiment:

- To perform a detailed analysis of alterations in the DNA methylation profile in the progeny of salt-stressed *Arabidopsis thaliana* plants;
- To examine changes in the progeny of salt-stressed *Arabidopsis thaliana* plants at the posttranslational histone modification level;
- To correlate the observed perturbations in the epigenetic profile with the expression of selected genes in the progeny of salt-stressed *Arabidopsis thaliana* plants.

2. A transgenerational heat stress experiment:

- To compare alterations in the small RNAome and transcriptome profiles under normal conditions and those in response to heat-shock in *Brassica rapa* plants, using the Illumina GAIIx sequencing platform;
- To validate the contribution of maternal- and paternal-derived small RNAs to the development of transgenerational epigenetic memory;
- To draw some conclusions regarding the involvement of small RNAs in the establishment of transgenerational memory.

3. The contribution of epigenetic factors to *Agrobacterium*-mediated plant transformation:

- To perform screening among mutants impaired in different epigenetic pathways in order to select candidates that are the most susceptible to stable *Agrobacterium*-mediated transformation;
- To examine factors contributing to genome integrity in the selected epigenetic mutants and relate them to the stable transformation efficiency;
- To develop a method for transient down-regulation of selected genes to elevate transformation efficiency in the model object *Arabidopsis thaliana.*

By using two different (although related) plant species, we demonstrate that transgenerational epigenetic inheritance of the memory of imposed stress is a cross-kingdom phenomenon in plants. We further provide evidence that the progeny of stressed plants suffer changes in all three components of the epigenetic module: DNA methylation, histone posttranslational modification (the transgenerational salt experiment, Chapter 3) and small RNA metabolism (the transgenerational heat stress experiment, Chapter 4). All the aforementioned perturbations in the epigenetic profile are related to fluctuations in the transcriptome profile in tissues of both parents and progeny of stressed plants.

Additionally, in the reverse genetic screening, using mutants compromised in either the transcriptional or posttranscriptional gene silencing pathways, two inhibitors of *Agrobacterium*-mediated stable transformation were revealed – *AGO2* and *NRPD1a.* We also demonstrate that transient down-regulation of the aforementioned genes using virus-induced gene silencing can be successfully used to elevate the stable transformation efficiency in plants. Finally, we provide additional evidence that supports an idea that

plants utilize similar epigenetic pathways against foreign nucleic acids irrespective of their origin.

Overall, the results presented herein provide a strong basis for further investigations to consider the components of epigenetic modules as vital regulators of plant genome stability in response to stress and as mediators of the transgenerational inheritance.

2. LITERATURE REVIEW

2.1. Epigenetic components involved in the plant stress response

Plants have developed a plethora of signaling pathways which allow them to quickly respond to the alterations in environmental conditions in order to reduce their negative impact. Emerging evidence indicates that vascular plants can memorize changes in the transcriptome profile after stress exposure and in some cases propagate it into the next generation; this phenomenon is termed 'transgenerational inheritance'. Curiously, short-term and transgenerational plasticity of plant phenotypes does not involve changes in the DNA sequence, but instead manifests itself in reversible changes of the chromatin structure that determine DNA accessibility for transcriptional factors. Chromatin re-shaping depends on epigenetic factors, such as DNA methylation, histone posttranslational modifications/replacements, and small RNA (smRNA) metabolism, which form a flexible self-reinforcing loop of gene regulation. In the following chapter, we will provide some examples of gene activity regulation through alterations in the epigenetic profile in response to environmental stimuli. Additionally, we will discuss a systemic propagation of acquired stress-induced epigenetic changes in the progeny and the possible contribution of epigenetic components to the process of plant adaptation and acclimation.

2.1.1. Stress perception and epigenetics

Despite numerous complex feedback controls that are known to be activated in response to stress, we are still missing a link between stress perception and the epigenetic machinery. Recent research sheds new light on this phenomenon, bringing epigenetic

factors into a complex stress-sensing mechanism (Kumar and Wigge 2010). A direct connection between the perception of ambient temperature fluctuations and modifications of the epigenetic landscape comes from the research of Kumar and Wigge (Kumar and Wigge 2010). Searching for genes that orchestrate the plant transcriptome in response to heat stress, the authors identified the nuclear *actin-related protein 6* (*arp6*) mutants with more than 5000 genes that were constitutively misregulated regardless of the ambient temperature. A product of the *ARP6* gene is a component of the SWR1 chromatin remodelling complex which substitutes histone H2A for the alternative histone variant H2A.Z in euchromatin nucleosomes (Kobor, Venkatasubrahmanyam et al. 2004, Deal, Topp et al. 2007). An increase in the ambient temperature was positively correlated with the eviction of H2A.Z from the nucleosomes at the 5′ region of heat-responsive genes. Intriguingly, it has recently been shown that ARP6 regulates the expression of phosphate starvation genes through mediating the deposition of histone variant H2A.Z at transcription-start sites (TSS) of the respective genes (Smith, Jain et al. 2010). Overall, the highest degree of diversification in amino acid sequence among histone H2A variants is observed in their C-termini (Bonisch and Hake 2012).

H2A.Z histone replacement has been found to be vital to control immunity of Arabidopsis plants against pathogen attacks (March-Diaz, Garcia-Dominguez et al. 2008, van den Burg and Takken 2009). Nevertheless, the result of the enrichment of histone variant H2A.Z at TSS is not unidirectional since it has both the negative and positive effect on the expression of encoded genes, thus suggesting the involvement of additional activators or repressors of gene activity (Guillemette, Bataille et al. 2005, March-Diaz, Garcia-Dominguez et al. 2008, Kumar and Wigge 2010). Modulation of the

transcriptional activity at TSS enriched with histone variant H2A.Z may also be a combinatorial consequence of the number of post-translational modifications at histone tails (Bonisch and Hake 2012), an exact nucleosome position relative to a positive regulatory DNA sequence (Marques, Laflamme et al. 2010) or an availability of other chromatin modifiers. For instance, the H2A.Z histone variant was found to act together with 16 different histone modifications at more than 3000 genes in the human genome (Wang, Zang et al. 2008). Taking into account a strikingly high conservation of the H2A.Z histone variant among different species (almost 80% interspecies identity (Bonisch and Hake 2012)), one can hypothesize that H2A.Z maintains the unique and specific functions that probably cannot be performed by other histones. Indeed, in budding yeast, the *htz1Δ* mutant which lacks the ability to deposit the corresponding yeast histone variant H2A.Z demonstrates a significant correlation of transcriptome profile with that in heat-stressed wild-type yeast (Kumar and Wigge 2010).

Recent studies in Zilberman and Henikoff laboratories have provided a persuasive proof that in Arabidopsis, the H2A.Z histone variant and DNA methylation reside in different genomic regions and mutually exclude each other from specific loci (Pearson's $r=-0.81$ for the quantitative distribution of DNA methylation and H2A.Z accumulation) (Zilberman, Coleman-Derr et al. 2008). Thus, we can hypothesize that the H2A.Z histone variant can be one of the direct epigenetic receptors of the ambient environmental conditions that trigger downstream alterations in the epigenetic landscape. The evidence for the aforementioned hypothesis can be obtained from the comparison of the epigenetic profile (DNA methylation, post-translational histone modifications, small RNA expression, etc.) before and after stress with respect to the H2A.Z distribution.

Additionally, transgenerational stress experiments on mutant lines of Arabidopsis *photoperiod-independent early flowering 1* (*pie1*) and *arp6* which are defective in H2A.Z deposition at specific loci can reveal the role of H2A.Z in the development of epigenetic memory.

2.1.2. DNA methylation and plant stress response

DNA methylation is a heritable epigenetic mark which involves a reversible chemical modification of cytosine residues with a methyl group that is covalently added to the C-5 position (Kovalchuk and Kovalchuk 2012). Being discovered almost a century ago (Johnson and Coghill 1925), DNA methylation has become a substantial focus of research in a number of species including bacteria, fungi, worms, insects, plants and mammals (Kovalchuk and Kovalchuk 2012).

Due to the sessile nature of plants and their inability to escape from unfavourable environmental cues that can jeopardize their homeostasis, vascular plants have developed a number of pathways of DNA methylation maintainance. The importance of DNA methylation in plants has been shown in a number of genetic functions, including transcription, replication, recombination, transposition, cell development and differentiation (Kovalchuk and Kovalchuk 2012, Mirouze, Lieberman-Lazarovich et al. 2012). Cytosine methylation is catalyzed by enzymes known as DNA methyltransferases (MTases) which utilize *S*-adenosyl-methionine as the methyl donor (Kovalchuk and Kovalchuk 2012). In mammals, symmetric CpG sites are usually preferred as targets for methylation, whereas in the plant genomes, the occurrence of methylated cytosines appears to arise virtually at any sequence, including symmetric methylation at both CpG

and CpHpG sites (where H = A, C, or T) and asymmetric methylation at CpHpH sites (Kovalchuk and Kovalchuk 2012). Consequently, only 2 to 8% of mammalian DNA is methylated, compared to up to 50% DNA methylation observed in higher plants (Zhu 2009).

De novo DNA methylation at asymmetric CpHpH sites in plants is catalyzed by methyltransferases DOMAINS REARRANGED METHYLASE 1 and 2 (DRM1 and DRM2, respectively) through the RNA-dependent DNA methylation pathway (RdDM) (Chinnusamy and Zhu 2009), whereas maintenance methylation of the symmetrical sequences CpG and CpHpG is performed by METHYLTRANSFERASE 1 (MET1) and CHROMOMETHYLASE 3 (CMT3), respectively (Henderson and Jacobsen 2007). Noteworthy, recent studies suggested a high level of redundancy between DNA methyltransferases that demonstrate the ability of MET1 and CMT3 to establish *de novo* methylation, whereas the maintenance of symmetrical methylation can be performed by DRM1 and DRM2 (Lister, O'Malley et al. 2008).

Plants also possess enzymes that counteract the activity of DNA methylases named 'DNA demethylases' (see Table 2.1). DNA demethylases such as DEMETER (DME), REPRESSOR OF SILENCING1 (ROS1) and DEMETER-LIKE (DML) PROTEINS DML2 AND DML3 belong to a small family of DNA glycosylases involved in DNA base excision repair (BER) and are the main enzymes which contribute to the active locus-specific and global DNA demethylation (Saze, Tsugane et al. 2012).

Genome-wide analysis of the distribution of methylated cytosines (meC) in a number of plant species indicates the enrichment of methylated DNA predominantly at repeats and transposons (on average, 90% of all sequences are methylated) where a

transcriptionally repressed chromatin state is maintained (Lippman, Gendrel et al. 2004, Vaughn, Tanurdzic et al. 2007, Li, Wang et al. 2008, Wang, Elling et al. 2009). A comparison of methylome and transcriptome data in Arabidopsis plants revealed that moderately expressed genes exhibit DNA methylation at the transcribed coding region, while both high and low expressed genes demonstrate a significantly lower level of methylation (Zilberman, Gehring et al. 2007, Cokus, Feng et al. 2008, Lister, O'Malley et al. 2008). An increase of methylation levels in coding sequences of actively transcribed genes is thought to be the outcome of small interfering RNA-mediated (siRNA) suppression of the expression of non-canonical promoters that reside within the coding region (Lauria and Rossi 2011). On the contrary, the occurrence of the meC at the 5′ region of the gene (including the promoter and part of the transcribed sequence) and at the 3′ region (including part of the coding sequence and the 3′ UTR) is negatively correlated with gene expression and may be involved in tissue-specific gene expression and pathogen response (Zilberman, Gehring et al. 2007, Gehring, Bubb et al. 2009, Zemach, Kim et al. 2010, Dowen, Pelizzola et al. 2012).

An additional level of transcriptome regulation orchestrated by DNA methylation is achieved through the modulation of alternative splicing sites (Zhou, Lu et al. 2012). Recently, it has been speculated that DNA methylation may contribute to the definition of exon-intron boundaries with preferably higher methylation levels of exons as compared to introns, and the enrichment of meC in DNA sequences wrapped around histone octamers rather than linker sequences (Chodavarapu, Feng et al. 2010). Using an immunoprecipitation technique, Chodavarapu et al. (2010) have demonstrated the higher enrichment of RNA polymerase II (Pol II) in the exon regions comparing to the intron

regions. A preferential deposition of nucleosomes at the exon sequences leads to the Pol II stalling at the intron–exon and exon–intron boundaries which results in precise mRNA splicing (Chodavarapu, Feng et al. 2010). Taking into account that at least ~42% of the intron-containing genes in *Arabidopsis* are alternatively spliced (Filichkin, Priest et al. 2010), DNA methylation accompanied by other chromatin marks undoubtedly provides a global and fine-tuned mechanism of gene expression regulation.

Stress-regulated alternative splicing in plants has been previously documented (Ali and Reddy 2008); nevertheless, it still remains to be elucidated how the epigenetic machinery contributes to this phenomenon.

A general overview of DNA methylation alterations in response to stress indicates stress-dependent changes of methylation at specific loci. For instance, osmotic stress triggers transient DNA hypermethylation at the repetitive heterochromatic loci in tobacco cell suspension culture (Kovarˇik, Koukalová et al. 1997), while aluminum, salt, paraquat and cold stresses initiate a decrease in CpG methylation in the coding region of the *GLYCEROPHOSPHODIESTERASE-LIKE* gene (*NtGPDL*) in tobacco plants (Choi and Sano 2007).

It appears that global demethylation leading to the activation of gene expression is an immediate response to stress that is more common in plants. The examples include global DNA hypomethylation after abiotic stress in a number of species: *Broynia dioica* (Galaud, Gaspar et al. 1993), maize (Steward, Ito et al. 2002), rice (Wang, Pan et al. 2011), *Trifolium repens L.* and *Cannabis sativa L*. (Aina, Sgorbati et al. 2004). Similarly, *Pseudomonas syringae*-challenged Arabidopsis plants (Pavet, Quintero et al. 2006) as well as virus infected tomato plants (Mason, Noris et al. 2008) exhibit DNA

hypomethylation at centromeric repeats and in several genomic regions involved in defence and stress responses, respectively. At the same time, *Mesembryanthemum crystallinum* plants exposed to salt stress demonstrated a twofold increase in CpHpG methylation (Dyachenko, Zakharchenko et al. 2006). Also, an age-dependent increase in global DNA methylation was correlated with the development of resistance in adult plants to the blight pathogen *Xanthomonas oryzae* in rice (Sha, Lin et al. 2005).

Unfortunately, the link between direct exposure to stress and global or sequence-specific DNA demethylation has not been established yet. The most plausible explanation of this phenomenon manifests from the studies showing the generation of ROS upon exposure to abiotic stress that results in oxidation of guanosine residues, which subsequently leads to the formation of 8-hydroxyguanosine (Dionisio-Sese and Tobita 1998). The occurrence of 8-hydroxyguanosine in the CpG sequences strongly suppresses methylation of adjacent cytosine residues (Cerda and Weitzman 1997). Nevertheless, this hypothesis of passive DNA demethylation does not include a rapid demethylation at the *NtGPDL* genomic locus that has been reported to occur as soon as 1 h after stress exposure (Choi and Sano 2007). The involvement of one type of DNA demethylases, DML3, in the active DNA demethylation process can also be excluded because in Arabidopsis seedlings, DML3 is negatively regulated by miR402 which is induced upon salt, dehydration or cold stress (Kim, Kwak et al. 2010). However, recently it has been suggested that DNA demethylation may be guided to specific loci by ROS3, an RNA-recognition motif containing protein which uses small RNAs as guides. ROS3 can interact with the DNA demethylase ROS1 and possibly guide it to target loci for

demethylation (Zheng, Pontes et al. 2008), thus suggesting an intriguing and dynamic interplay between DNA methylation and demethylation pathways.

A decrease in DNA methylation in mutants impaired in CpG maintenance methylation (*met1*) and non-CpG methylation (*drm1/drm2/cmt3* triple mutant) has been shown to result in a significantly higher resistance against bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) as compared to that in wild-type Arabidopsis plants, thus suggesting that DNA methylation, at least to some extent, suppresses plant defense mechanisms (Dowen, Pelizzola et al. 2012). A detailed analysis of the methylome profile in response to pathogen attack has revealed a number of transposable elements (TEs), pathogen responsive and protein-coding genes that were differentially methylated at the 5th day post infection in wild-type plants (Dowen, Pelizzola et al. 2012). Moreover, altered methylation of sequences encoding TEs was linked to their own expression and/or the expression of neighbouring genes as well as the accumulation of TE-associated 21-nt long siRNAs (Dowen, Pelizzola et al. 2012). The authors speculated that a pool of 21-nt long siRNAs can be used as non-cell-autonomous messengers that arise upon stress exposure in somatic tissues and eventually can shape the epigenome of gametes, which results in transgenerational stress memory. Taking into account the possible dual function of small RNAs in the process of DNA methylation, this is an intriguing hypothesis. Future research should address this interesting phenomenon and may reveal a link between the target demethylation pathway and plant response upon stress exposure.

In recent studies, the strong relationship has been established between DNA hypermethylation at particular loci in response to environmental cues and epigenetic

factors involved (Baek, Jiang et al. 2011, Tricker, Gibbings et al. 2012). For instance, the tandem repeat upstream of the start codon of a sodium transporter gene in Arabidopsis has been shown to be a putative small RNA-mediated target for methylation associated with salt stress tolerance (Baek, Jiang et al. 2011). A plausible relationship has been also reported between the transcriptional repression of two genes that control stomata development, the RdDM pathway and a decreased number of stomata in Arabidopsis plants cultivated under low humidity (Tricker, Gibbings et al. 2012).

Despite the undeniable role of DNA methylation in plant stress response, an increasing body of evidence indicates the involvement of histone occupation, octamer positioning and posttranslational histone modifications as the primary stress receptive elements that can act either in cooperation with (Baubec, Dinh et al. 2010, Bilichak, Ilnystkyy et al. 2012) or apart from DNA methylation (Lang-Mladek, Popova et al. 2010, Tittel-Elmer, Bucher et al. 2010).

Table 2.1. Plant DNA methyltransferases and demethylases

Gene Name	Target Sequence	Effects on Chromatin /Transcription	Effects of Mutation
METHYLTRANSFERASE1 (*MET1*)	CpG	Maintains the global methylation of symmetrical CpG sites; involved in the RdDM pathway/Repression	Inability to establish CpG methylation; a passive decrease of DNA methylation throughout generations
CHROMOMETHYLASE3 (*CMT3*)	Primarily CpHpG	Targets centromeric repeats and transposons; partially contributes to the establishment of DNA methylation at the CpG and CpHpG contexts/Repression	Loss of CpHpG methylation
DOMAIN REARRANGED METHYLTRANSFERASES (*DRM1, DRM2*)	CpG, CpHpG and CpHpH	*De novo* methylation of asymmetric sites; DRM2 is involved in *de novo* methylation of CpG sequences in the RdDM pathway/Repression	Loss of *de novo* DNA methylation
DEMETER (*DME*)	CpG, CpHpG and CpHpH	Demethylation of silenced promoter sequences through nucleotide excision repair (NER) pathway/Activation	Inability to activate imprinted genes; seed abortion
REPRESSOR OF SILENCING1 (*ROS1*)	CpG, CpHpG and CpHpH	Demethylation activity on methylated promoter sequences/Activation	Local hypermethylation and transcriptional gene silencing; reduced tolerance to genotoxic agents
DEMETER-LIKE (*DML*) *PROTEINS: DML2 AND DML3*	CpG, CpHpG and CpHpH	Demethylation of genes at the 5′ and 3′ regions, which leads to the reduced accumulation of both methylation at or near genes and a decrease in the number of stable epialleles /mostly unchanged	Hypermethylation of genes at the 5′ and 3′ regions

2.1.3. Chromatin fluctuations and plant stress response

The regulation of gene expression is not a one-dimensional process; it typically involves a tightly interwoven complex of chromatin modifiers that is connected to posttranslational histone modifications (PTM), ATP-dependent chromatin remodelling and histone variant replacement. In contrast to DNA methylation, histone modifications on the amino-terminal tails are highly variable and multifarious, but their role in chromatin regulation and gene expression is sometimes not completely obvious (see Table 2.2). The high complexity of encoded information carried by histone epigenetic marks originates from a large number of possible posttranslational modifications combined with different histone variants that together form a histone code of a cell (Chinnusamy and Zhu 2009, Kovalchuk and Kovalchuk 2012). Protruding from the globular nucleosome core, histone tails can undergo different PTMs such as: acetylation, methylation, phosphorylation, ubiquitination, sumoylation, biotinylation, carbonylation, glycosylation and ADP ribosylation catalyzed by a plethora of enzymes (Tariq and Paszkowski 2004, Kouzarides 2007, Liu, Lu et al. 2010, Lauria and Rossi 2011, Berr, Menard et al. 2012).

A wide range of histone modifications has been connected to the gene activity. Elevated gene expression correlates with the enrichment of acetylation, certain phosphorylation and ubiquitination (Sridhar, Kapoor et al. 2007, Zhang, Sridhar et al. 2007) in histone N-terminal regions, while down-regulation is linked to biotinylation and sumoylation (Camporeale, Oommen et al. 2007, Chen, Lv et al. 2010). Histone methylation plays a dual role in the modulation of gene activity depending on the lysine and arginine residues modified and a number of methyl groups attached to each lysine

residue. A genome-wide analysis of histone methylation marks in plants revealed that trimethylation of histone 3 lysine 4 and di-/trimethylation of histone 3 lysine 36 (H3K4me3 and H3K36me2/me3, respectively) are enriched in actively transcribed gene sequences, whereas H3K27me3 and H3K9me2 are the main gene silencing markers (Zhang, Clarenz et al. 2007, Wang, Elling et al. 2009). A few histone methylation modifications such as H3K27me1, H3K27me2 and H4K20me1 have been found to be accumulated in both transposon regions and constitutive heterochromatin regions (Roudier, Ahmed et al. 2011).

Increasing evidence indicates that a range of histone epigenetic marks co-interact with each other and form the combinatorial clusters of gene expression regulation (Strahl and Allis 2000, Berger 2007, Wang, Zang et al. 2008, Roudier, Ahmed et al. 2011). For instance, a genome-wide analysis of the distribution of 39 histone modifications in $CD4^{+}$T human cells revealed a common module consisting of 17 histone PTMs that were significantly enriched at 3,286 promoters of actively transcribed genes (Wang, Zang et al. 2008). A similar histone module has been recently described in Arabidopsis with 12 histone marks that have been found in about 90% of the genome (Roudier, Ahmed et al. 2011). *In silico* predictions of the distribution of the combinatorial cluster allowed the authors to define four main chromatin states in the Arabidopsis nucleus that mainly encompass active genes, repressed genes, silent repeat elements and intergenic regions. Furthermore, a strong association has been reported between the distribution of particular histone modifications and different categories of tissue-specific alternative splicing patterns (Zhou, Lu et al. 2012).

The association between chromatin marks and their fluctuations in response to stress is becoming a primary focus of plant epigenetic research. A growing body of evidence indicates that histone modifications are at the forefront of stress signal perception, modulation of gene activities and stress response. Nevertheless, such modifications at the histone tails may or may not be truly epigenetic in nature since the mechanism of propagation of histone code during DNA replication remains obscure, and it is thought that regardless of the circumstances, not all PTMs of histones are faithfully transmitted to daughter cells in the absence of the maintenance signal (Chinnusamy and Zhu 2009, Bonasio, Tu et al. 2010).

Table 2.2. Posttranslational histone modifications in plants

Enzyme category	Residue	Type of modification	Effect on the transcription
Histone acetyltransferase	Lysine	Acetylation	Activation
Histone deacetylases	Lysine	Deacetylation	Inhibition
Histone methyltransferases	Lysine	Methylation	Depends on the histone residue
Histone demethylases	Lysine	Demethylation	Depends on the histone residue
Ubiquitin ligase	Lysine	Ubiquitination	Activation
Ubiquitin protease	Lysine	Deubiquitination	Repression
Kinase	Serine/Threonine	Phosphorylation	Activation
Phosphatase	Serine/Threonine	Dephosphorylation	Inhibition
Arginine methyltransferases	Arginine	Methylation	Either activation or repression
Deiminase	Arginine	Demethylation	Either activation or repression

2.1.4. Histone acetylation and plant stress response

Environmental and endogenous cues can affect gene activity through alteration at the histone acetylation level. For instance, in Arabidopsis, histone deacetylases (HDAs), namely HDA6 and HDA19, catalyze deacetylation at several loci in response to abiotic and biotic stresses. Both genes are receptive to jasmonic acid (JA), and in the case of *HDA19* possibly, mediates the plant pathogen response through the JA-regulated *PATHOGENESIS-RELATED* (PR) genes (Zhou, Zhang et al. 2005, Wu, Zhang et al. 2008). Furthermore, studies on Arabidopsis knockouts revealed the involvement of the histone H4 deacetylase (HOS15) (for HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES) in cold stress response (Zheng, Pontes et al. 2008). Interestingly, *hos15* mutant plants demonstrated constitutive expression of stress-related genes, such as *COLD REGULATED 15A* and *ALCOHOL DEHYDROGENASE 1*, albeit they were unable to cope with cold stress unlike wild-type plants.

Overexpression of a histone deacetylase from Arabidopsis, *AtHD2C,* which is a member of the plant-specific HD2 family of HDA resulted in activating the abscisic acid (ABA) responsive genes and elevated salt and drought tolerance as compared to that in wild-type plants (Sridha and Wu 2006), thus suggesting that either histone acetylation suppresses plant stress tolerance, at least in the aforementioned examples, or HDAs demonstrate a locus-specific activity upon stress exposure. In support of the last statement, in the yeast *Saccharomyces cerevisiae*, it was shown that the stress-responsive mitogen-activated protein kinase Hog1 guides Rpd3 HDA to the promoters of stress related genes, which eventually leads to histone deacetylation, the entry of Pol II and initiation of gene expression (De Nadal, Zapater et al. 2004).

2.1.5. Histone methylation and plant stress response

Available data on the genome-wide histone modification distribution in Arabidopsis allowed combining certain histone acetylation (H3K56ac), methylation (H3K4me2 and 3, H3K9me2 and 3, H3K27me1, 2 and 3, H3K36me3 and H4K20me1) and ubiquitinylation (H2Bub) marks into one module, thus providing an explicit proof of a tight interplay between a number of histone modifications (Roudier, Ahmed et al. 2011). Therefore, it is safe to assume that alterations in certain chromatin marks upon stress exposure would bring a range of other modifications at the same locus. A typical example includes the accumulation of two repressive chromatin marks, H3K9me2 and H3K27me3, at the potent floral repressor, the *FLOWERING LOCUS C* (FLC) gene, after vernalization (Kim and Sung 2012). Similarly, recently it has been shown that the activation of the *PR1* gene in response to salicylic acid (SA) treatment or pathogen attack is correlated with the enrichment of permissive chromatin marks H3K4me2, H3K4me3 and H3ac in the promoter region (Mosher, Durrant et al. 2006, De-La-Pena, Rangel-Cano et al. 2012).

In a screen of genes required for the *accelerated cell death 11* (*acd11*) mutant phenotype which exhibits a constant autoimmune response regardless of pathogen perception, a histone lysine methyltransferase SET (Su(var)3-9, E(z) and the Trithorax-conserved) DOMAIN GROUP 8 (SDG8) gene was revealed (Palma, Thorgrimsen et al. 2010). The Arabidopsis SDG8 protein associated with methylations at H3K36 implements a regulatory function on the *lazarus* 5 resistance (*R*) gene which modulates strong defense responses upon pathogen attack. *sdg8* mutant plants failed to develop full

resistance to different strains of virulent *Pseudomonas* pathogens, indicating that SDG8 apparently targets a subset of *R* genes for activation (Palma, Thorgrimsen et al. 2010).

Recently, histone posttranslational modifications also have been shown to be involved in priming defence genes that permits an organism to respond faster and with a higher extent to biotic stresses (Jaskiewicz, Conrath et al. 2011). The same study also provides evidence of systemic epigenetic responses at the histone level in bystander leaves. Following the localized foliar infection by the pathogen *Pseudomonas syringae* pv. *maculicola*, the authors examined histone epigenetic marks at the promoter regions of stress-responsive transcription factors of the WRKY family proteins in distal untreated leaves. Three known *WRKY* promoters (*WRKY29, WRKY6* and *WRKY53*) demonstrated a significant accumulation of permissive chromatin marks: H3K4me2 and 3, H4K5ac, H4K8ac and H4K12ac, but failed to show an increase in transcription from the same genes without the direct stress application. These data allowed the authors to speculate that histone marks set a transcriptionally competent state of stress-related genes in systemic tissues which allows for the rapid initiation of transcription and, apparently, mediates SAR in response to pathogen attacks.

2.1.6. Histone phosphorylation/ubiquitination and plant stress response

In plants, phosphorylation of serine and threonine residues of histone tails is catalyzed by a wide range of kinases, the most prominent of which are Aurora and NIMA kinases that phosphorylate histone H3 at serine 10 and haspin-like kinase (a haploid germ cell-specific nuclear protein) with the phosphorylation activity at threonine 3 of histone

H3 (Houben, Demidov et al. 2007). The removal of phosphate groups from histone tails is catalyzed by the protein phosphatase 1 (PP1) family enzymes.

Similar to other histone epigenetic marks, histone phosphorylation has been linked to the modulation of gene activity, DNA damage repair, chromatin structure and apoptosis (Loury and Sassone-Corsi 2004, Houben, Demidov et al. 2007). For instance, phosphorylation of H2B and histone variant H2A.X at serine 14 was linked to the onset of apoptotic chromatin condensation and DNA fragmentation, respectively (Cheung, Ajiro et al. 2003, Thiriet and Hayes 2005). Phosphorylation of the H2A.X histone variant at serine 4 residues of the C-terminal tail generates a phosphorylated form known as γ-H2A.X (Redon, Pilch et al. 2002). The C-terminal tail domain projects out towards the front of the nucleosome and interacts with the linker DNA entering the nucleosome, thus making the C-terminus relatively accessible for diffusible factors, and its phosphorylation is believed to be a hallmark of DNA double-strand breaks. Indeed, the accumulation of γ-H2A.X initiates the accumulation of other components involved in DNA double-strand break repair and transcription (Thiriet and Hayes 2005, Lang, Smetana et al. 2012).

Histone monoubiquitination in Arabidopsis plants occurs at lysine 143 of histone H2B and lysine 121 of the histone variant H2A.1 with the help of the ubiquitin E3 ligase, HISTONE MONOUBIQUITINATION1 (HUB1) and polycomb group (PcG) proteins, the PRC1 RING-finger homologs AtBMI1A and AtBMI1B, respectively (Weake and Workman 2008, Bratzel, Lopez-Torrejon et al. 2010, Himanen, Woloszynska et al. 2012).

Overall, phosphorylation at Ser10 and Ser28 residues of histone H3 and monoubiquitination of H2B at Lys143 have been correlated with transcription activation (Khorasanizadeh 2004). For instance, global enrichment of H3 phosphorylated at Ser10

and phosphoacetylated histone H3 was sufficient to up-regulate stress-related genes in response to high salinity, cold and the exogenous ABA application in tobacco and Arabidopsis cell suspension cultures (Sokol, Kwiatkowska et al. 2007), whereas inducing monoubiquitination of histone H2B in Arabidopsis plants led to elevated plant tolerance to necrotrophic fungi (Dhawan, Luo et al. 2009).

2.1.7. ATP-dependent chromatin remodelling and plant stress response

Chromatin remodelling which utilizes the energy of ATP molecules for altering histone-DNA interactions was found to be an additional dynamic and vital process that modulates gene expression in response to stress (Gutzat and Mittelsten Scheid 2012). There are three main classes of the ATP-dependent chromatin remodelling complexes that are known to exist in plants: the imitation switch (ISWI) ATPases, the SWI/SNF ATPases and the chromodomain and helicase-like domain (CHD) ATPases (Kwon and Wagner 2007, Walley, Rowe et al. 2008).

Thus far, only a handful of the ATP-dependent chromatin remodelling proteins have been implicated in the plant stress response and development. For instance, the SWI/SNF class chromatin remodelling ATPase SPLAYED (SYD) was documented to be involved in the regulation of a range of stress-related and developmental processes (Bezhani, Winter et al. 2007, Walley, Rowe et al. 2008). A different member of the same class - SWI3B - is able to interact with the ABA co-receptor, HYPERSENSITIVE TO ABA1 (HAB1) and is a positive regulator of the ABA-mediated response (Saez, Rodrigues et al. 2008). Also, the SNF2/Brahma-type chromatin-remodelling protein

AtCHR12 was suggested to play a role in mediating the temporary growth arrest of Arabidopsis under stress conditions (Luo, Liu et al. 2012).

The involvement of chromatin remodelling distinct from DNA methylation and certain histone epigenetic marks in the activation of repetitive elements upon heat shock has been recently reported in Arabidopsis (Pecinka, Dinh et al. 2010, Tittel-Elmer, Bucher et al. 2010). Nucleosome loading was significantly but reversibly reduced at TE loci in response to the prolonged heat stress (Pecinka, Dinh et al. 2010). The participation of nucleosome occupancy rather than DNA or histone methylation in transcriptional regulation of TEs was further suggested by delayed re-silencing of heat stress-activated retrotransposons (TSI and ATHILA-related) in CHROMATIN ASSEMBLY FACTOR 1 (CAF-1) mutants (Pecinka and Mittelsten Scheid 2012).

2.1.8. The metabolism of small RNAs and plant stress response

A novel type of non-coding RNAs, so called small RNAs (smRNAs), has recently emerged and complemented the epigenetic mechanism of gene expression regulation. Ranging from 20- to 27-nt in length, smRNAs are vital regulators of global epigenome alterations during plant ontogenesis and periconception which covers gametogenesis, fertilization, and early zygotic development (Slotkin, Vaughn et al. 2009, Bourc'his and Voinnet 2010). Additionally, apart from being dynamically involved in promoting the formation of long-term memory, smRNAs also preserve genome integrity from the effects of potentially harmful genomic parasites like transposons. RNA silencing is a major mechanism of smRNA action by triggering either transcriptional (through DNA methylation) or posttranscriptional (through RNA degradation) gene silencing of

complementary DNA or RNA, respectively. In both pathways, smRNAs are used as the guides that direct effector proteins to the target nucleic acid molecules through base-pairing interactions (Carthew and Sontheimer 2009).

There are two major classes of smRNAs known in plants as small interfering RNAs (siRNAs) and micro RNAs (miRNAs). Whereas the former ones are processed from long double-stranded (dsRNA) or single-stranded RNAs (ssRNAs) with substantially perfect or near perfect hairpins, the later ones are generated from single-stranded stem-loop-like structures of precursor miRNAs that are folded - pre-miRNAs - through a two-step or sometimes multi-step process (Ramachandran and Chen 2008). The second strand of dsRNA molecules can be synthesized by either of six Arabidopsis RNA-dependent RNA polymerases (RDRs) that recognize aberrant decapped mRNAs (Brosnan, Mitter et al. 2007).

Massive amounts of data produced by the next-generation sequencing technologies revealed a variety of siRNAs expressed from endogenous loci. Three main classes of endogenous siRNAs have been put together regarding the loci from which they are generated: natural-antisense transcript-derived siRNAs (nat-siRNAs), ncRNAs produced from two overlapping and partially converging coding transcripts (Borsani, Zhu et al. 2005); heterochromatic or repeat-associated siRNAs (hc- or ra-siRNAs, respectively), dsRNAs generated from heterochromatin and DNA repeat loci (Guleria, Mahajan et al. 2011); trans-acting siRNAs (ta-siRNAs), miRNA-guided cleavage products of mRNA which are recognized and converted into dsRNAs by RDRs (Borsani, Zhu et al. 2005, Sunkar, Chinnusamy et al. 2007, Grativol, Hemerly et al. 2012).

DsRNA processing named ‘dicing’ is performed by one or more of the four Dicer-like proteins (DCL) with the Ribonuclease III-like activity. DCL1 generates 18–21 nt-long smRNAs, while DCL2, DCL3, and DCL4 produce 22, 24, and 21 nt-long smRNAs, respectively (Ruiz-Ferrer and Voinnet 2009). Following dicing, smRNAs with 3′ overhang ends are 2′-O-methylated by the methyltransferase HUA ENHANCER 1 (HEN1) that protects them from degradation. Later, smRNA duplexes can be either retained in the nucleus for the TGS pathway or exported to the cytoplasm, possibly through the exportin-5 homolog HASTY (HST) for PTGS. In the former scenario, smRNAs are picked by one of the PAZ and PIWI domain containing enzymes with endonucleolytic activities, ARGONAUTE 4 (AGO4), and incorporated into the RNA-induced transcriptional silencing complex (RITS) to promote sequence-specific DNA methylation. Besides AGO4 and siRNA, the RITS complex includes DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), the DDR complex composed of DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1), DEFECTIVE IN MERISTEM SILENCING 3 (DMS3), RNA-DIRECTED DNA METHYLATION 1 (RDM1) and Pol V (Law, Ausin et al. 2010, Kovalchuk and Kovalchuk 2012).

When siRNAs are guided from the nucleus, they are primarily picked by AGO6 and incorporated into the RNA-induced silencing complex (RISC) that scans the cell for complementary nucleic acids to execute its silencing function (Ramachandran and Chen 2008). The Arabidopsis genome encodes ten AGO proteins with the RNA-binding PAZ and RNase H-like PIWI domains that belong to three phylogenetic clades. Nevertheless, only a handful of AGOs has been ascribed to pathways in which they modulate gene activity (Vaucheret 2008).

2.1.9. Natural antisense transcript-derived siRNAs and plant stress response

Natural antisense transcript-derived siRNAs are a class of endogenous ncRNAs of 21-24-nt in length that fall into two groups regarding the origin of transcripts from which they are generated. Nat-siRNAs produced from mRNAs which are transcribed from opposite strands at the same locus are called *cis*-nat-siRNAs, while those generated from distinct genomic regions are named *trans*-nat-siRNAs. The members of both groups were found to regulate gene activity at the posttranscriptional level by guiding mRNA cleavage (Kovalchuk and Kovalchuk 2012).

cis-nat-siRNAs generated from convergently transcribed RNAs of *DELTA-1-PYRROLINE-5-CARBOXYLATE DEHYDROGENASE* (*P5CDH*) and *SIMILAR TO RCD ONE 5* (*SRO5*) genes were the first discovered nat-siRNAs in plants expressed in response to salt stress (Borsani, Zhu et al. 2005). Whereas the former gene encodes a constitutively expressed enzyme involved in proline metabolism, the later one is activated only in response to salt stress. When overlapping transcripts are generated, DCL2 and DICER RNA BINDING FACTOR (DRB) partners cleave the dsRNA duplex. Later, truncated *P5CDH* mRNA can become a substrate for the RNA-DEPENDENT RNA POLYMERASE6 (RDR6) together with SUPRESSOR OF GENE SILENCING3 (SGS3), which results in the generation of longer RNA duplexes. The formation of nat-siRNAs promotes the degradation of *P5CDH* mRNA, thus leading to proline accumulation, an important metabolite involved in developing salt stress tolerance (Borsani, Zhu et al. 2005).

Infection of Arabidopsis plants with the bacterial pathogen *Pseudomonas syringae* carrying effector avrRpt2 results in the generation of another *cis*-nat-siRNA - nat-siRNAATGB2 derived from the overlapping region of a Rab2-like small GTP-binding protein gene (*ATGB2*) and a *PENTATRICOPEPTIDE REPEATS* (*PPR*) protein-like gene (*PPRL*) (Katiyar-Agarwal, Morgan et al. 2006). It was shown that the production of this *cis*-nat-siRNA requires activities of HYL1, HEN1, RDR6, SGS3, and Pol IVa. The specific induction of 22 nt-long nat-siRNAATGB2 leads to silencing of the antisense gene *PPRL*, which apparently is a negative regulator of the pathogen signalling pathway. Therefore, the down-regulation of *PPRL* by nat-siRNAATGB2 plays a positive role in plant resistance against bacterial pathogens (Katiyar-Agarwal, Morgan et al. 2006).

The most recent genome-wide analysis of *cis*-nat-siRNAs has identified 17,141 and 56,209 unique nat-siRNA sequences in Arabidopsis plants exposed to biotic and abiotic stresses and abiotic stressed rice, respectively (Zhang, Xia et al. 2012). The biogenesis analysis revealed that the expression of 20-22-mer *cis*-nat-siRNAs is dependent on DCL1, whereas siRNAs ranging from 23 to 28 nt in length are produced by DCL3. Interestingly, the authors have revealed that many of the 21 nt-long nat-siRNAs were able to down-regulate their target transcripts, whereas the 24 nt-long siRNAs did not demonstrate a significant silencing activity.

The possible discrimination between the pathways in which smRNAs modulate gene activity comes from ten AGO proteins which demonstrate their preference to different siRNAs regarding their length and 5′ terminal nucleotide (Mallory and Vaucheret 2010). For instance, the majority of AGO1-associated smRNAs are either 21 or 22 nt in length and carry 5′ U, while most of the smRNAs associated with AGO2 are

21 nt-long and have 5′A. Furthermore, AGO4, AGO6, and AGO9 mostly precipitate with smRNAs which are 24 nt-long and have 5′ A, whereas AGO5 interacts with 24 nt-long smRNAs with 5′C (Mallory and Vaucheret 2010). Since AGO proteins have been found to act selectively in the distinct silencing pathways, smRNAs of different sizes and with distinct 5′ terminal nucleotides can repress gene expression either at the posttranscriptional level via PTGS or at the chromatin level through TGS depending on the AGO protein with which they interact. An example of siRNAs acting in the TGS pathway includes hc- and ra-siRNAs.

2.1.10. Heterochromatic and repeat-associated siRNAs and plant stress response

Heterochromatic and repeat-associated siRNAs (hc- and ra-siRNAs, respectively) are produced from transcripts generated at the heterochromatic regions and repetitive elements, respectively. Hc-siRNAs-mediated TGS plays an important role in defence against the proliferation of endogenous transposons and restriction of undesirable gene expression through DNA methylation and histone modifications.

A reinforcing heterochromatic loop begins from the generation of transcripts by Pol II, Pol III and Pol IV. Later, RNA from heterochromatic loci or repetitive elements is targeted by RDR2 to produce long dsRNAs followed by DCL3/DRB cleavage of dsRNA into 24 nt-long duplexes which are then methylated by HEN1. These 24 nt-long siRNAs are picked by AGO4 and incorporated into RITS that eventually mediates cytosine methylation of complementary DNA sequences through the RNA-dependent DNA methylation pathway (RdDM) (Zhang, Henderson et al. 2007, Haag and Pikaard 2011,

Kovalchuk and Kovalchuk 2012). The strand with the weaker hydrogen bonded 5′ end is preferentially selected as siRNA, while the opposite strand is degraded (Haag and Pikaard 2011). Then, the complete siRNA-guided RITS complex possibly interacts with another plant-specific polymerase – PoI V. Apparently, Pol V transcripts and the Pol V largest subunit bring the RITS complex into the proximity of chromatin to be modified, eventually enabelling recruitment specific chromatin modifiers (Haag and Pikaard 2011).

In plants, smRNA-directed cytosine methylation occurs within any sequence regions and is thought to be primarily performed by *de novo* methyltransferase DRM2 armed with the members of the DDR complex. Silencing at the targeted locus can be further reinforced by the accumulation of repressive chromatin marks. For instance, the removal of permissive chromatin marks (histone acetylation and H3K4me3) and the enrichment of repressive chromatin marks (H3K9me2 and H3K27me) contribute to transcriptional silencing at sites which are subject to Pol IV- and Pol V-mediated RdDM (Haag and Pikaard 2011).

The contribution of hc- and ra-siRNA to the immobilization of retrotransposons was further supported in the stress experiments that involved mutants impaired in the siRNA pathway (Ito, Gaubert et al. 2011). The authors demonstrated that heat-stressed Arabidopsis mutants *nrpd1, nrpd2* and *rdr2* significantly accumulate transcripts of a copia-type retrotransposon ONSEN which also generates extrachromosomal DNA copies. Following stress exposure, ONSEN transcripts and the extrachromosomal DNA copies gradually vanished; new ONSEN insertions were not detected in the genomic DNA of either wild-type or *nrpd1* plants. Enigmatically, high frequency retrotransposition was observed in the following generation of stressed mutant plants that

were compromised in the siRNA's metabolism. These results suggest either the occurrence of stress memory that was maintained throughout the development of mutant plants or an insufficient reinforcement of repressive chromatin marks that usually follows the Pol IV-mediated transposons transcription.

Interestingly, two recent reports in *Drosophila* and *Arabidopsis* have shown that smRNAs produced from transposon regions can affect the expression of endogenous genes, thus bridging TE and gene regulation networks. In the *Drosophila* early embryo, TE-originated PIWI-interacting smRNAs (piRNAs) can silence the *nanos* mRNA which is essential for a proper embryo segmentation (Rouget, Papin et al. 2010), while in *Arabidopsis*, *Athila-derived* smRNAs directly target *OLIGOURIDYLATE BINDING PROTEIN 1b* (*UBP1b*) mRNA – a component of stress granules involved in responses to certain abiotic stresses (McCue, Nuthikattu et al. 2012).

2.1.11. *Trans*-acting siRNAs and plant stress response

Trans-acting siRNAs (ta-siRNAs) are a class of plant siRNAs produced by the interaction between miRNA and siRNA pathways. The generation of ta-siRNAs starts with transcription of miRNA precursors (pre-miRNA) by Pol II. These pre-miRNAs contain miRNA sequences within a stem of a long imperfect RNA hairpin which is processed by DCL1 in the nucleus resulting in an imperfect RNA duplex with the 2-nucleotide 3′ overhangs on each strand. Most miRNAs are found to target mainly protein-coding mRNAs through the PTGS pathway, however, some miRNAs guide the cleavage of non-protein-coding primary transcripts of *trans*-acting siRNA (*TAS*) genes directing the formation of truncated RNA (Krasnikova, Milyutina et al. 2009). The

resulting cleavage product of *TAS* RNAs becomes a substrate for RDR6/SGS3 which leads to the generation of double-stranded *TAS* RNAs. SGS3 might stabilize the cleaved RNAs from degradation, while RDR6 synthesizes the second strand (Allen and Howell 2010). Subsequently, long double-stranded precursor ta-siRNAs (pre-ta-siRNAs) are cleaved by the cooperative action of DCL4 and DICER RNA binding factor4 (DRB4) at 21-nt long increments relative to the original cleavage site on both strands – a process called 'phasing'. Apparently, the generation of the uniform 21 nt-long siRNA occurs due to a precise slicing activity of DCL4 starting at the miRNA cleavage site. The exact sequence of miRNA-guided cleavage sets the entry point for DCL4 and thus for the phase of ta-siRNA. ds-ta-siRNAs are methylated by HEN1, and the RDR6-template strand is then loaded into the RISC complex with one of the AGO proteins (Kovalchuk and Kovalchuk 2012).

Most pre-ta-siRNAs have only a single miRNA target motif which is cleaved by miRNA-guided AGO1; however, there is a curious exception of these observations. For instance, mRNA transcribed from the *TAS3* gene contains two binding sites for miR390 within its sequence. One miR390 guides the AGO7-mediated cleavage of the 3′ side of *TAS3* RNA, whereas the second one interacts in a non-cleavage mode at a site near the 5′ terminus and is important for the production of ds-pre-ta-siRNA (Montgomery, Howell et al. 2008, Krasnikova, Milyutina et al. 2009). Subsequently, ta-siRNAs act *in trans* to reduce the expression of unrelated loci from which they are produced (Hsieh, Lin et al. 2009).

Arabidopsis ta-siRNAs are derived from eight loci that fall into four families: *TAS1*, *TAS2*, *TAS3* and *TAS4*. *TAS1*, *TAS2*, and *TAS4* ta-siRNA biogenesis is initiated

with either the miR173- (*TAS1* and *TAS2*) or miR828-guided (*TAS4*) cleavage at the 5′ terminus of the ta-siRNA-generating region. *TAS1/2* loci are transcribed by Pol II generating typical polyadenylated and capped transcripts. Enigmatically, *TAS* non-protein-coding transcripts are recognized by the silencing machinery as aberrant transcripts and are targeted for degradation. The *TAS1* family which consists of transcripts from three loci (*TAS1a*, *TAS1b* and *TAS1c*) codes for multiple ta-siRNAs with very similar sequences that are predicted to target the same mRNAs coding for unknown proteins. Whereas ta-siRNA – siR1511 produced from the *TAS2* transcript targets *PPR* mRNAs, ta-siRNAs processed from the *TAS3* transcript target the Auxin Response Factor family members ARF1, 2, 3 or 4 involved in the juvenile-to-adult transition in leaf development. Interestingly, miR828 which sets a cleavage point for *TAS4* transcripts is specifically involved in the direct regulation of the MYB transcription factor *MYB113*. In turn, the negative regulation of *MYB113* expression and related family members via miR828 can be further amplified by *TAS4* originated ta-siRNAs (Allen and Howell 2010). Since ta-siRNAs are mobile and can perform short-distance journeys across multiple cells, they can create a gradient of suppression activity in the proximate cells (Schwab, Maizel et al. 2009).

Recently, ta-siRNAs have been discovered to be involved in adaptation to phosphate (Pi) deficiency and cold stress in Arabidopsis and thermosensitive genic male sterile (TGMS) lines of wheat (*Triticum aestivum),* respectively (Hsieh, Lin et al. 2009, Tang, Zhang et al. 2012). Pi-deficient shoots of Arabidopsis accumulate *TAS4*-siR81(-) which is involved in the auto regulatory mechanism of PAP1/MYB75 and the biosynthesis of anthocyanin. In wheat, the *TAS3*-derived ta-siRNA-Auxin-Responsive

Factor (ARF) was significantly repressed at an early stage of spike development during cold stress that was correlated with the up-regulation of one of the *ARF* genes. Since in Arabidopsis, *TAS3a* ta-siRNAs have been shown to modulate *ARF3* gene expression which regulates late stages of flower development in a number of plants, the authors suggested that during cold treatment, an abnormal decline of ta-siRNA-*ARF* levels contributes to male sterility in the TGMS line through the negative regulation of *ARFs*. Nevertheless, it still remains to be elucidated how the expression of this and other ta-siRNAs is regulated during stress exposure.

2.1.12. Micro RNAs and plant stress response

Micro RNAs are typically 21 nt-long single-stranded RNAs which are generated by DCL1 from endogenous transcripts containing local hairpin structures. Compared to animals, the majority of miRNA genes in plants are located separately throughout the genome and are transcribed by Pol II into long pri-miRNAs that contain a 5′ cap and a 3′ poly (A) tail. Processing of pri-miRNAs is believed to take place shortly after a nascent transcript folds into the secondary hairpin-like structure that might involve some of the machineries responsible for capping, splicing and polyadenylation of protein-coding transcripts (Xie, Khanna et al. 2010). For instance, Arabidopsis mutants *cbp80/abh1* and *cbp20* of the cap-binding complex (CBC) accumulate elevated levels of pri-miRNAs concomitant with a decreased level of mature miRNAs (Kim, Yang et al. 2008, Laubinger, Sachsenberg et al. 2008).

The dsRNA arm of a hairpin loop is further recognized by DCL1, and in cooperation with the zinc finger-containing protein SERRATE (SE) and the dsRNA-

binding protein HYPONASTIC LEAVES1 (HYL1), mature miRNA/miRNA* duplexes are excised from the stem of the hairpin with a two-nucleotide overhang on each strand (Vazquez 2006). Subsequently, the mature miRNA/miRNA* duplexes are stabilized by methylation at the 2′-OH of the 3′ ends by HEN1 and are possibly exported to the cytoplasm through HASTY.

The last step of miRNA maturation involves a selective incorporation of the miRNA strand into the AGO1-containing miRNA-RISC complex. There are two known requirements for the miRNA-guided strand selection: (i) whichever strand is less stably paired at its 5′ end is incorporated into RISC and (ii) having a 5′-terminal uridine to be preferentially incorporated into AGO1 (Xie, Khanna et al. 2010). Subsequently, being a part of RISC, plant miRNAs target transcripts through the perfect (or near-perfect) pairing between miRNA and the mRNA transcript. Guided to the complementary transcript, AGO1 generates a single cut of the target mRNA phosphodiester backbone. Eventually, the truncated transcripts are either degraded by exonucleases or became a substrate for RDR enzymes (Kovalchuk and Kovalchuk 2012).

An emerging body of evidence suggests that miRNAs significantly contribute to plant stress response. For instance, Arabidopsis mutants *hen1* and *dcl1* that are partially compromised in miRNA metabolism are less stress tolerant as compared to wild-type plants (Sunkar and Zhu 2004). Recent studies further support the contribution of miRNAs to stress response in plants.

Photosynthesis results in the production of superoxide radicals which need to be scavenged as soon as they are generated to limit the production of more damaging hydroxyl radicals. Furthermore, a variety of environmental cues, such as drought,

salinity, high light, cold and heavy metals, lead to the elevated accumulation of reactive oxygen species (ROS). Therefore, plants have developed a highly sophisticated and fast-acting antioxidant system which involves the miRNA-mediated regulation of ROS scavenging enzymes (Sunkar, Chinnusamy et al. 2007). The genes encoding CU-ZN SUPEROXIDE DISMUTASE1 and 2 enzymes (*CSD1* and *CSD2*) which participate in detoxifying superoxide radicals are up-regulated in response to oxidative stress. Curiously, despite an increase in the amount of protein in response to stress, the nuclear run-on assay revealed that mRNA levels in these genes remain unchanged. Exposure to oxidative stress decreases miR398 transcription that normally guides the cleavage of cytosolic (*CSD1*) and plastidic (*CSD2*) gene mRNAs, resulting in the rapid accumulation of *CSD1* and *CSD2* transcripts allowing plants to cope with the burst in ROS production (Sunkar, Kapoor et al. 2006).

Many miRNAs have been found to be regulated in response to UV, cold, drought and salt stress; nevertheless only a few smRNAs have been shown to influence the pathways in which they modulate resistance or acclimation (Sunkar, Chinnusamy et al. 2007). For instance, miR399(a-f) and miR395 have been shown to modulate the activities of genes involved in phosphate and sulfate homeostasis, respectively. miR399(a-f) is induced in response to phosphate starvation and up-regulates a number of genes involved in phosphate metabolism *in trans* by targeting their suppressor – a ubiquitin-conjugating enzyme PHO2. The rates of sulfate translocation and accumulation during sulfur starvation are modulated by the miR395-mediated suppressing action on ATP sulfurylases (*APS1, APS3,* and *APS4*) and a low-affinity sulfate transporter (*AST68*).

2.1.13. Maintenance of the stress-induced epigenetic landscape and transgenerational epigenetic inheritance

Epigenetic components that modulate the transcriptome profile upon stress exposure can be both permanent and/or reversible in their nature. However, by definition, the subjects of epigenetic research are only chromatin alterations that are stable and faithfully inherited throughout mitosis and sometimes also meiosis.

Being flexible in its origin, epigenetic regulation of gene expression across generations is an attractive mechanism for mediating transgenerational plant response to stress, as compared to genetic changes such as point mutations, deletions, insertions and gross chromosomal rearrangements (Boyko and Kovalchuk 2011). Increasing evidence indicates that the propagation of the stress-induced epigenetic landscape to the next sexual generation, named "transgenerational epigenetic inheritance", is a cross-kingdom phenomenon of plant adaptation and acclimation to unfavorable environmental cues (Koturbash, Baker et al. 2006, Molinier, Ries et al. 2006, Pembrey, Bygren et al. 2006). Examples of this phenomenon include: an enhanced tolerance to NaCl and to a DNA-methylating agent - methyl methane sulfonate (MMS) in the offspring of Arabidopsis salt-stressed plants (Boyko, Blevins et al. 2010, Rahavi, Migicovsky et al. 2011); an elevated tolerance to heavy metals in the progeny of heavy metal-treated Arabidopsis and *Oryza sativa* plants (Rahavi, Migicovsky et al. 2011, Ou, Zhang et al. 2012); an increase in tolerance to several stressors in timberline plants as a result of adaptation to UV-B radiation (Turunen and Latola 2005); an elevated tolerance to chilling or freezing stresses in the progeny of cold-treated Arabidopsis plants (Blodner, Goebel et al. 2007); and the natural transgenerational adaptive plasticity to the maternal light environment in the

monocarpic herb *Campanulastrum americanum* (Galloway and Etterson 2007). Transgenerational stress adaptation is not only limited to abiotic stressors. Previously, we have shown that the offspring of *Nicotiana tabacum* plants challenged with *Tobacco mosaic virus* (TMV) developed a higher resistance not only against the same pathogen but also against *Pseudomonas syringae* and *Phytophthora nicotianae*. Furthermore, most of the ascribed transgenerational effects were accompanied by alterations in DNA methylation, posttranslational histone modifications and an increased frequency of homologous recombination events (Boyko, Blevins et al. 2010, Kathiria, Sidler et al. 2010, Bilichak, Ilnystkyy et al. 2012). Unfortunately, a mechanistic link between the acquired transgenerational stress tolerance and molecular/epigenetic factors that lead to this intriguing phenomenon has not been established yet.

It can be envisaged that systemic epigenetic signaling plays an important role in the transgenerational inheritance in plants since plants do not set aside a pre-determined germline linage in early development as animals do. Instead, angiosperms possess an undifferentiated state of spore mother cells (SMCs) developed from a sub-epidermal cell only late during ontogenesis (Dickinson and Grant-Downton 2009, Drews and Koltunow 2011). In Arabidopsis, the archespore, which is the first cell of the reproductive lineage, differentiates directly into the megaspore mother cell (MMC). Through meiosis, MMC gives rise to a tetrad of haploid megaspores, one of which survives (a functional megaspore), while the others degenerate. The functional megaspore develops into the haploid embryo sac (the female gametophyte) through three rounds of mitosis followed by cellularization. The complete embryo sac includes two gametes (the haploid egg cell

and the homo-diploid central cell), two synergids, and three antipodals (Schmidt, Wuest et al. 2011).

The development of the paternal germline begins from a pollen mother cell (PMC) that undergoes meiosis in the anthers, resulting in four haploid microspores. Each microspore gives rise to a larger vegetative cell and a smaller generative cell through an asymmetric division. The generative cell, which represents the male germline, undergoes a further symmetric partition to produce two identical sperm cells surrounded by vegetative cells (Calarco, Borges et al. 2012).

Most of the studies demonstrating the stress-induced transgenerational epigenetic inheritance in Arabidopsis were performed at the principal growth stage 1 (Boyes, Zayed et al. 2001) before inflorescence emergence and germline differentiation (Molinier, Ries et al. 2006, Pecinka, Rosa et al. 2009, Boyko, Blevins et al. 2010, Bilichak, Ilnystkyy et al. 2012). Therefore, the epigenetic memory engraved in chromatin of somatic cells upon stress exposure has to be systemically transmitted throughout a number of mitotic divisions to gametophyte initials and later survive meiosis and fertilization for being propagated into the next generation.

To date, there is no solid evidence confirming that epigenetic marks are reset at the gametophyte stage in plants, unlike in mammals where DNA undergoes several rounds of methylation and demethylation during germ cell proliferation and post-fertilization (Feng, Jacobsen et al. 2010). Moreover, recent advances in elucidating the molecular mechanisms underlying the formation of the epigenetic landscape in gametes revealed that both the sperm cell and the egg cell in angiosperms maintain a quiescent state concomitant with the enrichment of repressive chromatin marks at the euchromatin

regions (H3K9me2, H3K27me3 and DNA hypermethylation) (Calarco, Borges et al. 2012). Subsequently, the quiescence in both gametes with the respective chromatin marks is propagated into a zygote followed by the occurrence of the silent chromatin state in the embryo (Baroux, Raissig et al. 2011). In stark contrast, the global TE activation occurs in non-germline reproductive cells, such as a vegetative nucleus in pollen and a central cell in the embryo sac, that do not contribute genetic material to the progeny but generate smRNAs that are thought to reinforce silencing of transposable elements and imprinted genes in germ cells and the embryo (Baroux, Raissig et al. 2011). These observations lead us to the question: why do gamete nuclei require VN-derived smRNAs for the suppression of the already silenced TEs? We can speculate that either gametes do not possess the complete silencing machinery to suppress undesired transposition events as compared to somatic cells or smRNAs contribute to the adjustment of epigenetic landscapes later after fertilization occurs.

In a recent study using bisulfite sequencing of genomic DNA from Arabidopsis microspores and from their derivative sperm and vegetative cells, it was demonstrated that symmetric CpG and CpHpG methylation was largely retained in Arabidopsis sperm cells, whereas CpHpH methylation was vanished from at least 1,500 TEs. This was concomitant with the down-regulation of the RdDM methyltransferase DRM2 in sperm cells that is required for *de novo* CpHpH methylation guided by 24 nt-long smRNAs (Cao and Jacobsen 2002, Calarco, Borges et al. 2012). Nevertheless, it has been shown that stripping off CpHpH methylation still does not activate transposons in sperm cells (Slotkin, Vaughn et al. 2009), albeit homozygous mutants that are compromised in the RdDM pathway (*nrpd1*, *nrpd2* and *rdr2*) demonstrate the transgenerational mobility of

retrotransposons when plants are exposed to elevated temperature (Ito, Gaubert et al. 2011). This brings us to the conclusion that smRNAs derived from the transcriptionally active VN do not strictly suppress transposon activities in the sperm cells but rather modulate DNA methylation at the stage that follows periconception. Since there are no data confirming epigenetic memory resetting in the sperm and egg cells in plants, this hypothesis can possibly explain the mystery of drastic fluctuations in global DNA methylation observed immediately after stress as compared to that in the progeny of stressed plants.

Global DNA demethylation followed by transcription activation is the most common immediate plant stress response. In stark contrast, 10-12% global hypermethylation was observed in the untreated offspring of salt-stressed plants concomitant with the enrichment of repressive chromatin marks and transcription down-regulation (Boyko, Blevins et al. 2010, Bilichak, Ilnystkyy et al. 2012). We can hypothesize that such drastic fluctuations in the global DNA methylation profile is the consequence of a stress-induced increase in accumulation of smRNAs in the parental plants that eventually directs RdDM after periconception. Indeed, in a recent study, we demonstrated that the establishment of the transgenerational memory requires the functional siRNA biogenesis pathway because both *dcl2* and *dcl3* Arabidopsis mutants failed to maintain stress-induced epigenetic inheritance (Boyko, Blevins et al. 2010). Involvement of the smRNAs and RdDM pathway in the transgenerational epigenetic memory is further supported by changes in the distribution of DNA methylation in gene bodies and exon/intron regions in the progeny of salt-stressed Arabidopsis plants as compared to that in the unexposed progeny (Boyko, Blevins et al. 2010, Bilichak,

Ilnystkyy et al. 2012). DNA hypermethylation was further reinforced with the accumulation of the transcriptionally repressive histone modification mark H3K9me2 and the depletion of the permissive mark – H3K9ac. The highest level of correlation between repressive chromatin marks and mRNA levels was observed in the *MSH6* gene (r=-0.95 on average) that encodes a mismatch repair protein. MSH6, together with MSH2, are involved in the initial recognition of DNA errors, therefore the reduced expression of mismatch repair genes followed by lower levels of mismatch repair activities may result in a higher frequency of point mutations and, possibly, other genomic rearrangements in the progeny of stressed plants (Bilichak, Ilnystkyy et al. 2012). Indeed, we have previously documented an increase in the number of homologous recombination events as well as in the frequency of point mutations and microsatellite instability in the progeny of plants exposed to various stresses (Kathiria, Sidler et al. 2010, Yao and Kovalchuk 2011). These observations led us to hypothesize that plants may utilize epigenetic pathways to trigger locus-specific genome rearrangements, thereby forcing a rapid evolution of targeted sequences and associated phenotypes (Boyko and Kovalchuk 2011). Consistent with this hypothesis, previously (Meyers, Kaushik et al. 2005) it was suggested that the evolution of plant *R*-genes involved gene duplication and recombination events.

Overall, a rapid transgenerational adaptability of plants to new growth conditions cannot be explained by the stochastic heritable variability as it is generally suggested by Darwin's theory of evolution (Boyko and Kovalchuk 2011). The disproof of the random nature of genome variability in living organisms is manifested in multiple examples of prokaryotic systems as well as the existence of hypervariable loci in humans (Bjedov,

Tenaillon et al. 2003, Nolin, Brown et al. 2003, Foster 2007). In plants, studies by DeBolt confirmed that biotic and abiotic stresses could trigger gene copy number variations (CNV) in a non-stochastic way (DeBolt 2010). CNV initiation sites were most frequently found within stress response genes and transposable elements in the offspring exposed to the same stress over multiple generations, thereby supporting the non-random occurrence of rearrangements. The future analysis of the epigenome and genome profiles in the progeny of plants subsequently stressed over multiple generations will possibly reveal the contribution of epigenetic components to the microevolution in plants.

2.2. Manipulation of epigenetic factors and DNA repair machinery for transformation improvement of plants

Plant genetic engineering has emerged as a vital tool in contemporary biotechnology. The ability to introduce a foreign gene into the plant genome resulted in the development of a number of transgenic crops with beneficial traits (Kishore, Ahmad et al. 2008, Collinge, Jorgensen et al. 2010). Nevertheless, the improvement of economically important crops necessitates stable and predictable transgene expression which is usually hard to achieve in the field conditions and requires a tremendous investment of labour and time to select for the desired transgenic line (Curtin, Voytas et al. 2012). This is, mainly, due to three major challenges that are still to be addressed in plant biotechnology: (i) many important crops species remain recalcitrant to tissue culture regeneration; (ii) the transformation frequency – the number of transgenic plants (transgenics) generated in a single transformation round – is low; (iii) the low frequency of integration of the transgene into the desired position of the host genome to obtain

plants with predictable transgene expression (Barampuram and Zhang 2011, Husaini, Rashid et al. 2011). Although a tissue culture step is of great importance for plant transgenesis, herein we will focus on the latter two challenges.

Over the last decade, numerous methods of transgene delivery and targeted genome editing have been developed for plants (Rivera, Gomez-Lim et al. 2012). Overall the techniques of foreign DNA delivery fall into two major groups: indirect and direct DNA delivery. Whereas in the former approach, DNA is introduced into the host cell by means of bacterium-mediated transformation (e.g., *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*), in the latter one, bacterium cells are not used as mediators of plant transformation (Tzfira and Citovsky 2006, Barampuram and Zhang 2011). The most widely used indirect transformation methods include: biolistic, electroporation, ultrasound, silicon carbide fibers, microinjection, macroinjection, laser microbeams, and electrophoresis (Rivera, Gomez-Lim et al. 2012).

Although a great number of DNA delivery methods have been developed, the *Agrobacterium*-mediated plant transformation method still remains the primary tool for stable transformation of many dicotyledonous (dicot) and some monocotyledonous (monocot) crops (Hiei, Ohta et al. 1994, Leelavathi, Sunnichan et al. 2004, Hu, Chen et al. 2005). In fact, the inability to achieve high frequencies of transformation events mediated by *Agrobacterium* in monocots and recalcitrant plant species has prompted the development of specific direct DNA transfer methods (Barampuram and Zhang 2011, Rivera, Gomez-Lim et al. 2012). Nevertheless, advances in understanding of plant-microbe interactions, tissue culture, regeneration techniques, the development of new binary vectors and *Agrobacterium* strains have resulted in the adaptation of

Agrobacteium-mediated transformation techniques for recalcitrant monocots, such as rice (*Oryza sativa* L.) (Shah and Veluthambi 2010), maize (*Zea mays* L.) (Ishida, Saito et al. 1996, Ishida, Hiei et al. 2007), wheat (*Triticum aestivum* L.) (Cheng, Fry et al. 1997), barley (Tingay, McElroy et al. 1997), and other plants (Sood, Bhattacharya et al. 2011).

Agrobacterium tumefaciens belongs to the genus *Agrobacterium* that includes mostly saprophytic soilborne bacterial species which inhabit the rhizosphere (Escobar and Dandekar 2003, Păcurar, Thordal-Christensen et al. 2011). *Agrobacterium* has a natural ability to transfer a portion of its tumor-inducing (Ti) plasmid, termed transferred-DNA (T-DNA), and integrate it into the host genome leading to formation of a crown gall tumor.

The *Agrobacterium*-mediated plant transformation method has a number of advantages over other transformation techniques which include the ability to transfer large intact segments of DNA into the plant cell, predominantly simple transgene insertions and a low copy number of integration events (Barampuram and Zhang 2011). At the same time, there are still many economically important crop species and trees that are recalcitrant to *Agrobacterium*-mediated transformation (Păcurar, Thordal-Christensen et al. 2011).

Both transient and stable transformation processes are the outcome of the interaction between *Agrobacterium* and its hosts. Hence, two main approaches were undertaken to elevate the transformation efficiency in already transformable species and to increase a number of hosts for *Agrobacterium*-mediated transformation: (i) to identify or engineer highly virulent strains of *Agrobacterium* and (ii) to manipulate host factors involved in transformation either through the optimization of tissue culture conditions or

by directly affecting gene expression in the plant cell (Păcurar, Thordal-Christensen et al. 2011). The implementation of the first strategy has resulted in the development of highly virulent strains with a wide range of hosts including both dicots and monocots (e.g., hypervirulent strains carrying the Ti plasmid pTiBo542 and its derivatives) (Hood, Fraley et al. 1987, Komari 1989, Cheng, Lowe et al. 2004, Jones, Doherty et al. 2005). Nonetheless, enhancing *Agrobacterium* strains by supplying them with additional copies of *vir* genes has been suggested to reach its limit (Gelvin 2003, Păcurar, Thordal-Christensen et al. 2011). Alternative approaches involving the manipulation of host factors that participate in *Agrobacterium*-mediated plant transformation constitute a promising direction to explore.

The first attempt to identify plant genes involved in *Agrobacterium*-mediated plant transformation was done more than a decade ago, and utilized forward genetic screening among 3,000 *Arabidopsis thaliana* (Arabidopsis) T-DNA insertion mutants to reveal plants recalcitrant to *Agrobacterium* infection (Nam, Mysore et al. 1999). This study was followed by a larger-scale investigation which involved approximately 16,500 Arabidopsis mutants (Zhu, Nam et al. 2003). Overall, by using a combination of stable and transient root-based transformation assays, the authors identified more than 120 genes encoding proteins which were required to promote transformation (Gelvin 2009). It did not come as a surprise that products of most of identified genes were involved in key steps of *Agrobacterium* infection, i.e., bacterial attachment (an arabinogalactan protein), cytoplasmic trafficking of the T-DNA complex (actin-2 and actin-7), nuclear targeting (importin-α7 and importin-β3) and T-DNA integration/chromatin remodelling (histones H2A, H2B, H3, and H4) (Zhu, Nam et al. 2003). At the same time, the authors pointed

out that the screen was not saturating, suggesting that the potential for the discovery of new genes involved in *Agrobacterium*-mediated plant transformation is not exhausted. Indeed, the involvement of additional chromatin-related genes (24 genes in total) was revealed by using Arabidopsis mutants that carried RNA interference (RNAi) constructs (Crane and Gelvin 2007). Hence, it became apparent that the *Agrobacterium* T-DNA tightly interacts with host chromatin factors, albeit it has yet to be deciphered how particular chromatin proteins affect *Agrobacterium*-mediated transformation at the molecular level.

2.2.1. Plant epigenetic factors involved in *Agrobacterium*-mediated stable transformation

After the T-DNA enters the nucleus, chromatin proteins may mediate its integration into the genome. For example, histones (H2A, H2B, H3 and H4) can interact with the VirE2-interacting protein (VIP1), a protein which may associate with the T-DNA in the cytoplasm and help target it and attached *vir* proteins (the ‘‘T-complex’’) to the nucleus (Magori and Citovsky 2011). In addition, it was suggested that prior to integration into the genome, the T-DNA has to interact with host chromatin factors in order to promote local and transient chromatin decondensation required for an efficient integration to occur (Lacroix and Citovsky 2009). Studies on mutants that are resistant to *Agrobacterium*-mediated transformation (*rat* mutants) revealed a major contribution of histones to the T-DNA integration process (Crane and Gelvin 2007). For instance, H2A-1, an Arabidopsis mutant deficient in one of the replacement histones, demonstrates the *rat* phenotype, however, it is susceptible to transient transformation (Nam, Mysore et al.

1999, Mysore, Nam et al. 2000). Consistently, supplementing Arabidopsis and rice wild-type plants with an additional copy of the *HTA1* gene which encodes histone H2A-1 resulted in a 100% increase in the frequency of *Agrobacterium*-mediated stable transformation events in Arabidopsis and a 44% increase in rice (Table 2.3) (Mysore, Nam et al. 2000, Zheng, He et al. 2009). Moreover, another study showed that an individual overexpression of seven different *HTA* genes, one *HTR* and one *HFO* genes which code for H2A, H3 and H4 proteins, respectively, resulted in more than a twofold increase in the transformation of Arabidopsis root segments (Tenea, Spantzel et al. 2009). Curiously, in all cases examined, an increase in the frequency of stable transformation was paralleled by a higher level of transient *Agrobacterium*-mediated transformation, a process that involves T-DNA transfer to the nucleus but does not require T-DNA integration. The authors suggested that particular histones were able to increase transgene expression by protecting an incoming transgenic DNA from nucleolytic degradation and in this way, enhance the frequency of stable transformation events (Tenea, Spantzel et al. 2009).

In addition to histones, the histone-modifying enzymes and histone chaperons were also implicated in the *Agrobacterium*-mediated plant transformation process. The knockdown of both histone acetyltransferases (*HAF1* and *HAG3*) and histone deacetylases (*HST4*, *HDA2* and *HDA3*) attenuated the susceptibility to *Agrobacterium*-mediated root transformation in Arabidopsis (Crane and Gelvin 2007). The exact role of the histone acetylation balance and the effects of overexpression of histone-modifying enzymes on stable plant transformation have yet to be shown.

At the same time, the contribution of particular histone chaperons to the transformation process seems to be relatively obvious. Arabidopsis mutants impaired in the chromatin assembly factor 1 (CAF-1) complex were found to be highly susceptible to *Agrobacterium*-mediated stable transformation (Table 2.3) (Endo, Ishikawa et al. 2006). CAF1 is an evolutionary conserved complex that is involved in the deposition of H3/H4 histones onto the replicating DNA and nucleosome assembly after nucleotide excision repair. A loss of the functional CAF1 complex leads to an increase in the level of DNA double-strand breaks (DSBs), an upregulation of several DSB repair proteins involved in homologous recombination (HR) but not in non-homologous end joining (NHEJ), and an overall enhancement of HR frequency (Kirik, Pecinka et al. 2006, Schonrock, Exner et al. 2006). The cumulative contribution of the aforementioned factors along with a relatively loose chromatin structure resulted in a twofold increase in the T-DNA integration frequency observed in mutants (Endo, Ishikawa et al. 2006, Magori and Citovsky 2011). Hence, an alternative approach to elevate transformation frequency may be a transient down-regulation of genes that encode CAF1 subunits.

Table 2.3. Epigenetic-related genes which affect plant transformation

Gene	Gene function	Modulation of gene expression	Effects on plant transformation	Reference
HTA1	Histone H2A-1 involved in nucleosome assembly	Overexpression	A 2-fold increase in the frequency of *Agrobacterium*-mediated stable transformation of Arabidopsis	(Mysore, Nam et al. 2000)
HTA1	Histone H2A-1 involved in nucleosome assembly	Overexpression	Up to a 44% and 50% increase in *Agrobacterium*-mediated stable transformation and the frequency of GT events in rice, respectively	(Zheng, He et al. 2009)
HTA, *HTR* and *HFO*	Core histone proteins H2A, H3-11, and H4 involved in nucleosome assembly	Overexpression	A 2-fold increase in the transformation frequency of Arabidopsis root segments	(Tenea, Spantzel et al. 2009)
CAF-1	Histone chaperon, involved in nucleosome assembly	Knockout	A 2-fold increase in the T-DNA integration frequency	(Endo, Ishikawa et al. 2006)
RDR6	Biogenesis of siRNAs derived from posttranscriptionally-silenced transgenes	Down-regulation (RNAi)	An ~1.5-fold increase in the stable transformation frequency	(Dunoyer, Himber et al. 2006)
DRM1, DRM2, CMT3	The triple Mutant that is strongly deficient in CpHpG and CpHpH methylation	Knockout	An ~2-fold increase in the growth of crown gall in *Agrobacterium*-infected plants	(Gohlke, Scholz et al. 2013)
AGO4	RNA-dependent DNA methylation processes	Knockout	An ~2-fold increase in the growth of crown gall in *Agrobacterium*-infected plants	(Gohlke, Scholz et al. 2013)

2.2.2. A crosstalk between antiviral defence and *Agrobacterium*-mediated plant transformation

Previously, it has been suggested that the entire mechanism of genetic transformation facilitated by *Agrobacterium* resembles the retrovirus-mediated gene transfer (Magori and Citovsky 2011). As such, T-DNA invasion of the plant cell should trigger a similar plant defence response as seen upon viral infection (Zvereva and Pooggin 2012). RNA interference (RNAi) through small non-coding RNAs, such as small interfering RNAs (siRNAs, 21-24 nt-long), is believed to be a major antiviral defense system of plants (Ding and Voinnet 2007). 21 nt-long small RNAs were detected to be expressed from the *Agrobacterium* tryptophan 2-monooxygenase (*iaaM*) oncogene and the agropine synthase (*ags*) gene transcripts, in the infected tobacco plants at three days post infection, suggesting that *Agrobacterium*-mediated transformation can be inhibited by the host-mounted posttranscriptional gene silencing (PTGS) pathway (Dunoyer, Himber et al. 2006). Furthermore, an Arabidopsis mutant impaired in the RNA-dependent RNA-polymerase 6 (RDR6), which is required for biogenesis of siRNAs derived from posttranscriptionally-silenced transgenes, is more susceptible to *Agrobacterium*-mediated transformation as compared to wild-type plants. At the same time, mutants deficient in micro RNA biogenesis (*dicer-like1* and methyltransferase *hen1*) are recalcitrant to stable transformation, suggesting that micro RNAs are vital either for host cell proliferation during tumorigenesis or the initial stages of the interaction between the plant cell and the *Agrobacterium* cell or for both (Dunoyer, Himber et al. 2006).

In order to circumvent the host-mounted silencing defense, viruses developed suppressor proteins which act at different steps of the silencing pathway (Zvereva and Pooggin 2012). Although, an analogous suppressor of RNA silencing was previously revealed in a plant bacterial pathogen – *Pseudomonas syringae* (Navarro, Jay et al. 2008), none of the *Agrobacterium* effector proteins has been ascribed a similar role yet. Hence, by supplementing either the *Agrobacterium* or a host with a viral/bacterial suppressor of gene silencing, the transformation rate may be conceivably enhanced. Indeed, the overexpression of the *Turnip crinkle virus* P38 protein, which specifically inhibits the production of siRNAs from inverted repeats or sense transgenes in Arabidopsis plants, enhances the plant's susceptibility to a virulent *Agrobacterium* strain by twofold (Dunoyer, Himber et al. 2006). Moreover, the P19 suppressor of gene silencing from tombusviruses when it is overexpressed in tobacco cells enhances *Agrobacterium*-mediated transient transformation by 50-fold (Voinnet, Rivas et al. 2003). A similar implementation of gene silencing suppressors to counteract the host RNAi defence pathway during the genetic transformation of monocot and other species recalcitrant to *Agrobacterium* transformation possesses an additional promising approach to explore.

The epigenetic defence mechanism against the invading T-DNA is not limited to the RNAi pathway. Recently, DNA methylation has been shown to inhibit tumor growth in plants inoculated with the virulent *Agrobacterium* strain (Gohlke, Scholz et al. 2013). Consistently, the *ddc* triple mutant that is strongly deficient in CpHpG and CpHpH methylation displayed significantly enhanced growth of crown gall. Similarly, the *argonaut 4* (*ago4*) mutant impaired in the RNA-dependent DNA methylation pathway demonstrated a ~2-fold increase in crown gall growth that suggests an active contribution

of the transcriptional gene silencing pathway to host-mounted epigenetic defence against *Agrobacterium* transformation. This is in accord with the previous report where the pretreatment of tobacco leaf disks with the demethylating agent azacytidine enhanced transient transformation by 4- to 6-fold as compared to untreated controls (Palmgren, Mattson et al. 1993). Curiously, exposure of *Agrobacterium* cells to the same chemical prior to transformation resulted in a similar increase in the transient transformation rate, suggesting that azacytidine may activate the expression of *vir* genes through a passive demethylation process (occurring during the replication of *Agrobacteria*) in a bacterium cell before entering the plant cell (Palmgren, Mattson et al. 1993).

Overall, the involvement of epigenetic factors in *Agrobacterium*-mediated transformation is becoming quite obvious. A number of parallels can be drawn between the *Agrobacterium*- and virus-mediated transfer of nucleic acids to the plant cell (Dunoyer, Himber et al. 2006). The implementation of the knowledge obtained from the studies on the antiviral epigenetic defence mounted by hosts may possibly improve the efficiency of plant transgenesis.

2.2.3. The manipulation of the DNA repair machinery to elevate transformation rates

Upon its delivery to the chromatin labile region, T-DNA has to find a DNA molecule with a double-strand break (DSB) undergoing an active repair process in order to be covalently attached to the host gDNA. A recent line of evidence suggests that the efficiency of stable plant transformation can be significantly elevated by manipulating the DNA repair mechanism.

Although the mechanism of T-DNA incorporation into the plant genome still remains obscure (Gelvin 2010), it has been suggested that prior to integration, the single-stranded T-DNA is converted by host polymerases into double-stranded (ds) DNA intermediate (Chilton and Que 2003, Tzfira, Frankman et al. 2003). Subsequently, the DNA DSB repair machinery recognizes ds T-DNA molecules as genomic DSBs and mediates their integration through the DNA repair mechanism (Li, Vaidya et al. 2005). Hence, logically it can be assumed that an artificial elevation of the number of DSBs in the host genome should result in a higher frequency of T-DNA integration events. Indeed, the expression of the DNA endonucleases I-*Sce*I and I-*Ceu*I has been shown to increase the rate of T-DNA integration (Chilton and Que 2003, Tzfira, Frankman et al. 2003). Moreover, exposure of plant protoplasts to DNA damaging agents, such as the treatment with specific drugs (e.g., 3-aminobenzamide, mytomycin C and bleomycin) and various doses of X-ray and UV irradiation, resulted in up to a tenfold increase in the transformation rate using a direct DNA delivery system as compared to controls (Benediktsson, Spampinato et al. 1995). At the same time, a combination of both radiomimetic drug bleomycin and X-ray irradiation did not lead to a cumulative increase in the transformation rate of protoplasts and was comparable to that after treatment with either of the genotoxic agents (less than 3% of the relative transformation frequency) (Benediktsson, Spampinato et al. 1995). This suggests the existence of a DNA DSB saturation level which limits a further increase of the plant transformation rate. Nevertheless, the implementation of such an approach to improve plant transformation has a major drawback: DNA DSBs have a negative effect on genome stability and cell viability that affects the final yield and quality of transgenic plants.

2.2.4. Non-homologous end joining DNA repair and plant transformation

It is currently believed that *Agrobacterium vir* proteins that participate in the T-DNA delivery do not hold any DNA-repair activity and therefore, T-DNA integration is most likely mediated by host enzymes (Li, Vaidya et al. 2005). Hence, the modulation of the DNA DSB repair pathway by altering the quantity of host proteins required for an efficient repair process and their accessibility to DNA may present alternative approaches for increasing the frequency of T-DNA integration events.

In eukaryotes, DNA DSBs are repaired by two major pathways: the homologous recombination (HR) pathway which is active preferentially in the S and G2 phases and the non-homologous end joining (NHEJ) pathway which is active throughout the cell cycle (Mladenov and Iliakis 2011). Whereas the HR pathway utilizes long stretches of homologous DNA molecules to facilitate a precise repair of a lesion, the NHEJ pathway simply re-joins the DNA ends that were previously processed, thus altering the DNA sequence. Both pathways have been shown to participate in T-DNA integration in eukaryotic organisms, even though the integration of the transgene through the HR mechanism is preferred for the enhancement of plant transgenesis.

Although yeast is not a natural host for the *Agrobacterium* pathogen, it proved to be a vital model for providing evidence that the HR and NHEJ pathways can substitute each other in the T-DNA integration process. For instance, a loss of function mutation of either *Rad52* or *Ku70* genes resulted in a preferential integration of T-DNA through either the NHEJ pathway (van Attikum and Hooykaas 2003) or the HR pathway (van Attikum, Bundock et al. 2001), respectively. Furthermore, a significant shift from insertion-based integration (NHEJ) toward replacement events (HR) was observed in

rad50, *mre11* and *xrs2* mutants. At the same time, the *rad52 ku70* double mutant was recalcitrant to *Agrobacterium*-mediated stable transformation, suggesting that the presence of either of the proteins is absolutely required for genomic integration of the T-DNA in yeast (van Attikum and Hooykaas 2003).

The evidence for the involvement of DSB repair proteins in the T-DNA integration process was further provided in studies on Arabidopsis plants. Unlike in yeast, DNA DSBs in plants are predominantly repaired through the NHEJ rather than HR pathway (Ray and Langer 2002, Britt and May 2003). This led to the conclusion that the T-DNA is most likely integrated into the plant genome through NHEJ (Magori and Citovsky 2011). In plants, the canonical NHEJ (C-NHEJ) pathway is maintained through the activity of the four core proteins: the Ku70-Ku80 heterodimer, X-Ray Cross Complementation Protein 4 (XRCC4) and DNA ligase IV (Charbonnel, Gallego et al. 2010). The involvement of all four proteins in the process of *Agrobacterium*-mediated stable transformation has been studied in plants (Table 2.4), albeit the results for some of them are still controversial.

In contrast to yeast, a recent report demonstrated that mutations of the Arabidopsis *KU70* or *KU80* homologs did not result in an increase in T-DNA integration through the HR pathway but instead led to almost a fivefold decline in the relative transformation frequency utilizing the NHEJ pathway as compared to wild-type plants (Jia, Bundock et al. 2012). A similar two- to three-fold and 13-fold decrease in the stable transformation rate of the *ku80* Arabidopsis mutant was observed in the previous studies using the floral dip and root-based tumor-formation assays, respectively (Friesner and Britt 2003, Li, Vaidya et al. 2005). These data were further confirmed in *Oryza sativa*

(rice) plants where RNAi lines harbouring knock-down constructs against either *KU70* or *KU80* rice homologs demonstrated in average a 45% decrease in stable callus transformation (Nishizawa-Yokoi, Nonaka et al. 2012). At the same time, using a floral-dip method, no decrease in stable transformation was reported for the *ku80* Arabidopsis mutant in another study (Gallego, Bleuyard et al. 2003). The latter contradiction may simply have arisen from a different selection marker and experimental conditions used in the study as well as from the fact that the lower number of biological repeats was used.

The interaction of the Ku80 protein with the T-DNA was further revealed in additional experiments demonstrating that the T-DNA could be immunoprecipitated from transgenic Arabidopsis cells overexpressing the His-tagged Ku80 protein. Finally, supplementing Arabidopsis wild-type plants with an additional copy of the *KU80* gene resulted in a twofold increase in the stable transformation rate as compared to controls (Li, Vaidya et al. 2005). Since Ku80 acts as a heterodimer with the Ku70 protein, the simultaneous overexpression of both genes may conceivably have even a more pronounced effect on the frequency of stable transformation. Nevertheless, if this approach is utilized, the DNA DSB repair would predominantly occur through the NHEJ pathway and most probably may result in the poor quality of integration of the T-DNA into the host gDNA.

Curiously, a recent report revealed a protein involved in NHEJ repair that acts as an inhibitor of the stable T-DNA integration. Arabidopsis RNAi lines harbouring a knockdown construct against the *XRCC4* gene demonstrated a 50% increase in the stable transformation frequency as assessed using a root-based tumor-formation assay (Vaghchhipawala, Vasudevan et al. 2012). The XRCC4 protein plays a vital role in the

juxtaposition of the processed DSB ends and the stimulation of the ligase IV activity (Grawunder, Wilm et al. 1997). The authors proposed a model in which an efficient NHEJ pathway limits the availability of DSBs that occur spontaneously for T-DNA integration. Hence, decreasing the XRCC4 protein level may delay DSB repair through the NHEJ pathway, thus allowing the T-DNA to be successfully integrated into the gap. Moreover, the authors demonstrated an interaction of the *Agrobacterium virE2* protein with XRCC4 and provided additional evidence which suggests that *virE2* may titrate/exclude an active XRCC4 protein available for DSB repair and in this way, delay normal kinetics of DSB repair. This in turn allows more opportunity for the T-DNA to integrate into the host genome (Vaghchhipawala, Vasudevan et al. 2012). However, the delay in NHEJ repair does not exclude the possibility that plants may utilize an alternative HR repair pathway to fix DNA DSB. For instance, it has been shown that down-regulation of *XRCC4* in human somatic cells results in a decline of random integration events by 70% and a 33-fold increase in the number of gene targeting (GT) events using the homologous recombination-like mechanism (Bertolini, Bertolini et al. 2009). If such compensatory mechanism is also operational in plants, a transient down-regulation of the *XRCC4* gene by means of the RNAi system would provide a valuable technique to increase the frequency of GT-events in plant species.

The requirement of the DNA ligase IV, which acts downstream of XRCC4 in the NHEJ pathway, for T-DNA integration is controversial since it has been shown to be both vital and dispensable for *Agrobacterium*-mediated Arabidopsis transformation (Friesner and Britt 2003, van Attikum, Bundock et al. 2003). At the same time, knock-down of the DNA ligase IV homolog in rice resulted in a significant decrease in callus

transformation (Nishizawa-Yokoi, Nonaka et al. 2012). Further research is needed to clarify the contribution of DNA ligase IV to *Agrobacterium*-mediated transformation.

Table 2.4. DNA DSB repair genes involved in plant transformation

Gene	Function	Modulation of expression	The effect on transformation	Reference
MRE11	Functions early as part of the MRN complex in DNA damage sensing, signalling and the repair mechanism of both HR and NHEJ pathways	Knockout	A 10-fold increase in the frequency of GT-events/ An overall reduced frequency of *Agrobacterium*-mediated floral dip transformation	(Jia, Bundock et al. 2012)
XRCC4	Plays a vital role in the juxtaposition of the processed DSB ends	RNAi-mediated knockdown	A 50% increase in the stable transformation frequency	(Vaghchhipawala, Vasudevan et al. 2012)
Saccharomyces cerevisiae RAD54	Promotes strand invasion during HR in yeast	Overexpression	A 10-100-fold increase in the GT frequency	(Shaked, Melamed-Bessudo et al. 2005)
KU80	Involved in NHEJ	Overexpression	A 2-fold increase in *Agrobacterium*-mediated stable transformation	(Li, Vaidya et al. 2005)

2.2.5. Homologous recombination DNA repair and plant transformation

The improvement of plant transformation by increasing the number of transgenic plants obtained in a single transformation round does not necessarily guarantee a generation of transgenic plants with stable and predictable transgene expression in successive generations. Random multiple copy transgene integrations and truncations of the transgene sequence (possibly due to the involvement of the error-prone DNA repair process) negatively influence transgene expression partially due to a silencing effect (Eamens, Wang et al. 2008). Hence, the development of GT and genome-editing methods is widely explored in order to improve plant transformation.

In yeast, even short stretches of homology (~50 bp) between the transformation vector sequence and the endogenous DNA can result in the integration of DNA by the HR mechanism. In contrast, transgene integration in plants usually occurs randomly, irrespective of the presence of homology between the delivered DNA and host chromosomes (Hanin and Paszkowski 2003). The estimated ratio of transgene homologous integration to NHEJ-mediated insertion in plants is $1:10^3$ to $1:10^4$. Such negligible frequency of GT events requires screening of thousands of transgenic plants to identify rare homologous integration events (Shaked, Melamed-Bessudo et al. 2005). A few approaches have been proposed to elevate the frequency of GT events (Shaked, Melamed-Bessudo et al. 2005, Weinthal, Tovkach et al. 2010, Fauser, Roth et al. 2012).

The first approach encompasses the overexpression of genes involved in the HR repair process. For instance, Arabidopsis plants transgenic for the *Saccharomyces cerevisiae RAD54* gene are much more sensitive for HR-based transgene integration: the gene-targeting frequency was increased by two orders of magnitude in these plants

(Shaked, Melamed-Bessudo et al. 2005, Even-Faitelson, Samach et al. 2011). *RAD54* encodes a protein that is a member of the SWI2/SNF2 superfamily of chromatin remodelling proteins which promote strand invasion during the HR repair in yeast (Tan, Kanaar et al. 2003). A further enhancement of the frequency of GT events may be achieved through the co-expression of yeast proteins *RAD52*, *MIM* and *RAD51* which have been found to significantly enhance the rate of HR in the plant system (Johnson, Hellens et al. 2011).

An alternative approach conceivably has the most promising future in the applied genetic engineering of plants involves a direct editing of the genome by the endonucleases with the modular structure. Restriction enzymes such as meganucleases (Arnould, Chames et al. 2006), zinc-finger nucleases (ZFNs) (Zhang, Maeder et al. 2010), and transcription activator-like effector nucleases (TALEN) (Cermak, Doyle et al. 2011) can be specifically designed to recognise and introduce DSBs into the predicted locations within the host genome. Subsequently, a specific locus activated by DSB can be efficiently repaired through either the HR or NHEJ pathway, depending on the presence of homologous sequences in the delivered DNA (Puchta, Dujon et al. 1996, Salomon and Puchta 1998, Chilton and Que 2003, Fauser, Roth et al. 2012). For instance, by using ZFNs, the authors managed to achieve a 10^4- to10^6-fold enhancement in the rate of GT events over the frequencies of unassisted homologous recombination events in tobacco plants (Wright, Townsend et al. 2005). Similarly, 14% of tobacco protoplasts transformed with both TALENs and the promoter-less yellow fluorescent protein (YFP) donor template demonstrated a fluorescent signal, whereas protoplasts transformed with a donor construct alone showed no fluorescence (Zhang, Zhang et al. 2012). Moreover, an

elevated frequency of GT events in protoplasts allowed the recovery of regenerated calli with gene replacement under non-selective conditions with a weighted mean of 4%.

Overall, this strategy has great potential for plant transgenesis and GT technology since TALEN-mediated (or any other site-specific DNA restriction enzyme-assisted) modification of plant cells can be further utilized for routine recovery of high quality transgenic plants without selection markers or highthroughput screening. Additionally, by using the rare-cutting endonucleases, it is possible now to engineer a plant that has novel genetic variation and does not carry a transgene in a relatively short period of time (Curtin, Zhang et al. 2011, Li, Liu et al. 2012). Thus, recently rice resistant to the phytopathogenic bacterium *Xanthomonas oryzae* has been generated by using TALENs (Li, Liu et al. 2012). Additionally, to prevent the stable integration of the transgene which encodes an endonuclease for genome editing, this restriction enzyme can by transiently overexpressed using viral-based methods. Marton et al. (2010) showed that ZFNs can be transiently delivered to tobacco and petunia using a novel *Tobacco rattle virus* (TRV)-based expression system (Marton, Zuker et al. 2010). Since TRV has an ability to infect developing buds and penetrate the ovules, it enables ZFN-introduced mutations to be fixed in the gamete genome and transmitted to the next generation. Hence, the transgene is never introduced into the host genome; this can benefit the studies aimed at commercializing the resulting modified plants since the recovered plants should potentially be referred to as non-transgenic (Vainstein, Marton et al. 2011, Curtin, Voytas et al. 2012).

3. THE PROGENY OF *ARABIDOPSIS THALIANA* PLANTS EXPOSED TO SALT EXHIBIT CHANGES IN DNA METHYLATION, HISTONE MODIFICATIONS AND GENE EXPRESSION[1]

[1]**Chapter 3 has been published in its entirety:**

Bilichak, A., Y. Ilnystkyy, et al. (2012). "The progeny of Arabidopsis thaliana plants exposed to salt exhibit changes in DNA methylation, histone modifications and gene expression." PLoS One **7**(1): e30515.

3.1. ABSTRACT

Plants are able to acclimate to new growth conditions at relatively short time-scale. Recently, we showed that the progeny of plants exposed to various abiotic stresses exhibited changes in genome stability, methylation patterns and stress tolerance. Here, we performed a more detailed analysis of methylation patterns in the progeny of *Arabidopsis thaliana* (Arabidopsis) plants exposed to 25 and 75 mM sodium chloride. We found that the majority of gene promoters exhibiting changes in methylation were hypermethylated, and this group was overrepresented by regulators of the chromatin structure. The analysis of DNA methylation at gene bodies showed that hypermethylation in the progeny of stressed plants was primarily due to changes in the 5' and 3' ends as well as in exons rather than introns. All but one hypermethylated gene tested had lower gene expression. The analysis of histone modifications in the promoters and coding sequences showed that hypermethylation and lower gene expression correlated with the enrichment of H3K9me2 and depletion of H3K9ac histones. Thus, our work demonstrated a high degree of correlation between changes in DNA methylation, histone modifications and gene expression in the progeny of salt-stressed plants.

3.2. INTRODUCTION

Living organisms are frequently influenced by abiotic and biotic environmental factors. Apart from physiological changes in the exposed generation, stress also alters epigenetic marks that can potentially persist in the progeny. Epigenetic factors can contribute to both short-term (mitotic) and long-term (meiotic) inheritance of an altered gene expression without changing the primary DNA sequences (Saze 2008). The key factors that are implicated in epigenetic memory include, but are not limited to, DNA cytosine methylation, post-translational histone modifications and metabolism of small RNA molecules that can interact to form self-reinforcing loops (Boyko and Kovalchuk 2011, Mirouze and Paszkowski 2011, Paszkowski and Grossniklaus 2011). DNA methylation is largely responsible for regulating the transcriptional genome output as well as for directing the deposition of other epigenetic marks and chromatin remodelling (Zilberman, Gehring et al. 2007). Overall, slightly more than 20% of the Arabidopsis genome is methylated, with transposable elements (TEs) and DNA repeats representing the largest fraction of methylated sequences. Whereas TEs are heavily methylated throughout their whole sequence, non-TE genes that are expressed in a tissue-specific manner are primarily methylated at the gene promoter regions (Zhang, Yazaki et al. 2006). At the same time, methylation of coding regions does not usually result in gene silencing (Zhang, Yazaki et al. 2006, Cokus, Feng et al. 2008). Methylation of transcribed regions seems to primarily occur at CG sites, and there appears to be no obvious correlation between the level of gene-body methylation and gene expression (Zilberman, Gehring et al. 2007). Genes methylated within the coding sequence display moderate expression levels and are less likely to have tissue-specific expression (Zhang,

Yazaki et al. 2006, Vaughn, Tanurdzic et al. 2007, Zilberman, Gehring et al. 2007). Methylation in the coding sequence of these genes moderately correlates with the level of gene expression (Feng and Jacobsen 2011).

Alterations in DNA methylation have been suggested to be involved in the process of adaptation to stress in plants (Kovalchuk, Burke et al. 2003, Kovalchuk, Abramov et al. 2004, Chinnusamy and Zhu 2009, Urano, Kurihara et al. 2010). Our previous research also showed that stress exposure resulted in changes in DNA methylation and gene expression in unexposed progeny (Boyko, Blevins et al. 2010, Kathiria, Sidler et al. 2010). The persistence of cytosine methylation and its reversibility makes it an ideal mechanism controlling transgenerational response to stress. DNA methylation was also shown to direct the deposition of certain chromatin marks such as differentially modified histones. The analysis of the DNA methyltransferase and histone methyltransferase mutants revealed a tight link between DNA methylation and post-translational histone modifications (Lippman, May et al. 2003, Naumann, Fischer et al. 2005, Numa, Kim et al. 2010), suggesting that epigenetic regulation of gene expression is a complex mechanism of interaction between chromatin remodelling factors. In a mutant of *decrease in dna methylation1* (*ddm1*) which is responsible for the maintenance of cytosine DNA methylation in the heterochromatic regions, a decrease in DNA methylation is associated with gain of H3K4me and loss of H3K9me (Gendrel, Lippman et al. 2002). Additionally, the copia-like elements (TA2 and TA3) lose the H3K9me modification in the *chromomethylase3* (*cmt3*) and *dna methyltransferase* (*met1*) double mutants (Johnson, Cao et al. 2002). On the contrary, mutations of the KRYPTONITE gene that encodes a member of the Su(var)3-9 family of histone methyltransferases

causes depletion of H3K9me, loss of DNA methylation, and lower gene silencing (Jackson, Johnson et al. 2004).

Histone modifications provide another layer of epigenetic information that responds to the developmental and environmental cues in a fast and efficient manner. Among various histone modifications, histone acetylation acts directly by loosening histone association with DNA leading to transcriptional activation, whereas histone methylation helps recruit other effector proteins and their complexes, and thus either activating or repressing gene expression. For example, modifications at H3K9 have positive and negative effects on gene expression; whereas acetylation at H3K9 correlates with high gene expression and dimethylation of H3K9 acts as a repressive chromatin mark (Feng and Jacobsen 2011).

A correlation between gene expression, DNA methylation and histone modifications is not always obvious. Zhou et al. (2010) analyzed the genome-wide distribution of acetylation and demethylation of histone H3 lysine 9 (H3K9ac and H3K9me2) and correlated it with gene expression data (Zhou, Wang et al. 2010). They found that high levels of H3K9ac were primarily associated with actively transcribed genes and infrequently associated with transposons. In contrast, H3K9me2 was found to be primarily targeting TEs and occasionally – poorly transcribed non-TE genes. The authors found H3K9ac to cluster around transcription and translation start sites, whereas H3K9me2 was shown to span the entire coding region (Zhou, Wang et al. 2010). Lang-Mladek et al. (2010) analyzed changes in DNA methylation, histone acetylation and gene expression in response of somatic Arabidopsis tissue to heat stress (Lang-Mladek, Popova et al. 2010). The authors found a positive correlation between changes in the

level of gene expression and histone acetylation at a given locus but did not observe any correlation between the levels of gene expression and methylation. Unfortunately to date, no analysis of histone modifications in the progeny of stressed plants has been performed. Thus, there is no evidence whether changes in DNA methylation in progenies of stressed plants correlate with changes in histone post-translational modifications.

Here, we extended our previous work by performing a more detailed analysis of DNA methylation of progenies of salt-stressed plants. We followed this work by the analysis of histone modifications at a set of selected promoter- and gene-coding regions. Furthermore, we analyzed the expression of these genes and performed a correlation analysis of methylation patterns, gene expression and histone modifications. We found a high degree of correlation among the levels of methylation, histone modification status, and the level of mRNA in *SUVH2*, *SUVH5*, *SUVH6*, *SUVH8*, *UBP26*, *DRB2*, *WRKY22*, *ROS1*, *MSH6*, *UVH3* homolog, *APUM3* and *MOS6* in the progeny of salt-stressed plants. Our findings support previous reports on transgenerational changes in plants (Molinier, Ries et al. 2006, Pecinka, Rosa et al. 2009), they also provide new evidence of a tight correlation between epigenetic marks involved in stress response.

3.3. MATERIALS AND METHODS

3.3.1. An experimental set-up

In order to check the effect of NaCl stress, the Arabidopsis plants from line 11 (ecotype C24) (Swoboda, Gal et al. 1994, Ilnytskyy, Boyko et al. 2004) were germinated and grown for three weeks on sterile MS media supplemented with either 0, 25 or 75 mM NaCl. Then, the plants were transferred into soil and grown at 22°C under 12 h day/12 h night conditions and illumination at 100 μmol m^{-2} sec^{-1}. In every case, seeds from 20 plants were pooled together, and plants were propagated to the next generation under normal growth conditions. The seeds were germinated and grown on soil at 22°C under 12 h day/12 h night conditions and illumination at 100 μmol m^{-2} sec^{-1}. Tissue samples (leaves only) from these plants were harvested at three weeks after germination and were used for further analysis.

3.3.2. Immunoprecipitation analysis of methylated DNA

The Methyl-DNA immunoprecipitation (MeDIP) assay was performed to analyse DNA methylation (Zilberman, Gehring et al. 2007). Genomic DNA used for the analysis was prepared from 20 three-week-old progeny of salt-stressed *A. thaliana* plants using a Trizol reagent as published before (Boyko, Kathiria et al. 2007). DNA was sheared by sonication to 500- to 1,500-bp fragments followed by immunoprecipitation with antibodies against methylated cytosine (Zilberman, Gehring et al. 2007). 500 ng of control DNA and the entire immunoprecipitation reaction were amplified using the T7 RNA polymerase linear amplification protocol as described (Zilberman, Gehring et al. 2007). A 5 μg aliquot of RNA from each reaction was converted into double-stranded

DNA using the SuperScript Double-Stranded cDNA Synthesis kit (Invitrogen). Control and immunoprecipitated DNA were labelled with Cy3 and Cy5 fluorescent dyes, respectively.

Both samples with labelled DNA were hybridized to Whole Genome Tiling Array 2 (Cat. # C4348001-02-01, NimbleGen). The Array 2 contains probes, 90-nt long, covering the entire DNA sequence of chromosome 3 and the partial DNA sequences of chromosomes 2 and 4. The sequence of chromosome 2 consists of the region from nt position 9,687,916 to the end of chromosome at nt position 19,704,755. The sequence of chromosome 4 consists of the region from nt position 1,001 to nt position 6,133,069.

For the data normalization (performed by the Tukey-biweight scaling procedure) and statistical analysis, we used the R environment including the package Ringo (Toedling, Skylar et al. 2007). Furthermore, for the identification of the MeDIP-enriched regions, we followed an overall description made by Toedling and co-workers (Toedling, Skylar et al. 2007). After the preprocessing step we did a smoothing over individual probe intensities. We performed a sliding windows procedure (with 900 bp width) along the chromosomes and replaced the intensity at each genomic position by the median over the intensities of those reporters inside the window that is centered at this position. Next, we identified the MeDIP-enriched regions by taking into account that the region should contain at least three probe match positions and that the smoothed intensities of the reporters mapped to those regions exceed a defined threshold. This threshold is an upper bound for values arising from the underlying null distribution (the levels of smoothed reporters follow a mixture of two distributions, the null distribution of non-affected reporters and the alternative distribution for the values in the MeDIP-enriched regions),

thus smoothed probe levels larger than defined threshold are more likely to arise from the alternative (MeDIP enrichment) distribution and are taken as indicator for finding MeDIP-enriched regions. Array intensities for the MeDIP analysis were represented as $\log_2$ signal ratios of immunoprecipitated DNA to input DNA.

A more detailed analysis of methylation was done by using either 5- or 1-kb sequence of the promoter region and the coding sequence itself. The log2 ratio IP/INPUT values of individual reporters were taken into consideration for the analysis of the number of methylated reporters that are different between groups ("ct", "25" and "75"). Genomic regions (promoter and coding sequences) in which at least 5 reporters had different log2 IP/INPUT ratios between "75" and "ct" as well as "25" and "ct" were then taken into consideration. First the percentage of methylated reporters in each group was calculated. Next, the percentages of methylated reporters were intercompared for aforementioned groups and genomic regions in which the differences were over 50% for promoter regions and over 80% for coding sequence regions were short-listed (24 promoters and 22 coding region sequences).

To identify whether methylated regions in the "ct", "25 mM" and "75 mM" plants groups have different values, we performed the non-parametric statistical Wilcoxon rank-sum test. At first, we ranked all values in each array (ct, 25 mM, and 75 mM) and extracted a 1 percent tail on the right-hand side (high methylation) and the left-hand side (low methylation) followed by ranking the corresponding values of other arrays. The differences between "25" and "ct", "75" and "ct", and "75" and "25" were expressed in p-values. A separate analysis was performed for promoter regions (4584 regions), gene body regions (2179 regions) and all regions (6763 regions) (data not shown). A similar

analysis was performed for 0.1%, 0.5%, 5.0%, 10.0%, 15.0% and 20.0% tails (data not shown).

3.3.3. ChIP-qPCR analysis

All procedures for the ChIP analysis of histone modifications in the progeny of salt-stressed Arabidopsis plants were done according to the protocol described before (Saleh, Alvarez-Venegas et al. 2008) with minor modifications. Instead of using Salmon sperm DNA/protein A agarose beads, we found more convenient to use Protein G MagneticBeads (GenScript, cat.# - L002274). For immunoprecipitation, we used ChIP grade antibodies against acetyl H3K9 (Millipore, cat. # - 17-658) and methyl 2 H3K9 (Abcam, cat. # - ab1220). A no-antibody negative control was performed to measure the non-specific binding of DNA to the Protein G MagneticBeads. All quantitative measurements of precipitated DNA were performed using the qPCR technique with SsoFast EvaGreen Supermix (Bio-Rad, cat. #1725200) using either the promoter- or gene- specific primers. The promoter and transcribed region sequences were analyzed using EMBOSS CpGPlot software with a default settings in order to identify (if it was possible) and plot CpG islands that were used for further amplification (Larsen, Gundersen et al. 1992). Primers for the real-time quantitative PCR were designed using the Beacon Designer7 software (Table 3.3.1). The precipitated gDNA fragments were amplified under the following conditions: (1) 98°C for 2 min for one cycle; 98°C for 5 s, 48°C for 5 s, for 40 cycles; (2) melt-curve analysis – 65°C to 95°C for 5 s, with a 0.5°C increment. The optimization of the annealing temperature, melt-curve analysis, and gel analysis of amplicons were performed for each set of primers. The normalization was

done against ACTIN7 (AT5G09810). The average of four reactions (two dilutions per each of two DNA preparations stemming from two independent experiments) was obtained, and the normalized expression ratio was calculated using $2^{-\Delta\Delta CT}$ method.

3.3.4. MeDIP-qPCR

DNA precipitated through MeDIP was used for real time PCR of *SUVH2*, *SUVH6*, *WRKY22*, *MSH6*, *UBP26* and *UHV3* homolog genes. PCRs were performed as in "Chip-qPCR". Primers are listed in Table 3.3.1.

3.3.5. Real-time qPCR analysis

Approximately 80 mg of plant tissue was ground in liquid nitrogen and transferred to a chilled 1.5-mL Eppendorf tube, and 160 µL of TRIzol reagent (Invitrogen) was added. The remainder of the extraction was performed as per the manufacturer's protocol. Next, mRNA was purified and concentrated using the Oligotex mRNA Mini Kit (Qiagen, cat. # 70022). The quantity and quality of mRNA were measured in RNase-free double distilled water using a spectrophotometer. cDNA was then prepared from mRNA using the iScript Select cDNA synthesis kit (Bio-Rad, cat. # 170-8897) according to the manufacturer's protocol.

The real-time quantitative PCR was performed using SsoFast EvaGreen Supermix (Bio-Rad). cDNAs were amplified under the following conditions: (1) 98°C for 2 min for one cycle; 98°C for 5 s, 48°C for 5 s, 65°C to 95°C for 5 s; for 40 cycles; (2) melt-curve analysis - 65°C to 95°C for 5 s, with a 0.5°C increment. Primers for the real-time quantitative PCR were designed using the Beacon Designer7 program (Table 3.3.1). The

optimization of the annealing temperature, melt-curve analysis, and gel analysis of amplicons were performed for each set of primers. To evaluate the PCR efficiency, the standard curve was established using a series of cDNA dilutions. The expression of genes was related to the expression of RCE1 and tubulin. The average of four reactions (two independent experiments in two technical replicates) was obtained, and the normalized expression ratio was calculated using $2^{-\Delta\Delta CT}$ method.

3.3.6. Statistical treatment of the data

Statistical analyses were performed using MS Excel software and Microcal Origin 6.0. Standard errors or standard deviations were calculated. A statistically significant difference between the means was compared using either Student's t-test or single-factor ANOVA. Statistical analysis of the percentage of non-TE genes with differentially methylated regions was performed using single-factor ANOVA; since no replication of methylation analysis was performed, statistical analysis was done by comparing the percentage of hyper- and hypo-methylated regions in 25 mM and 75 mM plant groups (Figure 3.4.2). Statistical analysis of the percentage of TE-genes was performed between either hyper- or hypo-methylation groups and control group using data for both 25 and 75 mM plant groups (single-factor ANOVA).

Table 3.3.1. Primers used for the analysis of histone modifications and gene expression

Primer name	ChIP primers	qPCR primers
SUVH6-p-for	5′-TTATCCCTATTCCCTAGCATA-3′	
SUVH6-p-rev	5′-GTGTGATTCTTATCTTCTTCTG-3′	
SUVH6-t-for	5′-TTCGCCACAAGGATTATC-3′	
SUVH6-t-rev	5′-CATTCTCTGGTGTATTCATTAC-3′	
SUVH8-p-for	5′-ATTGCTTGACTAATGTTTCA-3′	
SUVH8-p-rev	5′-CAGAATAGACTTATCGGTTG-3′	
SUVH8-t-for		5′-ACATCAGCACCTCCTCAT-3′
SUVH8-t-rev		5′-CCAGCACTCGCATCATAA-3′
DRB2-p-for	5′-GCTTACAATAGTGGTGGATTATAG-3′	
DRB2-p-rev	5′-CGCTGCTAGTCAACTGAA-3′	
DRB2-t-for	5′-ACCAACCTGTGTTTACTG-3′	
DRB2-t-rev	5′-CTTCTCTGCTTGCTTCTT-3′	
APUM3-p-for	5′-CCCAGTTTCTTCTTAAAGTTTC-3′	
APUM3-p-rev	5′-AAATCCTAAAGATGACACCTT-3′	
APUM3-t-for	5′-ATAGTGGAAGTGGAGTAG-3′	
APUM3-t-rev	5′-TCATACATTGGAGAATAGTTAT-3′	
SUVH2-p-for	5′-ACCAAATAATTAGTACAGAAGAA-3′	
SUVH2-p-rev	5′-GTATGAACTTAAGATCGGAAT-3′	
SUVH2-t-for	5′-TTATTCGTATCTCAGAGC-3′	
SUVH2-t-rev	5′-CAGAATCCAATCCGTATA-3′	
SUVH5-p-for	5′-GAAGCCGAACGGGTGATA-3′	
SUVH5-p-rev	5′-TGATTCAATGTTGCATGATCTAGG-3′	
SUVH5-t-for	5′-ACGACATTACAATCATCAG-3′	
SUVH5-t-rev	5′-CTTGAAGACGAGTTTACC-3′	
ROS1-p-for	5′-AGAAGAAACGAAGCATCA-3′	
ROS1-p-rev	5′-CAGTAGAATCAATGGTTATGG-3′	
ROS1-t-for		5′-ACCTGCTTCTCTAATGTC-3′
ROS1-t-rev		5′-AACTTCAACTCGTCCTAA-3′
UVH3 homologue-p-for	5′-TGCTATGTGCCTGGTAAT-3′	

Table 3.3.1. Primers used for the analysis of histone modifications and gene expression (*continued*)

Primer name	ChIP primers	qPCR primers
UVH3 homologue-p-rev	5′-AATTCTTCACTTCGGTTCG-3′	
UVH3 homologue-t-for	5′-TTCGTGCTATATTGGTTC-3′	
UVH3 homologue-t-for	5′-AATAACTTTCGCCTCTTT-3′	
UBP26-p-rev	5′-CGAGTTTATTGGGACATT-3′	
UBP26-p-rev	5′-CGCTCTCTTATTTCAGATT-3′	
UBP26-t-for	5′-TGTTAGAGGCATCTGACT-3′	
UBP26-t-for	5′-CAGGTTTCCATAATTTGTTCT-3′	
WRKY22-p-for	5′-GTAATGAAGCAGAACCAA-3′	
WRKY22-p-rev	5′-AATAATCCGTCAGCAGTA-3′	
WRKY22-t-for	5′-CGACCACTATTGCTACTTAT-3′	
WRKY22-t-rev	5′-GCTAGATGATCCTCAACAG-3′	
MSH6-p-for	5′-GAGAGCGAGTATTATTAC-3′	
MSH6-p-rev	5′-ATTATGGAGTGAAGAGAT-3′	
MSH6-t-for	5′-GGTAATGTGGAAGAAGATA-3′	
MSH6-t-rev	5′-ATTCTCATCAACCAACTC-3′	
MOS6-p-for	5′-GAGTGGCAGGTTCGTTAT-3′	
MOS6-p-for	5′-CAAGAGCGTGTACTTAGGA-3′	
MOS6-t-for		5′-GAAATGTTGCTGGAGACT-3′
MOS6-t-for		5′-TTGAATTGAGACAGAAGAGG-3′
Actin7-for	5′-CAGTCCAAGAGAGGTATC-3′	
Actin7-rev	5′-AAGTGTGATGCCATATCT-3′	
RCE1-for		5′-CTGATGCATGGATATTACC-3′
RCE1-rev		5′-ACTGTGTTAATGTTAAAGAA-3′
Tubulin-for		5′-CTCAAGAGGTTCTCAGCAGTA-3′
Tubulin-rev		5′-TCACCTTCTTCATCCGCAGTT-3′

3.4. RESULTS

3.4.1. The progeny of stressed plants exhibit changes in DNA methylation

Our previous methylation analysis using cytosine extension assay showed that the genome of the progeny of stressed plants was hypermethylated in “25 mM plants” and “75 mM plants” by 12% and 10%, respectively.

To gain more detailed knowledge about a type of sequences in which changes in DNA methylation occurred, we analyzed methylation at the promoter and transcribed regions of all genes located on the NimbleGen Array #2. First, for the analysis of the promoter region, we used the 5 kb sequence 5' of a transcribed region. For the analysis of methylation at the transcribed region, we used the entire sequences of the transcribed region of each gene. We identified the number of methylated reporters (the region of 90 nt in length, see Materials and Methods for details) out of the total number of reporters which are present either in the 5-kb promoter region or in the transcribed region and compared these data between the progeny of control and stressed plants. To obtain a list of differentially methylated promoters and gene-body regions, we considered the regions to be hypermethylated if methylation changed from 0-50% in the progeny of control plants to 50-100% in the progeny of stressed plants. Similarly, we considered the regions to be hypomethylated if methylation changed from 50-100% in the progeny of control plants to 0-50% in the progeny of stressed plants. Out of 6,763 promoters and transcribed regions analyzed, there were 266 and 283 promoter regions in which methylation changes were observed in the progeny of plants exposed to 25 and 75 mM NaCl, respectively, as compared to the progeny of control plants; 170 promoter regions were similarly regulated in both plant groups exposed to 25 and 75 mM NaCl (Figure 3.4.1A). There were 434

and 451 differentially methylated gene-body regions in 25 and 75 mM plant groups, respectively; 304 regions were similar for both groups (Figure 3.4.1B).

To analyze whether differences in methylation between the progeny of stressed and control plants were significant, we performed the non-parametric statistical Wilcoxon rank-sum test using ranked data for ct, 25 mM and 75 mM plants groups in three regions: the promoter regions, the gene body regions and all regions. The analysis of a 1 percent tail in hypomethylated regions (the start of the rank) showed that neither 25 mM nor 75 mM plant groups were different from ct plants, although they were different from each other (data not shown). The analysis of a 1 percent tail in hypermethylated regions (the end of the rank) showed that both the 25 mM and 75 mM plant groups were different from ct plants, and the 25 mM and 75 mM plant groups were mostly similar to each other (data not shown). Further analysis showed that these similarities in hypomethylated regions and significant differences in hypermethylated regions were preserved even for a 10 percent tail (data not shown).

While comparing the lists of hyper- and hypomethylated regions, we found that the majority of genes and promoters exhibiting methylation changes in the progeny of stressed plants were hypermethylated. Namely, there was a 2.5-fold higher percentage of hypermethylated genes compared to hypomethylated genes in the progeny of plants exposed to 25 mM NaCl ($p=0.045$) and a 5-fold higher percentage – in the progeny of plants exposed to 75 mM NaCl ($p=0.003$) (Figure 3.4.2).

To further decrease the number of genes which have differentially methylated promoters, we decided to restrict the promoter size to a 1,000-nt sequence upstream of the transcribed region. A comparison of methylation levels between the progeny of

stressed and control plants showed 18 hypermethylated and 6 hypomethylated promoters exhibiting over 50% changes in methylation (Table 3.4.1, Table 3.4.2). Methylation changes were similar in the progeny of plants exposed to 25 and 75 mM NaCl in 12 out of 24 promoters being analyzed (Table 3.4.1, Table 3.4.2).

Similarly, to restrict the number of genes which were differentially methylated at the gene body, we considered only those genes with an over 80% increase or decrease in methylation, that is from 0-20% in the progeny of control to 80-100% of methylation in the progeny of stressed or from 80-100% in the progeny of control to 0-20% of methylation in the progeny of stressed. The analysis showed that there were 15 and 7 genes hypermethylated and hypomethylated at the transcribed regions, respectively (Table 3.4.3, Table 3.4.4). This again indicated that hypermethylation prevailed in the progeny. In 14 out of 22 genes being analyzed, hypermethylations or hypomethylations of a given gene were observed in the progeny of plants exposed to both 25 and 75 mM NaCl (Table 3.4.3, Table 3.4.4).

Since the cultivar used in this study was C24 and NimbleGen array was based on the sequence of cultivar Columbia, there was a possibility that substantial polymorphism would interfere with hybridization. We analyzed sequence polymorphism for 8 shortlisted genes and found an average of 1.6 substitutions in the average sequence length of ~3,500 nt (data not shown). Such a low percentage (0.05%) of sequence polymorphism unlikely interfered with hybridization between C24 DNA and Columbia DNA-based NimbleGen array.

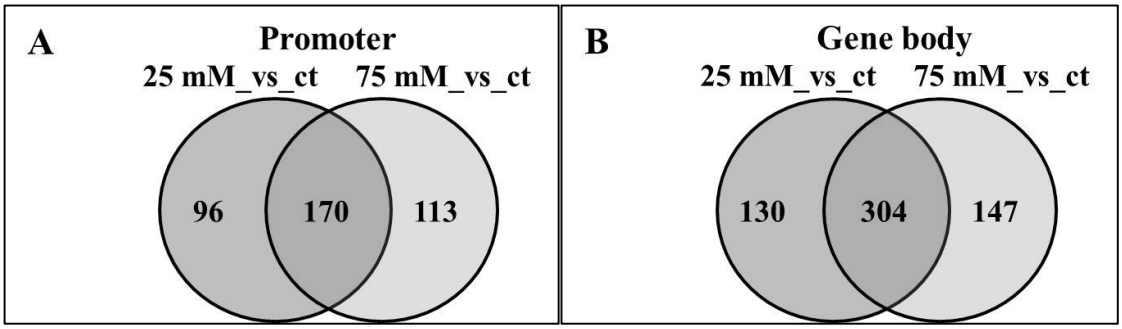

Figure 3.4.1. Venn diagrams showing the number of the similarly and differentially methylated promoter regions (A) and gene body regions (B) in the progeny of plants exposed to 25 and 75 mM NaCl as compared to the progeny of control plants

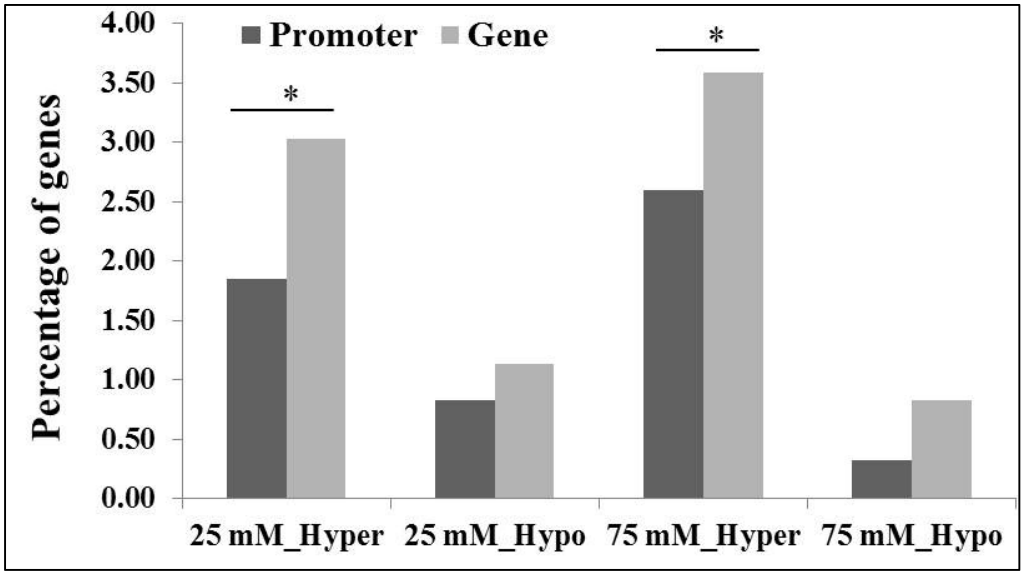

Figure 3.4.2. Percentage of differentially methylated genes

The figure shows the percentage of genes that are hyper- or hypomethylated at the promoter or gene body regions in the progeny of stressed (25 or 75 mM) plants as compared to the progeny of non-stressed control plants. "25_Hypo" and "75_Hypo" – stand for hypomethylated regions in the progeny of plants exposed to 25 and 75 mM NaCl, respectively. "25_Hyper" and "75_Hyper" – represent hypermethylated regions in the progeny of plants exposed to 25 and 75 mM NaCl, respectively. The asterisks denote a significant difference between the percentage of hypermethylated and hypomethylated regions ($p<0.05$; single-factor Anova).

Table 3.4.1. Promoters hypermethylated in the progeny of stressed plants

AGI	N of reporters	N of methylated reporters			% of methylated reporters			Gene symbol(function)
		ct	25	75	ct	25	75	
AT2G36490	11	0	6	0	0	55	0	*ROS1*
AT2G47275	7	0	0	5	0	0	71	*MICRORNA403* (*MIR403*)
AT2G25930	10	0	0	7	0	0	70	*EARLY FLOWERING 3* (*ELF3*)
AT2G24740	9	0	0	6	0	0	67	*SU(VAR)3-9 HOMOLOG 8* (*SUVH8*)
AT2G35160	7	0	0	5	0	0	71	*SU(VAR)3-9 HOMOLOG 5* (*SUVH5*)
AT2G45650	10	0	0	9	0	0	90	*AGAMOUS-LIKE 6* (*AGL6*)
AT3G55970	2	0	0	2	0	0	100	*JASMONATE-REGULATED GENE 21* (*JRG21*)
AT3G49430	10	0	8	6	0	80	60	Ser/Arg-rich protein 34a (*SRp34a*)
AT3G50500	10	0	5	6	0	50	60	*SNF1-RELATED PROTEIN KINASE 2.2* (*SNRK2.2*)
AT3G48057	12	0	11	11	0	92	92	*MICRORNA843A* (*MIR843A*)
AT3G25770	9	0	5	4	0	56	44	*ALLENE OXIDE CYCLASE* 2 (*AOC2*)
AT3G20340	9	0	9	8	0	100	89	downregulated by paraquat
AT3G23100	8	0	0	6	0	0	75	*XRCC4*
AT3G63010	7	0	0	5	0	0	71	*GA INSENSITIVE DWARF1B* (*GID1B*)
AT4G02150	10	0	8	6	0	80	60	*MODIFIER OF SNC1, 6* (*MOS6*)
AT4G02070	10	0	7	6	0	70	60	*MUTS HOMOLOG 6* (*MSH6*)
AT4G04695	10	0	0	8	0	0	80	(*CPK31*)
AT4G01250	10	0	0	5	0	0	50	(*WRKY22*)

The table shows the list of the genes that were hypermethylated at the promoter region. The promoter regions were defined as 1,000 nucleotides. The total number of reporters shows the number of reporters located on the array. The number of methylated reporters is the number of reporters for which the difference between enriched and input DNA was observed (see Materials and Methods for details). The percentage of methylated reporters reflects the percentage of reporters in which methylation has changed.

Table 3.4.2. Promoters hypomethylated in the progeny of stressed plants

AGI	N of reporters	N of methylated reporters			% of methylated reporters			Gene symbol(function)
		ct	25	75	ct	25	75	
AT2G25820	11	6	0	0	55	0	0	DREB subfamily A-4 of ERF/AP2 transcription factor family
AT2G28550	10	8	0	0	80	0	0	*RELATED TO AP2.7* (*RAP2.7*)
AT3G46710	7	4	0	0	57	0	0	disease resistance protein (CC-NBS-LRR class)
AT3G48900	11	7	0	0	64	0	0	DNA repair/chromatin binding (*UVH3 homolog*)
AT3G23240	11	7	0	0	64	0	0	*ETHYLENE RESPONSE FACTOR 1* (*ERF1*)
AT3G61650	10	6	0	2	60	0	20	*GAMMA-TUBULIN* (*TUBG1*)

The table shows the list of the genes that were hypomethylated at the promoter region. The promoter regions were defined as 1,000 nucleotides. The total number of reporters shows the number of reporters located on the array. The number of methylated reporters is the number of reporters for which the difference between enriched and input DNA was observed (see Materials and Methods for details). The percentage of methylated reporters reflects the percentage of reporters in which methylation has changed.

Table 3.4.3. Gene bodies hypermethylated in the progeny of stressed plants

AGI	N of reporters	N of methylated reporters			% of methylated reporters			Gene symbol(function)
		ct	25	75	ct	25	75	
AT2G28380	27	0	15	12	0	56	44	*DSRNA-BINDING PROTEIN 2* (*DRB2*)
AT2G29140	45	0	23	16	0	51	36	*ARABIDOPSIS PUMILIO 3* (*APUM3*)
AT2G42080	25	0	15	14	0	60	56	*DNAJ heat shock protein*
AT2G23740	65	0	57	56	0	88	86	*SU(VAR)3-9 HOMOLOG 6* (*SUVH6*)
AT2G33290	28	0	0	18	0	0	64	*SU(VAR)3-9 HOMOLOG 2* (*SUVH2*)
AT2G33340	55	0	33	31	0	60	56	*MOS4-ASSOCIATED COMPLEX 3B* (*MAC3B*)
AT2G23380	53	0	0	27	0	0	51	*CURLY LEAF* (*CLF*)
AT3G48050	63	0	39	35	0	62	56	DNA binding; Transcription elongation factor
AT3G11450	25	0	19	15	0	76	60	*DNAJ heat shock protein*, *MYB-like*
AT3G44880	31	0	18	17	0	58	55	*ACCELERATED CELL DEATH 1* (*ACD1*)
AT3G49600	70	0	37	32	0	53	46	*UBIQUITIN-SPECIFIC PROTEASE 26* (*UBP26*)
AT3G03420	19	0	11	10	0	58	53	Ku70-binding family protein
AT4G00450	83	0	66	65	0	80	78	*CRYPTIC PRECOCIOUS* (*CRP*)
AT4G04340	46	0	34	32	0	74	70	early-responsive to dehydration protein-related
AT4G08210	24	0	23	22	0	96	92	pentatricopeptide (*PPR*) repeat-containing protein

The table shows the list of the genes that were hypermethylated at the gene body region. The total number of reporters shows the number of reporters located on the array. The number of methylated reporters is the number of reporters for which the difference between enriched and input DNA was observed (see Materials and Methods for details). The percentage of methylated reporters reflects the percentage of reporters in which methylation has changed.

Table 3.4.4. Gene bodies hypomethylated in the progeny of stressed plants

AGI	N of reporters	N of methylated reporters			% of methylated reporters			Gene symbol(function)
		ct	25	75	ct	25	75	
AT3G32316	10	5	3	0	50	30	0	*AGAMOUS homolog*
AT3G28925	20	10	0	8	50	0	40	*ATSMC3* (*ARABIDOPSIS THALIANA STRUCTURAL MAINTENANCE OF CHROMOSOME 3*)
AT3G07520	38	19	0	0	50	0	0	*GLUTAMATE RECEPTOR 1.4* (*GLR1.4*)
AT3G50360	15	8	7	0	53	47	0	*CENTRIN2* (*ATCEN2*)
AT3G15790	17	10	9	0	59	53	0	*METHYL-CPG-BINDING DOMAIN 11* (*MBD11*)
AT4G04920	74	48	0	34	65	0	46	*SENSITIVE TO FREEZING 6* (*SFR6*)
AT4G02460	50	29	0	28	58	0	56	*POSTMEIOTIC SEGREGATION 1* (*PMS1*)

The table shows the list of the genes that were hypomethylated at the gene body region. The total number of reporters shows the number of reporters located on the array. The number of methylated reporters is the number of reporters for which the difference between enriched and input DNA was observed (see Materials and Methods for details). The percentage of methylated reporters reflects the percentage of reporters in which methylation has changed.

3.4.2. An increase in methylation in the progeny of stressed plants is primarily due to changes in the exons and at the 5' or 3' ends of the genes

Next, we tested whether there is a difference between the methylation level of the 5' end, the central part and the 3' end of the gene. Previous reports suggested that methylation in the coding regions differs, with central parts of the gene typically having higher methylation levels (Feng and Jacobsen 2011). The genes that are methylated in the central part of the gene body are typically moderately expressed, and methylation levels correlate positively with gene expression (Feng and Jacobsen 2011). Our analysis of methylation of 2,317 genes showed that the 5' and 3' ends of the genes (300-nt from either side) had approximately 25-fold lower levels of methylation as compared to the central part of the gene analyzed in control plants (Figure 3.4.3A; Table 3.4.5). We also found that the differences in methylation levels of the progeny of stressed plants as compared to the progeny of control plants were much more dramatic in the 5' and 3' ends of the genes rather than in the central part (Figure 3.4.3A, Table 3.4.5). Methylation levels in the entire gene body were 15.1% and 7.8% higher in the progeny of plants exposed to 25 and 75 mM NaCl, respectively, as compared to control plants. Methylation levels in the central part of the genes were 13.7% and 3.9% higher in the progeny of stressed plants exposed to 25 and 75 mM NaCl. On the contrary, in the 300-nt region of the 5' end of the gene, methylation levels was 37.3% and 81.5% higher in plants exposed to 25 and 75 mM NaCl, whereas at the 3' end, they were 37.6% and 69% higher.

Recent data on the analysis of DNA methylation in exons versus introns showed that methylation levels in exons are generally higher, and this may contribute to either exon definition or control of alternative gene splicing (Feng, Cokus et al. 2010, Laurent,

Wong et al. 2010). We analyzed whether methylation in the exon or intron of the genes differs in the progeny of stressed and control plants. We found that, in general, methylation levels in the exons were over 70% higher than those in the introns. A comparison between progenies of stressed and non-stressed plants showed that the increase in methylation in the progeny of salt-stressed plants was mainly due to changes in the exons. In the exons of the progeny of plants exposed to 25 and 75 mM NaCl, the level of hypermethylation was 21% and 17%, respectively. In the introns of plants exposed to 25 mM NaCl the level of hypermethylation was 5%, whereas in the introns of plants exposed to 75 mM NaCl we observed 8% hypomethylation, as compared to those in control plants (Figure 3.4.3B; Table 3.4.5).

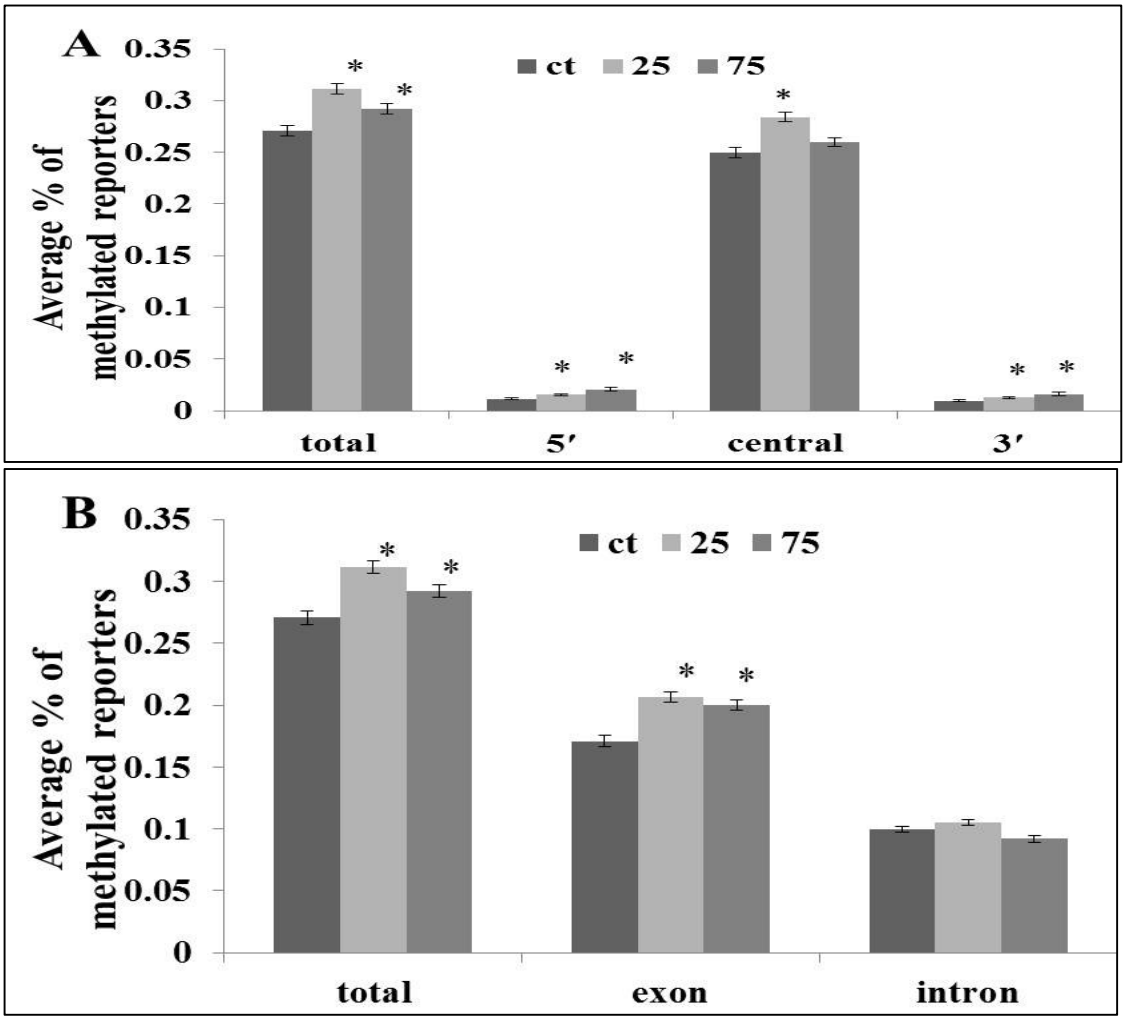

Figure 3.4.3. The average percentage of the differentially methylated reporters located in various parts of the gene body

The figure shows the average percentage of methylated reporters (with SE, calculated for over 2,000 genes) found in the coding sequences of genes in the progeny of control plants (Ct) and the progeny of stressed plants (25 and 75 mM). The asterisks denote a significant difference between the progeny of plants exposed to 25 or 75 mM NaCl and the progeny of control plants ($p<0.05$).

A. The average percentage of methylated reporters in the entire coding sequence, in the 300 nts of the 5' end, in the 300 nts of the 3' end, and in the central part of the gene.

B. The average percentage of methylated reporters in the entire coding sequence, in the exon and intron regions of the gene.

Table 3.4.5. Statistical analysis of differences in methylation levels in different parts of the gene body

Source of variation		Fold difference	F	P-value	F crit
TOTAL	25mM_ct	1.15	30.47	3.58E-08	3.84
	75mM_ct	1.08	8.30	0.004	3.84
	75mM_25mM	0.94	7.64	0.006	3.84
5' end	25mM_ct	1.37	9.97	0.002	3.84
	75mM_ct	1.82	22.79	1.87E-06	3.84
	75mM_25mM	1.32	6.04	0.014	3.84
Middle	25mM_ct	1.14	24.89	6.28E-07	3.84
	75mM_ct	1.04	2.16	0.140	3.84
	75mM_25mM	0.91	13.84	2.00E-04	3.84
3' end	25mM_ct	1.32	6.59	0.010	3.84
	75mM_ct	1.69	14.31	1.57E-04	3.84
	75mM_25mM	1.28	3.75	0.053	3.84
Exon	25mM_ct	1.21	34.12	5.54E-09	3.84
	75mM_ct	1.17	13.16	2.88E-04	3.84
	75mM_25mM	0.97	5.22	0.0224	3.84
Intron	25mM_ct	1.05	7.68	0.006	3.84
	75mM_ct	0.92	1.28	0.258	3.84
	75mM_25mM	0.84	16.07	6.2E-05	3.84

Single-factor ANOVA was used to identify significant differences between the progeny of plants exposed to 25 mM NaCl and the progeny of control plants (25mM_ct), between the progeny of plants exposed to 75 mM NaCl and the progeny of control plants (75mM_ct), and between the progeny of plants exposed to 75 mM NaCl and the progeny of plants exposed to 25 mM NACl (75mM_25mM). The analysis was performed for the entire sequence of the gene body as well as for the 5'/3' ends, the middle part, exon/intron regions.

3.4.3. The progeny of stressed plants have a large number of hypermethylated genes involved in the regulation of chromatin structure

While analyzing the aforementioned list of genes, we noticed that majority of them were involved in the regulation of chromatin structure. For example, genes that encode histone methyltransferases (HMTases), namely, *SUVH2*, *SUVH5*, *SUVH8*, were highly hypermethylated in the promoter region, the transcribed region or both in the progeny of exposed plants. SUVH2 is one of the main players among HMTases; together with SUVH4, it significantly contributes to mono- and dimethylation of H3K9 (Ebbs and Bender 2006) and heterochromatic gene silencing (Naumann, Fischer et al. 2005). The SUVH5 protein has the weak HMTase activity and is involved in methylation of H3K9 and CHROMOMETHYLTRANSFERASE3 (CMT3) - mediated non-CG methylation *in vivo*. A similar trend of hypermethylation in the coding region in the progeny of salt-stressed plants was observed in the *UBP26* gene. UBP26 and SUP32 catalyze H2B deubiquitination, and UBP26 is also required for heterochromatic histone H3 methylation and DNA methylation (Sridhar, Kapoor et al. 2007). The gene encoding a Polycomb repressive complex 2 (PRC2) subunit, CURLY LEAF (CLF), a histone-lysine N-methyltransferase, was also hypermethylated. A decrease in the CLF activity results in early flowering (Doyle and Amasino 2009).Intriguingly, genes that are involved in the transcriptional and posttranscriptional regulation of gene expression are also affected by stress conditions. For example, *ROS1*, a repressor of transcriptional gene silencing, also showed high levels of hypermethylation in the promoter region in “25 mM” and “75 mM” plants. The *ROS1* gene encodes a DNA glycosylase that functions by demethylating the target promoter DNA and, as a result, protects genes from potentially deleterious

methylation (Ponferrada-Marin, Martinez-Macias et al. 2010). Additionally, high levels of hypermethylation in the coding regions were observed in genes that are involved in post-transcriptional regulatory events – DOUBLE STRANDED RNA - BINDING PROTEIN (DRB2) and ARABIDOPSIS PUMILIO (APUM3). Arabidopsis DRB2, possibly, cooperates with DCL1 in specific tissues to mediate the metabolism of a subset of miRNAs (Curtin, Watson et al. 2008). APUM3 belongs to the Puf family proteins that have important roles in controlling gene expression at the post-transcriptional level by promoting RNA decay and repressing translation. The Pumilio homology domain (PUM-HD) is a conserved region within Puf proteins that binds with sequence specificity to the 3' untranslated region (UTR) of target mRNAs. It was suggested that these proteins might be involved in a wide range of post-transcriptional regulatory events allowing plants to respond rapidly to changes of environmental conditions (Tam, Barrette-Ng et al. 2010).

To confirm the methylation data obtained by MeDIP, we performed MeDIP-qPCR analysis for promoter and gene body regions of *SUVH5, SUVH6*, *MSH6*, *WRKY22, UBP26* and *UVH3* homolog. We partially confirmed methylation changes in all of these genes (Figure 3.4.4).We noted that MeDIP-qPCR and MeDIP data were more similar for the progeny of 25 mM stress as compared to 75 mM.

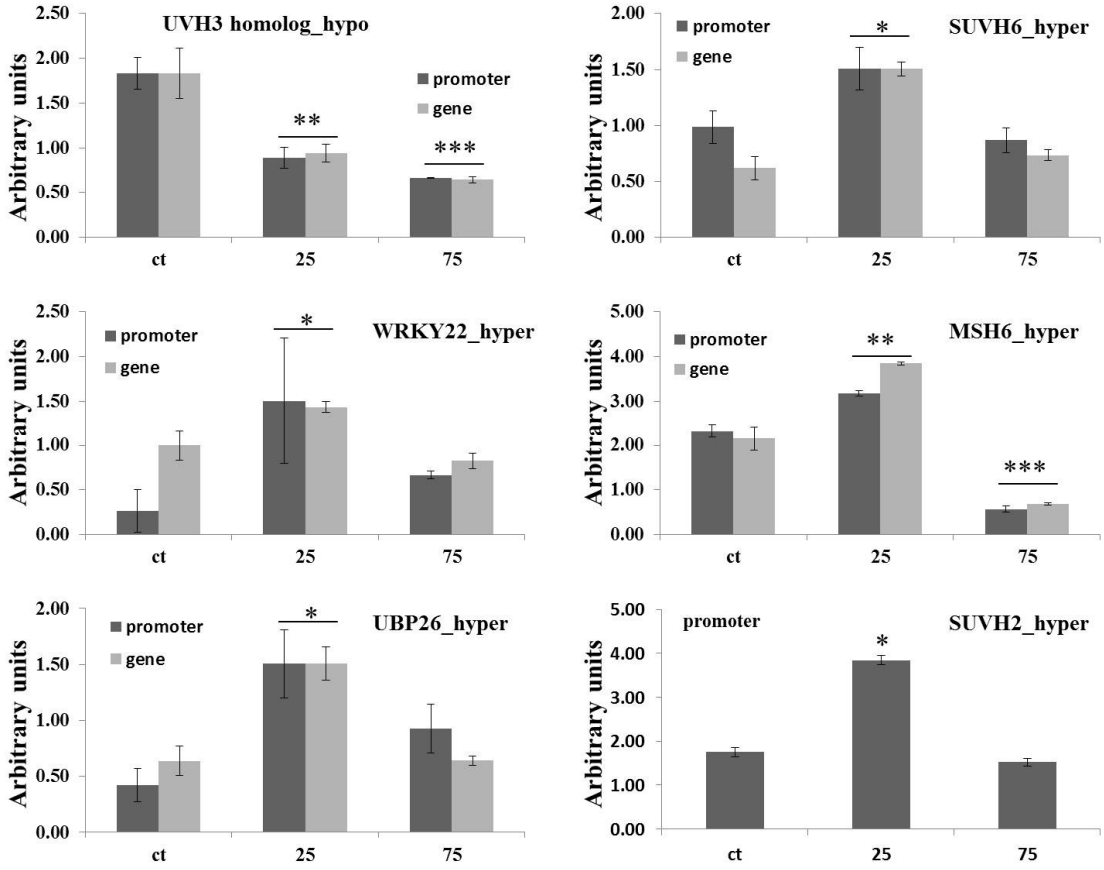

Figure 3.4.4. Analysis of methylation at the promoter and gene body regions of *SUVH2*, *SUVH6*, *WRKY22*, *MSH6*, *UBP26* and *UVH3* homolog genes as measured by MeDIP-qPCR

The Y-axis shows the methylation levels in average arbitrary units (calculated from two independent biological repeats and two technical repeats with SEM). The asterisks denote a significant difference between the progeny of stressed (25 and 75 mM) and control plants; one asterisk stands for $p<0.05$, two asterisks for $p<0.01$ and three for $p<0.001$ (Student's t-test).

3.4.4. Changes in DNA methylation correlate with changes in histone modifications

Being a part of the transcription regulation process, DNA methylation often correlates with specific histone modifications. Specifically, the promoter regions correlate with H3K9ac, whereas the transcribed regions correlate with H3K9me2 (Feng and Jacobsen 2011). We hypothesized that hypermethylated promoters in the progeny of stressed plants should have a lower level of H3K9ac and a higher level of H3K9me2. To test this hypothesis, we chose 12 genes from which 7 (*SUVH5*, *SUVH6*, *SUVH8*, *ROS1*, *MOS6*, *WRKY22*, *MSH6*) were hypermethylated at the promoter region, four genes (*SUVH2*, *UBP26*, *DRB2*, *APUM3*) were hypermethylated at the transcribed region, and one gene (*UVH3* homolog) was hypomethylated at the promoter region of at least one of the progenies of stressed plants (exposed to 25 or 75 mM NaCl). To analyze histone modifications associated with specific genomic regions, we performed the chromatin immunoprecipitation assay using anti-H3K9ac and anti-H3K9me2 antibodies followed by the quantitative PCR (ChIP-qPCR) analysis using both promoter- and gene-specific primers (Table 3.3.1). In the majority of the cases, we indeed found that the hypermethylated promoters were associated with a decrease in the level of H3K9ac (r=-0.6 on average, except for *SUVH8* and *WRKY22*) and an increase in the level of H3K9me2 (r=0.6 on average) in the progeny of stressed plants (Figure 3.4.5-3.4.8). The transcribed regions of these genes were also associated with similar histone modifications; methylation at the promoter region correlated negatively with H3K9ac (r=-0.5 on average) and correlated positively with H3K9me2 (r=0.6). The *SUVH2*, *UBP26*, *DRB2*, *APUM3* genes that were found to be hypermethylated at gene bodies

were also shown to have a lower level of H3K9ac and a higher level of H3K9me2 in both promoter and gene-body regions. The level of methylation correlated negatively with H3K9ac (r=-0.5 in promoter and gene-body regions) and correlated positively with H3K9me2 (r=0.7 and r=0.8 in promoter and gene-body regions, respectively). On the contrary, hypomethylation at the promoter region of a UVH3 homolog did not correlate with H3K9ac at the promoter or gene body region but negatively correlated with H3K9me2 at the gene body (r=-0.8) (Figure 3.4.6, 3.4.8). Additionally, we found a high degree of linear correlation (r=0.8 on average) between the accumulation of H3K9ac in the promoter and transcribed regions of analyzed genes. These experiments confirmed our hypothesis and showed a high degree of relationship between hypermethylation of the promoter or gene-body regions and the occurrence of repressive and permissive chromatin marks.

Since we found a correlation between promoter methylation and the associated chromatin marks, we hypothesized that the expression of these genes would also change in the progeny of stressed plants. The qPCR analysis indeed confirmed that in all cases, except for *WRKY22*, the genes hypermethylated at the promoter regions had lower levels of gene expression in the progeny of stressed plants (Figure 3.4.5-3.4.8). The *UVH3*-like gene hypomethylated at the promoter region in the progeny of plants exposed to 25 mM NaCl showed a higher level of mRNA. The correlation analysis between levels of specific histone modifications and mRNA expression showed a positive correlation between H3K9ac and mRNA levels (r=0.6 and r=0.7 on average for the promoter and gene-body regions, respectively) and a negative correlation between the levels of

H3K9me2 and mRNA (r=-0.7 and r=-0.5 on average for the promoter and gene-body regions, respectively).

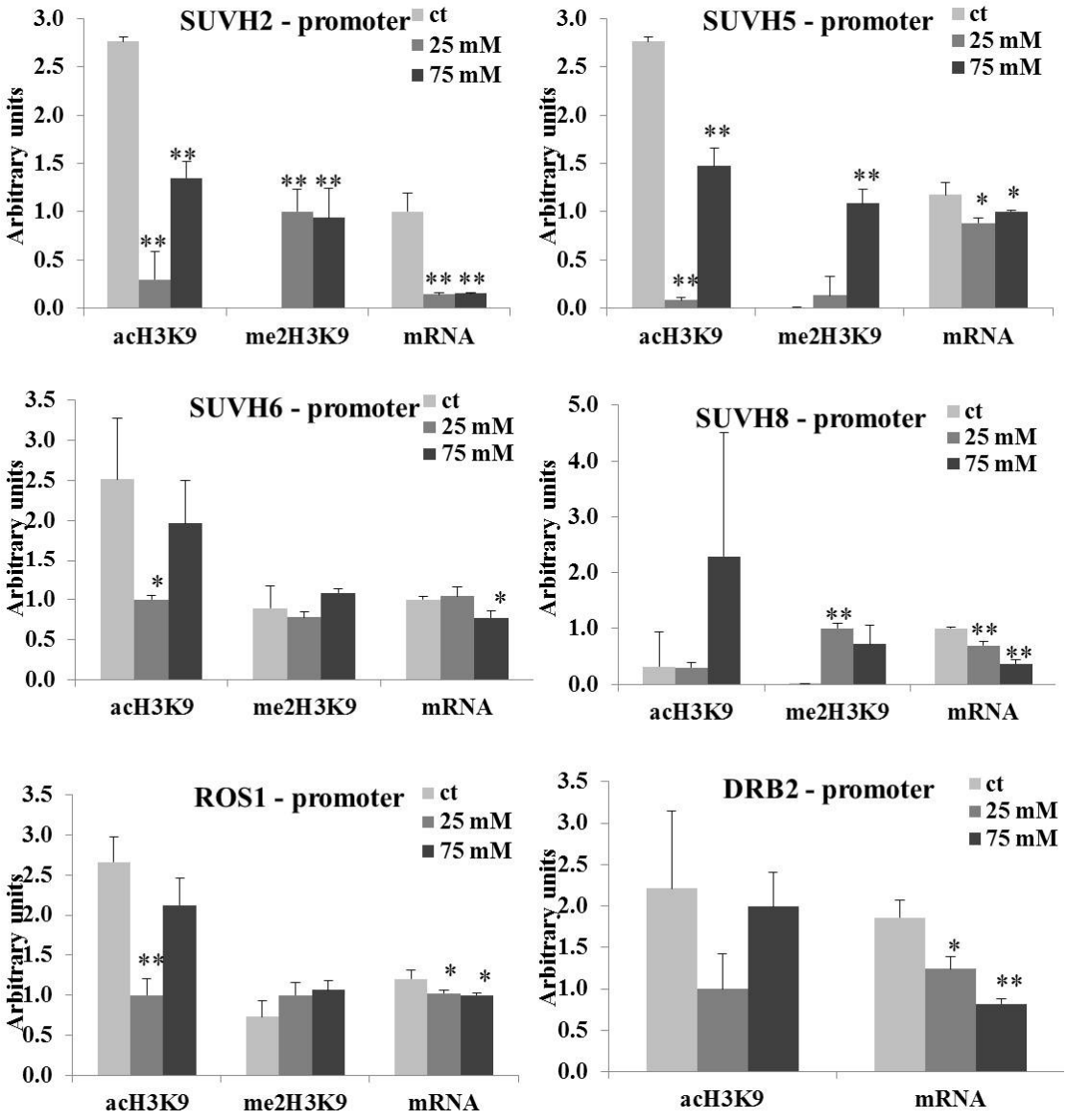

Figure 3.4.5. Histone modifications (H3K9me2 and H3K9ac) at the promoter regions of *SUVH2*, *SUVH5*, *SUVH6*, *SUVH8*, *ROS1* and *DRB2* genes

The figure shows the levels of H3K9me2 and H3K9ac observed at the promoter region of *SUVH2*, *SUVH5*, *SUVH6*, *SUVH8*, *ROS1* and *DRB2* genes. Each figure also shows mRNA levels for each of the genes. The Y-axis shows the levels of mRNA expression and H3K9me2/H3K9ac in average arbitrary units (calculated from three independent experiments with SD). The asterisks denote a significant difference between the progeny of stressed (25 and 75 mM) and control plants; one asterisk stands for $p<0.05$ and two asterisks for $p<0.01$.

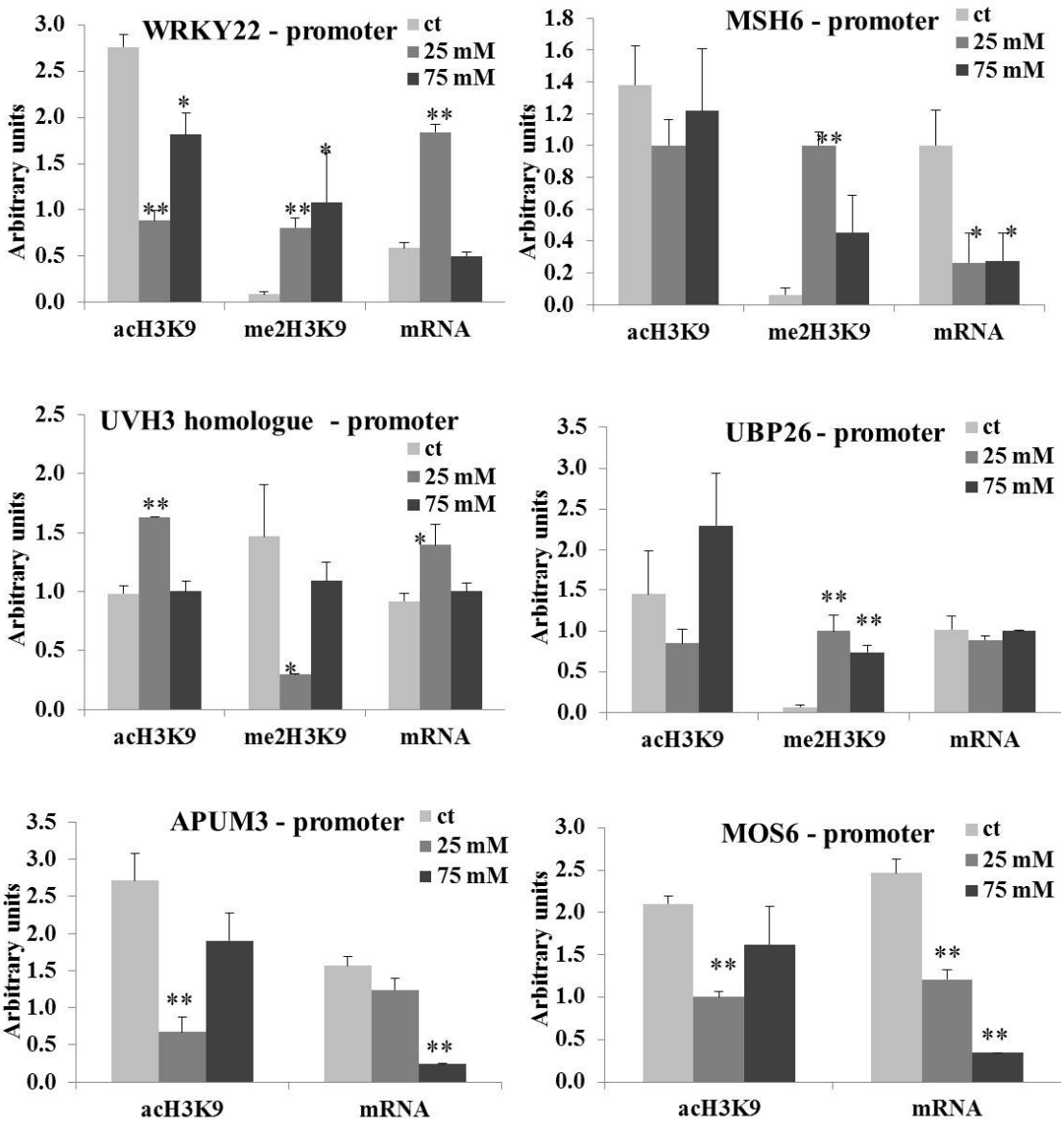

Figure 3.4.6. Histone modifications (H3K9me2 and H3K9ac) at the promoter regions of *WRKY22*, *MSH6*, *UHV3 homolog*, *MOS6*, *APUM3* and *UBP26* genes

The figure shows the levels of H3K9me2 and H3K9ac found at the promoter region of *WRKY22*, *MSH6*, *UVH3 homolog*, *MOS6*, *APUM3* and *UBP26* genes. Each figure also shows mRNA levels for each of the genes. The Y-axis shows the levels of mRNA expression and H3K9me2/H3K9ac in average arbitrary units (calculated from three independent experiments with SD). The asterisks denote a significant difference between the progeny of stressed (25 and 75 mM) and control plants; one asterisk stands for $p<0.05$ and two asterisks for $p<0.01$.

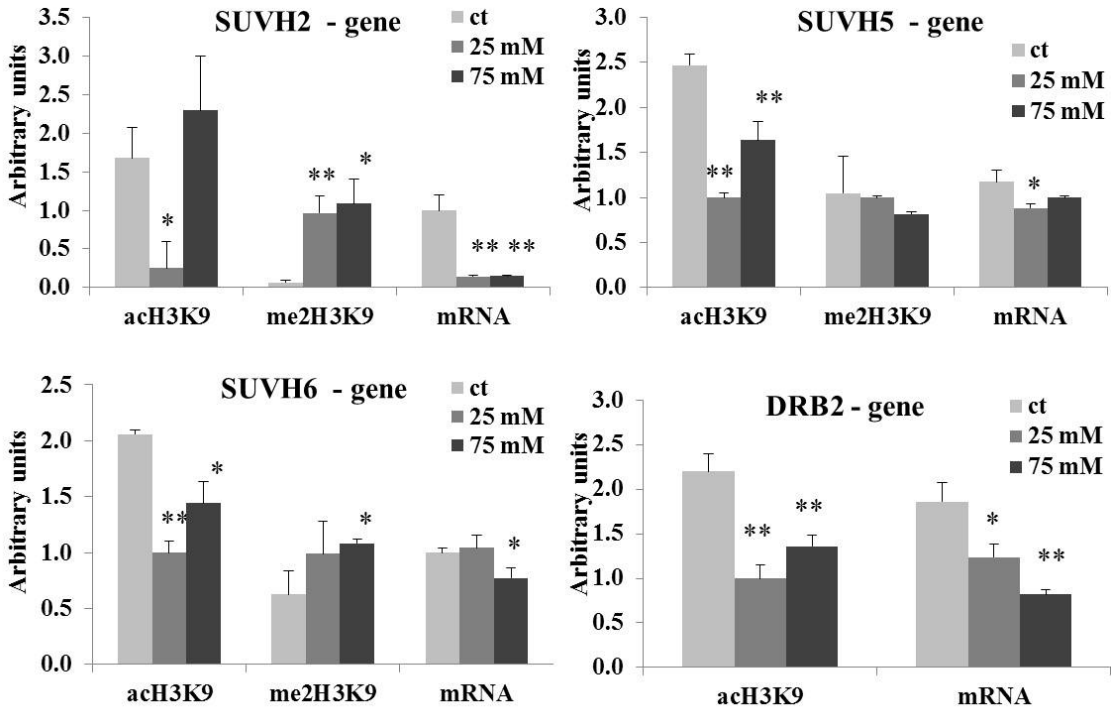

Figure 3.4.7. Histone modifications (H3K9me2 and H3K9ac) at the gene body regions of *SUVH2*, *SUVH5*, *SUVH6* and *DRB2* genes in the progeny of stressed plants

The figure shows the levels of H3K9me2 and H3K9ac found at the gene body regions of *SUVH2*, *SUVH5*, *SUVH6* and *DRB2* genes. Each figure also shows mRNA levels for each of the genes. The Y-axis shows the levels of mRNA expression and H3K9me2/H3K9ac in average arbitrary units (calculated from three independent experiments with SD). The asterisks denote a significant difference between the progeny of stressed (25 and 75 mM) and control plants; one asterisk stands for $p<0.05$ and two asterisks for $p<0.01$.

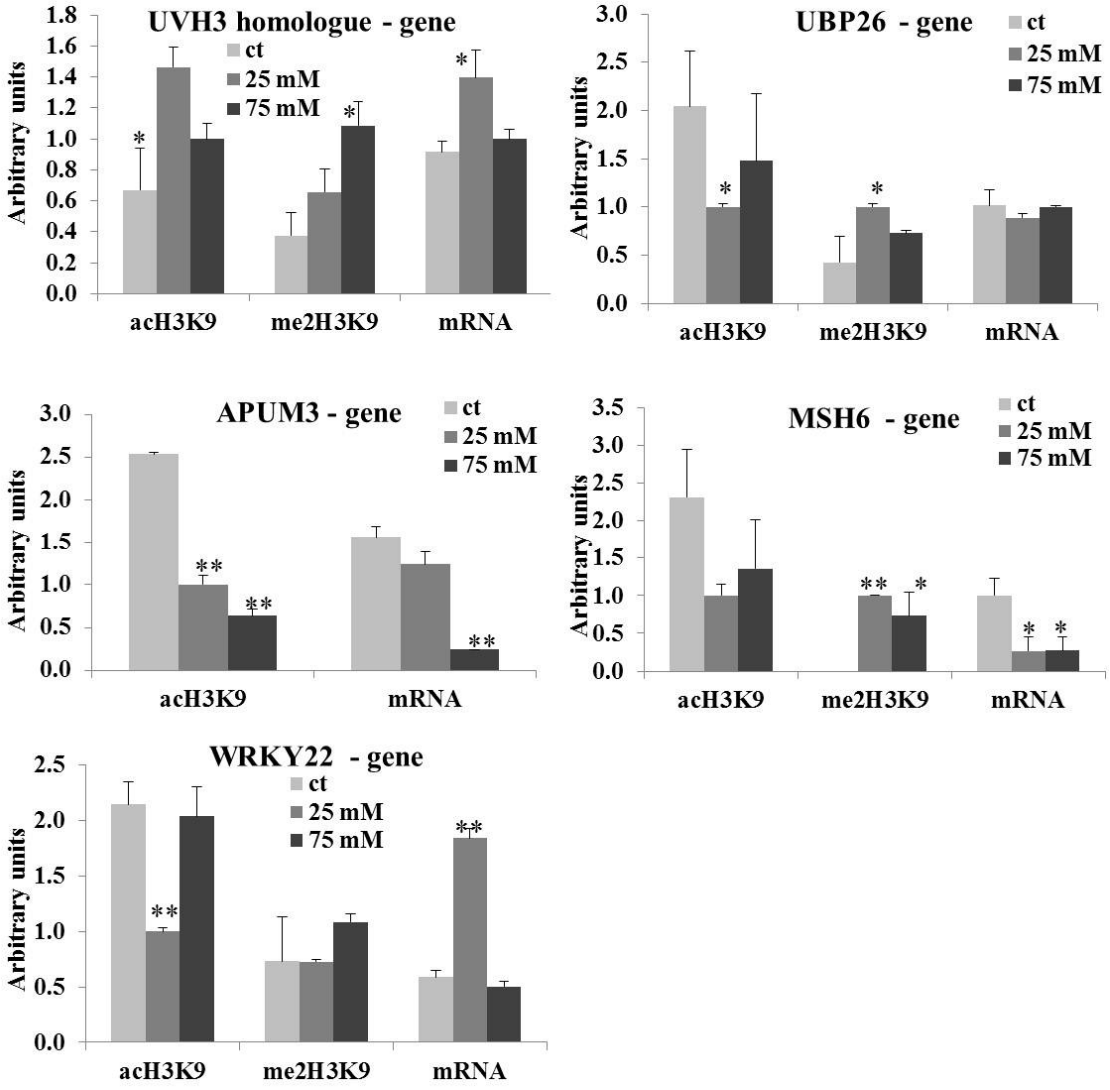

Figure 3.4.8. Histone modifications (H3K9me2 and H3K9ac) at the gene body regions of *UBP26*, *WRKY22*, *MSH6*, *UHV3 homolog* and *APUM3* genes

The figure shows the levels H3K9me2 and H3K9ac observed at the gene body regions of *WRKY22*, *MSH6*, *UVH3 homolog*, *MOS6*, *APUM3* and *UBP26* genes. Each figure also shows mRNA levels for each of the genes. The Y-axis shows the levels of mRNA expression and H3K9me2/H3K9ac in average arbitrary units (calculated from three independent experiments with SD). The asterisks denote a significant difference between the progeny of stressed (25 and 75 mM) and control plants; one asterisk stands for $p<0.05$ and two asterisks for $p<0.01$.

Table 3.4.6. Methylation in genes encoding proteins involved in siRNA biogenesis

Gene number	Gene name	Methylation
AT3G03300	DCL2	equally methylated
AT3G20550	DDL	slightly hypermethylated in 25 and 75 mM
AT3G43920	DCL3	equally methylated
AT2G32940	AGO6	slightly hypermethylated in 25 and 75 mM
AT3G23780	DRD2 (NRPD2)	slightly hypermethylated in 25 mM
AT2G40030	DRD3	equally methylated

3.5. DISCUSSION

Plants exposed to stress may pass the information about it as a dominant trait on to successive generations (Boyko, Blevins et al. 2010). Consequently, it can lead to an increased stress tolerance known as hardening phenomenon (Boyko and Kovalchuk 2011). Such transgenerational adaptation to stress may depend on a number of epigenetic marks that mediate heritable changes in DNA methylation and chromatin structure. The dynamic modifications of the chromatin structure are essential for the correct regulation of vital nuclear processes such as DNA transcription, replication, repair, and recombination (Mathieu, Reinders et al. 2007).

3.5.1. Exposure to stress results in changes in DNA methylation in the progeny

Somatic tissues of stressed plants may respond to stress with either a decrease or an increase in DNA methylation, depending on the genomic locus. It appears, however, that demethylation leading to the activation of gene expression is a more common immediate response to stress. In tobacco, the accumulation of several abiotic and biotic stress-induced transcripts was associated with an active demethylation process at given loci (Wada, Ohya et al. 2003, Choi and Sano 2007). Hemp and clover plants subjected to heavy metal stress also exhibited hypomethylation at several marker loci (Panella, Aina et al. 2004). Exposure to cold stress triggered demethylation in the DNA of the nucleosome core of the *ZmMI1* gene in root tissues of maize seedlings (Steward, Ito et al. 2002). Treatment with cold, salt and aluminum stress induced demethylation of the *NtGPDL* gene leading to higher tolerance to stress (Choi and Sano 2007). Similarly,

infection of Arabidopsis plants with *Pseudomonas syringae* (Pavet, Quintero et al. 2006) as well as infection of tomato plants with a virus (Mason, Caciagli et al. 2008) triggered DNA hypomethylation at centromeric repeats and in several genomic regions involved in defence and stress responses, respectively. At the same time, *M. crystallinum* plants exposed to high salinity conditions showed a two-fold increase in CNG methylation (Dyachenko, Zakharchenko et al. 2006). Similarly, an age-dependent increase in methylation was sufficient to mediate resistance to the blight pathogen *X. oryzae* in rice (Sha, Lin et al. 2005).

The information about methylation changes in the progeny of stressed plants is scarce. Verhoeven et al. (2010) demonstrated that methylation changes in a population of apomictic dandelion observed upon exposure to abiotic and biotic stresses was faithfully transmitted to the progeny (Verhoeven, Jansen et al. 2010). It was not possible, however, to deduce whether these changes were an increase or a decrease in methylation. Previously, we showed that the progeny of Arabidopsis plants exposed to different biotic (Kathiria, Sidler et al. 2010) and abiotic (Boyko, Blevins et al. 2010, Boyko, Golubov et al. 2010) stressors exhibited the higher frequency of homologous recombination, elevated tolerance to stress, and increased global DNA methylation.

Taking into consideration the abovementioned information, it can be hypothesized that a common response of plants to stress is demethylation of specific genomic regions followed by hypermethylation of the genome in the progeny (Boyko and Kovalchuk 2011).

Our analysis of methylation at the gene body showed that the 5' and 3' ends of the genes had a substantially lower level of methylation as compared to the central part of the

gene. A similar distribution of methylation was also observed before. Although Cocus et al. (2008) (Cokus, Feng et al. 2008) found a 5- to 8-fold difference and Lister et al. (2008) (Lister, O'Malley et al. 2008) observed an ~ 10-fold difference between methylation levels in the central part of the gene and at either the 5' and 3' ends of the gene, we found a 25-fold difference in this ratio. In our work, the increase in methylation at the transcribed regions in the progeny of stressed plants was much greater at the 5' and 3' ends of the gene rather than at the central part of the gene. It is not clear how methylation at the 5' or 3' end of the gene correlates with gene expression, but it can be hypothesized that increased methylation at these regions of the gene would negatively impact gene expression.

Another interesting result of our studies was the difference in methylation levels between exons and introns. We found that the level of methylation in exons was higher than that in introns. At the same time, the progeny of stressed plants had a higher increase in methylation in exons than in introns. As it is suggested by Feng and Jacobsen (2011), it is not clear what the role of methylation at gene bodies is since the expression of most of the genes does not change with a decrease in methylation at the gene body observed in mutants impaired in DNA methylation (Feng and Jacobsen 2011). It is proposed that methylation may regulate exon definition or/and splicing controlling the production of alternative transcripts (Feng and Jacobsen 2011). We hypothesize that an increase in methylation in exons in the progeny of stressed plants may control transcription, splicing or perhaps, the potential rearrangements, thus preventing reshuffling of exons. It remains to be shown whether the number of alternative transcripts and their frequency of occurrence decrease in the progeny of stressed plants. However, it should be noted that in

our experiments, we used C24 plants and the analysis of methylation was performed on microarrays that are based on DNA sequences from the Columbia ecotype, therefore, some of the changes in methylation may have either been over- or under-represented.

3.5.2. Changes in DNA methylation in the progeny of stressed plants correlate with changes in histone modifications

Changing DNA methylation is not the only way to epigenetically control gene expression in response to stress. It was recently demonstrated that activation of repetitive elements in heat-stressed Arabidopsis plants occurs without loss of DNA methylation but rather due to heterochromatin decondensation and nucleosome loss (Pecinka, Dinh et al. 2010). Changes in histone modifications were shown to be solely responsible for reactivation of silenced transgenes; exposure to several different abiotic stresses resulted in a release of transgene silencing without loss of DNA methylation via altering histone occupancy and inducing histone H3 acetylation (Lang-Mladek, Popova et al. 2010).

The level of DNA methylation frequently affects gene expression together with changes in histone code (Zaratiegui, Irvine et al. 2007). For instance, dimethylation of lysine 9 and lysine 27 of histone H3 (H3K9me2, H3K27me2) in plants (Iizuka and Smith 2003) together with hypermethylation of DNA are linked to the transcriptional repression, while dimethylation of lysine 4 and/or acetylation of lysine 9 of histone H3 (H3K4me2, H3K9ac) and hypomethylation of DNA of the promoter region are associated with an active gene. We attempted to find out whether changes in DNA methylation in the progeny of stressed plants are also paralleled by changes in histone modifications. Using the chromatin immunoprecipitation method (ChIP), we found a positive correlation

between the level of DNA methylation and the occurrence of the repressive chromatin H3K9me2 mark in the progenies of stressed plants. Additionally, a high level of H3K9me2 at a chosen DNA locus was paralleled by a decreased level of H3K9ac and gene expression. Until now, no data on changes in the level of H3K9ac or H3K9me2 in the progeny of stressed plants exist, however, changes in H3K9 modifications in stressed somatic tissues are well documented. Exposure to drought resulted in an increase in histone acetylation in the promoters of stress-induced genes (Kim, To et al. 2008). Also, exposure to UV-B triggered increase in histone acetylation in Arabidopsis plants and wheat (Casati, Campi et al. 2008, Cloix and Jenkins 2008). Similarly, Lang-Mladek (2010) showed that temperature and UV-B resulted in histone acetylation of a silent reporter gene (Lang-Mladek, Popova et al. 2010). Unfortunately, no information on changes in the progeny of these plants was provided.

H3K9 methylation in Arabidopsis plants is maintained by SET-domain proteins, including KRYPTONITE/SUVH4 (KYP/SUVH4), SUVH5, SUVH6 and SUVH2 (Jackson, Johnson et al. 2004, Vaillant and Paszkowski 2007). The *kyp* mutations cause a decrease in H3K9 methylation, loss of CNG DNA methylation, and reduced gene silencing (Jackson, Johnson et al. 2004). A similar correlation between DNA and histone methylation was shown in studies of *Neurospora crassa* (Tamaru and Selker 2001), further suggesting that H3K9 methylation is tightly linked to DNA methylation in different species.

In our studies, the Arabidopsis SU(VAR)3-9 homologs, namely *SUVH5, SUVH6, SUVH8*, were hypermethylated in the promoter regions and *SUVH2* - in the coding regions in the progenies of salt-stressed plants. The expression analysis showed a

decrease in the level of mRNA in these genes regardless of the fact that methylation changes were observed either in the promoters or gene bodies. It is possible that hypermethylation of these homologs may represent a protective mechanism against hypermethylation of the genome in the progeny of stressed plants. Recently, it was shown that *suvh2* mutant as well as mutants impaired in siRNA biogenesis exhibited increased rate of ONSEN activation when exposed to heat stress (Ito, Gaubert et al. 2011). Thus, decrease in the expression of SU(VAR)3-9 homologs may contribute to transposon activation.

Several other genes involved in either DNA repair or chromatin modifications showed altered methylation in the progeny of stressed plants, including *UBP26*, *MSH6* and *ROS1*. UBP26 protein facilitates heterochromatin formation by removing ubiquitin modifications of histone H2B; therefore, it is vital for endosperm development and flowering (Sridhar, Kapoor et al. 2007). It can also be hypothesized that hypermethylation of *UBP26* with the decrease of its expression levels may lead to local euchromatization events. Being part of the MutSα heterodimer complex, a mismatch repair protein, MSH6, together with MSH2 are involved in the initial recognition of DNA errors (Lario, Ramirez-Parra et al. 2011). Our analysis showed an inverse correlation between the level of repressive chromatin marks and expression of the *MSH6* gene. Reduced expression of mismatch repair genes followed by lower levels of mismatch repair activities may result in a higher frequency of point mutations and, possibly, other genomic rearrangements in the progeny of stressed plants. Indeed, that is exactly what was observed in the progeny of plants exposed to various stresses (Boyko and Kovalchuk 2010, Kathiria, Sidler et al. 2010, Yao and Kovalchuk 2011).

ROS1 gene encodes a member of the DEMETER (DME) family of DNA glycosylases that catalyzes the excision of methylated cytosines, thereby antagonizing the activity of DNA methyltransferases (Vaillant and Paszkowski 2007, Ponferrada-Marin, Martinez-Macias et al. 2010). The ChIP and qRTPCR analysis of this gene showed enrichment of the repressive chromatin mark H3K9me2 in both the promoter and coding regions paralleled by a slight depletion of mRNA levels and a decrease in the permissive chromatin mark H3K9ac. The decrease in *ROS1* expression may result in a lower ability of repairing DNA as well as removing methylated cytosines.Loss of the *ROS1* gene induces hypermethylation of cytosine residues within plant-specific CNG sequences (Tariq and Paszkowski 2004) and transcriptional silencing of transgenes, endogenous genes, and transposon sequences (Agius, Kapoor et al. 2006). These results are consistent with our data which show an increase in methylation of transposons in the progeny of salt-stressed plants (Boyko, Blevins et al. 2010).

The exact reason for *ROS1* transcriptional repression in the progeny of stressed plants is unknown. Possibly, in order to avoid demethylation of hypermethylated loci, the *ROS1* gene is partially silenced by the repressive chromatin marks. This effect can be related to the ROS1-mediated compensatory mechanism that has been shown to exist between the PolIV/RDR2/DCL3/AGO4 pathway and *ROS1* gene expression. This pathway is responsible for RNA – dependent DNA methylation (RdDM) in Arabidopsis and is required for *de novo* DNA methylation by the methyltransferase DOMAINS REARRANGED METHYLTRANSFERASE2 (DRM2) as well as for the maintenance of non-CG methylation by CMT3. It was observed that in *rdr2* and *drm2* mutant plants, genes that are normally demethylated by ROS1 accumulated CG and non-CG

methylation. The authors speculated that DNA hypermethylation was due to the *ROS1* down-regulation occurred in these mutants (Agius, Kapoor et al. 2006). Also, SUVH5 was suggested as a possible candidate that could mediate non-CG DNA methylation through CMT3 activity (Ebbs and Bender 2006). Therefore, silencing of the members of the HMTase family can possibly mediate down-regulation of *ROS1* expression through the PolIV/RDR2/DCL3/AGO4 pathway. The fact that we observed reduced expression of HMTases and *ROS1*, in part, supports this hypothesis. The future analysis of the chromatin marks of the ROS1- target loci in the *suvh* mutants may reveal a possible link between HMTases and DME proteins.

The exact mechanism of hypermethylation of specific genomic loci coding for chromatin modifiers in the progeny of stressed plants is still unknown (Saze 2008). Exposure to stress may result in the accumulation of specific siRNAs triggering *de novo* RdDM at non-CG sites in addition to programmed changes in methylation at symmetrical cytosines (Teixeira, Heredia et al. 2009). Thus, one of the possible directions that need to be explored to clarify the inheritance of epigenetic marks in stressed plants is RNA-dependent DNA methylation. Of note is the fact that the analysis of methylation among genes involved in small RNA biogenesis showed that *DCL2, DCL3* and *DRD3* were equally methylated in the progeny of control and stressed plants, whereas *DRD2, DDL, AGO6* were slightly hypermethylated in the progeny of stressed plants (Table 3.4.6). The future analysis of the global small RNA profiles with relation to potential genome targets for methylation and histone modifications in the progeny of stressed plants will allow better understanding of the mechanism of epigenetic transgenerational memory.

4. THE ELUCIDATION OF STRESS MEMORY INHERITANCE THROUGH EPIGENOME ALTERATIONS IN *BRASSICA RAPA* PLANTS

4.1. ABSTRACT

Plants are able to maintain the memory of stress exposure throughout their ontogenesis and faithfully propagate it into the next generation. This eclectic idea manifests itself in the reports of our lab and numerous other labs demonstrating the mitotic and meiotic transition of altered traits acquired by plants after stress treatment. Recent evidence argues for the epigenetic nature of this phenomenon. Superimposed on the DNA sequence, epigenetic factors have the potential to provide dexterity and plasticity in terms of modulating gene expression and response to the environmental signals. Small RNAs (smRNAs) are considered to be one of the most vital epigenetic factors since they both can affect gene expression at the place of their generation as well as maintain non-cell-autonomous DNA methylation. Their versatile nature make smRNAs ideal candidates for messengers of transgenerational epigenetic inheritance, which molecular mechanism is yet to be discovered. In the current study, we made an attempt to decipher the contribution of smRNAs to heat-shock-induced transgenerational inheritance in *Brassica rapa* plants using a massive parallel sequencing technology. To do this, we generated comprehensive profiles of a transcriptome and small RNAome (smRNAome) from somatic and reproductive tissues of stressed plants and their untreated progeny. We demonstrate that the highest tissue-specific alterations in the transcriptome and smRNAome profile were detected in tissues that were not directly exposed to stress, namely, in the endosperm and pollen. Importantly, for the first time, we revealed that the progeny of stressed plants exhibited the highest fluctuations at the smRNAome level, but not at the transcriptome level. Additionally, we uncovered the existence of transgenerationally transmitted and heat-inducible tRNA-derived small RNA

fragments in plants. Finally, we suggest that miR168 and *braAGO1* are involved in stress-induced transgenerational inheritance in plants.

4.2. INTRODUCTION

Plants constantly interact with environmental factors that can either benefit or jeopardize their homeostasis, depending on the intensity and nature of factors encountered. Significant environmental perturbations that exceed the optimum range of plant development can cause stress and trigger the onset of gene expression changes in plants. Stress-induced alterations in the transcriptome profile have been shown to be both stress- and tissue-specific, although the general stress response (GSR) that has been extensively studied in yeast and animals is also present in plants (Kreps, Wu et al. 2002, Kultz 2005, Dinneny, Long et al. 2008, Walley and Dehesh 2010, Iyer-Pascuzzi, Jackson et al. 2011). Previously, we demonstrated that the progeny of plants exposed to salt stress and *Tobacco mosaic virus* (TMV) exhibit a higher tolerance not only to the same stressor but also to methyl methane sulfonate, a genotoxic agent that induces DNA methylation, as compared to control progeny (Boyko, Blevins et al. 2010, Kathiria, Sidler et al. 2010). Hence, although plants may trigger immediate specific gene expression changes to cope with a particular stressor, transgenerational inheritance and responses to stress seem to involve non-specific priming of stress-responsive genes. Distinct epigenetic mediators of multigenerational inheritance of stress memory have been recently identified in two animal models – *Drosophila* (Seong, Li et al. 2011) and *C. elegans* (Buckley, Burkhart et al. 2012), which makes it tempting to argue for the existence of similar transgenerational mediators in plants.

Plants belonging to the genus *Brassica* are predominantly annual cool-season economically important crops whose cultivation is acutely affected by elevated temperatures and drought (Hall 2001, Yu, Wang et al. 2012). Heat shock (HS) stress can

severely influence reproductive tissues of plants that contribute to poor seed set yield (Zinn, Tunc-Ozdemir et al. 2010). On the other hand, the pre-treatment of plants under moderate HS conditions can protect them from an acute heat stress and provide a better stress tolerance, a phenomenon known as induced or acquired thermotolerance (Gurley 2000). The expression of heat-shock proteins regulated by heat stress transcription factors is believed to mediate the heat stress response and acquired thermotolerance in plants (Kotak, Larkindale et al. 2007, Yu, Wang et al. 2012). Curiously, the acclimation to abiotic factors and induced resistance to pathogens (Van Loon 1997) can be propagated into the next generation, a phenomenon known as transgenerational acquired tolerance (Boyko, Blevins et al. 2010, Holeski, Jander et al. 2012). Given the practical value of such response for its implementation in plant biotechnology and agriculture, there has been a remarkable interest in unraveling pathways involved in transgenerational acquired tolerance. Currently, two different mechanisms are proposed to account for the aforementioned phenomenon: maternal effects on seed size (Agrawal 2001) and epigenetic alterations that allow a vertical propagation of acquired traits without changing the underlying genomic DNA sequence (Jablonka and Raz 2009). Whereas the former mechanism has little support from multigenerational stress experiments on plants, the evidence favouring the second mechanism has been provided at the molecular level (Jablonka and Raz 2009, Boyko, Blevins et al. 2010, Bilichak, Ilnystkyy et al. 2012, Luna, Bruce et al. 2012, Rasmann, De Vos et al. 2012, Slaughter, Daniel et al. 2012).

Heritable fluctuations in DNA methylation, chromatin composition and smRNA metabolism are among the primary causes of transgenerational epigenetic inheritance (Holeski, Jander et al. 2012). Being reversible in their nature, acquired components of the

epigenetic machinery are usually prone to significant alterations during sexual plant development. In angiosperms, epigenetic factors have to survive the multifaceted process of gametogenesis and early embryogenesis that encompasses a significant restructuring of both cells and chromatin (Ibarra, Feng et al. 2012). Although methylation at CpG sequences found in vegetative cells is largely retained in sperm cells, embryo and conceivably in egg cell, methylation at asymmetric CpHpH (where H = A, C, or T) sequences is largely lost as compared to vegetative cells (Gehring, Bubb et al. 2009, Hsieh, Ibarra et al. 2009, Ibarra, Feng et al. 2012, Jullien, Susaki et al. 2012). Curiously, in all three cases – in sperm, egg cell and embryo, asymmetric DNA methylation is proposed to be restored through the RNA-dependent DNA methylation (RdDM) pathway guided by smRNAs generated by companion cells or tissues that do not contribute genetic material to the progeny. In turn, smRNAs have been shown to be vital environmental sensors, the expression of which is acutely affected by abiotic and biotic stressors (Khraiwesh, Zhu et al. 2012). Therefore, we hypothesized that perturbations in the expression of smRNAs caused by environmental fluctuations would eventually be imprinted in DNA methylation and transcriptome patterns in gametes and progeny.

In the current study, we used plants of the rapid-cycling *Brassica rapa* (*B. rapa*) cultivar R-o-18 which has the following advantages over other crop plant models: (i) a rapid life cycle; (ii) being self-fertile; (iii) the smallest genome size in the *Brassica* genus; (iv) the relatively close relationship to the model plant species, *Arabidopsis thaliana*; (v) the genome of the *B. rapa* accession Chiifu-401-42, a Chinese cabbage, has recently been sequenced and partially annotated and is now publically available (Wang, Wang et al. 2011, Bagheri, El-Soda et al. 2012).

In order to reveal the contribution of smRNAs to epigenetic transgenerational inheritance, plants were selectively exposed to temperature stress during the vegetative stage of plant ontogenesis followed by collecting somatic and reproductive tissues from control and exposed plants. To track the propagation of epigenetic memory into the following generation, seeds from control and stressed plants were germinated, and leaves from the progeny of stressed plants were collected. Subsequently, using the Illimina GAIIx sequencing technology, the genome-wide transcriptome and smRNAome profiles of the following tissues were generated and inter-compared: parental leaves, inflorescence meristem, pollen, unfertilized ovules, ovules at 24 hours post-fertilization, embryo, endosperm and leaf tissues of progeny plants. Transcriptome and smRNAome analysis enabled us to take a high-resolution snapshot of the status of reproductive and somatic tissues under normal conditions and after HS, which revealed the unknown before singularities in a tissue-specific response to stress in *B. rapa* plants. Additionally, for the first time, we have provided evidence that the most pronounced transgenerational stress-induced fluctuations occur at the smRNAome but not at the transcriptome level in plants. Finally, we suggest that miR168 and *braAGO1* are potential regulators of stress-induced epigenetic inheritance in plants.

4.3. MATERIALS AND METHODS

4.3.1. Plant cultivation

In the current work, R-o-18 a rapid-cycling self-compatible inbred line of *Brassica rapa* var. *trilocularis* (Roxb.) Hanelt (yellow sarson) was used (Williams and Hill 1986). Seeds were originally obtained from Dr. Joan E. Krochko, the NRC Plant Biotechnology Institute (Saskatoon, Saskatchewan, Canada).

4.3.1.1. Growing *B. rapa* plants for heat stress experiments and tissue harvesting

Seeds obtained from a single unstressed plant were germinated on *All purpose potting soil* (Plant *Etc*; Lethbridge, AB, Canada) mixed in the proportion 4:1 with vermiculite (The Professional Gardener Co LTD, AB, Canada) in 4x4 inch square pots. The soil mixture was pre-soaked once with Miracle-Gro fertilizer (Scotts Canada *Ltd.*, Mississauga, ON, Canada) and was maintained constantly moist with tap water. Rapid-cycling *B. rapa* parental plants (30 plants per group) and their progeny were cultivated in biochambers (Biofoot^{TM}, model GC-20, Winnipeg, MB, Canada) under continuous high-intensity cold light illumination (200 μmol m^{-2} s^{-1}) provided by fluorescent lamps and a 60 % relative humidity at 22°C as described previously (Daugherty and Musgrave 1994, Tel-Zur and Goldman 2007). The cultivation of plants under constant light did not affect their phenotypic appearance as compared to plants grown under a long-day photoperiod (16 h day, 8 h night). Two weeks post germination, before the appearance of apical inflorescence shoots, plants for the treated group were exposed to 42°C for 3h per day for

7 days in a row (Figure 4.3.1). Subsequently, plants from both control and treated groups and their progeny were grown in continuous light at 22°C.

In order to monitor the development of stress memory and follow its transmission to germ cells, 17 days after heat-shock treatment, leaves and up to 1 cm of the inflorescence meristem dissected from flower buds of control and stressed plants were harvested.

To follow the occurrence of epigenetic marks in reproductive tissues, pollen and unfertilized and fertilized ovules from both groups were harvested separately. The inflorescences were covered with plastic bags to prevent cross-pollination between treatment groups. Pollen from control and heat-shock treated plants was harvested separately using a vacuum manifold method (Johnson-Brousseau and McCormick 2004).

The unfertilized ovules containing mature embryo sacs were synchronized developmentally by emasculating flowers at stage 13 (Smyth, Bowman et al. 1990). Twenty four hours later, the ovules were collected from hand-dissected pistils (Le, Cheng et al. 2010). The fertilized ovules containing zygotes were harvested from siliques 24 hours after hand pollination of emasculated flowers (Le, Cheng et al. 2010). Finally, mature-green embryos and endosperms were dissected from seed coat and harvested separately.

In order to track the transmission of epigenetic marks to the next unstressed generation, the control and treated plants were propagated, and tissue samples from two-week-old seedlings were harvested. Each developmental stage was represented by two biological replicates that were harvested independently.

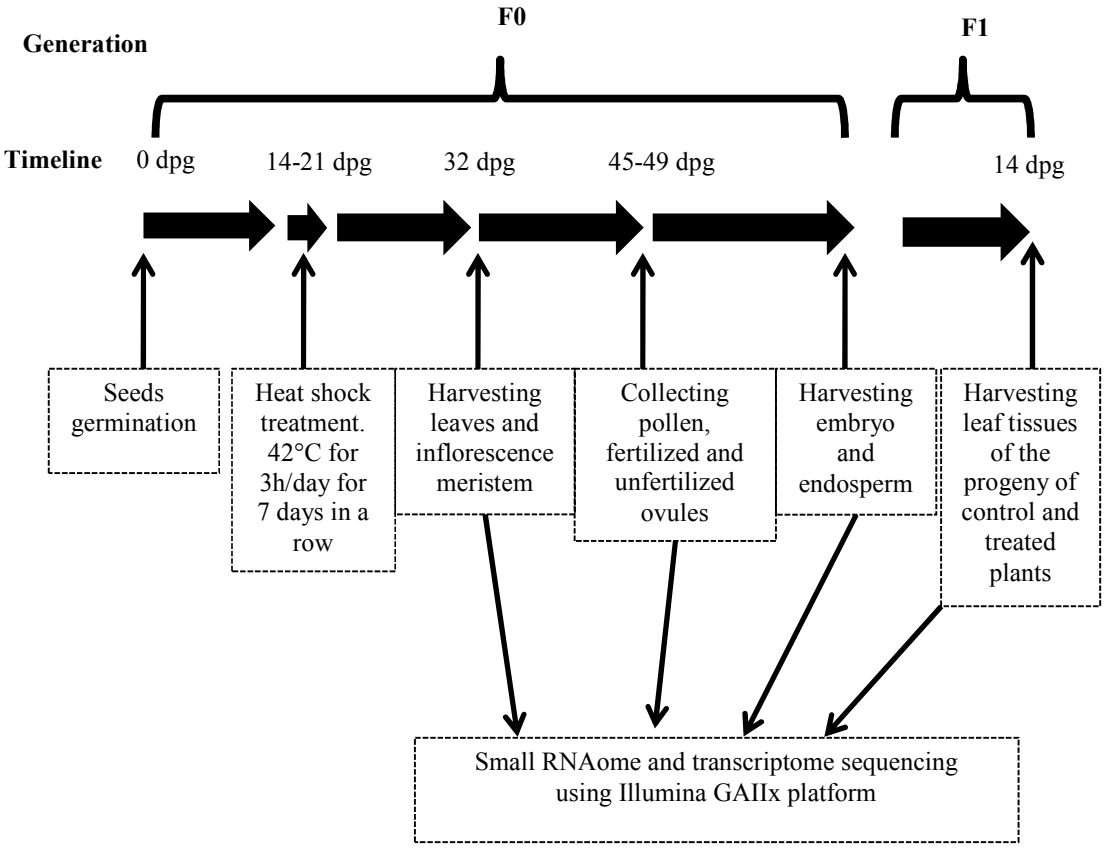

Figure 4.3.1. Experimental setup

Two groups were obtained for the experiment – "Control" and "Heat shock". Thirty *B. rapa* plants per group were used, and every tissue was harvested in duplicates. Dpg – days post germination. F0 – parental plants, F1 - progeny

4.3.2. Molecular techniques and methods used in this study

4.3.2.1. Total RNA isolation and purification

All tissue samples harvested from control and treated plants were subjected to total RNA isolation using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Two independent RNA samples per every tissue were prepared. Total RNA was further purified and concentrated using the RNA Clean-Up and Concentration Kit (Norgen Biotek Corp., Ontario, Canada).

The quality and concentration of every sample were quantified using the NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc.) and the absorption ratios at 260/280 and 260/230 nm wavelengths. Agarose gel electrophoresis was performed to verify RNA sample integrity.

4.3.2.2. mRNA deep sequencing

The mRNA libraries were prepared from 16 samples of total RNA in 2 biological replicates according to the TruSeq RNA sample Prep v2 LS protocol (Illumina, San Diego, CA, U.S.A.). Briefly, mRNA was purified from the total RNA samples using poly-T oligo-attached magnetic beads followed by mRNA fragmentation, first and second strand cDNA synthesis. Later, the overhangs resulting from the fragmentation of double-stranded (ds) cDNA were repaired to form blunt ends. A single 'A' nucleotide was added to the 3′ ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. Multiple indexing adapters were ligated to the ends of ds cDNA to prepare them for hybridization onto a flow cell followed by a PCR amplification step. The libraries were quantified using the qPCR technique and

analyzed on a Bioanalyzer 2100 (Agilent Technologies) using a DNA specific chip. Subsequently, the libraries were normalized and pooled together followed by flow-cell cluster generation using a cBot fully automated clonal cluster generation system for Illumina sequencing.

Single end multiplexed sequencing was done using the Illumina GAIIx platform with the total of 100 cycles.

4.3.2.3. Deep sequencing of small non-coding RNAs

Small non-coding RNA libraries were generated from 16 samples of total RNA in 2 biological replicates using the TruSeq small RNA library construction kit according to the manufacturer's protocol (Illumina, San Diego, CA, U.S.A.). Briefly, the 3′ and 5′ adapters were ligated to small RNAs from the total RNA sample followed by reverse-transcription PCR amplification. PCR was performed with two primers that anneal to the ends of adapters and contain indexes. Subsequently, the libraries with unique indexes were pooled together, the cDNA was gel-purified using a TBE PAGE gel and then concentrated by ethanol. Following a successful library quality control by qPCR, flow cell cluster generation was performed using a cBot.

Single end multiplexed sequencing was done using the Illumina GAIIx platform with the total of 36 cycles.

4.3.2.4. Northern blot analysis of small non-coding RNAs

Confirmation of small RNA sequencing data was performed using a non-radioactive northern blot method as described previously (Kim, Li et al. 2010). Briefly, 3

ug of total RNA was separated on a 15% urea PAGE (National diagnostics, USA), transferred to a positively charged nylon membrane (Roche). The pre-hybridization was performed with ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) at 37 °C for at least 30 min in hybridization oven followed by hybridization step with the DIG-labeled probe in ULTRAhyb Ultrasensitive Hybridization Buffer at 37 °C overnight with slow rotation. The DIG-labeled miR168 probe - AB492 (Table 4.3.1) was synthesized by Eurofins MWG Operon (Huntsville, AL, USA). Subsequently, the membrane was washed and incubated with Anti-Digoxigenin-AP Fab fragments (Roche) followed by detection using CDP-Star (Roche). The membrane was photographed using the FluorChem HD2 MultiImage™ Light Cabinet (Cell Biosciences Pty *Ltd*, Heidelberg, Australia), and the bands were quantified using the Image J program (NIH, http://rsbweb.nih.gov/ij/).

4.3.2.5. cDNA synthesis and qPCR gene expression analysis

To confirm the results of transcriptome sequencing, 500 ng of total RNA from every tissue in 2 biological replicates was DNase I treated, purified, converted into cDNA and quantified with qPCR as previously described (see Section 3.3.5. Real-Time qPCR Analysis). Gene expression was confirmed for four differentially expressed genes in the progeny: Bra029235, Bra031065, Bra029719, Bra040903 and Bra032254 (*AtAGO1* homolog). The normalization was done against four *B. rapa* housekeeping genes: *GAPDH*, *TUBULIN*, *EF1α* and *UBC* (Qi, Yu et al. 2010). For primer sequences see Table 4.3.1.

Statistical significance between treatment groups was evaluated using the two-tailed paired Student's t-test (α=0.05) and performed using JMP 10.0 software (SAS Institute Inc).

Table 4.3.1. Primers and probes used in this study

Primer/Probe Name	Sequence	Description
AB492	5′-DigN-TCCCGACCTGCACCAAGCGA-DigN-3′	DIG labelled probe for northern blot detection of miR168
AB445	5′-TTGGAATTGTCGAGGGACTC-3′	Forward qPCR primer – *braGAPDH*-AF536826
AB446	5′-GAGCTGTGGAAGCACCTTTC-3′	Reverse qPCR primer – *braGAPDH*-AF536826
AB447	5′-CTCGATGGCCTCAACCTTTA-3′	Forward qPCR primer – *braTUBULINE* - D78496
AB448	5′-ATGTTGCTCTCGGCTTCTGT-3′	Reverse qPCR primer – *braTUBULINE* - D78496
AB449	5′-GAGCATACCGGTCTCCACAC-3′	Forward qPCR primer – *braEF1a* - GO479260
AB450	5′-AAAGAGGCCATCAGACAAGC-3′	Reverse qPCR primer – *braEF1a* - GO479260
AB451	5′-TAACTGCGACTCAGGGAATCTT-3′	Forward qPCR primer – *braUBC* - GO479262
AB452	5′-TCATCCTTTCTTAGGCATAGCG-3′	Reverse qPCR primer – *braUBC* - GO479262
AB493	5′-TCATGATTCTGGGAGGGAAG-3′	Forward qPCR primer – *braAGO1* - Bra032254
AB494	5′-TGGCACATCTGAGCAAGTTC-3′	Reverse qPCR primer – *braAGO1* - Bra032254

4.3.3. Bioinformatic treatment of deep sequencing data

4.3.3.1. mRNA deep sequencing data analysis

Base calling and demultiplexing of transcriptome sequencing reads were performed using the Consensus Assessment of Sequence and Variance (CASAVA) v 1.6 and Novobarcode software (http://www.novocraft.com/). FastQC v 0.10.1 software was used for the preliminary quality check. In the following step, the reads were mapped to the genome, and *de novo* splice site prediction was performed using TopHat v 2.0.4 beta software (Trapnell, Pachter et al. 2009). *De novo* predicted splice-sites obtained were used to perform transcript assembly for each sample separately using Cufflinks v 2.0.2 (Trapnell, Williams et al. 2010). The assemblies were merged using a cuffmerge tool in Cufflinks software with the reference file containing *B. rapa* predicted genes (Wang, Wang et al. 2011, Trapnell, Roberts et al. 2012). The aim of this analysis was to compare our *de novo* assembly with the transcriptome that was previously *in silico* predicted (Wang, Wang et al. 2011).

The merged transcript assembly was used to assess differentially expressed features between treatments with a cuffdiff tool in Cufflinks software (Trapnell, Roberts et al. 2012). The q-value Benjamini-Hochberg method (Benjamini and Hochberg 1995) below 0.05 was considered as a significant difference of gene expression between treatment groups.

4.3.3.2. Deep sequencing data analysis of small non-coding RNAs

Base calling and demultiplexing of sequencing reads generated by the Illumina GAIIx platform was performed using the CASAVA v 1.8.1. software

(http://support.illumina.com/downloads/casava_181.ilmn). Then, the sequencing reads were processed using adapter trimming Cutadapt v 1.1 software (Martin 2011) with options specified to search for adapter sequences anywhere within the sequencing read and to retain only the sequences that were longer than 17 nucleotides; quality trimming was performed with a Sanger quality cutoff score of 20.

Summary statistics and run quality data were collected from the adapter trimmed libraries using FastQC v 0.10.1 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Samples that passed quality control tests were aligned to the *B. rapa* genome (accession number AENI01000000) (Wang, Wang et al. 2011) using Bowtie v 2 2.0.0 - beta2 aligner run (Langmead, Trapnell et al. 2009). The reads that could be aligned to the genome were further classified based on feature classes. The alignment was performed in a stepwise fashion. The reads that could be aligned to features of a certain class were counted and excluded from subsequent alignments. In this way, the pool of sequences was gradually depleted. The mapping process continued until the remaining reads could not be assigned to any of the known mapping categories, and were labeled as "unclassified". Both unique reads and reads that matched multiple loci (cutoff $\geq$ 50 loci for reads mapped to multiple loci) were considered for this analysis.

The alignment to *B. rapa* mature and passenger strand miRNA sequences was performed using conservative and predicted miRNA sequences described in (Yu, Wang et al. 2012). A novel miRNA prediction was done by using MiRDeep-P software (Yang and Li 2011) followed by its alignment to both predicted *trans*-acting siRNAs (ta-siRNAs) by using the UEA sRNA workbench (Stocks, Moxon et al. 2012) and non-

coding RNA genes, structured *cis*-regulatory elements and self-splicing RNAs from Rfam database, v 10.01 (Burge, Daub et al. 2012). The remaining reads were aligned to repeats and predicted genes (*B. rapa* gene database v 1.2). As a result, all small RNAs were sorted into 7 groups: miRNA candidates, gene-aligned small RNAs, conserved miRNAs, Rfam v 10.01, ta-siRNA candidates, transposon-aligned and unclassified small RNAs.

To perform statistical comparisons, sequence reads were collapsed to unique tags after adapter trimming using a fastx_collapser program from the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Raw read counts assigned to unique tags were compared between treatments and tissues. Normalization and statistical tests were performed using DESeq bioconductor package as described in the user's manual (Anders and Huber 2010). Reads with the sum raw counts ≤ 5 across all libraries that participated in a particular comparison were excluded from the analysis. The cutoff value for significance was $q < 0.2$ (the Benjamini-Hochberg method) (Benjamini and Hochberg 1995).

Precise mapping of all assorted small RNAs was done using MicroRazerS v 1.0 software with default settings (i.e. the first 16 nt-long ones were matched, no mismatch allowed) (Emde, Grunert et al. 2010). Only tags that were considered to be significantly changed ($q < 0.2$) were annotated. Due to the repetitive nature of some tag sequences, some single tag sequences had multiple annotations.

4.4. RESULTS

4.4.1. The examination of gene expression in *B. rapa* parental leaves, inflorescence meristem, pollen, unfertilized ovules, 24-hour post-fertilization ovules, embryo, endosperm and leaf tissues of progeny plants after heat shock treatment

The species *B. rapa* encompasses various economically important vegetable crops whose cultivation is acutely affected by heat, often in a combination with drought or other stresses (Yu, Wang et al. 2012). Although, considerable research efforts have been directed towards identifying genetic mechanisms of plant stress response, the improvement of crops is hindered by the lack of gene expression database related to heat stress response. More importantly, every tissue counters stress in a unique and definite way which leads to the development of the tissue-specific differential gene expression profile (Prandl, Kloske et al. 1995, Nylander, Svensson et al. 2001). Unfortunately, a publicly available gene expression database for economically important crops has not been developed. Notably, it is still vague how reproductive tissues in plants respond to stress compared to somatic tissues and to what extent oscillations in the parental transcriptome are transmitted to untreated progenies.

Hence, we profiled the transcriptomes of reproductive and somatic tissues in response to HS stress in *B. rapa* plants and in the untreated progeny. Three main goals were set: 1) to generate the first comprehensive gene expression database of somatic and reproductive tissues of *B. rapa*; 2) to assess alterations in gene expression pattern in *B. rapa* plants subjected to HS treatment; 3) to monitor stress-induced transgenerational changes in the transcriptome profile observed in plants.

The transcriptome libraries were generated from the total RNA of somatic and reproductive tissues of control and exposed parental *B. rapa* plants as well as from the untreated progeny (Table 4.4.1). Subsequently, the libraries were sequenced using the Illumina GAIIx platform followed by primary and secondary data analysis. In the experiment, 16 sequencing libraries were generated in two biological replicates with an average of 11,723,384 reads per library and genome-matched reads comprised on average 67.70% of the total reads (Table 4.4.1). Unfortunately, whole genome transcriptome sequencing data for *B. rapa* species that we could use to compare our data with are not available yet.

Table 4.4.1. The characteristics of transcript reads of control and heat shock treated tissues of *B. rapa* plants

Library ID	Description	The total number of library reads	Percentage of reads mapped to gDNA
CT Leaves	Leaf samples from untreated plants	8,913,668	64.13
TR Leaves	Leaf samples from exposed plants	8,111,316	64.48
CT Inflorescence meristem	Inflorescence meristem of untreated plants	13,992,893	65.29
TR Inflorescence meristem	Inflorescence meristem of exposed plants	21,031,116	78.38
CT Pollen	Mature pollen from untreated plants	6,128,711	66.54
TR Pollen	Mature pollen from exposed plants	14,386,477	81.49
CT Unfertilized ovules	Ovules from emasculated flowers of untreated plants	10,112,352	62.28
TR Unfertilized ovules	Ovules from emasculated flowers of exposed plants	15,684,059	74.35
CT Fertilized ovules	24-hour seeds from emasculated pollinated flowers of untreated plants	16,766,263	73.99
TR Fertilized ovules	24-hour seeds from emasculated pollinated flowers of exposed plants	10,461,971	67.54
CT Embryo	Mature green embryo dissected from seeds of untreated plants	9,261,796	67.67
TR Embryo	Mature green embryo dissected from seeds of exposed plants	10,167,508	67.58
CT Endosperm	Mature green endosperm dissected from seeds of untreated plants	9,867,388	61.58
TR Endosperm	Mature green endosperm dissected from seeds of exposed plants	10,393,140	63.92
CT Progeny	Leaf samples from the progeny of untreated plants	12,352,719	61.89
TR Progeny	Leaf samples from the progeny of exposed plants	9,942,780	62.11
Average		11,723,384	67.70

4.4.1.1. Heat stress treatment resulted in tissue-specific alterations in the transcriptome in *B. rapa* plants

Previous reports have shown an explicit proof of tissue-specific changes in gene expression in *A. thaliana* plants subjected to biotic and abiotic stress (Kreps, Wu et al. 2002, Ma and Bohnert 2007). In our study, we observed unique tissue-specific alterations in mRNA accumulation in heat-shock treated *B. rapa* plants, with a nearly even representation of the number of up- and down-regulated genes (Figure 4.4.1). Whereas in the leaves of parental plants that were directly exposed to stress, we detected 572 differentially expressed genes as compared to untreated controls (the Benjamini-Hochberg method, $q<0.05$, Figure 4.4.1A); in the inflorescence meristem, derived from the exposed shoot apical meristem, there were only 75 differentially expressed genes as compared to untreated controls (Figure 4.4.1B). Both paternal and maternal reproductive tissues responded to HS with little differential expression of genes (3, 80 and 28 differentially expressed genes for pollen, unfertilized ovules and fertilized ovules, respectively, Figures 4.4.1C, D and E).

Enigmatically, the highest oscillations in gene expression was observed in tissues that were not directly exposed to HS – the endosperm (6533 differentially expressed genes, $q<0.05$, Figure 4.4.1G) as compared to control tissues. Such behaviour of the endosperm could possibly be attributed to global genome demethylation and a decrease in the expression of the silencing-related genes as compared to other tissues that were previously reported to occur in the *Arabidopsis* endosperm under normal conditions (Hsieh, Ibarra et al. 2009, Jullien, Susaki et al. 2012). Considerable oscillations of gene expression were also observed in the embryo, albeit to a lesser extent than that in the

endosperm (1323 differentially expressed genes, $q<0.05$, Figure 4.4.1F). Leaves collected from the progeny of stressed plants demonstrated a moderate response to HS (117 differentially expressed genes, $q<0.05$, Figure 4.4.1E).

The analysis showed that despite the fact that drastic changes in gene expression were observed in the embryo and endosperm tissues, these changes were not passed on to the progeny. This suggests that the reversal of changes in gene expression takes place either during the final steps of seed maturation or later throughout seed germination and plantlet development. Additionally, unfertilized and 24-hour post-fertilization ovules also responded to HS treatment with moderate changes in the gene expression profile (Figure 4.4.1D and E), therefore it may be suggested that alterations in the transcriptional activity of genes occur during stages of cell division/expansion of seed development and proceed into green embryo and endosperm tissues (Le, Cheng et al. 2010). Noteworthy, since we did not control the actual fertilization of ovules in emasculated flowers that were pollinated manually, we were unable to rule out the possibility that fertilized ovule tissue samples could have been contaminated with unfertilized embryo sacs. This could eventually affect the sequencing outcome in ovule tissue samples at 24 hours post-fertilization. Nevertheless, a comparison of unfertilized and fertilized ovule transcriptoms from control plants revealed 791 significantly differentially expressed genes ($q<0.05$, the Benjamini-Hochberg method) with 603 being up-regulated in fertilized ovules (data not shown), which suggests that in our experiment, fertilized and unfertilized ovule samples were indeed different. However, it does not completely rule out the presence of unfertilized ovules in fertilized ovule samples.

A unique tissue-specific pattern of transcriptome fluctuations following HS stress was further observed in the comparison analysis (Figure 4.4.2). The most pronounced overlap of differentially expressed genes was seen between embryo and endosperm (1240 commonly changed genes, Figure 4.4.2C and D) and then between embryo and leaves (51 commonly changed genes, Figure 4.4.2E). The untreated progeny of plants stressed by HS had the highest overlap of differentially expressed genes with the endosperm (31 commonly changed genes, Figure 4.4.2D) followed by the inflorescence meristem (15 commonly changed genes, Figure 4.4.2E) and embryo (13 commonly changed genes, Figure 4.4.2E). Surprisingly, embryo as well as unfertilized and fertilized ovules of stressed parental plants had the lowest number of common differentially expressed genes, which can conceivably be attributed to the overall quiescent response of maternal reproductive organs to stress (Figure 4.4.2B and C).

Transcriptome sequencing data were further confirmed in the progeny of stressed and non-stressed plants for four differentially expressed genes using the qPCR method (Table 4.4.2, $q<0.05$, the Benjamini-Hochberg method).

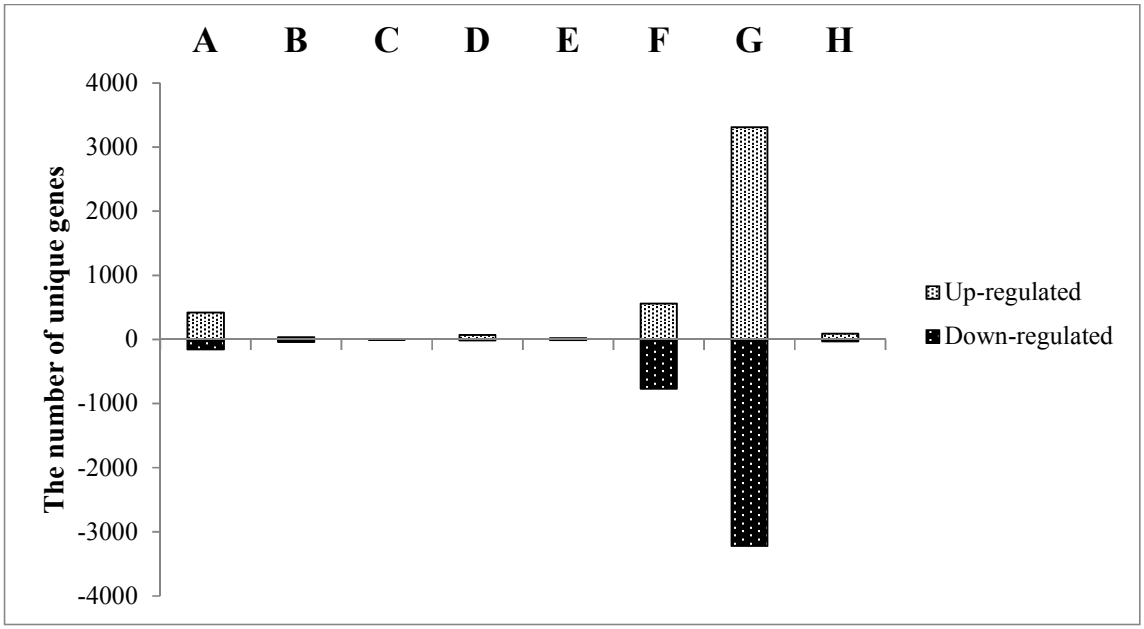

Figure 4.4.1. The effect of exposure to elevated temperature on gene expression in tissues of parental plants and leaves of untreated progeny plants of *Brassica rapa*

A – Leaves, **B** – Inflorescence meristem, **C** – Pollen, **D** – Unfertilized ovules, **E** – Fertilized ovules, **F** – Embryo, **G** – Endosperm, **H** – Progeny. Bars represent the number of unique differentially expressed genes in response to HS in parental tissues and progeny, the Benjamini-Hochberg method, $q<0.05$.

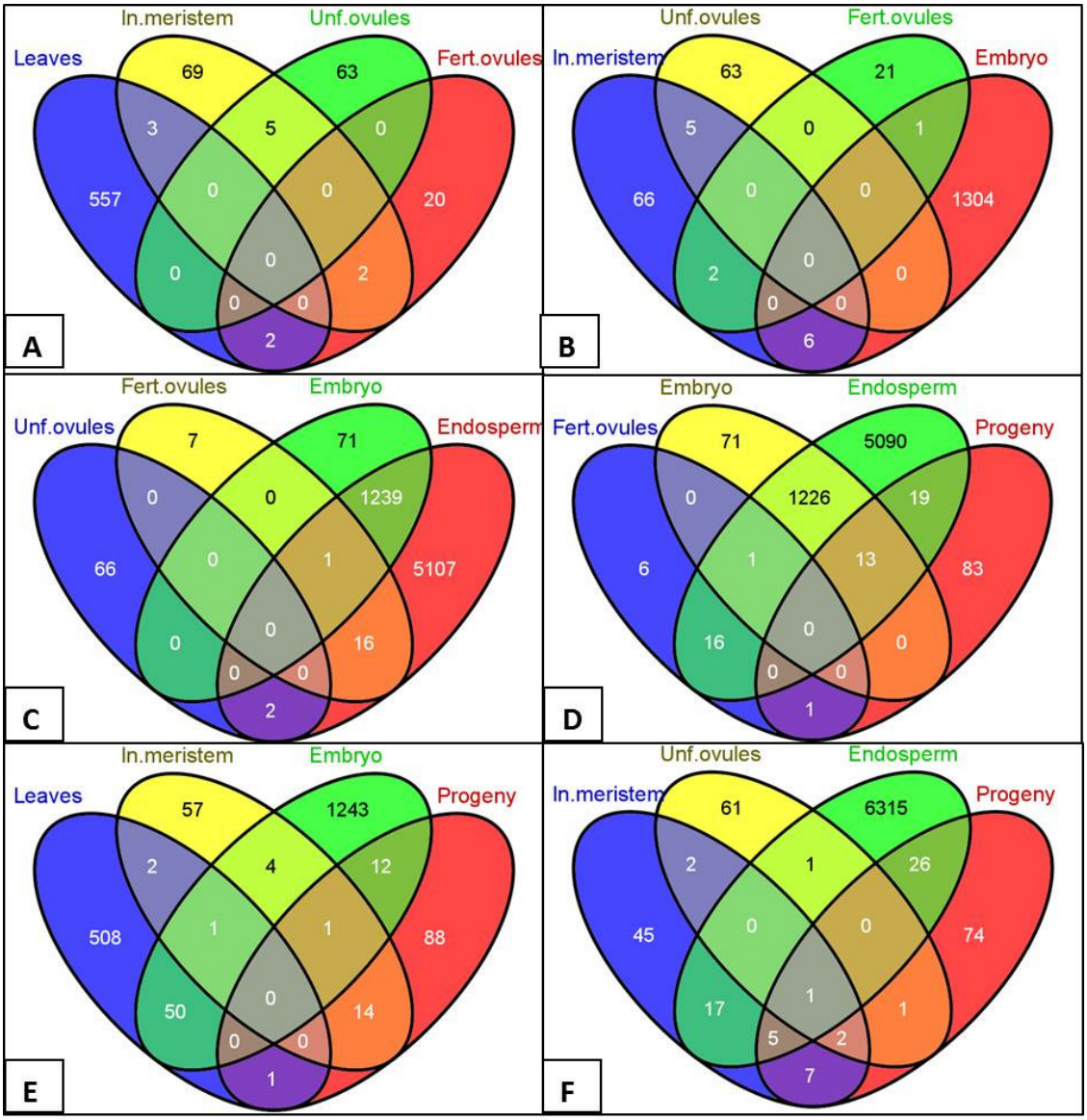

Figure 4.4.2. Venn diagrams representing common genes the expression of which was significantly changed in selected tissues

Gene IDs of differentially expressed genes were used to create the Venn diagrams using the Venny program (Oliveros). Labels: In. meristem – Inflorescence meristem, Unf. ovules – Unfertilized ovules, Fert. ovules – Fertilized ovules.

Table 4.4.2. Validation of transcriptome sequencing data in the leaves of progeny of control and stressed plants of *B. rapa* using qPCR

Gene ID	log2 Fold change TR vs CT, sequencing	q-value, sequencing	log2 Fold change TR vs CT, qPCR	p-value, qPCR	Significant	SWISS-PROT annotation
Bra029235	5.98	1.50E-04	5.37	5.03E-04	Yes	BAT42_CAEEL BTB and MATH domain-containing protein 42 OS=*Caenorhabditis elegans*
Bra031065	5.96	1.92E-03	3.79	9.04E-03	Yes	TI10A_ARATH Protein TIFY 10A OS=*Arabidopsis thaliana*
Bra029719	-4.65	3.43E-02	-3.71	2.25E-03	Yes	NLTP6_ARATH Non-specific lipid-transfer protein 6 OS=*Arabidopsis thaliana*
Bra040903	-3.53	4.37E-07	-3.24	1.69E-02	Yes	UBP13_ARATH Ubiquitin carboxyl-terminal hydrolase 13 OS=*Arabidopsis thaliana*

qPCR gene expression analysis was done as described previously (see Section 3.3.2.5. cDNA synthesis and qPCR gene expression analysis). The average of four reactions (two dilutions per each of two cDNA preparations stemming from two independent tissue samples) was obtained, and the normalized expression ratio was calculated using $2^{-\Delta\Delta CT}$ method. The statistical significance of sequencing reads and qPCR expression analysis was assessed using the Benjamini-Hochberg method ($q<0.05$) and Student's t-test ($p<0.05$), respectively.

4.4.1.2. Gene ontology annotation of differentially expressed genes in tissues of *B. rapa* plants subjected to heat shock

Differentially expressed genes in all tissues were further grouped into gene ontology categories according to the biological process they participate in using a Blast2GO v 2.6.2 software with default settings (Conesa, Gotz et al. 2005). As expected, the majority of genes in all tissues fall into the gene ontology (GO) category "response to stress" (Figure 4.4.3 and Figure 4.4.4), with the only exception of unfertilized ovules (Figure 4.4.3C) where the GO category "response to stress" was absent. Apparently it can be due to the overall moderate transcriptome oscillations in maternal reproductive tissue in response to HS stress (Figure 4.4.1D).

Differentially expressed genes in the leaves, embryo and endosperm tissues fell into the largest number of GO categories – 11 in total (Figure 4.4.4A and B). In contrast, altered genes in unfertilized and fertilized ovules belonged only to 8 and 7 GO categories, respectively (Figure 4.4.3C, D). The GO categories "response to stress" and "protein metabolic process" were some of the largest GO categories observed among selected tissues. Curiously, the GO categories "response to stress", "response to biotic stimulus" and "response to abiotic stimulus" were the most pronounced in the leaves of progeny of stressed plants, which probably suggests the existence of the selective mechanism of transgenerational gene priming in plants (Figure 4.4.4C) (Luna, Bruce et al. 2012, Slaughter, Daniel et al. 2012).

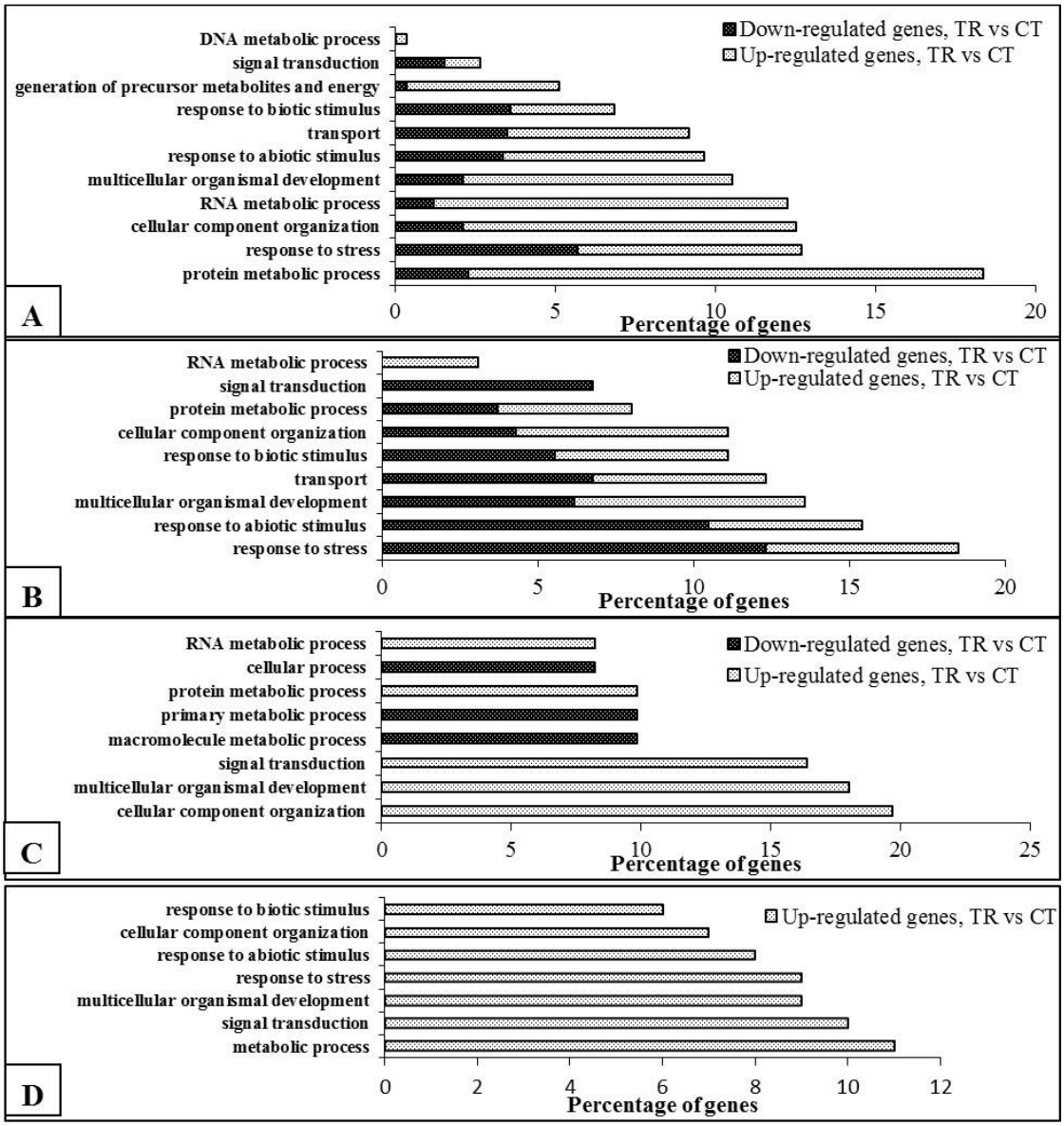

Figure 4.4.3. Gene ontology annotation of differentially expressed genes in selected *B. rapa* tissues

A – Leaves, **B** – Inflorescence meristem, **C** – Unfertilized ovules, **D** – Fertilized ovules. Coding sequences of differentially expressed genes (TR vs CT) were extracted from the *B. rapa* transcriptome database v 1.2 and loaded as a FASTA file into Blast2GO v 2.6.2 software for the NCBI BLAST similarity search using blastx option (Conesa, Gotz et al. 2005). Further, the recovered ontologies were annotated and grouped into gene ontology categories using default settings. Gene ontology (GO) nodes were combined into the most prominent categories using a GO-slim-TAIR tool and represented as the percentage of up- and down-regulated genes in the corresponding GO category.

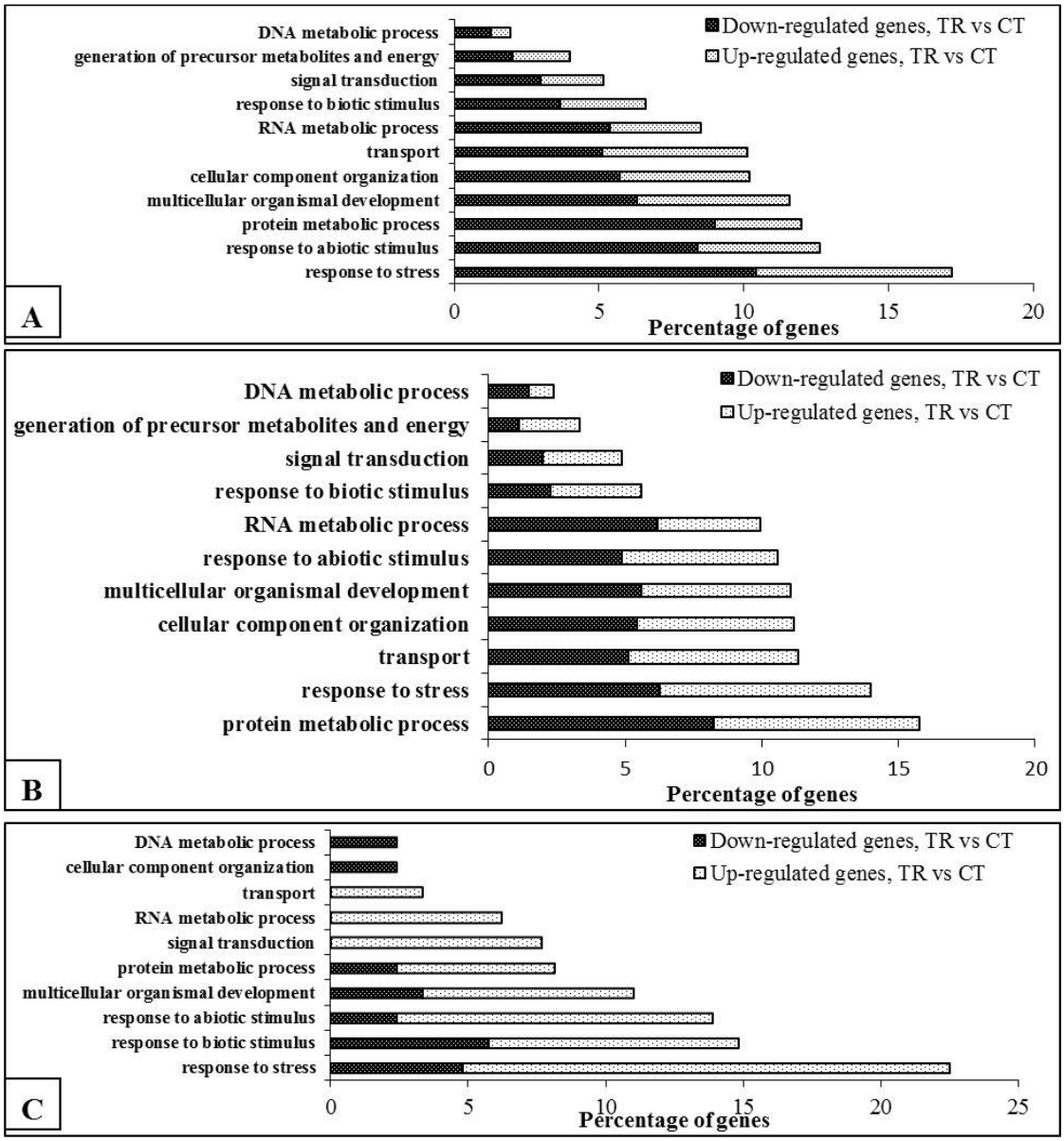

Figure 4.4.4. Gene ontology annotation of differentially expressed genes in selected *B. rapa* tissues

A – Embryo, **B** – Endosperm, **C** – Progeny. Coding sequences of differentially expressed genes (TR vs CT) were extracted from the *B. rapa* transcriptome database v 1.2 and loaded as a FASTA file into Blast2GO v 2.6.2 software for the NCBI BLAST similarity search using blastx option (Conesa, Gotz et al. 2005). Further, the recovered ontologies were annotated and grouped into gene ontology categories using default settings. Gene ontology (GO) nodes were combined into the most prominent categories using a GO-slim-TAIR tool and represented as the percentage of up- and down-regulated genes in the corresponding GO category.

4.4.2. Small RNA transcriptome analysis of *B. rapa* parental leaves, inflorescence meristem, pollen, unfertilized ovules, 24-hour post-fertilization ovules, embryo, endosperm and leaf tissues of progeny plants after heat shock treatment

The reproduction of angiosperms encompasses a specific and multifarious chain of events that leads to the production of highly specialized male and female gametes during late post-embryonic development directly from established somatic cell lineages (Dickinson and Grant-Downton 2009, Grant-Downton and Rodriguez-Enriquez 2012). The essential requirement for the smRNA pathways to operate and control somatic and reproductive development has been shown previously in *dicer-like1* (*dcl1*) and *argonaute1* (*ago1*) null mutants impaired in miRNA metabolism (Golden, Schauer et al. 2002, Vaucheret, Vazquez et al. 2004, Nodine and Bartel 2010). Hence, it is safe to assume that alterations in the smRNAome profile in somatic tissues would result in differential smRNA expression in gametes and eventually in progeny. Unfortunately, to date the information about possible influences of adverse environmental factors on smRNA metabolism in plant gametes is scarce. Moreover, a possible impact of such smRNAs on the progeny of stressed plants is unknown.

Illumina small RNA sequencing was performed to compare alterations in the smRNA transcriptomes in somatic and reproductive tissues of *B. rapa* plants and in the progeny in response to HS treatment. We hypothesized that it will allow us to reveal possible messengers of transgenerational stress memory inheritance in plants. For smRNA sequencing, we used the same tissues as for mRNA sequencing. The experiment contained 16 sequencing libraries (8 from stressed and 8 from non-stressed plants) in two biological replicates. An average of 4,873,042 reads per library was achieved. Genome-

matched reads comprised on average 52.68% of the total reads (Table 4.4.3), and it was comparable with the previously published report on Chinese cabbage (56.96% of genome-mapped smRNA reads) (Wang, Li et al. 2012). Strikingly, we observed drastic fluctuations in the percentage of genome-mapped sequencing reads: the highest percentage was observed in the inflorescence meristem (79.57% mapped reads on average) and the lowest one – in leaves (25.71% mapped reads on average). These unclassified reads may originate from intragenic or other unannotated regions of the genome. This is not surprising as the *B. rapa* genome has only recently been sequenced and is still poorly annotated.

Table 4.4.3. Small RNA sequencing libraries generated from the total RNA of control and heat shock treated tissues of *B. rapa* plants

Library ID	Description	The total number of library reads	Percentage of aligned reads to gDNA elements
CT Leaves	Leaf samples from untreated plants	3,380,794	25.22
TR Leaves	Leaf samples from exposed plants	3,483,667	26.20
CT Inflorescence meristem	The inflorescence meristem of untreated plants	9,127,684	80.29
TR Inflorescence meristem	The inflorescence meristem of exposed plants	4,831,492	78.84
CT Pollen	Mature pollen from untreated plants	5,465,212	80.18
TR Pollen	Mature pollen from exposed plants	5,221,604	74.63
CT Unfertilized ovules	Ovules from emasculated flowers of untreated plants	4,474,129	44.49
TR Unfertilized ovules	Ovules from emasculated flowers of exposed plants	2,462,572	69.45
CT Fertilized ovules	24-hour seeds from emasculated pollinated flowers of untreated plants	14,151,236	49.94
TR Fertilized ovules	24-hour seeds from emasculated pollinated flowers of exposed plants	2,660,009	44.44
CT Embryo	Mature green embryo dissected from the seeds of untreated plants	3,827,441	53.64
TR Embryo	Mature green embryo dissected from the seeds of exposed plants	4,927,259	53.87
CT Endosperm	Mature green endosperm dissected from seeds of untreated plants	1,991,580	39.67
TR Endosperm	Mature green endosperm dissected from seeds of exposed plants	5,001,153	52.51
CT Progeny	Leaf samples from the progeny of untreated plants	2,895,793	40.84
TR Progeny	Leaf samples from the progeny of exposed plants	4,067,056	33.19
Average		4,873,042	52.68

4.4.2.1. Mapping and general compositional analysis of small RNA libraries

A general compositional analysis of smRNA libraries revealed substantial variations in relative smRNA enrichment among tissues, whereas differences between control and exposed groups within the same tissue were not so pronounced (Figure 4.4.5). Unexpectedly, the overwhelming majority of aligned sequencing reads were mapped to gDNA gene regions (30.29% of library reads on average) followed by those ones mapped to transposons (15.88% of library reads on average) and miRNAs (4.76% of library reads on average). The remaining classified reads comprised of Rfam database (v 10.01) mapped smRNAs (1.38% of library reads on average), ta-siRNA candidates (0.23% of library reads on average) and miRNA candidates (0.14% of library reads on average). Curiously, we observed considerable differences in the relative smRNA library composition between parental somatic tissues such as leaves and the inflorescence meristem and between paternal and maternal reproductive tissues. The most abundant fraction of smRNAs in leaves was unclassified (74.33% of library reads on average in control and treatment groups) followed by gene-mapped smRNAs (13.71% of library reads on average) and transposon-mapped smRNAs (6.66% of library reads on average). Conversely, in the inflorescence meristem, gene-mapped smRNAs represented the major fraction of sequencing reads (38.31% of library reads on average) followed by transposon-mapped smRNAs (25.77% of library reads on average) and unclassified ones (20.15% of library reads on average). Whereas the highest enrichment of gene-mapped smRNAs among libraries was observed in pollen (53% of library reads), the inflorescence meristem had the highest relative expression of transposon-mapped smRNAs and miRNAs (25.77% and 12.84% of library reads on average, respectively).

Both unfertilized and fertilized ovules had the comparable relative level of gene-mapped smRNAs (31.29% and 29.70% on average, respectively) and miRNAs (2.69% and 3.14% on average, respectively). In the embryo and endosperm tissues, almost similar levels of gene-mapped smRNAs (28.66% and 28.24% on average, respectively) and miRNAs were observed (2.27% and 1.98% on average, respectively). At the same time, both control and exposed embryo tissues had almost a 1.5-fold higher relative number of transposon-mapped smRNAs as compared to endosperm tissues (22.19% and 15.12% on average, respectively). The leaf tissues from the progeny demonstrated on average 21.06%, 11.81% and 4.66% of library reads mapped to such prominent genomic elements as genes, transposons and conservative miRNAs, respectively.

The most pronounced alterations in the library composition after HS stress were observed in unfertilized ovule tissues with a 9.27% and 8.15% increase in the number of transposon- and gene-mapped smRNA reads as compared to control, respectively (Figure 4.4.5). Changes in the endosperm were less pronounced – there was a 3.85% and 8.24% increase in the number of transposon- and gene-mapped smRNA sequences, respectively. Curiously, the progeny of stressed plants had a detectable decrease in the number of smRNA reads mapped to genes (6.66% as compared to control) and to transposons (3.61% as compared to control, Figure 4.4.5), which was in accordance with the overall up-regulation of gene expression observed in the progeny of stressed as compared to control plants (Figure 4.4.1H). Surprisingly, leaf tissues that were directly subjected to stress suffered only minor oscillations in the smRNA library composition as compared to control, albeit both control and treated smRNA libraries had the highest relative amount

of unclassified reads among all tissues that conceivably could contain responsive smRNAs (Figure 4.4.5).

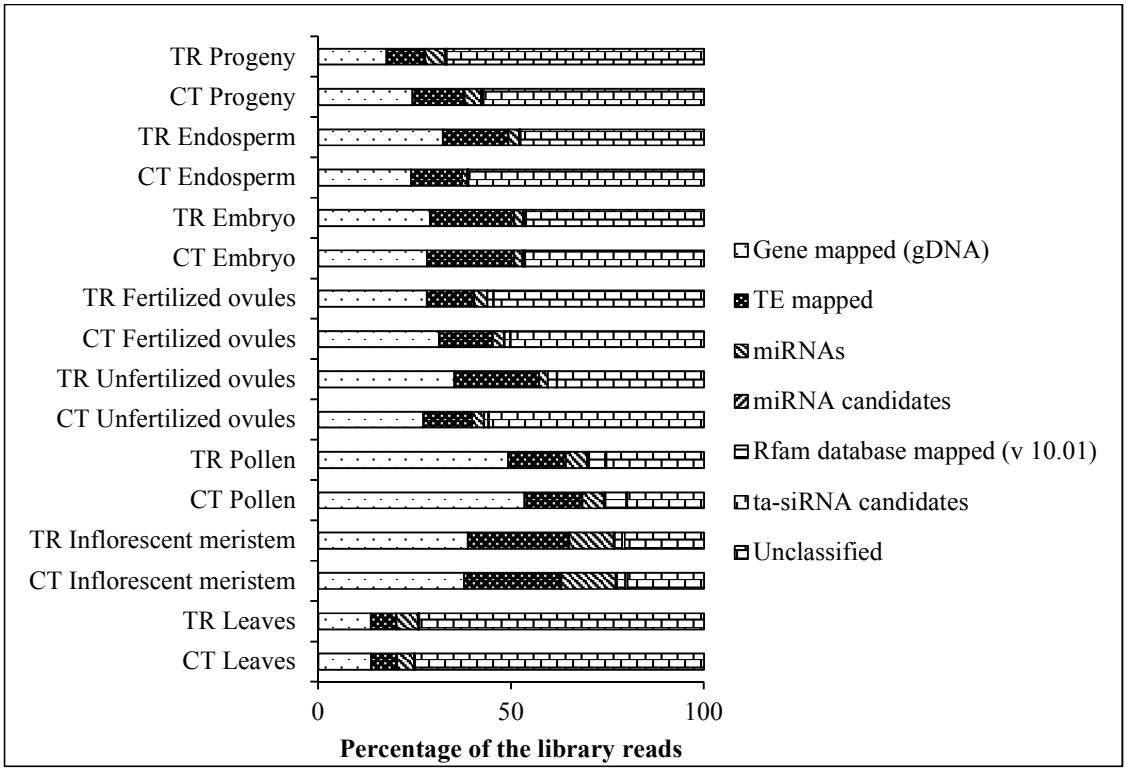

Figure 4.4.5. A general compositional analysis of small RNA libraries sequenced from the total RNA of corresponding tissues

The length of stacked bars represents the percentage of smRNA fraction occupied by a specific smRNA type in the corresponding library.

4.4.2.2. The length distribution and the 5′ nucleotide bias of small RNA libraries

Previous reports have shown a conservative pattern of the smRNA length distribution in plants which is compatible with DICER-dependent transcriptome processing (Rajagopalan, Vaucheret et al. 2006, Fahlgren, Howell et al. 2007, Moxon, Jing et al. 2008, Szittya, Moxon et al. 2008, Song, Wang et al. 2010, Chi, Yang et al. 2011). We observed the overall bimodal length distribution of smRNA sequencing reads in all tissues (Figure 4.4.6) with the major peak at 24 nucleotides (40.19% of all reads on average) and a smaller shoulder at 21 nt (18.30% of all reads on average), which was consistent with published data on *B. rapa* plants (He, Fang et al. 2008, Wang, Li et al. 2012). Patterns for the 21- and 24-mers distribution were dissimilar between tissues. In a stark contrast to other tissues, the 21 nt smRNA fraction was predominant in leaves (28.06% on average) followed by 24 nt-long smRNAs (22.20% on average, Figure 4.4.6A). Also, an equivalent accumulation of 21 and 24 nt-long reads was observed in the leaves of two-week-old progenies (28.42 and 29.55% for 21 and 24 nt-long reads, respectively, Figure 4.4.6H). This is partially in agreement with the previous demonstrating 21-mers composed the major fraction of the smRNA library from the four-week-old seedlings of *B. rapa* (subsp. *oleifera*) followed by the 24 nt-long fraction (39.9 and 22.94% of 21 and 24 nt-long reads of the total smRNA library, respectively) (He, Fang et al. 2008). Curiously, whereas paternal (Figure 4.4.6C) and maternal (Figure 4.4.6D, E) reproductive tissues had a different distinguishable pattern of smRNA library length distribution, the length of smRNA reads derived from the inflorescence meristem (Figure 4.4.6B), embryo (Figure 4.4.6F) and endosperm (Figure 4.4.6G) tissues was

highly similar. Sequencing reads from pollen contained both the 21 (14.98% on average) and 24 nt-long (20.12% on average) fractions of smRNAs plus an additional biologically undefined 17 nt-long (17.09% on average) fraction. Such pattern was not observed in smRNA reads of unfertilized and fertilized ovules (Figure 4.4.6D and E, respectively); only a small percentage of smRNAs belonged to 21-mers (8.16% on average), with the majority of reads belonging to 24 nt smRNA fraction (58.14% on average). None of these tissues except pollen responded to HS with drastic detectable fluctuations in the relative smRNA length distribution (Figure 4.4.6C). The most prominent alterations were observed in the 24 nt-long smRNA fraction (a 1.4 fold increase as compared to control, Student's t-test, α=0.05), which usually corresponds to the smRNA fraction deriving from heterochromatic genomic regions in angiosperms (Axtell 2013).

The length distribution analysis of sequencing reads mapped to the prominent genomic sequence categories revealed that the majority of gene- (Figure 4.4.7) and transposon-mapped (Figure 4.4.8) smRNAs fell mostly into the 24 and 21 nt-long categories in all tissues except pollen. In male reproductive tissues, a vast amount of low range small RNAs (19-mers and smaller) observed in the total smRNA library were solely mapped to gene regions in gDNA (17.09% and 16.97% of the relative fraction of 17 nt-long reads in the total smRNA and gene-mapped smRNA libraries, respectively, Figure 4.4.7C), which suggests that a tissue-specific transcriptome degradation process takes place in pollen. Similar results were previously reported in mature Arabidopsis pollen where 16 nt-long reads were prevailing in the smRNA sequencing library. Unfortunately, the authors did not explain the origin of small size smRNAs in pollen (Grant-Downton, Le Trionnaire et al. 2009).

Transposable element (TE)-mapped smRNAs in male tissues were distributed between a major peak of 24 nt-long reads (3.96% of the total reads on average) and an unusually broad but minor peak stretching from 17 to 22 nt in length (Figure 4.4.8C). Previous reports on smRNA metabolism in Arabidopsis pollen (Slotkin, Vaughn et al. 2009) give us a reason to speculate that low range smRNAs can apparently be non-cell autonomous silencing signals generated in the vegetative nucleus to suppress the TE activity in sperm cells. We also observed the redistribution of smRNAs following HS in pollen. The fractions of 19 and 20 nt-long reads suffered a decline in the relative expression (a 1.5-fold decline on average), whereas the 24 nt-long smRNA portion demonstrated a 1.4-fold increase upon HS treatment as compared to control (Student's t-test, α=0.05).

A detailed examination of the prevailing 24 nt-long fraction of sequencing reads revealed a common strong bias for adenine at the 5′ terminal nucleotide in all tissue libraries of *B. rapa* plants, which is in accordance with previous studies on Arabidopsis and *B. napus* plants (Figure 4.4.9) (Mi, Cai et al. 2008, Zhao, Wang et al. 2012). The highest degree of conservation at the 5′ terminal nucleotide was observed in leaf tissues (69% on average of the 24-nt long reads had the 5′-A terminal nucleotide, Figure 4.4.9A), whereas the lowest degree of conservation was found in pollen (51%, Figure 4.4.9C). Since in our experiment, smRNAs were not directly co-precipitated with ARGONAUTE proteins, we can only speculate about their biogenesis and support our conclusions by indirect evidence. According to the previous report on Arabidopsis, 24 nt-long smRNAs with the dominant 5′-A terminal nucleotide satisfy the requirements for AGO4-processed smRNAs that belong to repeat-associated and heterochromatic siRNAs

(Mi, Cai et al. 2008). Eventually, these smRNAs may guide sequence-specific alterations of gene and/or transposon activity in the genome through the RNA-dependent DNA methylation process (Axtell 2013). Noteworthy, the rest of sequencing reads demonstrated a lower bias for a particular nucleotide at the 5´ terminus; and HS stress had no significant effect on the accumulation of 24 nt-long reads with the 5´- A terminal nucleotide in all tissue libraries as compared to control (data not shown).

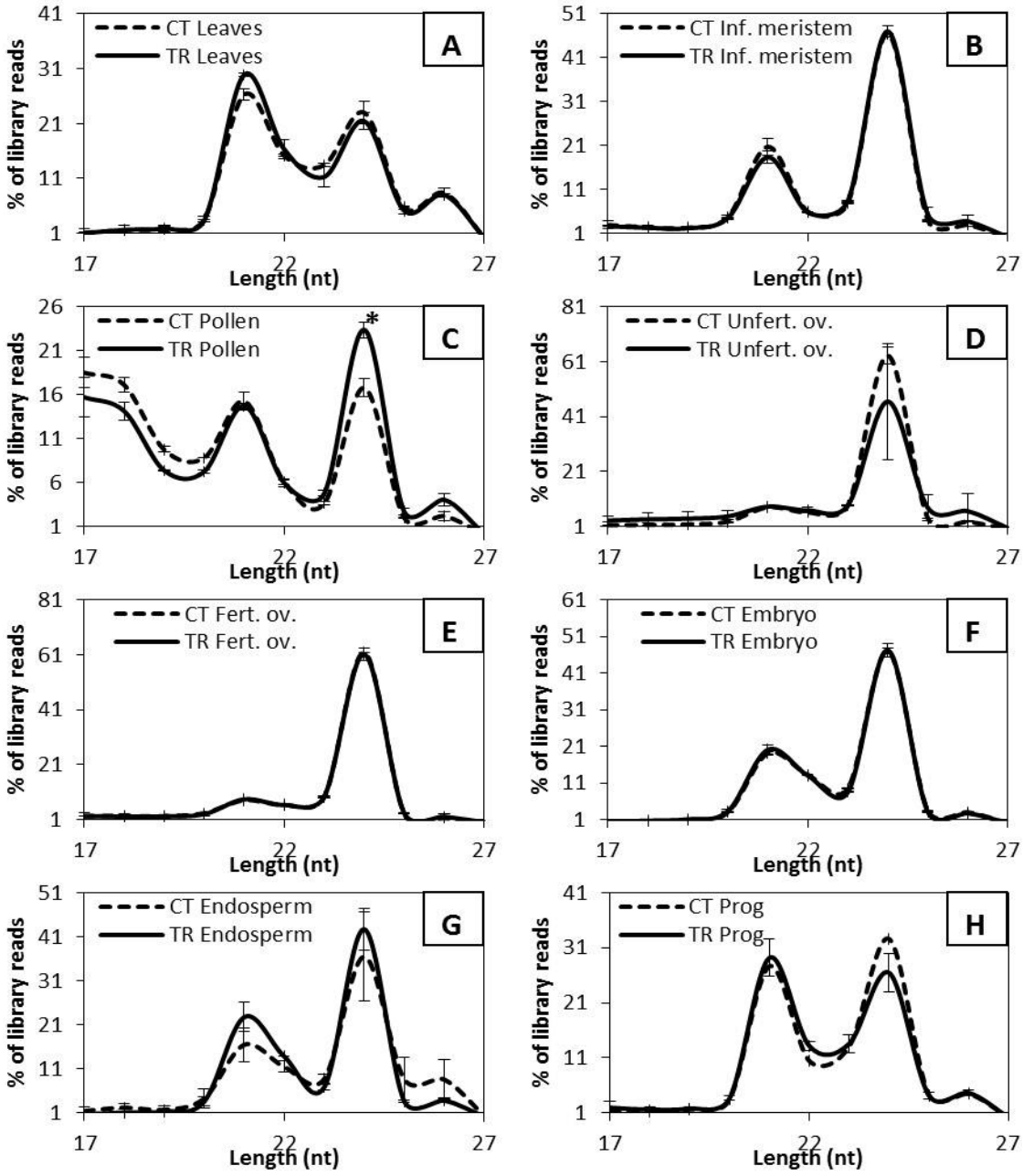

Figure 4.4.6. The relative length distribution of sequencing reads from the total small RNA library of the corresponding tissues

A – Leaves, **B** – Inflorescence meristem, **C** – Pollen, **D** – Unfertilized ovules, **E** – Fertilized ovules, **F** – Embryo, **G** – Endosperm, **H** – Progeny. The values represent the mean ± SD of the relative enrichment of smRNA fraction in the total library of the corresponding tissue. An asterisk shows a statistically significant difference as compared to the control library of the corresponding tissue (Student's t-test: α=0.05, t=2.45).

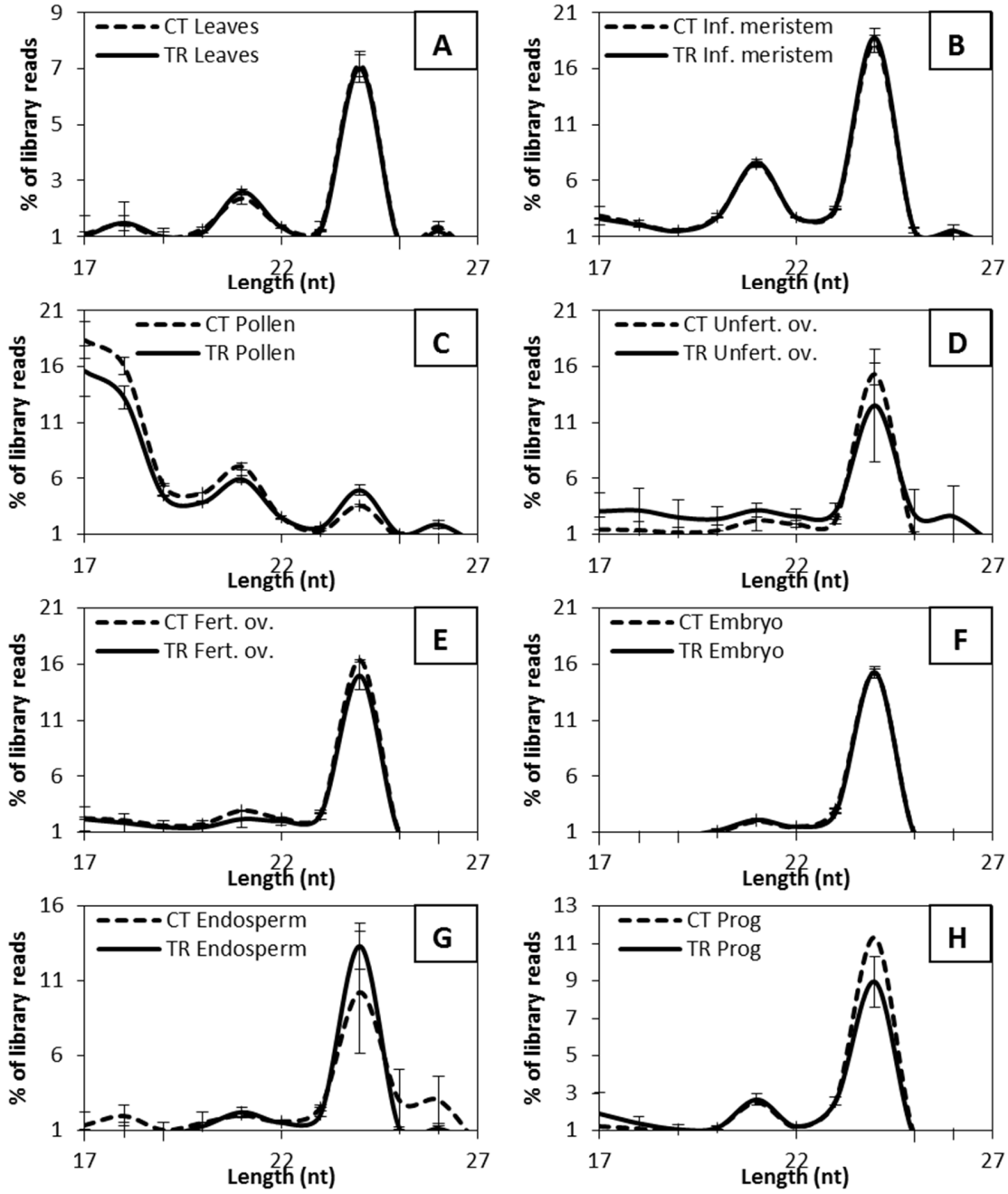

Figure 4.4.7. The relative length distribution of sequencing reads mapped to gene regions in the total small RNA library of the corresponding tissue

A – Leaves, **B** – Inflorescence meristem, **C** – Pollen, **D** – Unfertilized ovules, **E** – Fertilized ovules, **F** – Embryo, **G** – Endosperm, **H** – Progeny. The values represent the mean ± SD of the relative enrichment of smRNA fraction in the total library of the corresponding tissue.

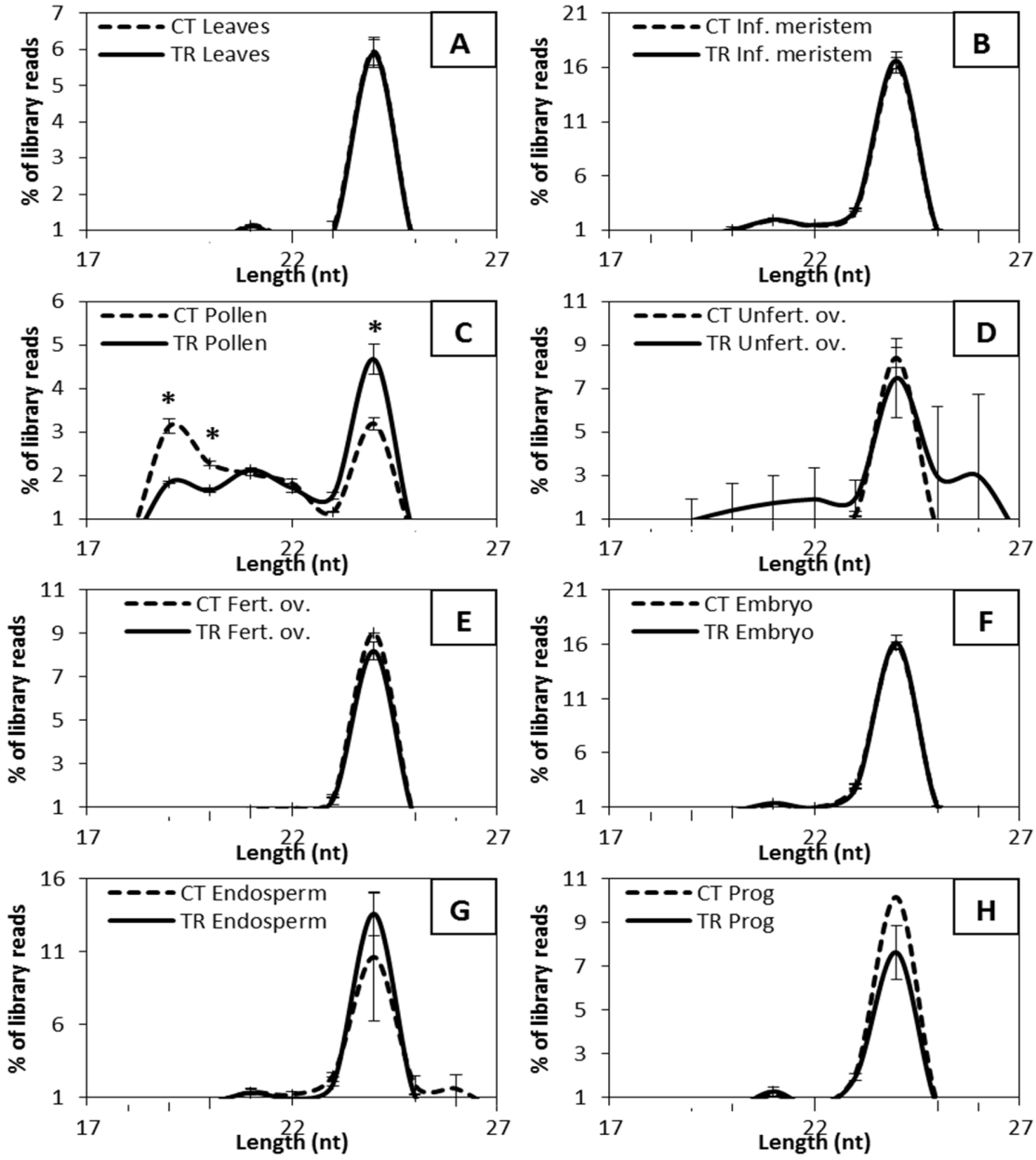

Figure 4.4.8. The relative length distribution of sequencing reads mapped to transposon element regions in the total small RNA library of the corresponding tissue

A – Leaves, **B** – Inflorescence meristem, **C** – Pollen, **D** – Unfertilized ovules, **E** – Fertilized ovules, **F** – Embryo, **G** – Endosperm, **H** – Progeny. The values represent the mean ± SD of the relative enrichment of smRNA fraction in the total library of the corresponding tissue. The asterisks show a statistically significant difference as compared to the control library of the corresponding tissue (Student's t-test: α=0.05, t=2.45).

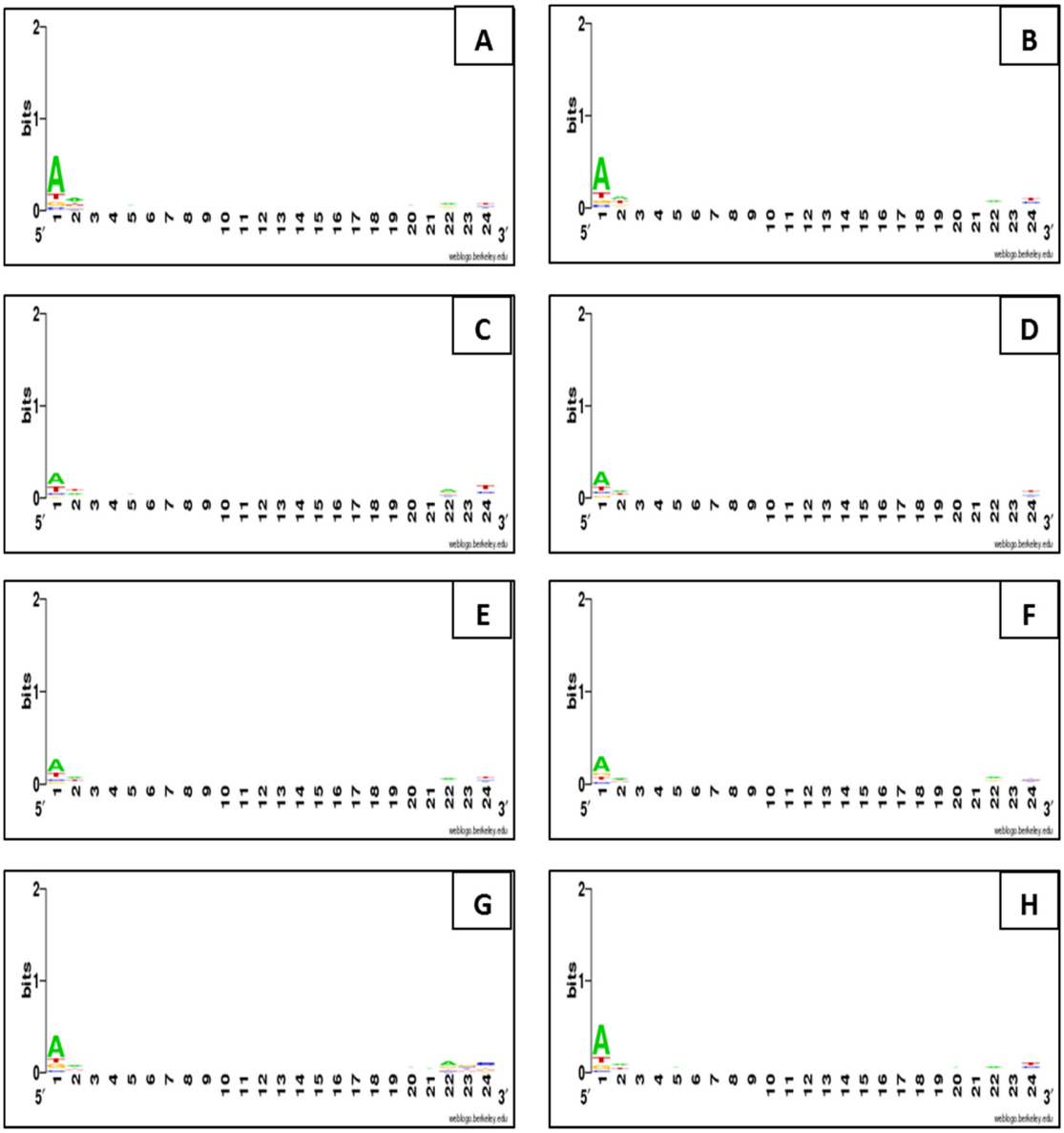

Figure 4.4.9. The relative nucleotide bias at each position of small RNAs in 24 nt-long small RNA reads

A – CT Leaves, **B** – CT Inflorescence meristem, **C** – CT Pollen, **D** – CT Unfertilized ovules, **E** – CT Fertilized ovules, **F** – CT Embryo, **G** – CT Endosperm, **H** – CT Progeny. The graphics were made by using the 24 nt-long sequencing reads from the corresponding control library and a WebLogo software (Crooks, Hon et al. 2004). The sequence conservation at each position is indicated by the overall height of the stack of symbols (T, A, C, and G), while the relative frequency of each nucleotide is represented by the height of the corresponding symbol.

4.4.2.3. Pollen, endosperm and leaf tissues of progeny plants demonstrate significant alterations in the small RNAome profile in response to heat shock

Despite the fact that we did not detect significant fluctuations in the sequence read length distribution in response to HS, considerable changes in differential expression of smRNAs were observed in all tissues except unfertilized ovules (Figure 4.4.10D). Surprisingly, the most striking alterations were detected in systemic tissues that were not directly exposed to stress such as pollen (621 differentially expressed smRNAs, Figure 4.4.10C), the endosperm (385 differentially expressed smRNAs, Figure 4.4.10G) and more importantly, in leaf tissues of the progeny (376 differentially expressed smRNAs, Figure 4.4.10H). Minor changes were recorded in leaves (12 differentially expressed smRNAs, Figure 4.4.10A), the inflorescence meristem (15 differentially expressed smRNAs, Figure 4.4.10B), fertilized ovules (31 differentially expressed smRNAs, Figure 4.4.10E) and embryo (8 differentially expressed smRNAs, Figure 4.4.10F). Strikingly, none of smRNAs responded to HS in unfertilized ovules (Figure 4.4.10D, the Benjamini-Hochberg method, $q<0.2$).

The detailed mapping and analysis of differentially expressed smRNAs showed a lower representation of gene-mapped siRNAs as compared to those ones derived from transposable elements in all tissues (566 and 823 in total for genes and transposons, respectively). Whereas in the endosperm, 86.75% of all differentially expressed smRNAs were up-regulated (Figure 4.4.10G), in pollen and leaves of the progeny, 59.58 and 89.89% of all differentially expressed smRNAs were down-regulated, respectively (Figure 4.4.10C and H). MiRNAs comprised a minor fraction with only 59 of them being differentially expressed *in toto*.

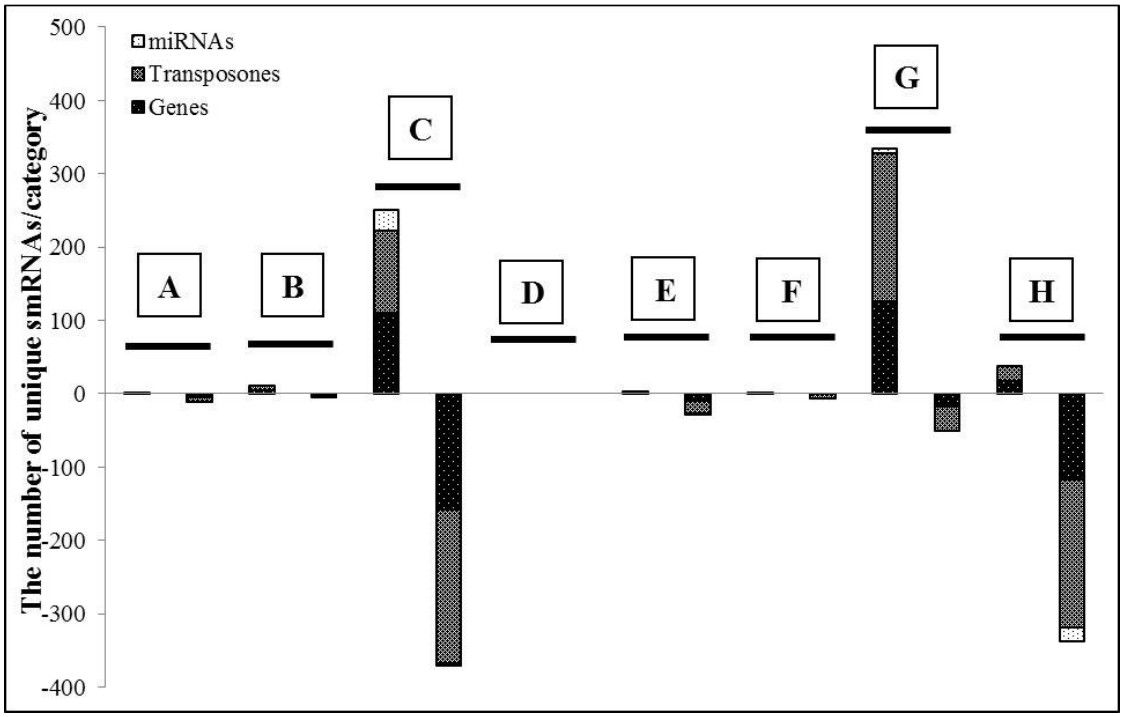

Figure 4.4.10. Differentially expressed small RNAs, TR vs CT

A – Leaves, **B** – Inflorescence meristem, **C** – Pollen, **D** – Unfertilized ovules, **E** – Fertilized ovules, **F** – Embryo, **G** – Endosperm, **H** – Progeny. The stacked bars represent the number of unique differentially expressed smRNAs per category in the corresponding tissue. SmRNAs were divided into 3 categories regarding genome regions they were mapped to: miRNAs, transposon and gene regions. The first and second stacked bars show the number of smRNAs with the positive and negative log2fold change values (TR vs CT) in corresponding tissue, respectively (the Benjamini-Hochberg method, $q<0.2$).

4.4.2.4. Differentially expressed siRNAs are mapped to genes that are unique for every tissue

To reveal the commonalities in action of altered siRNAs mapped to gene regions, we extracted their gene IDs and used them for inter-tissue comparisons. Mature siRNAs in contrast to mature miRNAs do not possess a strict sequence conservation, even in closely related plant species. This is largely due to the fact that many siRNAs are generated from heterochromatic loci which overlap with transposons or transposon remnants. Alterations in transposon location and copy number that occur during plant evolution could alter the type of siRNAs produced and their average expression levels in different plants (Ma, Coruh et al. 2010).

Additionally, a number of genes in the vicinity of active TEs can become targets of the RNA-dependent DNA methylation (RdDM) pathway due to either the spread of silencing from transposon sequences or read-through transcription (Cropley and Martin 2007, Slotkin and Martienssen 2007). In the second scenario, gene transcripts are targeted for degradation by the RNA interference (RNAi) pathway, which leads to the accumulation of 24 nt-long products of mRNA decay. These 24 nt-long siRNAs can eventually direct the RdDM machinery to active transposon loci to initiate the recruitment of chromatin silencing marks through DNA methylation (Law and Jacobsen 2010, Wierzbicki, Cocklin et al. 2012). Since TEs are known to get activated in response to environmental perturbations (Grandbastien 1998) we hypothesized that the levels of TE- and gene-derived siRNAs would be affected by stress exposure, and moreover this response is expected to be tissue-specific.

In accordance with our hypothesis, in our study, differentially expressed siRNAs were mostly tissue-specific, with a small number of siRNAs overlapping between pollen and endosperm (32 common genes, Figure 4.4.11B, C and D), pollen and leaf tissues in the progeny (16 common genes, Figure 4.4.11C and D), and endosperm and leaf tissues in the progeny (14 common genes, Figure 4.4.11C and D). No overlap was found between embryo and any other tissue as there were no changes in siRNAs expression in response to HS in the embryo (Figure 4.4.6F). An intriguing hypothesis to explain the lack of differentially expressed siRNAs in *B. rapa* plants in response to HS was proposed recently in Arabidopsis (Mosher and Melnyk 2010). Maternal-specific global DNA demethylation that naturally occurs triggers transcriptional activation of the transposon array in the endosperm concomitant with the generation of the heterochromatic siRNAs that subsequently act *in trans* to reinforce transposon silencing in the young embryo.

The number of differentially expressed siRNAs in fertilized ovules was very small (11 gene-mapped siRNAs) despite a substantially larger number of them found in pollen (267 gene-mapped siRNAs, Figure 4.4.10C and E). In angiosperms, the maternal gametophyte (the embryo sac) encompasses the haploid egg cell and the homodiploid central cell, while the paternal gametophyte (pollen grain) contains two haploid sperm nuclei completely enclosed within the vegetative cell (tricellular mature pollen grain). Since the vegetative nucleus does not contribute genetic material to the next generation, it is logically to assume that a vast majority of altered smRNAs induced by heat may originate from the vegetative nucleus only traces of which are transferred to the ovules upon fertilization. In agreement with this statement, a previous report demonstrated that

the burst of transient TE reactivation and siRNA expression naturally occurring in pollen is limited to the vegetative nucleus (Slotkin, Vaughn et al. 2009).

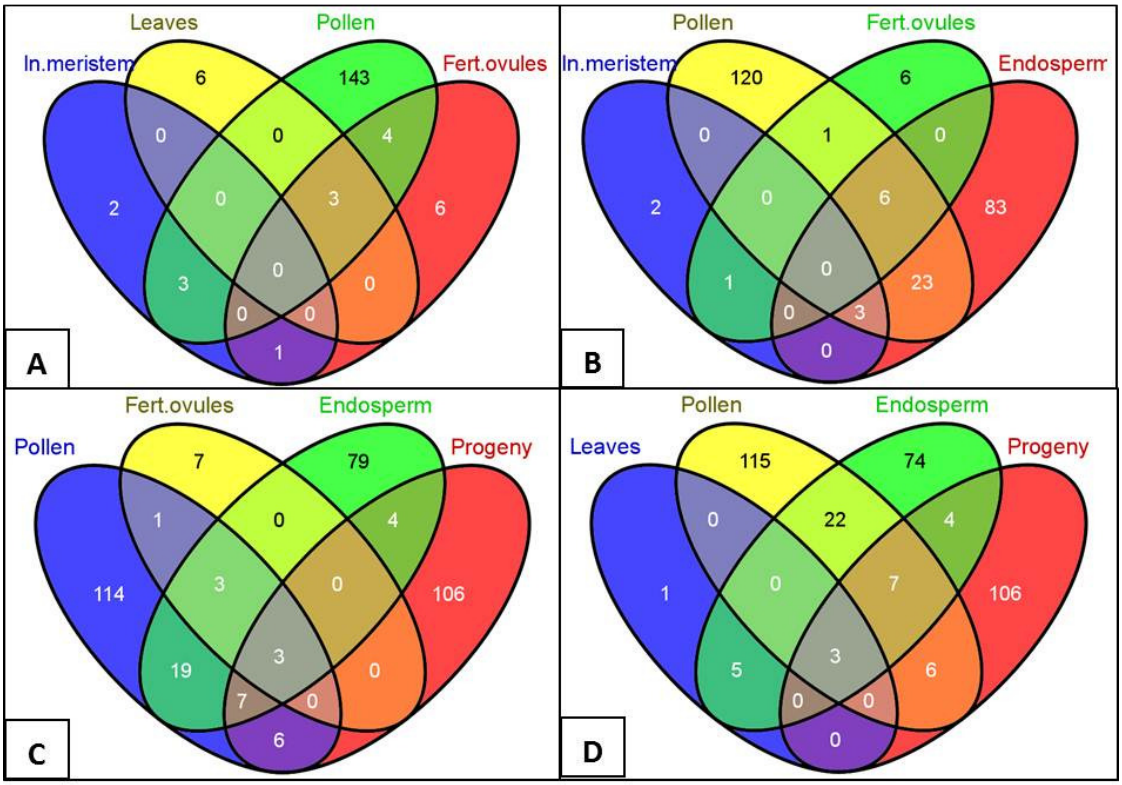

Figure 4.4.11. Venn diagrams representing common genes mapped to the significantly altered siRNAs among tissues of *B. rapa* plants exposed to heat stress

Gene IDs of putative gene-mapped siRNA targets were used to create the Venn diagrams using the Venny program (Oliveros). Labels: In. meristem – Inflorescence meristem, Fert. ovules – Fertilized ovules.

4.4.2.5. Expression hot spots of small non-coding RNAs observed in *B. rapa* tissues after heat shock treatment

In our study we observed hot spots of changes in the expression of smRNAs involved in stress response common for leaves, pollen, fertilized ovules, endosperm and leaves in the progeny (the Benjamini-Hochberg method, $q<0.2$, Table 4.4.4). Despite the fact that the commonly regulated smRNAs were mapped to three different genes (Bra003466, Bra018314 and Bra030669), the sequences of these smRNAs were highly similar because they were produced from the predicted tRNAs which reside in the intron region of the three aforementioned genes. tRNA-derived RNA fragments (tRFs) comprise a novel class of smRNAs discovered recently in plants (Hsieh, Lin et al. 2009, Chen, liu et al. 2011, Hackenberg, Huang et al. 2012) and *in silico* predicted to have a regulatory role in gene expression through the miRNA pathway (Loss-Morais, Waterhouse et al. 2013). Using a psRNATarget software, the putative mRNA targets of most of the tRFs were predicted in *B.rapa* transcriptome (data not shown) (Dai and Zhao 2011). It still remains to be shown whether we can observe a correlation between the expression of tRFs and the mRNA level of their putative targets.

The existence in plants of transgenerationally transmitted, heat-responsive tRFs is a novel finding that adds one more variable to environmentally induced epigenetic responses to stress.

Table 4.4.4. Common stress-responsive hot spots mapped to differentially expressed siRNAs in leaves, pollen, fertilized ovules, endosperm and leaves of the progeny of heat-treated *B. rapa* plants

Tissue	Gene ID	siRNA origin	Average siRNA expression log2 FC, TR vs CT
Leaves	Bra003466	Intron/tRNA-Gly	-1.57
	Bra018314	Intron/tRNA-Ala	-1.71
	Bra030669	Intron/tRNA-Gly	-1.71
Pollen	Bra003466	Intron/tRNA-Gly	2.40
	Bra018314	Intron/tRNA-Ala	-0.18
	Bra030669	Intron/tRNA-Gly	2.40
Fertilized ovules	Bra003466	Intron/tRNA-Gly	-6.46
	Bra018314	Intron/tRNA-Ala	-6.46
	Bra030669	Intron/tRNA-Gly	-6.46
Endosperm	Bra003466	Intron/tRNA-Gly	8.18
	Bra018314	Intron/tRNA-Ala	8.31
	Bra030669	Intron/tRNA-Gly	8.18
Progeny	Bra003466	Intron/tRNA-Gly	6.95
	Bra018314	Intron/tRNA-Ala	2.59
	Bra030669	Intron/tRNA-Gly	4.61
SWISS-PROT annotation Bra003466	BECN1_ARATH Beclin-1-like protein OS=Arabidopsis thaliana GN=At3g61710 PE=2 SV=2		
SWISS-PROT annotation Bra018314	N/A		
SWISS-PROT annotation Bra030669	CIA2_ARATH Protein CHLOROPLAST IMPORT APPARATUS 2 OS=Arabidopsis thaliana GN=CIA2 PE=2 SV=1		

The common differentially expressed smRNAs (the Benjamini-Hochberg method, $q<0.2$) were mapped to the genome and positional coordinates on the *B. rapa* chromosomes were extracted. Subsequently, using the region coordinates obtained, genes and tRNAs were allocated in the BRAD – *Brassica* Genome Browser v 1.2 (http://brassicadb.org/cgi-bin/gbrowse/Brassica/)

4.4.2.6. The functional annotation of gene-mapped siRNAs

To examine common biological pathways that are presumably affected by differentially expressed smRNAs mapped to genes, their putative targets were further annotated and classified according to the biological process they were involved in using the Blast2GO v 2.6.2 software with default settings (Figure 4.4.7). To do this, we included only the data sets of the three tissues that demonstrated the maximum smRNAome disequilibrium after HS – pollen, endosperm and progeny. Due to the repetitive nature of the *B. rapa* genome, a number of differentially expressed sequencing reads were mapped to multiple genomic loci. Also, a vast majority of siRNAs originated from the intron sequences, apparently do not have an ability to regulate gene expression at the posttranscriptional level, excluding alternative transcripts that rarely occur in plants.

Overall, whereas the genes involved in “response to stress” were the predominant putative targets in pollen and the leaves of progeny tissues (19.13 and 19.78%, respectively, Figure 4.4.12A and C), in endosperm, smRNAs mapped to the genes involved in “RNA metabolic process” and “transport” were the most enriched.

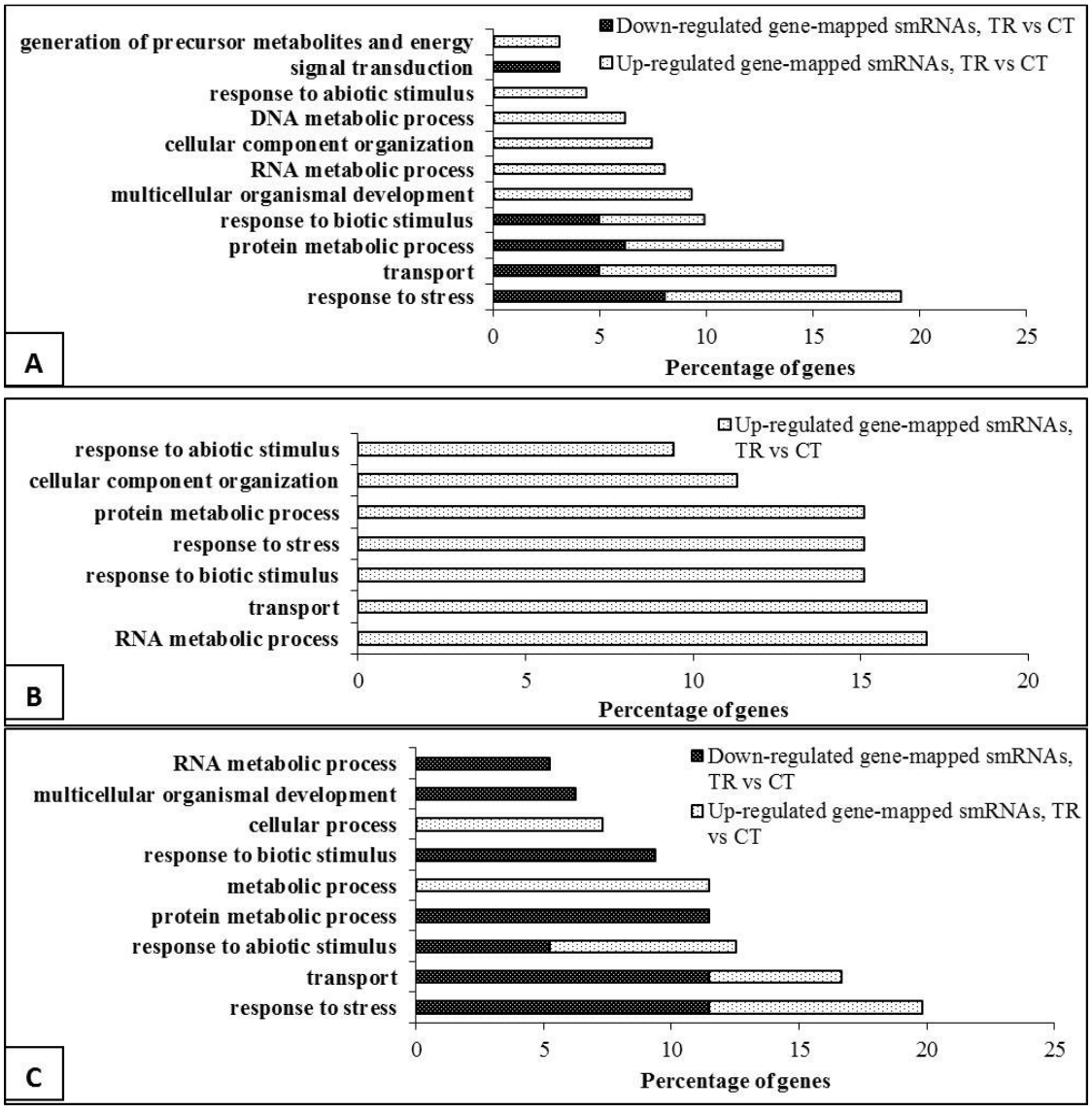

Figure 4.4.12. Gene ontology annotation of putative siRNA targets

A – Pollen, **B** – Endosperm, **C** – Progeny. Coding sequences of putative targets of differentially expressed gene-mapped siRNAs were extracted from the *B. rapa* transcriptome database v 1.2 and loaded as a FASTA file into the Blast2GO v 2.6.2 software for the NCBI BLAST similarity search using a blastx option (Conesa, Gotz et al. 2005). Further, the recovered ontologies were annotated and grouped into gene ontology categories using default settings. Gene ontology nodes were combined into the most prominent categories using a GO-slim-TAIR tool and represented as bar graphs.

4.4.2.7. Class I transposons are the major source of the TE-derived small RNAs in *B. rapa* tissues

In our experiment, the overwhelming majority of smRNAs change in response to HS in all tissues derived from genomic repeats (Figure 4.4.5). Hence, in order to reveal a tissue-specific pattern of stress response, we decided to proceed with a detailed analysis of repeat-associated sequencing reads and group them into TE categories.

Repeat-originated smRNAs recovered from our dataset revealed a complex picture where small non-coding RNAs that responded to HS mapped to various classes of mobile elements, including retroviral-like elements, DNA transposons, simple and low-complexity repeats (Figure 4.4.13). In consistency with the previous reports (Kumar and Bennetzen 1999, Pereira 2004), a vast majority of smRNA reads was mapped to Class I retrotransposons such as long tandem repeat (LTR), short interspersed nucleotide elements (SINEs) and long interspersed nucleotide elements (LINEs), that comprised 40 to 100% of all TE-derived reads, depending on the tissue type. The remaining fraction combined Class II transposable elements including DNA transposons, simple repeats, rolling-circle transposons, satellites and unknown ones. More importantly, we observed a tissue-specific response to HS with regard to differential transposon-derived siRNA expression. The overall suppression of LTR-mapped siRNA expression in pollen (76.4% as compared to control, Figure 4.4.13C) persisted through fertilized ovules, embryo (76.9 and 87.5% in fertilized ovules and embryo, respectively, as compared to control Figure 4.4.13D and E) and finally resulted into a considerable decline in the untreated progeny (91.6%, Figure 4.4.13G). At the same time, a subtle up-regulation in the overall pool of TE-mapped siRNAs was detected in the inflorescence meristem (9 out of 10 unique

siRNAs were up-regulated, Figure 4.4.13B) and a major overexpression in the endosperm (269 out of 307 unique differentially expressed siRNAs were up-regulated, Figure 4.4.13F). The last observation can be attributed to the developmental relaxation of silencing of transposable elements taking place in the endosperm tissues under normal conditions (Gehring, Bubb et al. 2009, Hsieh, Ibarra et al. 2009). Overall a decline in TE-derived siRNAs observed in the majority of examined tissues after stress treatment suggests the existence of effective epigenetic mechanisms that suppress the undesirable activity of mobile elements.

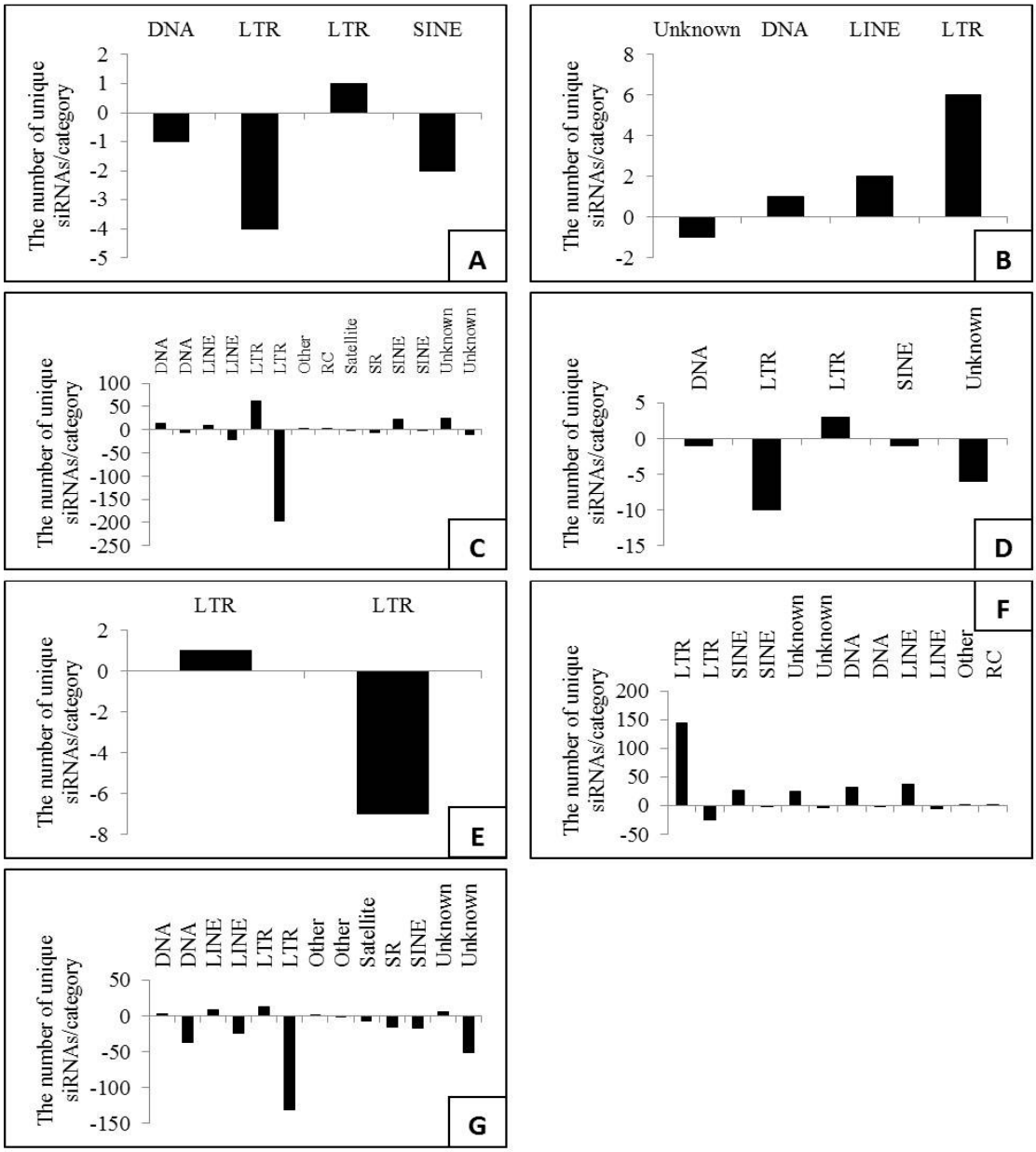

Figure 4.4.13. The distribution of differentially expressed siRNAs among transposon classes in the corresponding tissue

A – Leaves, **B** – Inflorescence meristem, **C** – Pollen, **D** – Fertilized ovules, **E** – Embryo, **F** – Endosperm, **G** – Progeny. The bar graphs represent the number of unique small RNAs per TE category. Whereas, the first bar of every category represents the number of up-regulated siRNAs, the second bar shows the number of down-regulated siRNAs, TR vs CT. LTR – long tandem repeats, SINE – short interspersed nucleotide elements, LINE – long interspersed nucleotide elements, SR – simple repeats, RC – rolling-circle transposons.

4.4.2.8. Differentially expressed miRNAs that are commonly changed in different tissues

The analysis of siRNA fractions of sequencing reads was followed by a detailed examination of those reads mapped to conservative plant micro RNA (miRNA) sequences described in the recent paper (Yu, Wang et al. 2012) and those ones that were *de novo* predicted by the MiRDeep-P software (Yang and Li 2011). Unique sequences mapped to miRNAs constituted a minor fraction of differentially expressed smRNAs that responded to HS regardless of the tissue sample (Figure 4.4.10). The largest number of altered mature miRNA sequences were observed in pollen (31 miRNAs, 4.99% of all altered smRNAs, Table 4.4.5), in endosperm (6 miRNAs, 1.56% of all altered smRNAs, Table 4.4.6) and in progeny (20 miRNAs, 5.32% of all altered smRNAs, Table 4.4.7).

Only one, miRNA was differentially expressed in the inflorescence meristem and fertilized ovules as compared to controls (Table 4.4.8) and none of altered miRNAs was observed in unfertilized ovules, embryo and leaves of heat stressed *B. rapa* plants (the Benjamini-Hochberg method, $q<0.2$). Curiously, the up-regulation of miRNA transcriptome expression was not passed on to the progeny (Figure 4.4.10C and G), instead, HS treatment resulted in a global decrease in the expression of smRNAs in leaves of offspring (Figure 4.4.10).

Our analysis allowed us to predict one novel miRNA in the pool of differentially expressed smRNAs in pollen (miR22711, Table 4.4.5) and two novel miRNAs in the progeny (miR31241, miR315691, Table 4.4.7), suggesting that the potential for the sequencing-based discovery of novel miRNAs in *B. rapa* plants is not exhausted.

MicroRNAs are known to play an important role in plant stress response since they act as global regulators of gene expression (Kruszka, Pieczynski et al. 2012). More importantly, recently miRNAs have been implicated in a non-cell autonomous mode of action (Carlsbecker, Lee et al. 2010, Marin, Jouannet et al. 2010). This raises a question whether miRNAs generated in directly exposed tissues can be mobilized to distinct systemic organs, such as reproductive tissues, and modulate the inheritance of transgenerational stress memory. Hence, we performed the commonality analysis of miRNAs between sequencing libraries of parental plants and untreated progeny. We observed a unique pattern of tissue response to stress, with only a few overlapping differentially expressed mature miRNAs among pollen, endosperm and leaf tissues of the progeny belonging to three microRNA gene families: bra-miR167, bra-miR390 and bra-miR168 (Figure 4.4.14B, Table 4.4.9). Curiously, the members of miR167 and miR390 gene families have been implemented in the regulation of auxin response factors (ARFs) in Arabidopsis which are transcription factors that bind to auxin response elements in the promoters of early auxin response genes (Tiwari, Hagen et al. 2003, Mallory, Bartel et al. 2005, Montgomery, Howell et al. 2008). MiR390 has a peculiarity in its mode of action since the regulation of target *ARF3* and *ARF4* transcripts is performed indirectly through *trans*-acting siRNAs (ta-siRNAs) generated from the cleaved *TAS3* RNA (Montgomery, Howell et al. 2008). Nevertheless, we did not observe differential expression of the corresponding ta-siRNAs either in the pollen and endosperm or leaf tissues of the progeny, albeit *TAS3* has been shown to be present and functional in the *B. rapa* genome (Yu, Wang et al. 2012).

Among the three overlapping microRNA gene families, differential expression of miR168 regulating *ARGONAUTE 1* (*AGO1*) expression in Arabidopsis was the most intriguing (Vaucheret, Vazquez et al. 2004). *AGO1* has been shown to be vital for plant development due to its unique and essential role in microRNA metabolism. Hence, the discovery of altered expression of miR168 in parental *B. rapa* plants exposed to heat stress and in untreated progeny makes it tempting to speculate about its role in trangenerational epigenetic inheritance of stress memory. Consequently, the members of the miR168 microRNA gene family were selected for further analysis, and their expression was related to the *AGO1* transcript level in both parental plants exposed to stress and untreated progeny.

Table 4.4.5. The significantly differentially expressed miRNAs in pollen of *B. rapa* plants treated with heat shock

miRNA gene family	Mature differentially expressed miRNAs	Log2FC, TR vs CT	q-value	Length, nt
bra-miR158	bra-miR158a-2	-2.10	1.12E-01	19
bra-miR159	bra-miR159a-1	1.24	8.64E-04	21
bra-miR162	bra-miR162a	1.02	3.13E-02	21
bra-miR166	bra-miR166a-1	1.31	1.57E-01	19
	bra-miR166a-1	1.68	1.82E-08	21
	bra-miR166a-1	1.79	1.11E-04	20
	bra-miR166a-1	2.44	4.05E-02	21
bra-miR167	bra-miR167a-1	1.19	4.86E-02	19
	bra-miR167a-1	1.91	3.01E-05	21
	bra-miR167a-1*	1.44	5.69E-03	21
bra-miR168	bra-miR168a-1	1.18	7.47E-02	21
	bra-miR168a-1	1.19	4.10E-02	20
	bra-miR168a-3	1.06	1.39E-02	21
bra-miR1885	bra-miR1885a	1.08	6.82E-02	22
bra-miR319	bra-miR319c-1	1.47	1.56E-02	21
bra-miR390	bra-miR390a-1	2.32	1.66E-02	21
	bra-miR390a-1*	1.96	1.91E-01	20
bra-miR393	bra-miR393a	1.41	4.51E-02	21
	bra-miR393a	3.34	8.08E-06	22
bra-miR395	bra-miR395a-1	1.92	7.44E-02	21
bra-miR396	bra-miR396a	0.83	5.56E-02	21
	bra-miR396b	2.34	7.47E-02	21
bra-miR398	bra-miR398b-1	2.59	1.48E-15	21
bra-miR403	bra-miR403	0.79	2.89E-02	21
	bra-miR403	1.46	9.83E-03	20
bra-miR408	bra-miR408a	1.58	9.14E-02	20
	bra-miR408a	2.02	2.16E-03	21
bra-miR5718	bra-miR5718	1.32	1.91E-04	22
	bra-miR5718*	1.04	1.27E-02	21
bra-miR827	bra-miR827	1.02	1.90E-01	21
	Predicted miR22711	-1.34	1.95E-01	22

Differentially expressed smRNAs mapped to conservative mature miRNAs (TR vs CT, $q<0.2$, the Benjamini-Hochberg method). * - denotes a complementary strand of the corresponding mature miRNA.

Table 4.4.6. The significantly differentially expressed miRNAs in the endosperm of *B. rapa* plants treated with heat shock

miRNA gene family	Mature differentially expressed miRNAs	Log2FC, TR vs CT	q-value	Length, nt
bra-miR167	bra-miR167a-1	4.37	0.05	20
	bra-miR167a-2	5.63	0.04	21
	bra-miR167a-1*	5.29	0.04	21
bra-miR168	bra-miR168a-3	6.48	0.05	21
bra-miR171	bra-miR171a-1	14.89	0.06	21
bra-miR390	bra-miR390a-1	3.78	0.19	21

Differentially expressed smRNAs mapped to conservative mature miRNAs (TR vs CT, q<0.2, the Benjamini-Hochberg method). * - denotes a complementary strand of the corresponding mature miRNA.

Table 4.4.7. The significantly differentially expressed miRNAs in the progeny of *B. rapa* plants treated with heat shock

miRNA gene family	Mature differentially expressed miRNAs	Log2FC, TR vs CT	q-value	Length, nt
bra-miR1140	bra-miR1140	-2.20	1.02E-01	20
	bra-miR1140	-1.80	9.64E-02	21
	bra-miR1140*	-1.74	8.68E-02	22
bra-miR165	bra-miR165a	-2.48	6.29E-03	20
	bra-miR165a	-2.20	4.38E-02	21
	bra-miR165a	-2.43	1.16E-01	21
bra-miR166	bra-miR166c*	-2.05	5.01E-02	21
bra-miR167	bra-miIR167d	-3.29	1.35E-01	21
bra-miR168	bra-miR168a-1	-1.57	1.58E-01	21
bra-miR319	bra-miR319a-1	-3.74	4.13E-07	20
	bra-miR319a-1	-4.45	2.73E-10	21
	bra-miR319a-3	-4.32	5.43E-02	20
	bra-miR319a-3	-5.79	1.85E-08	21
	bra-miR319b*	-13.73	2.27E-03	19
	bra-miR319c-1	-1.93	5.73E-02	21
bra-miR390	bra-miR390a-1	-2.69	4.01E-04	21
	Predicted miR31241	-2.18	5.99E-02	20
	Predicted miR31241	-1.83	7.77E-02	21
	Predicted miR315691	-3.29	1.35E-01	20
	Predicted miR315691	-1.67	1.62E-01	21

Differentially expressed smRNAs mapped to conservative mature miRNAs (TR vs CT, q<0.2, the Benjamini-Hochberg method). * - denotes a complementary strand of the corresponding mature miRNA.

Table 4.4.8. The significantly differentially expressed miRNAs in the inflorescence meristem and fertilized ovules of *B. rapa* plants treated with heat shock

miRNA gene family	Mature differentially expressed miRNAs	Log2FC, TR vs CT	q-value	Length, nt	Tissue
bra-miR396	bra-miR396a	-2.50	0.03	17	Inflorescence meristem
	bra-miR396a	-2.01	0.08	17	Fertilized ovules

Differentially expressed smRNAs mapped to conservative mature miRNAs (TR vs CT, q<0.2, the Benjamini-Hochberg method).

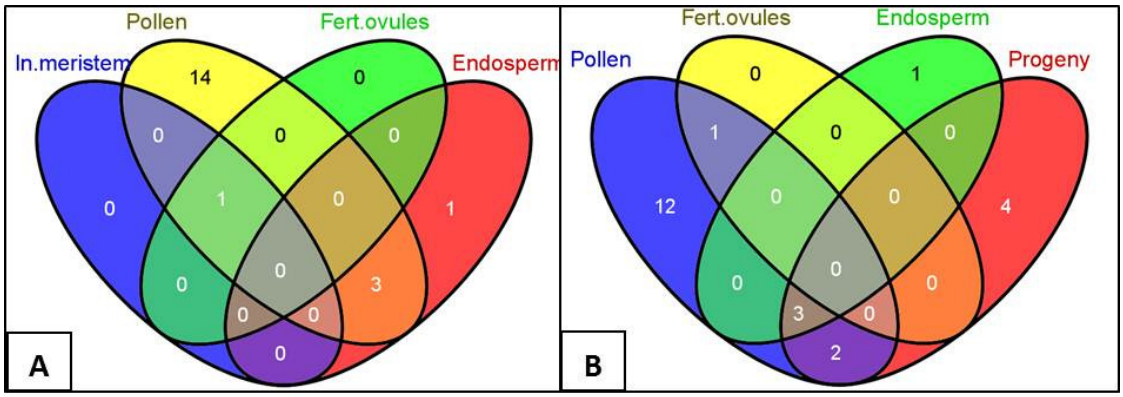

Figure 4.4.14. Venn diagrams representing the common microRNA gene families of differentially expressed mature miRNAs among tissues in heat-stressed *B. rapa* plants as compared to controls

Differentially expressed smRNAs mapped to conservative miRNAs (TR vs CT, q<0.2, the Benjamini-Hochberg method) were grouped into classes and used for the generation of Venn diagrams using the Venny software. Labels: In. meristem – Inflorescence meristem, Fert. ovules – Fertilized ovules.

Table 4.4.9. Common significantly altered microRNA gene families in pollen, endosperm and leaves of the progeny of heat-stressed plants

The miRNA gene family	miRNA	Tissue	Log2 fold change, TR vs CT	q-value
bra-miR167	bra-miR167d	Pollen	1.19	5.00E-02
		Endosperm	4.37	5.00E-02
		Progeny	-3.29	1.30E-01
bra-miR390	bra-miR390a-1	Pollen	2.32	2.00E-02
		Endosperm	3.78	1.90E-01
		Progeny	-2.69	4.00E-04
bra-miR168	bra-miR168a-1	Pollen	1.18	4.00E-02
	bra-miR168a-3	Endosperm	6.48	5.00E-02
	bra-miR168a-1	Progeny	-1.57	1.90E-01

Differentially expressed smRNAs mapped to conservative mature miRNAs (TR vs CT, $q<0.2$, the Benjamini-Hochberg method)

4.4.2.9. MiR168 is a putative messenger of transgenerational epigenetic inheritance

Simply detecting miRNAs in stressed parental tissues and untreated progeny does not provide evidence that they are functional as smRNAs, largely acting as guides through RNA binding to target RNA and DNA substrates (Grant-Downton and Rodriguez-Enriquez 2012). Hence, we proceeded with the examination of putative targets of differentially expressed miRNAs in parental tissues and the progeny. The psRNATarget software with default settings was used to predict and retrieve gene IDs of putative miRNA gene-targets from the *B. rapa* CDS library v 1.1 (Dai and Zhao 2011). Subsequently, we searched for putative gene-target IDs in the differentially expressed gene dataset of the corresponding tissue and annotated them using the SWISS-PROT database (Bairoch and Apweiler 2000). Since the endosperm was the tissue that was more transcriptionally responsive to stress at the level of gene and smRNA expression (see Figure 4.4.1G and Figure 4.4.10G for endosperm transcriptome and smRNAome expression, respectively), it conceivably led to the highest number of smRNA/mRNA target pairs identified. Six smRNA/mRNA target pairs were identified in the endosperm of stressed plants (Table 4.4.10), the most intriguing of them was bra-miR168/*AGO1* (Max identity=95%, E-value=2E-99 for *atAGO1* and *braAGO1* BLAST search). Curiously, the expression of *braAGO1* was also significantly altered in the embryo of stressed plants as compared to controls (Table 4.4.12, the Benjamini-Hochberg method). Overall, we observed a bimodal tissue-specific trend in the expression of *braAGO1* after HS stress in parental plants. Whereas an insignificant down-regulation was observed in leaves, unfertilized and fertilized ovules, the up-regulation was detected in the

inflorescence meristem and pollen (Table 4.4.12, the Benjamini-Hochberg method). The absence of negative correlation for significantly overexpressed miR168 and *braAGO1*, observed in our study in pollen, is in contrast with the previous report showing that the regulation of *AGO1* expression by miR168 is active in mature Arabidopsis pollen grains (Grant-Downton, Hafidh et al. 2009).

The expression of *braAGO1* was similar in the progeny of treated and untreated plants (Table 4.4.12, the Benjamini-Hochberg method), albeit a significant down-regulation of miR168 was detected (Table 4.4.12, the Benjamini-Hochberg method). An inverse correlation for miR168 expression and *atAGO1* transcript levels under stress conditions is not always obvious in Arabidopsis, because both *atAGO1* and miR168 promoters are activated under abiotic stress conditions, suggesting that an increase in the miR168 level is essential for retaining a stable *AGO1* transcript level during stress response (Li, Cui et al. 2012). We also observed a moderate negative correlation for bra-miR168a-1 and *braAGO1* expression in all tissues of stressed parental plants and untreated progeny of *B. rapa* (Pearson's r = -0.66, Table 4.4.12).

Unexpectedly, in the most of differentially expressed miRNAs, we did not find an obvious negative correlation with their putative gene targets in the endosperm (Table 4.4.10). On the contrary, both miRNAs and their target genes demonstrated an increase in the expression following HS. It is possible that these miRNAs function at the level of translational inhibition rather than the level of mRNA cleavage, although the latter mechanism prevails in plants (Axtell 2013). The large-scale comparison of differentially expressed miRNAs and their putative targets together with the analysis of the corresponding protein levels can further clarify the picture.

A detailed analysis of mature miRNA sequences belonging to the miR168 gene family revealed a common 20 nt-long core sequence between 3 miRNAs with only a variable 3´-terminal nucleotide (Table 4.4.11). Curiously, whereas in the Arabidopsis genome, there are two copies of miR168 gene, the *B. rapa* genome encodes 5 different copies of miR168 gene, which is apparently due to the whole genome triplication and a subsequent fractionation of one copy since its divergence from the *A. thaliana* lineage at least 5 – 9 million years ago (Table 4.4.11) (Wang, Wang et al. 2011). At this point it is not clear which of these copies are transcriptionally active, albeit a considerable domination of mature bra-miR168a-2 reads generated from miRNA genes resided at chromosomes A01 and A03 was observed in all smRNA sequencing libraries except pollen, embryo and endosperm (data not shown) as compared to bra-miR168a-1 and bra-miR168a-3. The validation of the expression of miR168 was performed using smRNA Northern blot analysis with a probe designed to recognize the consensus sequence of the three mature miRNAs (Figure 4.4.15).

Overall, at this stage, it is speculative whether the members of the bra-miR168 gene family play a substantial role in transgenerational stress memory inheritance, and whether the *braAGO1* gene is a vital regulator that epigenetically orchestrates global gene expression in *B. rapa* plants. Evidence in favour of this idea manifests itself in the studies in model systems - *Caenorhabditis elegans* and *Arabidopsis thaliana* where the putative homolog of *atAGO3* maintains multigenerational epigenetic inheritance, and the disruption of *atAGO1* gene leads to severe phenotypic abnormalities and misregulated gene expression, respectively (Vaucheret, Vazquez et al. 2004, Kurihara, Kaminuma et al. 2009, Buckley, Burkhart et al. 2012). Noteworthy, we observed a significant inverse

Pearson's correlation between the log2fold change in *braAGO1* expression (TR vs CT) and a number of differentially expressed genes in every tissue of parental stressed *B. rapa* plants and leaf tissues of the progeny (r = -0.89), which conceivably may suggest for the global regulatory function of *braAGO1* in *B. rapa* plants.

Table 4.4.10. Changes in the expression of miRNAs and their putative targets in response to heat stress in the endosperm of *B. rapa* plants

miRNA	miRNA log2FC, TR vs CT	Gene ID of the putative target	Gene log2FC, TR vs CT	Predicted miRNA's mode of action	SWISS-PROT annotation
bra-miR167	4.37	Bra015704	0.86	Translation inhibition	TM1L2_XENLA TOM1-like protein 2 OS=Xenopus laevis GN=tom1l2 PE=2 SV=1
bra-miR167	4.37	Bra002277	-1.25	mRNA cleavage	TDRD3_CHICK Tudor domain-containing protein 3 OS=Gallus gallus GN=TDRD3 PE=2 SV=1
bra-miR167*	5.29	Bra025064	5.84	mRNA cleavage	GDL82_ARATH GDSL esterase/lipase At5g45670 OS=Arabidopsis thaliana GN=At5g45670 PE=2 SV=1
bra-miR167*	5.29	Bra005019	2.28	Translation inhibition	RCA_ARATH Ribulose bisphosphate carboxylase/oxygenase activase, chloroplastic OS=Arabidopsis
bra-miR168	6.48	Bra032254	-1.56	mRNA cleavage	AGO1_ARATH Protein argonaute OS=Arabidopsis thaliana GN=AGO1 PE=1 SV=1
bra-miR171a-1	14.89	Bra039431	0.79	Translation inhibition	PRS6A_BRACM 26S protease regulatory subunit 6A homolog OS=Brassica campestris GN=TBP1 PE=2 SV=1

Gene target prediction for miRNAs was done using the psRNATarget program for the *Brassica rapa* CDS library v 1.1 (Dai and Zhao 2011). Log2FC – log2fold change, TR vs CT. Statistical significance was calculated using the Benjamini-Hochberg method, $q<0.2$. * - denotes a complementary strand of the corresponding mature miRNA.

Table 4.4.11. The expression profile of mature miRNAs of miR168 class in tissues of heat-stressed *B. rapa* parental plants and untreated progeny

miRNA	Sequence of mature miRNA	Position of the miRNA gene on the chromosome	Tissue	Log2FC, TR vs CT	q-value
bra-miR168a-1	5′-TCGCTTGGTGCAGGTCGGGA-3′	A01:5386102..5386238 (+strand) A03:23663743..23663871 (+strand) A06:25071499..25071634 (- strand) A08:10924553..10924689 (+ strand) A09:12975267..12975402 (+ strand) E-value=0.001	Leaves	0.58	1
			Inflorescence meristem	0.05	1
			Pollen	1.19*	0.04
			Unfertilized ovules	-0.54	1
			Fertilized ovules	-0.62	1
			Embryo	0.61	1
			Endosperm	3.42*	0.41
			Progeny	-0.58*	0.93
bra-miR168a-2	5′-TCGCTTGGTGCAGGTCGGGAC-3′	A01:5386102..5386238 (+strand) A03:23663743..23663871 (+ strand) E-value =4E-04	Leaves	0.61	1
			Inflorescence meristem	0.06	1
			Pollen	1.18*	0.07
			Unfertilized ovules	-0.88	1
			Fertilized ovules	-0.89	1
			Embryo	0.51	1
			Endosperm	3.37*	0.29
			Progeny	-1.57*	0.16
bra-miR168a-3	5′-TCGCTTGGTGCAGGTCGGGAA-3′	A06:25071499..25071634 (- strand) A08:10924553..10924689 (+ strand) A09:12975267..12975402 (+ strand) E-value = 4E-04	Leaves	0.51	1
			Inflorescence meristem	0.30	1
			Pollen	1.06*	0.01
			Unfertilized ovules	-0.81	1
			Fertilized ovules	-0.42	1
			Embryo	0.56	1
			Endosperm	6.48*	0.05
			Progeny	-0.71*	0.85

The asterisks denote a significant difference in the expression as compared to controls (the Benjamini-Hochberg method, $q<0.2$). Positions of miRNA genes were retrieved using the BRAD – *Brassica* Genome Browser v 1.2

Table 4.4.12. Validation of bra-miR168a-1 and *braAGO1* expression in tissues of heat-shock-stressed *B. rapa* parental plants and untreated progeny

Tissue	log2FC TR vs CT, bra-miR168a-1 expression, sequencing	q-value, sequencing	Normalized log2FC TR vs CT, bra-miR168a-1 expression, Northern Blot	log2FC TR vs CT *braAGO1* expression, sequencing	q-value, sequencing	log2FC TR vs CT *braAGO1* expression, qPCR	p-value, TR vs CT, qPCR
Leaves	0.58	1	3.15	-0.08	1	0.74*	2.13E-02
Inflorescence meristem	0.05	1	-0.07	0.41	1	0.69*	6.00E-04
Pollen	1.19*	0.04	0.36	0.64	1	1.08*	2.01E-04
Unfertilized ovules	-0.54	1	-0.21	-0.08	1	-0.81*	5.04E-03
Fertilized ovules	-0.62	1	0.64	-0.04	1	0.70*	1.26E-04
Embryo	0.61	1	2.50	-0.77*	1.29E-02	-0.60*	4.12E-03
Endosperm	3.42*	0.41	1.16	-1.56*	1.36E-04	-0.81*	1.23E-03
Progeny	-0.58*	0.93	-1.12	0.06	1	0.57*	2.03E-02
Correlation, *braAGO1* expression vs bra-miR168a-1, sequencing data	Pearson's r = -0.66						

The asterisks denote a significant difference in the expression as compared to controls (the Benjamini-Hochberg method, $q<0.2$ and the Student's t-test, $P<0.05$ for sequencing and qPCR data, respectively). Log2FC – estimated log2 value of fold change for treated plants versus controls.

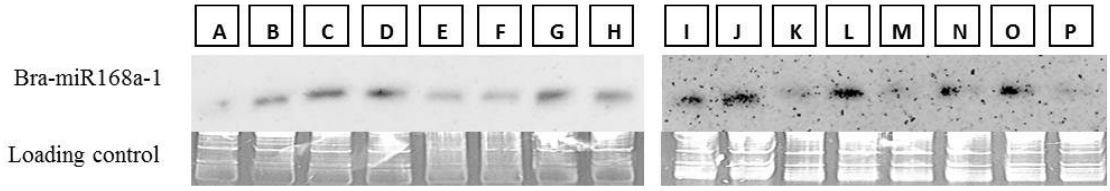

Figure 4.4.15. Small RNA Northern blot of bra-miR168 in heat-shock-stressed *B. rapa* parental tissues and untreated progeny

A – CT Leaves, **B** – TR Leaves, **C** – CT Inflorescence meristem, **D** – TR Inflorescence meristem, **E** – CT Pollen, **F** – TR Pollen, **G** – CT Unfertilized ovules, **H** – TR Unfertilized ovules, **I** – CT Fertilized ovules, **J** – TR Fertilized ovules, **K** – CT Embryo, **L** – TR Embryo, **M** – CT Endosperm, **N** – TR Endosperm, **O** – CT Progeny, **P** – TR Progeny. Total RNA was used as a loading control. The probe was designed to hybridize to the consensus sequence of the mature bra-miR168 miRNAs.

4.5. DISCUSSION

The main focus of this research was investigation of modulation of stress responses and substantial mitotic and meiotic propagation of stress memory in *B.rapa* plants. Global transcriptome and smRNAome profiling in HS-stressed parental and untreated progeny tissues manifested itself in the following key findings: a) heat stress treatment results in a tissue-specific gene expression response of the aerial part of *B. rapa* plants, with the endosperm and embryo being the most transcriptionally labile tissues, and reproductive tissues being the least labile ones as compared to controls; b) major alterations of the transcriptome profile in the endosperm and embryo is not propagated into the next generation, albeit a considerable differential expression of genes is also detected in the progeny of stressed plants as compared to controls; c) most of the altered genes are unique for every tissue examined, with the highest degree of overlap being observed between embryo and endosperm; d) genes involved in stress response and protein metabolic process constitute the major fraction of the altered transcriptome in all tissues except ovules; e) the general composition of smRNA libraries is unique for every tissue examined and is not acutely affected by HS treatment; f) a vast majority of smRNA sequencing reads are 21 and 24 nt-long in all tissue libraries of *B. rapa* plants; g) 24 nt-long sequencing reads constitute a predominant fraction of gene- and transposon mapped smRNAs; h) pollen, endosperm and progeny of heat-stressed *B. rapa* plants demonstrate the highest alterations in the smRNAome profile as compared to controls; i) MiR168 and *braAGO1* gene are putative mediators of stress-induced transgenerational epigenetic memory in *B. rapa* plants.

4.5.1. Embryo and endosperm demonstrate the most pronounced oscillations in the trancriptome profile after heat shock stress

A tissue-specific perturbation of gene expression in response to stress exposure has been previously shown in a number of plant species including *Arabidopsis* (Prandl, Kloske et al. 1995, Nylander, Svensson et al. 2001, Iyer-Pascuzzi, Jackson et al. 2011), wine grape *Vitis vinifera* (Tillett, Ergul et al. 2011), diploid cotton *Gossypium arboretum* (Zhang, Yao et al. 2013), *Nicotiana plumbaginifolia* (Castresana, de Carvalho et al. 1990) and *Brassica napus* (Dong, Keller et al. 2004). In the current study, we observed tissue-dependent fluctuations of the transcriptome in response to stress in *B. rapa* plants, by using the massive parallel sequencing technology (Figure 4.4.1 and Figure 4.4.2). For the first time to our knowledge, we demonstrated a comprehensive profile of gene expression following heat shock in somatic and reproductive parental tissues and in the untreated progeny of plants. More importantly, we found that the highest oscillations of gene expression were observed not in parental tissues that were directly exposed to stress (such as leaves) but in developmentally distant untreated seeds, suggesting the existence of a mitotically and meiotically transmitted signal of plant stress response (Figure 4.4.1). A handful of messengers have been implicated in heat stress response (HSR) in plants that include reactive oxygen species (ROS) (Larkindale and Huang 2004, Larkindale, Hall et al. 2005), Ca^{2+} cations (Liu, Sun et al. 2005), and phytohormones such as abscisic acid (ABA), salicylic acid (SA) and ethylene (Larkindale and Huang 2004, Larkindale, Hall et al. 2005, Larkindale, Mishkind et al. 2007). At present, among them only ROS was shown to mediate systemic signalling of heat stress (Miller, Schlauch et al. 2009), albeit the rest of mediators can also contribute to long-distance signalling in plants (Heil and

Ton 2008, Jung, Tschaplinski et al. 2009). Regardless of the signal's nature, in our experiments, their action resulted in the priming of an array of genes in somatic and reproductive tissues of stressed plants that enigmatically culminated in a burst of transcription changes in the embryo and endosperm (Figure 4.4.1). Alternatively, less pronounced HSR of gene expression observed in the inflorescence meristem and reproductive tissues of stressed *B. rapa* plants indicates a more stringent regulation of gene expression in these tissues as compared to the embryo and endosperm (Figure 4.4.1).

Unfortunately, reports indicating transcriptome changes in reproductive tissues of plants in response to stress are scarce. A single study conducted on mature pollen treated with 0°C for 72 hours reported insignificant oscillations in the transcriptome profile of pollen as compared to vegetative leaf tissue (Lee and Lee 2003). This is consistent with our data demonstrating only three genes to be differentially expressed following HS treatment in pollen (Figure 4.4.1C). Transcriptional quiescence of pollen is presumably due to the lack of resources in the pollen grain required to initiate stress response, suggesting that messengers of transgenerational epigenetic inheritance are deposited throughout gametogenesis and are not obtained during pollen migration. On the other hand, the lack of substantial oscillations in the transcriptome profile in exposed ovules as compared to controls can be simply due to the abortion of severely affected ovules to facilitate shunting of resources from reproductive activities into metabolic reactions that increase stress tolerance (Sun, Hunt et al. 2004, Young, Wilen et al. 2004, Hedhly 2011). The remaining survived ovules, apparently acquired an epigenetic signal that was transmitted to the embryo and endosperm. Curiously, 94.58% and 19.48% of

differentially expressed genes were common in the stressed embryo and endosperm, respectively, suggesting that the additional maternal genome contributes to substantial fluctuations in the endosperm transcriptome (Figure 4.4.2C and D). This finding is in agreement with the previous report demonstrating that the maternal genome in the Arabidopsis endosperm is substantially less methylated than the paternal genome in the CpG context. At the same time, there were no significant differences in DNA methylation between parental genomes in the Arabidopsis embryo (Ibarra, Feng et al. 2012).

Differential DNA methylation at cytosine residues is responsible for tissue-specific patterns of gene expression and effective responses to stress (Zhang, Kimatu et al. 2010). In studies on human tissues, a significant correlation was reported between DNA hypomethylation and tissue-specific transcription (Schilling and Rehli 2007). Unfortunately, similar systemic investigations in plants are not available yet (Zhang, Kimatu et al. 2010), albeit a drastic response of endosperm to heat sock observed in our study can be attributed to the naturally occurring genome-wide demethylation mediated by DEMETER (DME) DNA glycosylase that was recently reported to occur in Arabidopsis and maize (Gehring, Bubb et al. 2009, Hsieh, Ibarra et al. 2009, Waters, Makarevitch et al. 2011). Nevertheless, taking into account that in our experiment, plants were exposed to heat stress during the vegetative stage for a relatively short period of time, the question remains: how do the progeny of stressed plants inherit the memory of stress exposure at the molecular level? In invertebrate animals *Drosophila* and *C. elegans,* mediators of transgenerational inheritance, include the ACTIVATION TRANSCRIPTION FACTOR-2 (ATF-2) that functions in hetero-chromatin nucleation (Seong, Li et al. 2011) and an Argonaute protein that associates with small interfering

RNAs in the germ cells of progeny (Buckley, Burkhart et al. 2012). Numerous examples of transgenerational inheritance in angiosperms undoubtedly suggest the existence of analogous messengers in plants (Boyko, Blevins et al. 2010, Bilichak, Ilnystkyy et al. 2012, Luna, Bruce et al. 2012, Rasmann, De Vos et al. 2012, Slaughter, Daniel et al. 2012). As such, they presumably act to prime the stress-specific genes for providing faster and more pronounced changes in transcription if akin exposure is encountered by offspring of stressed plants (Kathiria, Sidler et al. 2010, Luna, Bruce et al. 2012). Consistent with this notion, we detected a higher enrichment of stress-related genes in the fraction of differentially expressed genes in the progeny of stressed plants as compared to the parental tissues, which argues against a stochastic nature of epigenome variability (54.29% of stress-related genes out of the total number of differentially expressed genes, Figure 4.4.4C).

Recently, we have shown that plants impaired in smRNA metabolism lack the ability to transmit memory of stress exposure to the next generation (Boyko, Blevins et al. 2010). Additionally, previous reports have provided an explicit proof that smRNAs of virtually all size classes are mobile and are able to move over long distances in Arabidopsis and presumably in other plant species (Lin and Chiou 2008, Pant, Buhtz et al. 2008, Dunoyer, Brosnan et al. 2010, Dunoyer, Schott et al. 2010, Molnar, Melnyk et al. 2010). Hence, systemic epigenetic signals initiated by smRNAs in response to stress can seemingly make an imprint on the chromatin in germline cells and eventually be transferred to the following generation. To further scrutinize the mechanisms underlying transgenerational inheritance via smRNAs in plants, we sequenced smRNAome of the progeny and parental *B. rapa* plants subjected to HS.

4.5.2. Pollen exhibits tissue-dependent smRNAome fluctuations in response to heat shock

Regulatory small RNAs are vital components of the plant transcriptome used both to fine-tune gene expression and to guard the genome against an undesirable activity of exogenous or endogenous nucleic acids (Axtell 2013). Currently, two main classes of mature smRNAs that differ functionally and in their mode of biogenesis are recognized in plants, namely, small interfering RNAs (siRNAs) and microRNAs (miRNAs) (Axtell 2013). Whereas siRNAs predominantly derive from the dsRNA precursors generated by the RNA-dependent RNA (RDR) polymerase from a truncated RNA, miRNAs originate from single-stranded RNAs that possess an intramolecular, self-complementary hairpin structure. Both classes regulate gene expression; siRNAs primarily act at the transcriptional level by guiding DNA methylation at complementary loci, while miRNAs act at the post-transcriptional level either by targeting complementary transcripts for degradation or by translational inhibition.

In numerous flowering plants examined to date, 24 nt-long heterochromatic siRNAs (hc-siRNA) comprise the overwhelming majority of smRNA transcriptome (Nobuta, Lu et al. 2008, Wu, Zhou et al. 2010, Korbes, Machado et al. 2012, Axtell 2013). In our study, a vast amount of smRNAs were in the 24 nt-long fraction followed by the 21 nt-long fraction of reads in all libraries except leaves (Figure 4.4.6). The prevailing dominance of 21-mers in leaf tissues of parental plants and progeny of *B. rapa* plants is apparently due to the high redundancy of 21 nt-long reads, specifically in leaves (data not shown).

Mapping of smRNAs to transposable elements and genic regions confirmed the previous findings that the 24 nt smRNAs encompass a major fraction of siRNAs for all tissues except pollen (Figure 4.4.7 and Figure 4.4.8). Pollen-derived sequencing reads demonstrated a singularity of smRNA length distribution mapped to the gene and transposon regions as compared to other *B. rapa* tissues. Gene-mapped smRNAs demonstrated the accumulation of sequencing reads with a length less than 19 nt (Figure 4.4.7C), conceivably the products of mRNA degradation or yet undiscovered pollen-specific regulatory RNAs. Previously, using a microarray transcriptome analysis of mature Arabidopsis pollen, it has been shown that less than half of the transcripts are present in pollen as compared to the vegetative tissue (Pina, Pinto et al. 2005). Curiously, the major peak of smRNA reads spanning a region from 17 to 19 nucleotides in the gene-mapped smRNAs was shifted towards the 19 nt-long reads in the control transposon-mapped smRNA pollen library (Figure 4.4.8C). In Arabidopsis pollen, the 21-22 nt-long smRNAs are predominantly generated in the vegetative nucleus, that is sacrificed by allowing rampant transposon expression concomitant with global DNA demethylation (Slotkin, Vaughn et al. 2009, Calarco, Borges et al. 2012). Subsequently, these 21-22 nt siRNAs guide RNA-dependent DNA methylation at non-symmetrical CpHpH sequences in sperm cells in order to reinforce silencing of transposons. In our experiment, 21-22 nt smRNA reads remained at the same level after HS as compared to controls, although the 19, 20 and 24 nt-long smRNAs demonstrated significant oscillations in the expression as compared to controls (Figure 4.4.8C). We also observed a reciprocal response in the smRNA read distribution in pollen, with the down-regulation of 19 and 20 nt-long fractions and the up-regulation of 24 nt-long fractions of smRNAs following HS. Neither

unfertilized nor fertilized ovules responded with significant fluctuations in the smRNA length distribution, albeit the smRNA pathways have been shown to be functional in Arabidopsis egg cells (Figure 4.4.6D and E) (Wuest, Vijverberg et al. 2010). Curiously, the fertilization of the embryo sac resulted in slight perturbations in the smRNA profile with a vast majority of repeat-derived sequencing reads (19 out of 27 unique sequencing reads) as compared to controls and unfertilized ovules (Figure 4.4.10D and E). Whereas a comparison of unique altered sequencing reads between pollen and fertilized ovules returned only one common siRNA, seven out of 14 genes were found to be a common source of siRNAs in pollen and fertilized ovules. This finding partially confirms a previous report that the vegetative nucleus which does not contribute genetic material to the progeny is the primary source of smRNAs in pollen (Calarco, Borges et al. 2012). Also it provides new evidence of a transcriptionally quiescent response of the embryo sac to stress in plants that can be a prerequisite for the maintenance of genome stability in the harsh environmental conditions.

4.5.3. MiR168 is a putative messenger of transgenerational stress memory inheritance in *B. rapa* plants

Whereas siRNAs, with a few exceptions (Dunoyer, Brosnan et al. 2010, McCue, Nuthikattu et al. 2012), are known to suppress predominantly TE activity in the genome, miRNAs are well-characterized regulatory elements of gene expression in plants and animals (Axtell 2013). The biogenesis of miRNAs starts with the primary miRNA transcripts (pri-miRNA) that are transcribed from specific non-protein-coding MIR genes by Pol II and fold to form imperfectly paired stem-loops (Martinez de Alba, Jauvion et al.

2011). The pri-miRNA is further processed into the pre-miRNA intermediate and mature miRNA with the help of the RNAseIII protein DCL1 and several other proteins, including the Cap-binding proteins CBP20 and CBP80/ABH1, the zinc finger protein SERRATE (SE), the double-stranded RNA-binding protein DRB1/HYL1, and the smRNA export protein HASTY (Zhu 2008, Xie, Khanna et al. 2010). Eventually, mature miRNAs are picked up by ARGONAUTE (AGO) proteins and act in *trans* as the sequence-specific guides to modulate the stability and expression of partially complementary mRNAs.

The Arabidopsis genome encodes 10 AGO proteins, most of which demonstrate a clear bias towards a specific class of smRNAs depending on the size and 5′-terminal nucleotide composition (Vaucheret 2008). Curiously, one of the AGO proteins – AGO1 – plays a principal role in both the siRNA- and miRNA-guided modulation of gene activity, which is further supported by severe developmental defects exhibited by Arabidopsis plants carrying hypomorphic and null *ago1* alleles (Bohmert, Camus et al. 1998, Morel, Godon et al. 2002, Kidner and Martienssen 2004). As a result of the global importance of AGO1 in plant homeostasis and development, its expression is firmly modulated in both an AGO1- and a PNH/ZLL/AGO10-specific way by negative feedback loops involving miR168 and AGO1-derived siRNAs (Vaucheret, Vazquez et al. 2004, Vaucheret, Mallory et al. 2006, Mallory, Hinze et al. 2009, Mallory and Vaucheret 2009). Recently, the cyclophylin protein SQUINT (SQN) (Smith, Willmann et al. 2009) and the F-box protein FBW2 (Earley, Smith et al. 2010) have been also implicated in the regulation of AGO1. Moreover, the evolutionary conservation and importance of AGO1 in the antiviral defence system in plants resulted in the development of p19-mediated RNA-

silencing suppression by *Tombusvirus* that elevates miR168 expression in virus infected plants and subsequently leads to a decrease in the level of AGO1 protein and limits the effect of host-mounted RNA-silencing defence (Varallyay, Valoczi et al. 2010). Therefore, the AGO1 protein in Arabidopsis is highly versatile, even though it is currently unknown how it distinguishes between certain classes of smRNAs (McCue, Nuthikattu et al. 2012).

A negative feedback loop of AGO1 regulation mediated by miR168 is of particular interest since miR168 expression is altered by numerous environmental perturbations such as salt, drought, cold, heat, hypoxia, UVB stresses and ABA treatments in a number of plant species including poplar (*Populus trichocarpa*), tobacco (*Nicotiana tabacum*), Arabidopsis, maize (*Zea mays*), and rice (*Oryza sativa*) (Li, Oono et al. 2008, Ding, Zhang et al. 2009, Jia, Wang et al. 2009, Jia, Mendu et al. 2010, Zhou, Liu et al. 2010, Li, Cui et al. 2012, Sunkar, Li et al. 2012). In our study we observed a differential expression of bra-miR168 following HS in parental tissues that was negatively correlated with *braAGO1* transcript levels in the corresponding tissues (Pearson's r=-0.66, Table 4.4.12). More importantly, the down-regulation of bra-miR168 along with other stress-responsive miRNAs was subsequently observed in the untreated progeny of stressed *B. rapa* plants (Table 4.4.7).

Mature miRNAs belonging to the three conserved MIR gene families: miR167, miR390 and miR168 were common among differentially expressed miRNA datasets of pollen, endosperm and progeny (Table 4.4.9). MiR167 and miR390 have been previously implemented in the regulation of auxin response transcription factors (ARFs), and they are responsive to abiotic stress in Arabidopsis plants (Covarrubias and Reyes 2010,

Khraiwesh, Zhu et al. 2012, Kinoshita, Wang et al. 2012). Our work has shown that these miRNAs also respond to heat stress in systemic tissues of *B. rapa* plants with the up-regulation of mature miRNA levels (Tables 4.4.5, 4.4.6, 4.4.7) (Sunkar, Li et al. 2012). Unexpectedly, the overwhelming majority of differentially expressed smRNAs were down-regulated in the progeny of stressed plants as compared to controls (338 out of 376, Figure 4.4.10), which was concomitant with a slight up-regulation of gene expression (Figure 4.4.1). Taking into consideration that smRNAs act strictly by down-regulation of gene expression, we can speculate that their progressive depletion provides a capacity for an organism to up-regulate the required gene expression without the ultimate smRNA-mediated transcript degradation. Furthermore, the removal of such post-transcriptional restraints along with locus-specific demethylation can be the cause of transgenerational priming of stress-responsive genes previously described in offspring of stressed plants (Luna, Bruce et al. 2012).

Curiously, the examination of bra-miR168a-1 accumulation in response to stress revealed developmental oscillations in its expression that culminated in overexpression in the endosperm and a decline in the progeny (Table 4.4.12). On the contrary, a decrease in bra-miR168 accumulation did not lead to a significant over expression of *braAGO1* transcripts as compared to controls (sequencing data), which suggests that the transcriptional activity of the *braAGO1* promoter remained constant in systemic tissues regardless of stress. Hence, consistent with the negative feedback loop mechanism of AGO1 regulation, it brings us to the conclusion that miR168 can be mitotically or meiotically transmitted from leaves to pollen after stress exposure. Alternatively, miR168 can presumably be conveyed to the gametes through the phloem since both mature

miR168 and a passenger strand along with other miRNAs have been previously detected in the phloem sap of *B. napus* plants (Buhtz, Springer et al. 2008). In our experiment, only the mature miR168 strand was detected.

Recently, it has been hypothesized that in addition to being important for the establishment of imprinted expression, endosperm hypomethylation also provides a mechanism to reinforce silencing of transposable elements in adjacent embryo tissues by supplying smRNAs (Mosher, Melnyk et al. 2009, Mosher and Melnyk 2010, Wuest, Vijverberg et al. 2010). Nevertheless, in our experiment, we failed to detect the significantly altered smRNAs common for the embryo and endosperm of exposed plants. Hence, we proposed an alternative hypothesis whereby the considerably overexpressed smRNAs conveyed to embryo might perform their regulatory activity followed by the rapid degradation. A comparison of the putative predicted gene targets of differentially expressed miRNAs in the endosperm and the significantly altered transcripts in the embryo revealed only one common target – Bra032254 which is an Arabidopsis *AGO1* homologue (E-value = 2e-99, Max identity = 95%, Table 4.4.10). Although it still remains to be validated whether *braAGO1* maintains the same functions as Arabidopsis homologue, it is an interesting finding that conceivably suggests that miR168 and AGO1 are possible bandmasters of transgenerational stress memory inheritance in plants. Consistent with this notion, we observed an inverse Pearson's correlation for the expression of *braAGO1* gene and alterations in the transcriptome profile of the corresponding tissue in parental exposed plants and untreated progeny (r=-0.89, for the total number of differentially expressed genes and *braAGO1* log2fold changes, TR vs CT in the corresponding tissue). At the same time, we did not observe a comparable with

braAGO1 Pearson's correlation for the other epigenetic-related genes: putative *DNA-DIRECTED RNA POLYMERASE E*, r=0.65; putative methyltransferase *CMT2*, r=0.63; and putative lysine-specific demethylase *JMJ14*, r=-0.67.

For the first time to our knowledge, by using a massive parallel sequencing technology, we provide evidence of transgenerational stress memory inheritance both at the transcriptome and smRNAome levels in plants. More importantly, we also suggest that miR168 is a possible messenger that mediates meiotic epigenetic inheritance in plants. Further transgenerational stress experiments involving the Arabidopsis hypomorphic *ago1* mutants will shade new light on its contribution to epigenetic inheritance in plants.

4.6. SUMMARY

The current work was aimed at analyzing the influence of stress on the mRNA and smRNA transcriptome profile of somatic and reproductive tissues in plants and finding putative messengers of trangenerational stress memory, the occurrence of which we previously observed in Arabidopsis plants (Boyko, Blevins et al. 2010, Bilichak, Ilnystkyy et al. 2012). In the process, we also assembled the comprehensive smRNA and mRNA libraries for seven aerial *B. rapa* tissues: leaves, inflorescence meristem, pollen, unfertilized ovules, 24-hour-post fertilization ovules, embryo and endosperm under normal conditions and in response to HS. Generated sequencing data will be subsequently uploaded into publicly available sequencing depositories.

Overall, we observed that direct and paternal HS treatments of *B. rapa* plants had a profound tissue-specific effect on the smRNA and mRNA abundance. The highest oscillations in the transcriptome profile were observed not in the directly exposed tissues such as leaves but in the endosperm and embryo. This is a novel finding demonstrating a global priming of genes in plant systemic tissues. The data obtained in this work also reveal that plants can maintain the epigenetic memory of a single relatively short-term, mild stress exposure during the complete life cycle at the levels of gene and smRNA expression.

In the current study, we also provide a new evidence of trangenerational inheritance of stress exposure in plants. Using Illimina GAIIx sequencing, we demonstrate that stress-induced transgenerational inheritance is more pronounced at smRNA levels rather than at gene expression levels. This novel finding partially supports our hypothesis of smRNAs as putative transgenerational epigenetic messengers, albeit

alterations in their expression can be the outcome but not the cause of chromatin fluctuations that were not examined in this study.

We have suggested that miR168 with its target *AGO1* are putative messengers of transgenerational epigenetic inheritance in plants. After overexpression in directly exposed leaves, the level of miR168 can be conceivably maintained throughout multiple cell divisions and/or eventually be conveyed to pollen. Along its journey, it triggers the modulation of *braAGO1* expression in tissues, which results in global oscillations in gene expression. Eventually, as a consequence of the self-regulatory feedback loop, the expression of miR168 culminates in the embryo and endosperm, thus significantly reducing the levels of *braAGO1* transcripts and along with global DNA hypomethylation, (Gehring, Bubb et al. 2009) inducing mis-regulation of genes in exposed plants. A decline in *braAGO1* expression in the embryo and endosperm required for miR168 stabilization (Vaucheret, Mallory et al. 2006) leads to a significant decrease in miR168 accumulation, which eventually brings *braAGO1* transcripts to the control level in untreated progeny. Unfortunately, due to the lack of necessary equipment for performing laser capture dissection (Casson, Spencer et al. 2005), we were unable to separate an egg mother cell and central cell. Therefore, we were not able to distinguish fluctuations in the transcriptome and smRNAome in these two cells and in the rest of the ovule. Thus, it is vague to speculate why a significant overexpression of miR168 observed in pollen (sequencing data) was not passed on to fertilized ovules. The hypothesis of the involvement of miR168 and *AGO1* in transgenerational epigenetic memory inheritance in plants awaits further confirmation.

5. APPLIED PLANT EPIGENETICS: REVEALING THE CONTRIBUTION OF THE EPIGENETIC MACHINERY TO PLANT DEFENCE SYSTEMS AGAINST *AGROBACTERIUM*-MEDIATED TRANSFORMATION

5.1. ABSTRACT

The epigenetic pathway involved in the regulation of foreign and parasitic nucleic acid activities that is termed RNA silencing is part of antiviral defence in plants. We hypothesized that the RNA silencing machinery is also involved in the protection against a single-stranded T-DNA of *Agrobacterium tumefaciens*, a plant pathogen that is widely used in plant transgenesis. Here, we provide evidence that plants utilize a similar small interfering RNA pathway against both viruses and *Agrobacterium* pathogens. Using mutants compromised in either transcriptional or posttranscriptional gene-silencing pathways, two inhibitors of stable transformation were revealed – *AGO2* and *NRPD1a.* Mutants of these genes exhibit a decrease in global DNA methylation and elevated levels of DNA strand breaks. We further demonstrate that the *Tobacco rattle virus* (TRV) - based virus-induced gene silencing (VIGS) technique can be successfully used to transiently down-regulate the expression of both genes in reproductive organs of Arabidopsis, thus allowing to increase transformation rate. Additionally, we provide evidence that infection of Arabidopsis with an empty TRV vector increases the number of transgene integration events almost by threefold. These findings further support the idea that plants utilize similar epigenetic pathways against foreign nucleic acids irrespective of their origin.

5.2. INTRODUCTION

The advancement of plant transgenesis techniques that allow the delivery and expression of foreign genes in plant cells resulted in engineering of transgenic plants with enhanced pest resistance, elevated tolerance to harsh environmental conditions, and the superior quality of seeds and fruits (Herrera-Estrella, Simpson et al. 2005, Tsaftaris, Polidoros et al. 2008). Among transformation techniques available at the present time *Agrobacterium tumefaciens* (*Agrobacterium*) – mediated transgene delivery is considered to be the most efficient and reproducible method of dicotyledonous plants (dicots) transformation (Windels, Buck et al. 2008). *Agrobacterium* is a natural plant pathogen that exploits a horizontal gene-transfer system to deliver a portion of its DNA in a single-stranded form, known as transferred DNA (T-DNA), into plant cells (Tzfira and Citovsky 2006). The integration of T-DNA into genomic DNA (gDNA) conceivably requires broken DNA and eventually leads to the development of tumours with the altered auxin/cytokinin balance and overexpression of opines produced by the T-DNA-encoded enzymes (Escobar and Dandekar 2003). *Agrobacterium* can thrive in the resulting tumour by metabolizing opines. This unique ability of *Agrobacterium* to deliver a portion of its DNA to the plant genome has been widely exploited for transient and stable plant transformation by using the oncogene-free or 'disarmed' T-DNAs. However, transgenes within the T-DNA are often either poorly expressed or not expressed at all in the plants predominantly owing to the RNA silencing of the transgenes (Dunoyer, Himber et al. 2006).

Methylation of transgenic DNA directed by viral RNA was discovered two decades ago in transgenic tobacco plants, long before the role of the RNA interference

(RNAi) and small RNAs (smRNAs) was known (Wassenegger, Heimes et al. 1994). Nowadays, two major overlapping pathways are recognized that epigenetically modulate endogenous and exogenous gene activities: posttranscriptional gene silencing (PTGS) and transcriptional gene silencing (TGS). Both pathways have been implemented in counteracting viral infection, preventing transposon mobility, and regulating endogenous gene and transgene expressions (Raja, Sanville et al. 2008, Castel and Martienssen 2013).

The inhibiting action of the TGS and PTGS defence pathways on transgene expression is one of the main challenges for plant genetic engineering. The solution may come from studies performed by using viral plant pathogens that evolved a wide variety of viral suppressors of RNA silencing to counteract both the TGS and PTGS pathways in plants (Burgyan and Havelda 2011). For instance, the overexpression of the *Turnip crinkle virus* P38 protein in *Arabidopsis thaliana* (Arabidopsis) plants specifically inhibits the production of small interfering RNAs (siRNAs) from inverted repeats or sense transgenes and significantly enhances the plant's susceptibility to the virulent *Agrobacterium* strain as compared to control plants (Dunoyer, Himber et al. 2006). Furthermore, co-expression of the P19 protein of tombusviruses that prevents RNA silencing by binding viral siRNAs with high affinity (Silhavy, Molnar et al. 2002) enhances the expression of proteins from T-DNA by 50-fold (Voinnet, Rivas et al. 2003). Hence, it is obvious that plants utilize the similar epigenetic defence pathways against viruses and *Agrobacterium*-mediated transformation, however unlike viruses, *Agrobacterium* did not evolve suppressors of RNA silencing (at least it was not discovered yet).

Curiously, the mode of action of *Agrobacterium* to deliver its T-DNA into the plant cell closely resembles that of viral plant pathogens belonging to the viral *Geminiviridae* family (geminiviruses). For instance, both *Agrobacterium* and geminiviruses deliver ssDNA that does not encode any polymerases and therefore depends on the host machinery for replication and transcription (Buchmann, Asad et al. 2009). At the same time, whereas geminiviruses carry and transfer a circular ssDNA, *Agrobacterium* transfers a linear T-DNA to its host, albeit recently it has been shown that complex extrachromosomal T-DNA structures, including circular T-DNA molecules, are formed in *Agrobacterium*-infected plant cells immediately after infection (Krupovic, Ravantti et al. 2009, Singer, Shiboleth et al. 2012). This finding allowed the authors to speculate that prior to integration, a single-stranded linear T-DNA is converted into a double-stranded form by host polymerases, followed either by integration, circularization or formation of extrachromosomal structures that eventually may integrate into the plant genome. The behaviour of the *Agrobacterium* T-DNA further overlaps with biogenesis of the viral DNA whereby ssDNA is copied by complementary strand replication (CSR) to double-stranded covalently closed circular DNA (cccDNA) that is later reproduced by rolling circle replication (RCR) and recombination-dependent replication (RDR), thus generating large amounts of heterogeneous linear dsDNAs (Paprotka, Deuschle et al. 2011). Although both processes take place in the nucleus, unlike the *Agrobacterium* T-DNA associated with bacterial proteins, the viral DNA has not been reported to have any integrase activity (Liu, Fu et al. 2011). Nevertheless, a recent systematic search for sequences similar to the viral circular ssDNA in publicly available databases of eukaryotic genome revealed that virus-related sequences of geminiviruses, nanoviruses

and circoviruses have been frequently transferred to a broad range of eukaryotic species, including plants, fungi, animals and protists (Liu, Fu et al. 2011). To be retained across generations, such transfer had to occur either in somatic predecessors of gametes or directly in germ cells, which resembles the ability of *Agrobacterium* to deliver the T-DNA to the nucleus of both somatic cells and female gametes (Ye, Stone et al. 1999). Hence, bearing in mind that geminiviruses and *Agrobacteria* utilize similar intracellular pathways to invade their host cells, we hypothesized that both pathogens trigger an analogous epigenetic immune response in plants.

Recent reports have provided an explicit proof of the epigenetic nature of plant defence pathways against geminiviruses (Raja, Sanville et al. 2008, Buchmann, Asad et al. 2009, Rodriguez-Negrete, Carrillo-Tripp et al. 2009, Paprotka, Deuschle et al. 2011, Zhang, Chen et al. 2011). Moreover, both the TGS and PTGS pathways have been implemented in counteracting the viral infection. A number of epigenetic mutants impaired in the maintenance of global DNA methylation and small RNA metabolism were highly susceptible to the *Cabbage leaf curl virus* (CaLCuV, genus *Begomovirus*) and *Beet curly top virus* (BCTV, genus *Curtovirus*) belonging to geminiviruses (Raja, Sanville et al. 2008). Similarly, an increase in *Agrobacterium*-mediated plant transformation efficiency may possibly be achieved by manipulating the expression of genes that have been shown to participate in antiviral epigenetic defence. We hypothesized that mutants that are highly susceptible to viral infection might also be prone to *Agrobacterium*-mediated transformation.

Indeed, by using a floral dip transformation method, we revealed two inhibitors of stable plant transformation – *AGO2* and *NRPD1a* that are involved in the PTGS and TGS

pathways, respectively. We found a threefold increase in the rate of stable transformation in both mutants as compared to controls. An increase in transformation rates was paralleled by a decrease in global DNA methylation and an increase in the levels of DNA strand breaks, two marks that are assumed to favour high rates of *Agrobacterium*-mediated transformation (Magori and Citovsky 2011, Gohlke, Scholz et al. 2013). Using a *Tobacco rattle virus* (TRV)-mediated virus-induced gene silencing (VIGS) system, we further demonstrate that the transient disruption of either *AGO2* or *NRPD1a* genes in Arabidopsis wild-type (wt) plants can also considerably enhance a stable plant transformation efficiency. At the same time, we provide new evidence that an infection of Arabidopsis wt plants just with empty TRV leads to almost a threefold increase in transgene copy number in transgenic plants obtained after using *Agrobacterium*-mediated floral dip transformation method. At the same time, the amount of recovered transgenic plants was comparable to that in controls. Our findings further support the notion that defence pathways against virus and *Agrobacteria* infections overlap in plants.

5.3. MATERIALS AND METHODS

5.3.1. Plant cultivation

In this study, the following homozygous mutant *Arabidopsis thaliana* (Arabidopsis) lines of ecotype Columbia (Col-0) background were used: *ago2-1* (Lobbes, Rallapalli et al. 2006), *dcl3-1* (Blevins, Rajeswaran et al. 2006), *dcl4-2* (Blevins, Rajeswaran et al. 2006), *ddm1-2* (Jeddeloh, Stokes et al. 1999), *nrpd1a-3* (Onodera, Haag et al. 2005), *rdr2-2* (Vazquez, Vaucheret et al. 2004) and *rdr6-1* (Xie, Johansen et al. 2004). The Arabidopsis Col-0 was used as a wild-type (wt) control in all experiments.

Transgenic Arabidopsis line 15d8 (ecotype Col-0) homozygous for the luciferase-based recombination reporter construct (Ilnytskyy, Boyko et al. 2004) was crossed with either *ago2-1* or *nrpd1a-3* mutants and plants homozygous for the recombination reporter transgene, and the respective mutations were selected and used for further analysis. F3 and F4 generations derived from these crosses were used in all experiments.

5.3.1.1. Growing Arabidopsis wild-type and mutant plants for transformation experiments

Seeds from homozygous mutants and wt plants were planted on *All purpose potting soil* (Plant *Etc*; Lethbridge, AB, Canada) mixed with vermiculite in proportion: 4:1 (The Professional Gardener Co LTD, AB, Canada) in 2x2 inch square pots. Soil mixture in the pots was pre-soaked once with Miracle-Gro fertilizer (Scotts Canada *Ltd.*, Mississauga, ON, Canada) and was maintained continuouslly moist with tap water. Seeds were incubated at 4°C in darkness for 2 days to break dormancy, and then moved to a

growth chamber (Biochambers, model SPRS-1115, Winnipeg, MB, Canada). Over the duration of the experiment, plants were grown in high light conditions (32.8 $\mu Em^{-2}s^{-1}$) at 22°C under a 16-hour light regime and at 18°C under an 8-hour dark regime under a constant humidity of 65%.

5.3.2. Plant transformation techniques and screening for transgenic plants

5.3.2.1. *Agrobacterium*-mediated floral dip transformation

Arabidopsis stable transformation was performed using the previously described *Agrobacterium*-mediated floral dip method with some modifications (Clough and Bent 1998). The *Agrobacterium tumefaciens* (*Agrobacterium*) strain GV3101 (pMP90) (Csaba and Jeff 1986) carrying the binary vector pCAMBIA3301 was used in all stable transformation experiments. Bacteria were grown to stationary phase in liquid culture at 28°C, 180 rpm in sterilized YEP (10 g tryptone, 5 g yeast extract, 5 g NaCl per litre water) carrying added rifampicin (25 μg/ml), gentamycin (25 μg/ml) and kanamycin (50 μg/ml). Cultures were typically started from a 1:100 dilution of smaller overnight cultures and grown for about 18–24 h. Cells were harvested by centrifugation for 20 min at room temperature at 4000 g and then re-suspended in the infiltration medium to a final OD_{600}=0.80 prior to use. The floral dip inoculation medium contained 5.0% sucrose and 0.005% Silwet L-77 (Lahile seeds, USA). For floral dip, the inoculum was added to a beaker, plants were inverted into this suspension so that all floral buds were submerged, and then plants were removed after 3–5 seconds of gentle agitation. Plants were left covered in the dark for two days in the growth chamber, after that the cover was removed. Plants were grown for a further 3–5 weeks until siliques were brown and dry,

keeping the bolts from each pot together and separated from neighbouring pots using garden wires and supporting sticks. Seeds were harvested from each plant separately and if necessary, were dried overnight at 37°C and used for screening of T1 transgenic plants with a glufosinate ammonium solution (see below).

5.3.2.2. Screening for transgenic Arabidopsis plants using glufosinate ammonium

Glufosinate ammonium is an active ingredient of several non-selective systemic herbicides that interferes with the biosynthetic pathway of the amino acid glutamine and with ammonia detoxification. Resistance to glufosinate ammonium is conferred by the bacterial *BIALOPHOS RESISTANCE* gene (*BAR*) encoding the enzyme phosphinotricin acetyl transferase (PAT) (Weigel and Glazebrook 2006).

T1 seeds obtained from floral dip-transformed plants were planted at a high density in 2-inch dip pots filled with soil mix and pre-soaked with a fertilizer. After 2 days of incubation at 4°C in the dark, the pots with seeds were moved to the growth chamber, and the seeds were germinated under the same conditions as described previously (see Section 5.3.1.1. Growing Arabidopsis wild-type and mutant plants for transformation experiments). Two or three days post germination, seedlings were sprayed with 1000x dilution of Liberty 150CN Herbicide and Crop Desiccant stock solution (Aventis CropSciense Canada Co, Canada, 150 g/L concentration of glufosinate ammonium in the stock solution) once a day for 7 days in a row. Plants resulting from stable transformation received a transfer DNA (T-DNA) region harboring the *BAR* gene present in the pCAMBIA3301 plasmid appeared to be green healthy seedlings after 1-2

weeks of herbicide application, whereas the non-transgenic plants demonstrated bleaching of cotyledons and delay in growth. The healthy-looking transgenic plants were transplanted into separate pots and grown for two weeks followed by tissue collection for transgene copy number analysis and RNA isolation.

5.3.2.3. *Tobacco rattle virus*-mediated virus-induced gene silencing in Arabidopsis plants

Transient suppression of specific Arabidopsis genes was achieved using the previously described *Tobacco rattle virus* (TRV)-mediated virus-induced gene silencing (VIGS) method with some modifications (Burch-Smith, Schiff et al. 2006, Wang, Cai et al. 2006). The genome of TRV is conveniently cloned into two binary vectors - pTRV1 and pTRV2 that are used for *Agrobacterium*-mediated virus delivery (Liu, Schiff et al. 2002). Whereas the pTRV1 plasmid carries almost the whole viral genome, the pTRV2 plasmid encodes the coat protein from the genomic RNA and two non-structural proteins from the subgenomic RNAs. Cloning of the targeted gene fragments is performed in the pTRV2 plasmid. Silencing of the Arabidopsis *PHYTOENE DESATURASE* (*PDS*) gene was used as a positive control of the successful development of VIGS in infected plants. All plasmids in *E. coli* cultures were obtained from the ABRC stock centre (stock numbers: CD3-1039, CD3-1040, and CD3-1043 for pTRV1, pTRV2, and pTRV2-*PDS* respectively).

For the VIGS protocol, separate colonies of *Agrobacterium* GV3101 (pMP90) carrying the pTRV1 and recombinant pTRV2 constructs were inoculated into 3 ml Luria-Bertani (LB) medium containing rifampicin (25 µg/ml), gentamycin (25 µg/ml) and

kanamycin (50 µg/ml) and shaken at 28°C overnight. Cultures (1 ml) were transferred into 100 ml YEBi medium (the composition per 100 ml of solution: 0.5 g beef extract, 0.1 g yeast extract, 0.5 g bacto-peptone, 0.2 ml of 1 M $MgSO_4$ (pH 7.0), 10 ul of 200 mM acetosyringone, 1 ml of 1M MES and antibiotics: rifampicin, gentamycin and kanamycin in the aforementioned concentrations) and grown until OD_{600} was between 0.8 and 1.2. Subsequently, *Agrobacterium* cells were collected by centrifugation and re-suspended in MMAi solution (the composition per 100 ml of solution: 0.5 g MS salts (Phytotechnology laboratories, KS, USA), 0.195 g MES, 2.0 g sucrose, 100 µl of 200 mM acetosyringone, pH 5.6) into suspensions of OD_{600}=4.0 followed by shaking at a speed of 50 rpm for 2 hours. The suspensions of *Agrobacterium* carrying the pTRV1 and pTRV2 recombinant constructs were mixed at a 1:1 ratio to lead to a final OD_{600}=2.0 of each culture.

The delivery of *Agrobacterium* suspension was performed with a needleless 1 ml syringe into two leaves of two- to three-leaf-stage plants, infiltrating the entire leaf from the abaxial side of the leaf. The plants were left covered overnight. Symptoms of viral infection were observed approximately 1 week after virus introduction. Infected and control plants were left to flower and eventually were stably transformed using a floral dip method.

5.3.3. Molecular techniques and assays

5.3.3.1. Total DNA, RNA and cDNA preparations

gDNA was isolated from 100 mg of leaf samples using the cetyltrimethylammonium bromide method (Doyle and Dickson 1987) that was modified according to the DArT protocol (http://www.diversityarrays.com/sites/default/files/pub/

DArT_DNA_isolation.pdf) (Ziemienowicz, Shim et al. 2012). Subsequently, gDNA was treated with RNaseA (a final concentration: 20 µg/ml) for 30 min at 37°C followed by purification using the phenol-chloroform method and precipitation with ethanol (Sambrook and Russell 2006).

Total RNA from Arabidopsis plants was isolated as described previously (see Section 4.3.2.1. Total RNA isolation and purification). After RNA isolation, 500 ng of total RNA was treated with the DNase I (Thermo Scientific), purified, converted into cDNA and either quantified with qPCR as previously described (see Section 3.3.5. Real-Time qPCR Analysis) or used for PCR amplification of the target gene fragment and cloning into the pTRV2 plasmid. Gene expression was quantified for the following genes: *KU70*, *RAD51*, *AGO2* and *NRPD1a*. Normalization was done against two housekeeping genes: *TUBULINE* and *RCE1* (Seki, Narusaka et al. 2001). For primer sequences see Table 5.3.1

5.3.3.2. Southern blot analysis of the transgene copy number in T1 transgenic plants

To test for the transgene copy number, Southern blot analysis was performed as described previously (Ziemienowicz, Shim et al. 2012). Six microgram of gDNA was digested with FastDigest PstI in a 200 µl reaction mixture containing 1xFastDigest buffer (Thermo Scientific) and 12 ul of the enzyme. The reactions were incubated at 37°C for 2 hours. The digested DNA was purified using the phenol/chloroform method and precipitated with ethanol (Sambrook and Russell 2006). The digested and purified gDNA was separated on a 0.8% (w/v) agarose gel at 220 V for 3 h in 1xTAE buffer. The gel was

rinsed in distilled water, depurinated for 15 min in 0.25 N HCl, rinsed in water, denatured for 30 min in 0.4 N NaOH, rinsed again in water, neutralized for 15 min in 0.5 M Tris-HCl (pH 7.5) containing 3 M NaCl, and soaked in transfer buffer (10x SSC; 1.5 M NaCl and 0.15 M sodium citrate) for 10 min. DNA transfer onto a positively charged nylon membrane (Roche) was performed for 2.5 h using a vacuum blotter (Appligene). DNA was then cross linked to the membrane at 120 mJ/cm^2. UV treatment of the membrane was repeated twice using a Spectrolinker (Spectromics Corp.). The probe was prepared with the PCR DIG Probe Synthesis kit (Roche) according to the protocol provided by the manufacturer using primers AB136 and AB139 for the GUS-specific probe (Table 5.3.1). Hybridization with the GUS-specific probe was performed at 55°C in the DIG EasyHyb solution (Roche). The probe was detected using AP-conjugated anti-DIG antibodies (Roche; diluted 1:2,500) in blocking solution (1% (w/v) Blocking Reagent (Roche) in maleic acid buffer (0.1 M maleic acid, 0.5 M NaCl, pH 7.5)) and a substrate - CDP Star (Roche). Pictures of the membrane were taken with FluorChem HD2 (Alpha Innotech).

5.3.3.3. Plasmid construction

Floral dip stable transformation was performed using the pCAMBIA3301 binary vector generated by replacing the hygromycin resistance gene in the original pCAMBIA2301 plasmid with the *BAR* gene. The *BAR* gene was amplified as an *XhoI* fragment from pFGC5941 (ABRC accession number 1004952070) using primers AB026 and AB027 (Table 5.3.1).

In order to minimize the off-target effects, *AGO2* and *NRPD1a* sequences for VIGS-mediated gene down-regulation that were cloned into pTRV2 plasmids were

picked up using the "RNAi Scan" program (Xu, Zhang et al. 2006). To generate pTRV2-*AGO2*, a cDNA fragment was PCR amplified using Arabidopsis ecotype Col-0 cDNA and primers AB286 and AB287 (see Table 5.3.1 for primer sequences). The resulted PCR product (567 bp) was cloned into *EcoRI*- *XbaI*-cut pTRV2. To obtain pTRV2-*NRPD1a* plasmid, the Arabidopsis *NRPD1a* cDNA fragment was PCR amplified from Arabidopsis cDNA using primers AB304 and AB305 (200 bp) and cloned into pTRV2 digested with *XbaI* and *BamHI* (see Table 5.3.1 for primer sequences). All recombinant constructs were verified by sequencing.

5.3.3.4. Measuring the levels of DNA strand break using the ROPS assay

Measurments of single- and double-strand breaks were performed using the random oligonucleotide primed synthesis (ROPS) assay as described (Basnakian and James 1996). In the ROPS assay, the Klenow fragment is used to catalyze random oligonucleotide-primed synthesis from the re-annealed 3´OH ends of single-stranded (ss)DNA. The DNA is first denatured at elevated temperatures followed by a re-association step. The ssDNA serves as its own primer by randomly re-associating with itself or other ssDNA molecules. Under the controlled reaction conditions, the incorporation of [^{3}H]-dCPT into a newly synthesized DNA by the Klenow fragment is proportional to the initial number of 3´OH ends (breaks).

The isolated gDNA (1 µg) aliquot was heat-denatured at 100°C for 5 min and then chilled on ice. The 25 µl of reaction mixture for one sample contained the following components: 1 µg of heat denatured DNA, 2.5 µl of 0.5 mM 3 dNTPs (dGTP, dATP and dTTP mix) (Fermentas), 2.5 µl of 10x Klenow fragment buffer (New England Biolabs), 5

units of the Klenow enzyme (New England Biolabs), 0.1 µl of [^{3}H]-dCPT (PerkinElmer; Boston, MA, USA). The reaction mix was incubated at 25°C for 60 min and stopped by 2.5 µl of 100 mM EDTA pH 8.0. Later, the whole reaction mix was transferred to 25 mm DE-81 ion-exchanging filter papers (Whatman). The filter papers were air-dried followed by a washing step with 500 mM sodium phosphate buffer (pH 7.0) for 3x10 min. The filters were air-dried again, transferred to vials containing 5 mL of scintillation cocktail each, and radiation levels were assessed using a scintillation counter (Beckman LS 5000CE; Fullerton, CA, USA) as ^{3}H counts per minute (CPM). Three biological and two technical repeats were done per each Arabidopsis mutant. Results are expressed as a mean of the three biological repeats.

5.3.3.5. Analysis of global genome methylation

A cytosine extension assay was used to measure the levels of global genome methylation (Pogribny, Yi et al. 1999). The DNA is digested with one of the methylation-sensitive restriction endonucleases, and the incorporation of radioactively labeled [^{3}H]-dCPT nucleotides in the resulting overhangs is detected. In this study two isoschizomers were used – HpaII and MspI – which recognize the C↓CGG sequence. Whereas the restriction activity of the HpaII endonuclease is blocked by methylation of the internal cytosine in the recognition sequence, the MspI enzyme is sensitive to methylation of the external cytosine. The incorporation of [^{3}H]-dCPT is proportional to the initial number of recognition sites containing unmethylated cytosines, thus the higher the level of methylation at restriction sites, the lower the incorporation of [^{3}H]-dCPT by the *Taq* polymerse. Hence, the HpaII enzyme was used to assess global methylation at the CpG

sites, whereas the MspI enzyme was used to quantify global CpHpG methylation (where H = A, C, or T) (Boyko and Kovalchuk 2010).

Total gDNA was digested with 10 units of either of the methylation-sensitive restriction endonucleases: HpaII or MspI (Fermentas) in 1x enzyme buffer in a total volume of 20 µl overnight at 37°C. After the digestion, 10 µl of each mix was added to 1.5 mM $MgCl_2$, 0.5 units of *Taq* polymerase (Fisher Scientific), and 0.1 µl of [^{3}H]-dCPT (PerkinElmer; Boston, MA, USA) in a final volume of 25 µl. The samples were incubated at 56°C for 1 hour. Later, the whole reaction volume of each sample was applied to 25 mm DE-81 ion-exchanging filter paper (Whatman), air-dried and washed 3 times for 10 min each with 500 mM sodium phosphate buffer (pH 7.0). Subsequently, the filters were thoroughly air-dried, transferred to vials containing 5 mL of scintillation cocktail, and the radiation levels were measured by a scintillation counter (Beckman LS 5000CE; Fullerton, CA, USA) as CPM. As a background control, each sample was incubated in the absence of any enzyme and subjected to identical reaction conditions. The measurements of radioactivity using samples of gDNA of a mutant background were related to readings from wt-control samples, with controls being taken as 100%. Three biological and two technical repeats were done per each Arabidopsis mutant.

5.3.4. Analysis of homologous recombination frequency in mutant Arabidopsis plants

Arabidopsis plants of line 15d8 carry in the genome two non-functional overlapping halves of the luciferase gene that are substrates for homologous recombination (HR) (Ilnytskyy, Boyko et al. 2004). A single recombination event in the

recombination cassette that restores luciferase activity can be visualized *in vivo* with a CCD camera as a luminescent spot. HR frequency was calculated by relating the number of events to the total number of plants scored. Each experiment was repeated at least 3 times with 32 plants per group.

5.3.5. Statistical treatment of the data

The statistical significance was confirmed by two-tailed paired Student's t-test with α=0.05 and single factor ANOVA. The statistical analysis was performed using the JMP 10.0 software (SAS Institute Inc).

Table 5.3.1. Primers used in this study

Primer	Sequence	Description
AG392	5′-TCTGTGATGGCTTGTCTAT-3′	Forward qPCR primer – *AtKU70* - AT1G16970
AG393	5′-AGGCTGAATCCGTTGTAT-3′	Reverse qPCR primer – *AtKU70* - AT1G16970
AG394	5′-TGGTGTGGCTGTTGTTAT-3′	Forward qPCR primer – *AtRAD51* - AT5G20850
AG395	5′-TCTCCTCTGCTCTTCCTT-3′	Reverse qPCR primer – *AtRAD51* - AT5G20850
AG390	5′-ACAGAAGCGGAGAGCAACAT-3′	Forward qPCR primer – *Tubulin* - AT5G62690
AG391	5′-TCCTCATCCTCGTAGTCACCTT-3′	Reverse qPCR primer – *Tubulin* - AT5G62690
RCE1.for	5′-CTGATGCATGGATATTACC-3′	Forward qPCR primer – *RCE1* - AT4G36800
RCE1.rev	5′-ACTGTGTTAATGTTAAAGAA-3′	Reverse qPCR primer – *RCE1* - AT4G36800
AB275	5′-AATAATGATGGAAGTGATAA-3′	Forward qPCR primer – *AtAGO2* - AT1G31280
AB276	5′-AAGAGTGTAGTAATGAGT-3′	Reverse qPCR primer – *AtAGO2* - AT1G31280
AB121	5′-AAAGGGAATATCGGGAAG-3′	Forward qPCR primer – *AtNRPD1a* - AT1G63020
AB122	5′-CCAAAGGACAAAGAAACTG-3′	Reverse qPCR primer – *AtNRPD1a* - AT1G63020
AB286	5′-AAAGAATTCGAGGAGAACAAGGTCGTG-3′	Primers for amplification of the *AtAGO2* fragment from cDNA. Primers include *EcoRI* and *XbaI* recognition sequences. L=567 bp
AB287	5′-AAATCTAGAACCTCGAAACCTTCTTGG-3′	
AB304	5′-AAATCTAGACACACTGTTGGACAATAAAG-3′	Primers for amplification of the *AtNRPD1a* fragment from cDNA. Primers include *XbaI* and *BamHI* recognition sequences. L=567 bp
AB305	5′-AAAGGATCCCAACAGAAGTCGTTGGGAGG-3′	
AB136	5′-GGTCAATAATCAGGAAGTG-3′	Primers for synthesis DIG-labeled GUS probe for Southern blot analysis
AB139	5′-GTATTCGGTGATGATAATC-3′	
AB026	5′-AAACTCGAGTTAGATCTCGGTGACGGGCA-3′	Primers for amplification of a *BAR* gene from pFGC5941 plasmid and cloning it into pCAMBIA2301 plasmid instead of hygromycin gene. *XhoI* recognition sequences added
AB027	5′-AAACTCGAGATGAGCCCAGAACGACGCCC-3′	

5.4. RESULTS

5.4.1. Genes involved in small RNA metabolism inhibit *Agrobacterium*-mediated plant transformation

We chose several Arabidopsis epigenetic mutants (Table 5.4.1) that have been shown to vary in their response to viral infection and are deficient in either the TGS (*ddm1-2, dcl3-1*, *nrpd1a-3* and *rdr2-2*) or PTGS pathways (*ago2-1*, *dcl4-2* and *rdr6-1*) and performed reverse genetic screening to reveal the contribution of epigenetic machinery to *Agrobacterium*-mediated plant transformation process. To do this, we used the floral dip method of Arabidopsis transformation that relies on the ability of *Agrobacterium* cells to deliver T-DNA to the egg cell in the plant embryo sac. Subsequently, T1 seeds obtained after transformation are germinated on soil, and the selection of transgenics is performed using glufosinate ammonium.

Consistent with our hypothesis, half of the epigenetic mutants in our study demonstrate susceptible to *Agrobacterium* transformation phenotype (*ago2-1*, *dcl4-2*, *nrpd1a-3* and *rdr2-2*), with *ago2-1* and *nrpd1a-3* Arabidopsis mutants having the highest transformation efficiency (a threefold increase in transformation over wt plants, Student's t-test: α=0.05, Figure 5.4.1). At the same time, in our study, *dcl3-1* and *ddm1-2* mutants that, demonstrated moderately enhanced and severe disease symptoms in response to geminivirus infection, respectively (Raja, Sanville et al. 2008) had the transformation efficiency that was comparable to that of wild-type plants. Furthermore, the discrepancy was observed between the transformation efficiency of *dcl4-2* and *rdr2-2* mutants and their susceptibility to geminivirus infection previously reported by Raja et al. (2008) (Raja, Sanville et al. 2008). Whereas in our study, loss-of-function *dcl4-2* and *rdr2-2*

mutants demonstrated a twofold although statistically insignificant increase in the transformation efficiency as compared to wt control plants (Student's t-test: $p>0.1$, Figure 5.4.1), in the study of Raja et al. (2008), mutant susceptibility to geminivirus infection was similar to that in wt plants (Raja, Sanville et al. 2008). Overall, our results provide evidence for an increased susceptibility of reproductive tissues to *Agrobacterium* infection in epigenetic mutants, but at the same time, they demonstrate the discrepancy between the plant defence pathways against geminivirus and *Agrobacterium* infection.

Table 5.4.1. Genes examined in this study for their role in *Agrobacterium*-mediated stable plant transformation

Gene	Locus number	Protein function	Reference
ARGONAUTE 2, *AGO2*	AT1G31280	Involved in the antiviral defence pathway, has high affinity to viral siRNAs	(Harvey, Lewsey et al. 2011, Wang, Jovel et al. 2011)
DICER-LIKE 3, *DCL3*	AT3G43920	The RNase III family protein. Required for the generation of 24 nt-long siRNAs from endogenous and viral transcripts	(Curtin, Watson et al. 2008, Fan, Dai et al. 2012)
DICER-LIKE 4, *DCL4*	AT5G20320	The RNase III family protein. Catalyzes processing of trans-acting 21 nt-long siRNAs and viral transcripts	(Yoon, Yang et al. 2010, Wang, Jovel et al. 2011)
DECREASED DNA METHYLATION 1, *DDM1*	AT5G66750	A SWI2/SNF2-like chromatin remodelling protein involved in cytosine methylation in CpG and non-CpG contexts	(Jeddeloh, Stokes et al. 1999)
NUCLEAR RNA POLYMERASE D1A, *NRPD1a*	AT1G63020	One of two alternative large subunits of RNA polymerase IV. Involved in RNA dependent DNA methylation	(Eamens, Vaistij et al. 2008)
RNA-DEPENDENT RNA POLYMERASE 2, *RDR2*	AT4G11130	Synthesizes a second strand using a single-stranded (ss) RNA as a template. Involved in the biogenesis of heterochromatic siRNAs	(Willmann, Endres et al. 2011)
RNA-DEPENDENT RNA POLYMERASE 6, RDR6	AT3G49500	Synthesizes a second strand using ssRNA as a template. Involved in the biogenesis of ta-siRNAs and nat-siRNAs	(Willmann, Endres et al. 2011)

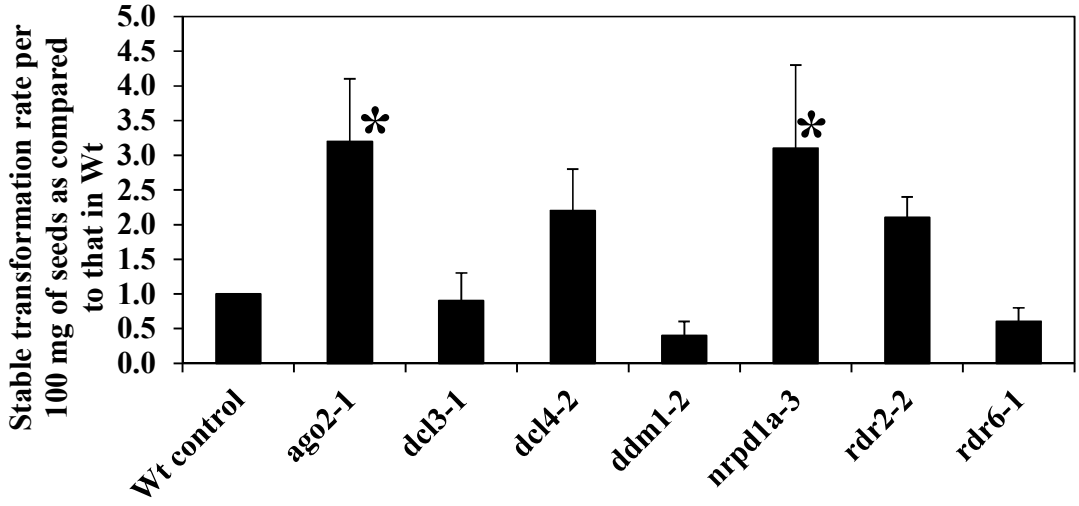

Figure 5.4.1. Reverse genetic screening among selected epigenetic mutants identifies two gene-inhibitors of *Agrobacterium*-mediated stable plant transformation – *AGO2* and *NRPD1a*

The analysis of stable plant transformation efficiency among selected epigenetic mutants using a floral dip method. The stable transformation rates per 100 mg of seeds in wt control plants were standardized to 1.0. The floral dip transformation was repeated at least 3 times for every mutant and transformation rate per 100 mg of seeds was normalized to its corresponding control. The values represent the mean ± SEM. The asterisks denote a statistically significant difference as compared to wt control plants. Student's t-test: $\alpha=0.05$, $t=2.09$, $P<0.05$.

5.4.2. An elevated transformation efficiency in loss-of-function *ago2-1* and *nrpd1a-3* mutants coincides with a decrease in global DNA methylation and an increase in the levels of DNA strand breaks

Based on the results of our reverse genetic screening (see in the previous section), both *ago2-1* and *nrpd1a-3* mutants were chosen for further analysis of molecular components that are affected to favour a higher transformation efficiency as compared to wt control plants. Argonaute 2 (AGO2) is one of the ten Argonaute proteins that act in the RNA-induced silencing complex (RISC) to target the cleavage of viral RNA through base pairing with small RNAs (Harvey, Lewsey et al. 2011, Zhang, Singh et al. 2012). Moreover, it has been suggested that AGO2, among other Arabidopsis AGO proteins, has evolved to specialize in antiviral defense (Jaubert, Bhattacharjee et al. 2011). This notion was further supported by studies on *Nicotiana benthamiana* plants where *NbAGO* with similarity to the Arabidopsis *AGO2* gene has been demonstrated to be vital for antiviral defense against *Tomato bushy stunt virus* (TBSV) (Scholthof, Alvarado et al. 2011). In contrast to AGO2 which acts predominantly in the PTGS pathway, NRPD1a is involved in the TGS pathway. *NRPD1a* encodes one of two alternative largest subunits of a putative plant-specific RNA polymerase IV (Pol IV), involved in the silencing of thousands of retrotransposons, endogenous repeats, invading DNA viruses, transgenes and some protein-coding genes through the RNA-dependent DNA methylation (RdDM) pathway (Huettel, Kanno et al. 2006, Zhang, Henderson et al. 2007, Mosher, Schwach et al. 2008, Raja, Sanville et al. 2008). Hence, both of the genes seem to have evolved as the components of the immune system that guards the plant cells against the undesirable activity of endogenous (*NRPD1a*) and exogenous nucleic acids (*AGO2* and *NRPD1a*).

Curiously, more recently, both AGO2 and NRPD1a have been shown to directly mediate DNA double-strand break (DSB) repair in Arabidopsis (Wei, Ba et al. 2012). Both *ago2* and *nrpd1a* loss-of-function mutants were greatly compromised in DNA DSB repair that was concomitant with a decline in the level of DSB-induced small RNA (diRNA). It was suggested that diRNAs can guide the repair machinery to the DNA DSB sites that resemble the RdDM pathway in its mode of action (Wei, Ba et al. 2012). Unfortunately, the authors did not directly measure the levels of single-strand breaks or DSB in DNA in the mutants, instead, they provided evidence of the levels of phosphorylated histone H2AX (γ-H2AX) in response to γ-irradiation that were comparable to those in wild-type plants. γ-H2AX is a molecular marker for monitoring DSBs and their repair (Amiard, Charbonnel et al. 2010, Wei, Ba et al. 2012). Neither wt plants nor mutants had detectable γ-H2AX foci in the nucleus of untreated plants. In our study, by using the ROPS assay which provides cumulative information regarding the levels of both single- and double-strand breaks in DNA, we observed a significant increase in the levels of DNA strand breaks in both mutants as compared to wt plants grown under normal conditions (a 6- and 9-fold increase as compared to wt controls in *ago2-1* and *nrpd1a-3*, respectively, Student's t-test: α=0.05, Figure 5.4.2). Altogether, these data may suggest that both *ago2-1* and *nrpd1a-3* mutants have the elevated levels of single-strand DNA breaks.

Previously, mutants impaired in the maintenance DNA methylation pathways or *de novo* DNA methylation pathways have been shown to be hyper-susceptible to geminivirus infection which was concomitant with the reduced levels of DNA methylation at the viral DNA (Raja, Sanville et al. 2008). Treatments of *Nicotiana*

tabacum plants with the DNA demethylating agent 5-azacytidine increased both transient and stable *Agrobacterium*-mediated transformation (Zhu, Hughes et al. 1991, Palmgren, Mattson et al. 1993).

Whereas a decrease in DNA methylation in *pol iv* mutants is well-documented (Onodera, Haag et al. 2005), the effect of *ago2* mutation on global DNA methylation is still unknown. Using a cytosine extension assay, we confirmed a significant global DNA demethylation in both the CpG and CpHpG contexts in the *nrpd1a-3* Arabidopsis mutant (a 2.00- and 1.90-fold decrease in CpG and CpHpG methylation, respectively, Student's t-test: α=0.05, Figure 5.4.3) We also revealed that *ago2* deficient plants demonstrated a significant decrease in global DNA methylation in both examined regions (a 1.75 and 2.05-fold decrease in CpG and CpHpG methylation, respectively, Student's t-test: α=0.05, Figure 5.4.3). Taking into account the results of previous reports on geminivirus infection (Raja, Sanville et al. 2008, Paprotka, Deuschle et al. 2011), it is reasonable to speculate that DNA methylation can act in *cis* through direct methylation of the *Agrobacterium* T-DNA in the plant cell. The exact mechanism of T-DNA and geminivirus DNA methylation and its role in the prevention of T-DNA integration into the plant genome is unknown. Moreover, a decrease in global DNA methylation may result in the chromatin de-condensation and a subsequent increase in recombination levels (Melamed-Bessudo and Levy 2012). Recombination levels can also increase in response to the elevated DNA strand breaks (Endo, Ishikawa et al. 2006).

Recently, in our lab, we have provided evidence that an increase in the frequency of DNA recombination can be a possible cause of higher rates of stable transformation in tobacco explants (Boyko, Matsuoka et al. 2009, Boyko, Matsuoka et al. 2011).

Nevertheless, in the current study, we observed that the level of DNA homologous recombination (HR) events per plant in the leaves of *ago2-1* and *nrpd1a-3* mutant plants was comparable to that in wt plants (Student's t-test: α=0.05, Figure 5.4.4.A). The expression of the *RAD51* gene involved in the HR DNA repair pathway was similar in mutants and control plants, whereas the expression of the *KU70* gene participating in the non-homologous end joining (NHEJ) DNA repair pathway was significantly higher in the *nrpd1a-3* mutant (Student's t-test: α=0.05, Figure 5.4.4.B).

Both HR and NHEJ pathways appear to compete for a template during the DNA DSB repair process, with the NHEJ pathway prevailing in plants (Mladenov and Iliakis 2011). The lack of increase in the frequency of HR events in *ago2* and *nrpd1a* mutants suggests the involvement of the NHEJ pathway in the T-DNA integration process, although we did not directly examine the levels of NHEJ events in *ago2-1* and *nrpd1a-3* mutants. Overall, our data suggest that an increase in the stable transformation efficiency of *ago2-1* and *nrpd1a-3* mutants can be due to cumulative effects of both a deficiency in the *de novo* DNA methylation pathway and an increase in the levels of DNA strand breaks.

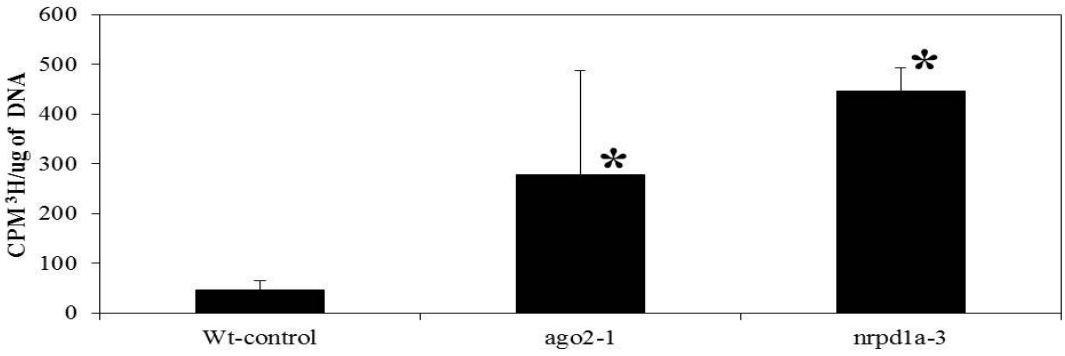

Figure 5.4.2. *ago2-1* and *nrpd1a-3* mutants exhibit an elevated level of DNA strand breaks as assessed using the ROPS assay

The analysis of DNA strand break levels (radioactive counts, CPM ^{3}H) in the genome of *ago2-1* and *nrpd1a-3* mutant plants. The higher the radioactive count, the more single- and double-strand breaks are present in the genome. Two independent experiments were performed, and the radioactivity of each sample was counted twice. The values represent the mean ± SD. The asterisks show a statistically significant difference as compared to wt controls. Student's t-test, α=0.05, t=2.26.

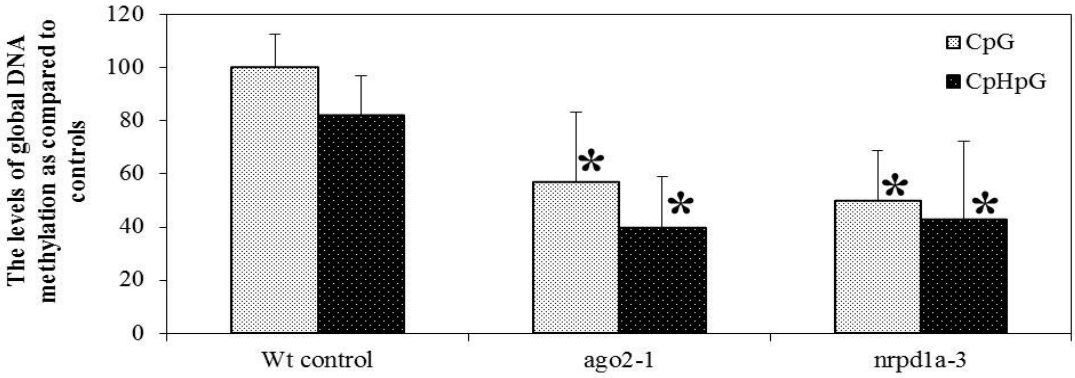

Figure 5.4.3. *ago2-1* and *nrpd1a-3* mutants demonstrate a decrease in the levels of global DNA methylation both in the CpG and CpHpG contexts as assessed by the cytosine extension assay

The average CPM reading for a wt control sample (global CpG methylation) was standardized to 100% and used for normalization of the rest of samples. The cytosine extension assay for each experiment was repeated three times, and readings were taken at least twice per each reaction. The values represent the mean ± SD. The asterisks denote a statistically significant difference as compared to the corresponding wt control plants. Student's t-test: α=0.05, t=2.13.

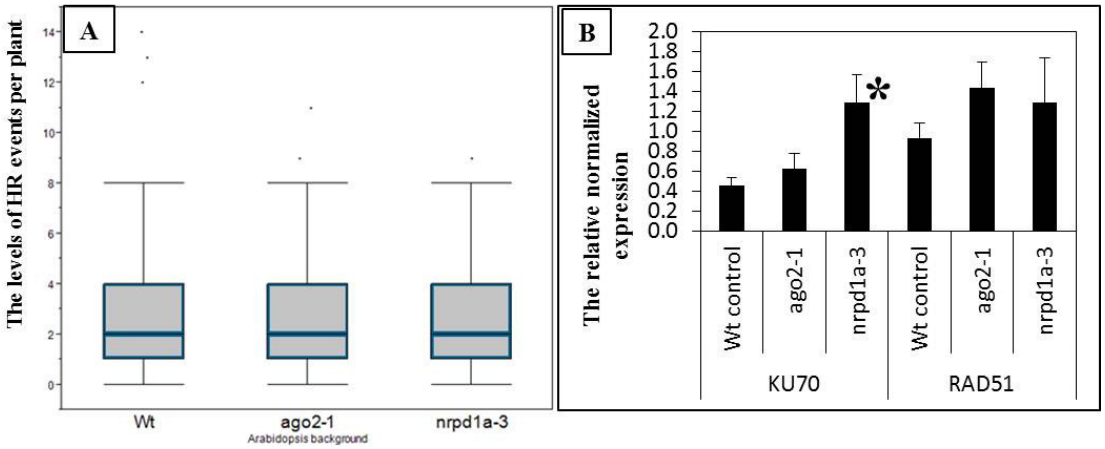

Figure 5.4.4. DNA recombination rates in *ago2-1* and *nrpd1a-3* mutants are comparable to those in wt plants

The analysis of the frequency of DNA HR events (the average number of HR events per plant (**A**) and expression levels of *KU70* and *RAD51* genes (**B**) in *ago2-1* and *nrpd1a-3* mutant plants. For the assessment of HR frequency, *ago2-1* and *nrpd1a-3* mutant plants were crossed to wt plants carrying a luciferase-based construct (15d8 line) for HR analysis and the homozygous lines for both the transgene and specific mutations were obtained. Scoring HR events was performed twice with 32 plants per group. The values represent the mean ± SD. An asterisk denotes a statistically significant difference as compared to the corresponding wt control. Student's t-test: $P<0.05$.

5.4.3. Transient down-regulation of the *AGO2* and *NRPD1A* genes enhances stable *Agrobacterium*-mediated plant transformation

The transient manipulation of host factors involved in the plant transformation process is one of the approaches to increase the efficiency of plant transgenesis (Boyko, Matsuoka et al. 2011). Hence, in our study, we made an attempt to transiently down-regulate both the *AGO2* and *NRPD1a* genes to develop a method that would result in the enhanced transformation efficiency but at the same time would allow generation transgenic plants without undesirable changes in the expression of target genes. The following criteria were taken into account to choose the transient down-regulation technique: (i) it has to occur in the female gametes; (ii) it has to be strong enough to suppress gene expression to such level that would lead to the enhanced susceptibility to *Agrobacterium*-mediated transformation; (iii) it has to be gene-specific and last during the whole plant life cycle; (iv) gene silencing should not be propagated into the following generation. The aforementioned criteria are completely satisfied by a method of virus-induced gene silencing (VIGS) mediated by a *Tobacco rattle virus* (TRV). VIGS is a gene down-regulation technique which exploits the natural ability of viruses to trigger the plant's immune response directed at silencing of viral genes. Cloning of a gene fragment into the replicating virus vector allows for transient homology-dependent down-regulation of the targeted endogenous gene. TRV-based VIGS is one of the most efficient and widely-used gene silencing tools in Arabidopsis plants at the present time (Pflieger, Blanchet et al. 2008). Moreover, TRV has been shown to invade the ovules of infected *N. benthamiana* plants, albeit unlike other viruses and it is not seed-transmitted (Wang, MacFarlane et al. 1997, Martin-Hernandez and Baulcombe 2008). Hence, we

hypothesized that infection of Arabidopsis wt plants with TRV carrying fragments of either the *AGO2* or *NRPD1a* genes would allow for transient disruption of their expression and as a result an increase in plant transformation rates.

The genome of TRV is conveniently cloned into two binary vectors, one of which carries almost a complete viral genome, whereas the other one encodes the coat protein from the genomic RNA and two non-structural proteins from the subgenomic RNAs and is used as a cloning vector. To avoid the off-targeting effects, the sequences of both the *AGO2* and *NRPD1a* genes used for cloning into the TRV vector were carefully picked up using the "RNAi Scan" software (Xu, Zhang et al. 2006). *Agrobacterium* cells were transformed with the TRV plasmids and delivered into Arabidopsis wt seedlings (Burch-Smith, Schiff et al. 2006). In our study, consistent with a previous report (Burch-Smith, Schiff et al. 2006), silencing of a *Phytoene desaturase* (*PDS*) gene, used to control for the efficient development of VIGS in infected plants became apparent at 12-14 days post infection (Figure 5.4.5). On the other hand, in contrast to the report by Burch-Smith et al. (2006), we observed a severe initial growth delay in empty TRV-infected plants which almost vanished at four weeks post infection. Whereas TRV-*AGO2*-infected plants did not develop any symptoms of infection, the TRV-*NRPD1a* inoculated plants demonstrated a slight delay in growth detectable at two weeks post infection (Figure 5.4.5). Nevertheless, at a later stage, the TRV-*NRPD1a* infected plants were indistinguishable from their untreated counterparts (Figure 5.4.5). The successful down-regulation of both the *AGO2* and *NRPD1a* genes in Arabidopsis wt plants was confirmed using real-time PCR (Student's t-test, α=0.05, Figure 5.4.8A and B).

Following VIGS optimization in Arabidopsis, infected plants were left to flower and then were stably transformed using a floral dip method. Consistent with our hypothesis, transient down-regulation of both the *AGO2* and *NRPD1a* genes resulted in a considerable increase in the number of transgenic plants per 100 mg of seeds (a 5.94- and 3.47-fold in TRV-*AGO2* and TRV-*NRPD1a* infected plants, respectively, Figures 5.4.6 and 5.4.7). At the same time, we observed a variation in transformation efficiency of infected plants which was apparently due to differences in the development of VIGS among plants. To prove this hypothesis, we examined the expression of the *AGO2* and *NRPD1a* genes in infected plants with regard to their transformation efficiency. Indeed, we observed a threshold of changes in the mRNA level in both genes, which correlated with the development of an increased susceptibility to *Agrobacterium* transformation phenotype. Whereas in plants infected with TRV-*AGO2,* the elevated levels of transformation were observed in those ones where the expression level of *AGO2* transcript was less than 15% (Student's t-test, α=0.05, Figure 5.4.8A), in plants infected with TRV-*NRPD1a*, a substantial increase in transformation rates was only found in plants with the remaining *NRPD1a* transcript being ~3% (Student's t-test, α=0.05, Figure 5.4.8B). A more stringent down-regulation of the *NRPD1a* gene required to develop the increased susceptibility to *Agrobacterium* transformation phenotype partially explains the elevated but statistically insignificant changes in transformation rates of TRV-*NRPD1a* as compared to the TRV-*AGO2* group of plants (Figure 5.4.7).

An increase in the number of transgenic plants can also lead to the elevated number of transgene integrations per genome. Such an increase in the frequency of transgene integration events can be due to both a more open chromatin structure caused

by lower levels of global DNA methylation and a higher chance of T-DNA replication inside the host nucleus as a result of an altered defence mechanism in mutants. We examined six transgenic plants from each group using a Southern blot analysis (Figure 5.4.9). Indeed, in TRV-*AGO2* and TRV-*NRPD1a* transgenic plants, a high transformation rate was paralleled by an almost threefold increase in the number of transgenes per genome (Figure 5.4.10). Surprisingly, transgenic plants originated from empty TRV-infected plants also had an increased, although not significantly, number of transgene integrations (Student's t-test, α=0.05). This finding is partially in agreement with the previous report (Voinnet, Rivas et al. 2003) suggesting that viruses can counteract the plant defence pathways. These pathways may reduce plant susceptibility to *Agrobaterium*-mediated transformation. Initially, it has been shown that the co-expression of a viral suppressor of gene silencing, the p19 protein of *Tomato bushy stunt virus* (TBSV), prevents the onset of PTGS in the infiltrated *Nicotiana benthamiana* tissues and allows a high level of *Agrobacterium*-mediated transient expression (Voinnet, Rivas et al. 2003). It still remains to be shown whether a weak viral suppressor of gene silencing – 16K from the TRV virus – can cause a similar effect.

Finally, we examined the propagation of *AGO2* and *NRPD1a* silencing into the progeny of infected plants. Consistent with the previous report, we did not observe either the transmission of TRV RNA to transgenic progenies of infected plants (data not shown) (Martin-Hernandez and Baulcombe 2008) or down-regulation of both genes in the corresponding groups (Figure 5.4.11A and B). On the contrary, all the four examined T1 transgenic plants obtained after floral-dip transformation with pCAMBIA3301 of the TRV-*AGO2* infected plants demonstrated a significant up-regulation of *AGO2* expression

as compared to the T1 transgenic progeny of uninfected plants. At the same time, only one out of the four examined T1 transgenic plants obtained after floral-dip transformation with pCAMBIA3301 of the TRV-*NRPD1a* infected plants had an elevated expression of the *NRPD1a* gene (Student's t-test, α=0.05). This is an interesting finding that conceivably suggests a tight transgenerational regulation of *AGO2* gene expression in plants.

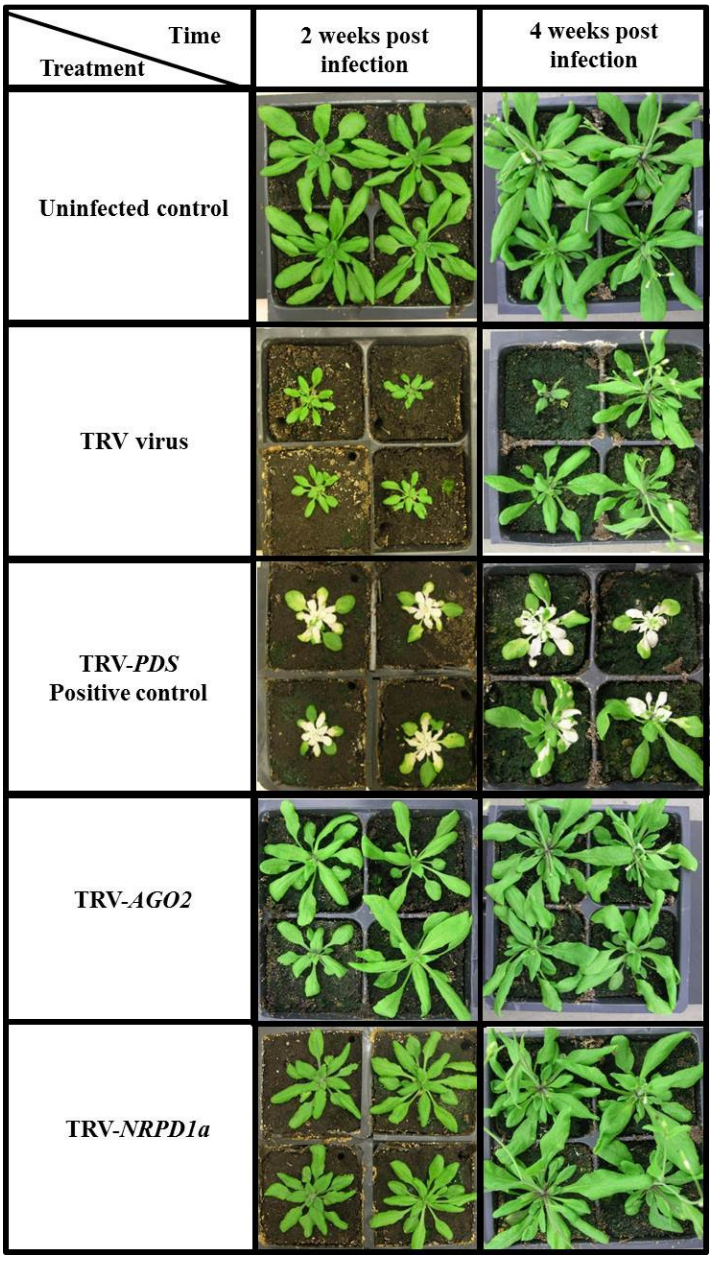

Figure 5.4.5. Optimization of TRV-mediated VIGS in Arabidopsis plants

Seedlings of Arabidopsis wt plants at the two- to three-leaf stage were infected with either of the following constructs: the empty TRV virus, TRV virus carrying a fragment of the *PDS* (positive control), *AGO2* or *NRPD1a* genes. Symptoms of virus-mediated suppression of gene activity became apparent at 2 weeks post infection (TRV-*PDS*, 2 wpi), albeit plants infected with the empty TRV virus demonstrated a severe delay in growth as compared to uninfected controls already at 1 wpi (pictures not shown). Later, most of the empty TRV-infected plants recovered from infection (TRV virus, 4 wpi).

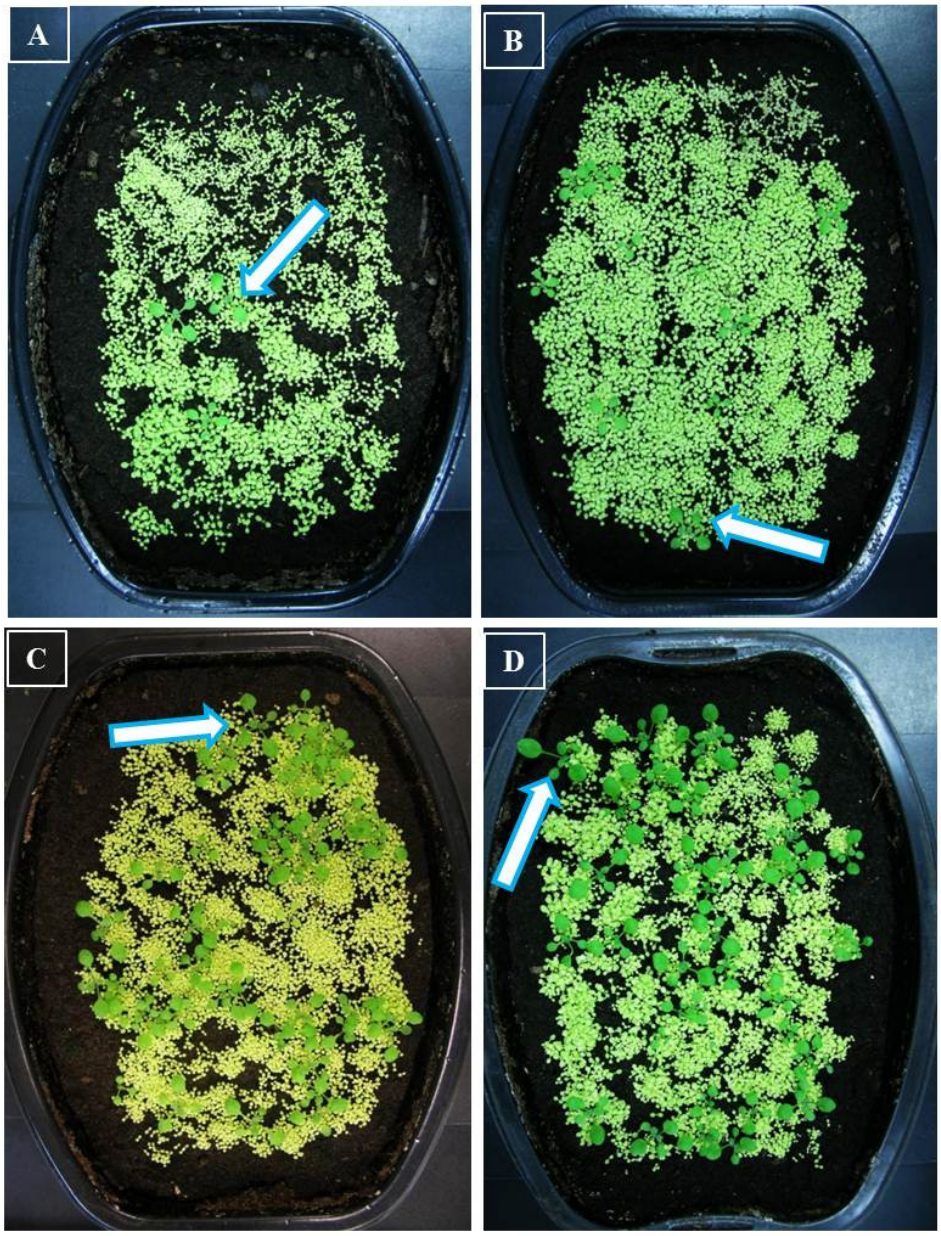

Figure 5.4.6. *In solium* selection of transgenic plants obtained after VIGS-mediated stable transformation using glufosinate ammonium

A, **B**, **C** and **D** represent selection plates with transgenic plants obtained after transformation of untreated plants, plants infected with the empty TRV virus, TRV virus carrying an *AGO2* fragment and TRV virus carrying an *NRPD1a* fragment, respectively. The arrows point at the representative transgenic plants which were scored in every plate containing 100 mg of seeds harvested from individual T0 plants.

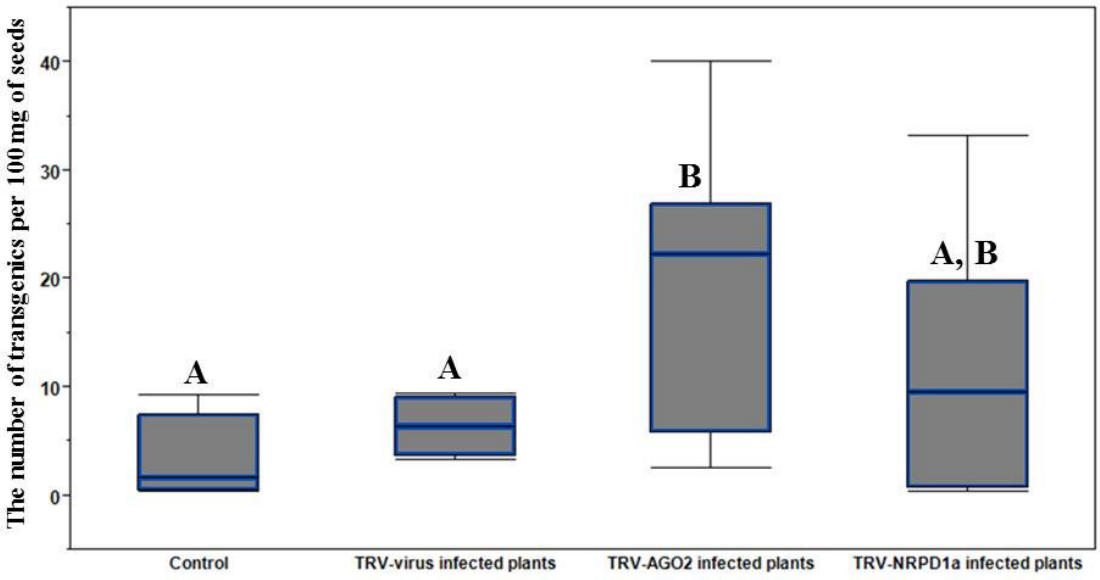

Figure 5.4.7. Box-plots demonstrating a variation in transformation efficiency of the empty TRV, TRV-*AGO2* and TRV-*NRPD1a* infected Arabidopsis wt plants

The analysis of the stable transformation efficiency of plants infected with either the empty TRV virus, a virus with a fragment of the *AGO2* gene or a virus with a fragment of the *NRPD1a* gene. Two independent experiments were performed. A minimum of 4 plants per group were analyzed in each experiment. The box-plots that are not connected by the same letter are significantly different. Nonparametric Wilcoxon method, $P<0.05$.

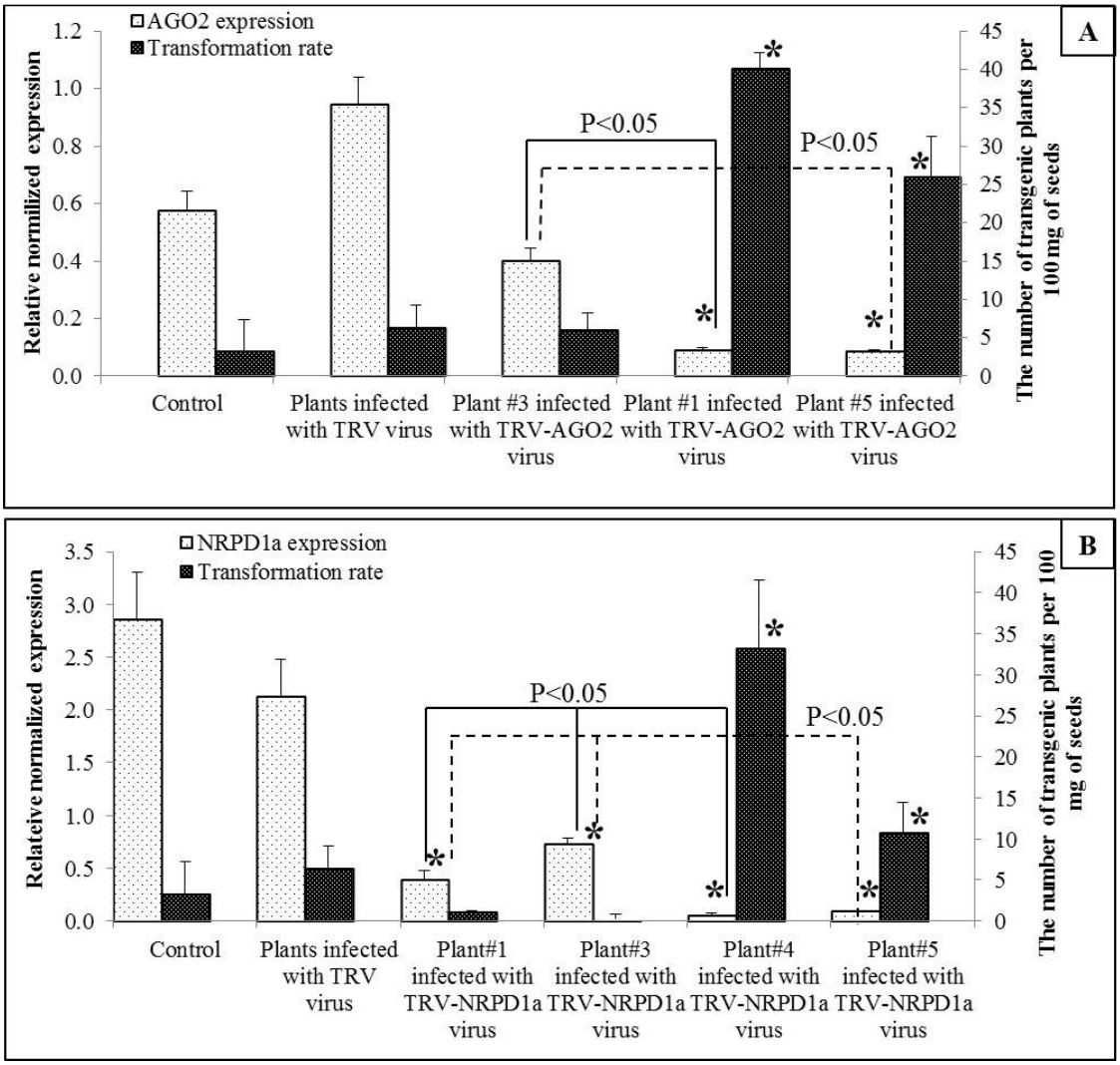

Figure 5.4.8. Steady-state mRNA level of target genes and average transformation rates in empty TRV-, TRV-*AGO2*- and TRV-*NRPD1a*-infected Arabidopsis wt plants

The real-time PCR analysis of VIGS-mediated transient down-regulation of the *AGO2* (**A**) and *NRPD1a* (**B**) genes in Arabidopsis wt TRV-infected plants. Three plants in "Control" and "Plants infected with TRV virus" groups were examined, respectively. Each measurement was done in duplicates. The values represent the mean ± SEM. The asterisks denote a statistically significant difference as compared to its corresponding control. Student's t-test (two tail), α=0.05, t=2.26, P<0.05.

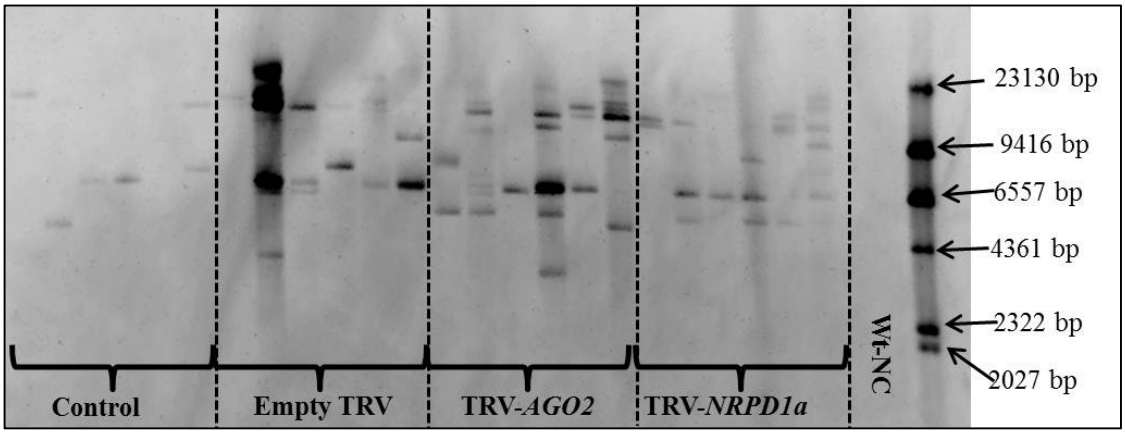

Figure 5.4.9. Southern blot analysis of the transgene copy number in T1 transgenic plants obtained using the VIGS-mediated stable transformation method

The analysis of transgene copy number in T1 transgenics obtained using the VIGS-mediated stable transformation method. gDNA was isolated from T1 transgenic plants and digested with a PstI restriction enzyme which has a single recognition site within T-DNA derived from pCAMBIA3301. The DIG-labelled probe (1222 bp-long) was designed to hybridize to a GUS-gene sequence at the 3´ region of the PstI recognition site. Six transgenic plants per group were analyzed. wt-NC – wild type negative control, M – DIG labelled DNA marker (Roche).

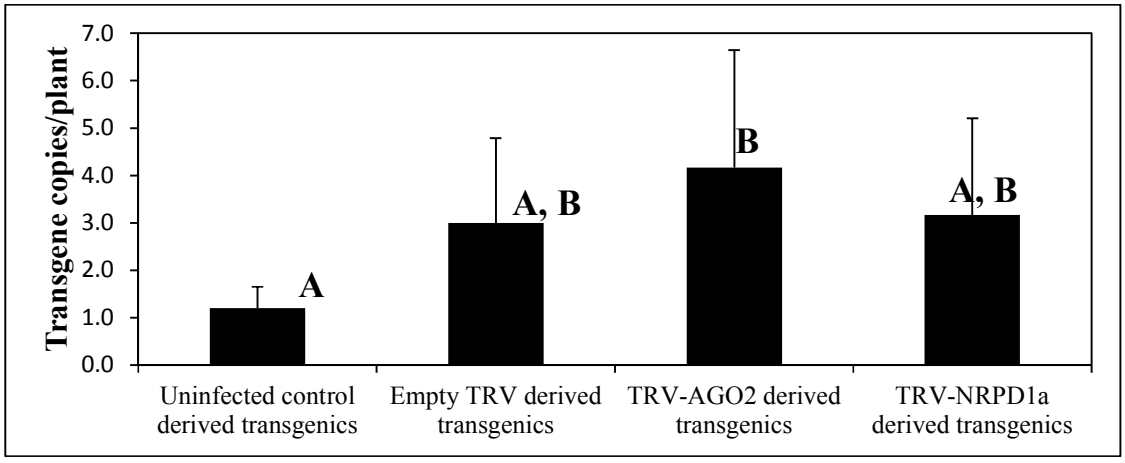

Figure 5.4.10. Transgene copy number in T1 Arabidopsis transgenic plants as analyzed by Southern blot

The analysis of the transgene copy number in transgenics obtained using the VIGS-mediated stable transformation method. The bars that are not connected by the same letter are significantly different. Student's t-test (two tail), single factor ANOVA, $\alpha=0.05$, $t=2.09$, $N=6$, $P<0.05$.

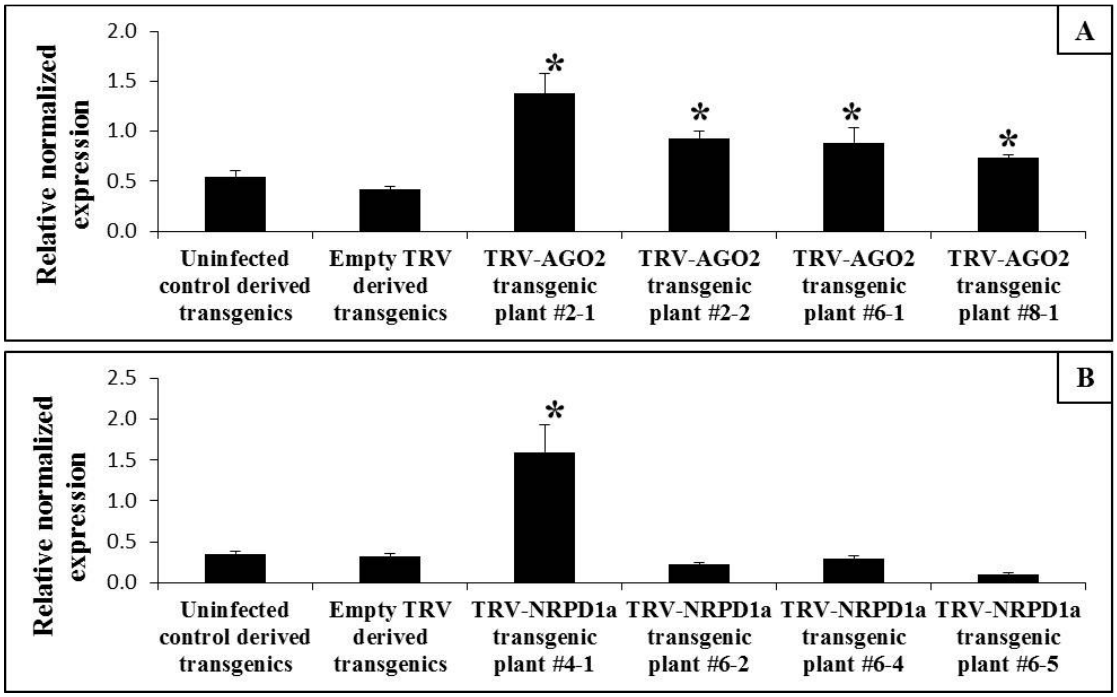

Figure 5.4.11. The analysis of propagation of VIGS-mediated silencing of the *AGO2* (A) and *NRPD1a* (B) genes into the T1 generation of Arabidopsis transgenic plants obtained using the VIGS-mediated stable transformation method

Three plants in "Uninfected control derived transgenics" and "Empty TRV derived transgenics" groups were examined, respectively. Each measurement was done in duplicates. The values represent the mean ± SEM. The asterisks denote a statistically significant difference as compared to transgenic plants derived from uninfected control plants. Student's t-test, $\alpha=0.05$, $t=2.26$, $P<0.05$.

5.5. DISCUSSION

This work is aimed at deciphering the contribution of epigenetic factors to the plant defence system against *Agrobacterium*-mediated transformation and further transient manipulation of these factors to favour a higher stable transformation efficiency. We found that a) both the *AGO2* and *NRPD1a* genes considerably inhibit *Agrobacterium*-mediated plant transformation; b) a significant increase in stable transformation efficiency of the *ago2-1* and *nrpd1a-3* mutants can be the cumulative outcome of a decrease in global DNA methylation and an increase in the level of DNA strand breaks; c) the TRV-mediated VIGS technique can be successfully used to manipulate the expression of target genes in plants in order to favour a higher stable transformation efficiency; d) transient down-regulation of the *AGO2* and *NRPD1a* genes results in an increased transformation efficiency of Arabidopsis wt plants partially due to an elevated number of transgene integrations per genome in transgenic plants; e) infection of Arabidopsis wt plants with the empty TRV virus also increases the number of transgene copies per genome in stable transformants.

5.5.1. The *AGO2* and *NRPD1a* genes contribute to the plant defence system against *Agrobacterium*-mediated plant transformation

Previously, it has been noted that the process of *Agrobacterium*-mediated genetic transformation resembles retrovirus-mediated gene transfer (Magori and Citovsky 2011). In our study, we complemented this idea and provided further evidence that plants utilize similar epigenetic components to defend the genome against undesirable exogenous nucleic acid activities. Here, we made a parallel to geminivirus infection which has been

shown to be inhibited by both the PTGS and TGS pathways in plants (Hohn and Vazquez 2011).

It does not come by surprise that both transcriptional and posttranscriptional barriers can mediate plant epigenetic immune responses to alien DNA since PTGS can act as the primary pathway to generate 24 nt-long small RNAs that eventually target the complementary DNA sequences for methylation. Also previously, it has been shown that both DNA viruses, *Cauliflower mosaic virus* (CaMV) (Moissiard and Voinnet 2006) and *Cabbage leaf curl virus* (CaLCuV) (Blevins, Rajeswaran et al. 2006), trigger the accumulation of a wide range of small RNA species derived from the viral transcriptome, with the 24 nt-long RNA species being predominant. Nevertheless, the *dcl3* mutant, a mutant impaired in the 24-mers generation, demonstrated either the same (Blevins, Rajeswaran et al. 2006) or moderately enhanced (Raja, Sanville et al. 2008) symptoms of CaLCuV infection as compared to wt controls. This suggests that other Dicer-like proteins may compensate for the absence of *DCL3* in the *dcl3* mutant. We observed no increase (*dcl3-1*) or an insignificant increase in transformation rates (*dcl4-2*) of single *DCL* mutants as compared to controls (Figure 5.4.1).

Curiously, unlike in the case with the geminivirus where delivery of the virus into the *ddm1* mutant caused severe infection symptoms (Raja, Sanville et al. 2008), in our experiment, *Agrobacterium*-mediated floral dip transformation of *ddm1-2* led to a slight insignificant decrease in the transformation rate as compared to controls. This is in agreement with the previous report demonstrating that the *ddm1* mutant, a SWI2/SNF2-like chromatin-remodelling protein which is necessary for DNA methylation and heterochromatin maintenance is resistant to transient and stable root transformation

(Crane and Gelvin 2007). The authors have speculated that DDM1 along with other chromatin remodelling proteins is not directly involved in transformation; instead, the transformation process requires DNA synthesis and mitosis in growing infected tissues.

It is generally accepted that DCL-mediated processing of the viral RNA alone is not enough to circumvent virus infection and the virus-derived small RNA products have to be loaded into the RNA induced silencing complex (RISC) to target the viral RNA for degradation (Jaubert, Bhattacharjee et al. 2011). In turn, the activity of RISC depends on one of the ten Argonaute (AGO) proteins, which developed a significant degree of diversification and specialization to perform the gene silencing function (Vaucheret 2008). Curiously, among all AGO proteins, only AGO2 has been shown to be vital for resistance against RNA viruses: *Potato virus* X, *Turnip crinkle virus*, and *Cucumber mosaic virus* (Harvey, Lewsey et al. 2011, Jaubert, Bhattacharjee et al. 2011). Unfortunately, the importance of AGO2 in mediating an antiviral response against DNA containing geminiviruses has not been shown yet. Although, in our study, we did not examine transformation efficiency of the rest of the *ago* mutants, here for the first time, we have provided evidence of the vital role of the AGO2 protein in suppressing *Agrobacterium*-mediated plant transformation (Figure 5.4.1).

An increase in the floral dip stable transformation rate of the *ago2-1* mutant was concomitant with a decrease in the level of global DNA methylation and an increase in the level of DNA strand break (Figures 5.4.2 and 5.4.3). The same trend was observed in the mutant of the *NRPD1A* gene which is the largest subunit of plant-specific Pol IV and is involved in the generation of 24 nt-long small RNAs from transposable elements (TEs) and TE-associated genes. Curiously, that for a long period of time, the *AGO2* and

NRPD1a genes have been known to act in two separate pathways: PTGS and TGS, respectively. The recent report provided an intriguing evidence of the involvement of both genes in the DNA DSB repair process (Wei, Ba et al. 2012). Although it still remains to be shown whether small RNAs generated from the DNA DSB sites can indeed guide the DNA repair machinery, the participation of both genes in the antiviral defence and DNA repair processes made them suitable candidates for regulating the *Agrobacterium*-mediated stable transformation process in plants. In the same report, the authors have speculated that ssDNA protruding from DSBs might serve as a template for Pol IV-mediated expression of double-stranded (ds) small RNA precursors. This notion was further supported by an example of the filamentous fungus *Neurospora crassa*, where the RNA-dependent RNA polymerase (RDR) homologue QDE-1 can generate dsRNAs from ssDNA (Lee, Aalto et al. 2010). Since we observed an elevated level of DNA strand breaks in both mutant backgrounds, we may suggest that both the *ago2* and *nrpd1a* mutants lost the ability to recognize both the endogenous ssDNA and single-stranded T-DNA from *Agrobacterium tumefaciens* delivered upon transformation. The latter would result in the phenotype susceptible to *Agrobacterium* transformation. It still remains to be shown whether plant-specific Pol IV can use *Agrobacterium* single-stranded T-DNA as a template to produce transcripts for the RdDM pathway.

5.5.2. Transient down-regulation of the *AGO2* and *NRPD1a* genes elevates stable transformation efficiency of Arabidopsis wt plants

One of the approaches to improve plant transformation frequency is to manipulate the expression of host genes involved in the transformation process. Since we discovered

that both the *AGO2* and *NRPD1a* genes inhibited *Agrobacterium*-mediated plant transformation, we decided to check whether the transient disruption of their expression in Arabidopsis wt plants would also lead to the enhanced transformation efficiency.

The VIGS method to disrupt gene activity in somatic tissues is widely implemented for a number of plant species (Purkayastha and Dasgupta 2009), although it has not been shown to be efficient in the gametes yet. In our study, we favoured a floral deep Arabidopsis transformation method over methods for transforming somatic tissue, therefore, it was vital for us that efficient silencing of the examined genes would occur in ovules – the target of *Agrobacterium* transformation (Ye, Stone et al. 1999). Among viruses such as *Apple latent spherical virus*, *Cabbage leaf curl virus, Potato virus X* and *Turnip yellow mosaic virus* (Purkayastha and Dasgupta 2009) which can trigger VIGS in somatic Arabidopsis tissues, only TRV has been shown to penetrate into the embryo sac of *N. benthamiana* plants. In our experiment, we indirectly confirmed this by providing evidence that infection of Arabidopsis wt seedlings with the TRV virus carrying the fragment of either the *AGO2* or *NRPD1a* gene can cause silencing of target genes in systemic tissues and subsequently increase stable transformation efficiency using the dipping method wherby ovules are infected by *Agrobacterium*.

Infection of Arabidopsis wt plants with either the empty TRV or TRV carrying fragments of genes resulted in a threefold increase in transgene copy number integrations as compared to controls irrespective of the construct used. Since we did not analyze the transgene copy number in transgenics obtained after transformation of the *ago2-1* and *nrpd1a-3* null mutants, we cannot rule out the possibility that the inactivation either of the *AGO2* or *NRPD1a* genes may contribute to an increase in the number of transgene

integration events. However, we observed such increase in plants infected with TRV alone, therefore, we hypothesize that this increase is caused mainly by the viral infection.

The TRV-based VIGS technique has been previously implemented to show the inhibiting role of the X-RAY CROSS COMPLEMENTATION PROTEIN4 (XRCC4) in the stable *Agrobacterium*-mediated transformation process of *N. benthamiana* plants (Vaghchhipawala, Vasudevan et al. 2012). Unfortunately, the authors did not provide data on how native TRV affects transformation efficiencies and the number of transgene integrations as compared to untreated controls. TRV is known to encode a 16K suppressor of RNA interference which can inhibit the PTGS pathway in plants (Martínez-Priego, Donaire et al. 2008). The introduction of TRV into plant cells may result in the compromised PTGS defence pathway that eventually leads to a higher number of successfully integrated copies of T-DNA.

Previously, it was reported that the double mutant *nrpd1a nrpd1b* defective in the activity of both Pol IV and Pol V exhibits the *ago9* phenotype which is characterized by two independent female gametophytes developing in the same ovule at a frequency of 44.03% (Olmedo-Monfil, Duran-Figueroa et al. 2010). At the same time, all *ago9* null mutants were fertile and did not show signs of ovule or seed abortion. Unfortunately, the authors did not demonstrate whether the appearance of ovules of the single *pol iv* and *pol v* mutants was affected. Since female reproductive organs are the primary target for *Agrobacterium* transformation (Ye, Stone et al. 1999, Desfeux, Clough et al. 2000), alterations in the composition of ovules of the *nrpd1a-3* mutant may possibly contribute to the increased transformation rates observed in our study. It still remains to be shown

whether either of the *ago2-1* or *nrpd1a-3* null mutants exhibits any abnormalities in the development of ovules.

Summarizing, based on the available data regarding the contribution of epigenetic-related genes to *Agrobacterium*-mediated plant transformation and the results that are presented herein, a following model of T-DNA processing in a plant cell is proposed (Figure 5.5.1).

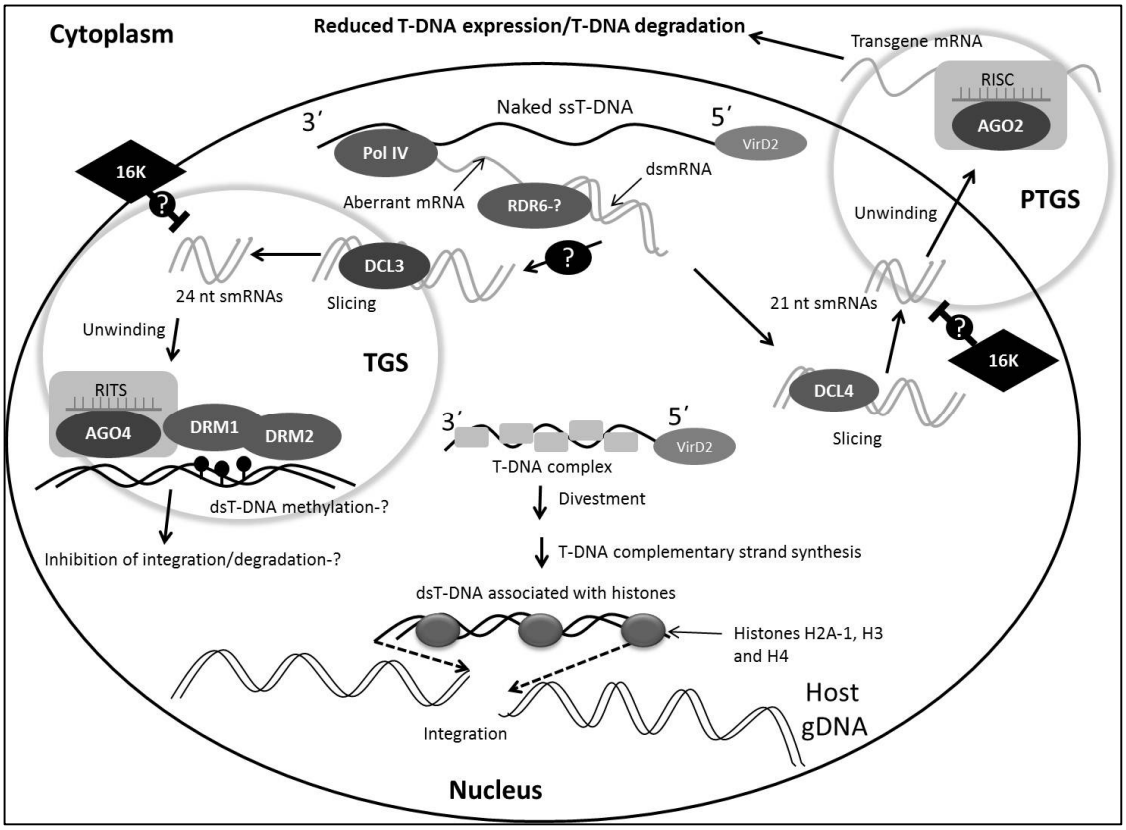

Figure 5.5.1. A proposed model of epigenetic processing of the *Agrobacterium* T-DNA in a plant cell

The single-stranded T-DNA after the stripping off of bacterial proteins may be transcribed by Pol IV that generates an aberrant mRNA. A truncated mRNA is recognized by RDR6 and converted into ds mRNA that can be processed either by DCL3 or DCL4. Whereas in the former scenario the TGS pathway takes place, in the latter case the PTGS pathway is involved. DCL3 slices ds mRNA into 24 nt-long duplexes that after unwinding are used to guide the RNA-induced transcriptional silencing (RITS) complex together with the DRM1 and DRM2 DNA methylases to ds T-DNA in the RdDM pathway. Ds T-DNA methylation may eventualy inhibit T-DNA stable integration. In the case of DCL4, dicing of ds mRNA leads to the generation of 21 nt-long smRNAs engaged into the RNA-induced silencing complex (RISC) to guide the degradation of complementary T-DNA transcript. Both pathways may probably be inhibited by the TRV suppressor of gene silencing – 16K. A successful integration of T-DNA into the host genome conceivably involves the association of T-DNA with histone proteins.

5.6. SUMMURY

Our study demonstrated the contribution of the epigenetic machinery to the plant defence system against stable *Agrobacterium* transformation. In the reverse genetic screening, two genes – *AGO2* and *NRPD1a* – involved in the PTGS and TGS pathways, respectively, were found to be inhibitors of stable plant transformation. The involvement of these two genes in the antiviral defence (that was demonstrated previously) made it possible to speculate that plants utilize similar pathways against exogenous nucleic acids. Our data demonstrate that an increase in stable transformation efficiencies of both mutants is possibly the cumulative outcome of both the reduced level of global DNA methylation and the increased level of DNA strand breaks. Alternatively, the deficiency in the small RNA pathway which acts as an immune system against invading nucleic acids may result in an increase in the stable transformation rate observed in the *ago2* and *nrpd1a* mutants.

The manipulation of host factors to favour higher transformation efficiencies is a favourable approach used in the contemporary biotechnology. We decided to develop a method which would allow transient down-regulation of the *AGO2* and *NRPD1a* genes to increase transformation efficiency of wild-type plants. To do this, we implemented the TRV-based VIGS technique that proved to be efficient in elevating the average number of stable transformants obtained in a single transformation round. Additionally, we revealed that TRV itself can compromise the plant defence pathway against *Agrobacterium* transformation, resulting in a considerably higher number of transgene integration events as compared to controls. This discovery further supported the notion that similar defence pathways are involved in protection against viral nucleic acids and T-

DNA delivered by *Agrobacterium*. Overall, our study provides an approach that can be implemented to further decipher the contribution of the rest of epigenetic players to *Agrobacterium*-mediated stable plant transformation.

6. FUTURE DIRECTIONS

Our results demonstrate that exposure of plants to different abiotic stresses results in heritable alterations in the epigenetic profile. At the same time, we observed tissue-specific perturbations in smRNA expression in *B. rapa* plants which were, surprisingly, more pronounced in unexposed tissues such as pollen, embryo and endosperm. It will be important to reveal the molecular nature of a signal that mediates these drastic fluctuations in distant tissues. Previously, the siRNA pathway was reported to prevent transgenerational retrotransposition in heat-stressed Arabidopsis plants (Ito, Gaubert et al. 2011). Hence, comparing the epigenetic profiles of seed tissues in mutants impaired in the siRNA pathway that were cultivated under normal and stress conditions would possibly reveal the contribution of small RNAs to stress-induced inter-tissue communication.

Additionally, based on our results, further research is definitely required to decipher a role of the *AGO1* gene and miR168 in transgeneretional epigenetic inheritance of stress memory in plants. To do this, mutants of either of the genes can be subjected to transgenerational stress experiments. The regulation of both genes may also occur at the transcriptional level as well. Hence, it is of vital importance to monitor DNA methylation/histone modification profiles in these two genes at the promoter and gene body regions after stress exposure both in parents and in progeny of stressed plants.

Another important finding of our research was that it revealed the existence of transgenerationally transmitted and heat-inducible tRNA-derived small RNA fragments in plants. Since three detected tRNA fragments were mapped to gene introns, they did not escape our attention, albeit other tRNA-derived small RNAs were also sorted out during

the mapping process. Their mode of action was suggested to resemble that of miRNAs (Loss-Morais, Waterhouse et al. 2013). Hence, in the future, it would be interesting to monitor the expression of tRNA-derived small RNAs in all tissues and correlate it with mRNA levels of putative targets.

While performing a reverse genetic screen among mutants that are deficient in different epigenetic pathways, we revealed two inhibitors of stable *Agrobacterium*-mediated transformation. A list of epigenetic-related genes involved in plant immune response against *Agrobacterium* infection is by no means exhausted. Hence, in order to completely comprehend plant defence responses to the introduced transgene, it is of vital importance to continue reverse genetic screening among the rest of the epigenetic mutants. Furthermore, our work and reports from other labs provided evidence that viruses can compromise plant defence pathways against *Agrobacterium* pathogens. However, this phenomenon is thought to be caused solely by distinct proteins encoded in the virus genome – suppressors of gene silencing. Transient overexpression of such suppressors may be one of the approaches to enhance transformation of recalcitrant plant species. Moreover, since all suppressors isolated from different viruses are dissimilar in their mode of action, the use of them in the combination or generation of a universal suppressor protein may result even in a more pronounced enhancement of plant transformation.

7. BIBLIOGRAPHY

Agius, F., A. Kapoor and J. K. Zhu (2006). "Role of the Arabidopsis DNA glycosylase/lyase ROS1 in active DNA demethylation." Proc Natl Acad Sci U S A **103**(31): 11796-11801.

Agrawal, A. A. (2001). "Transgenerational consequences of plant responses to herbivory: an adaptive maternal effect?" Am Nat **157**(5): 555-569.

Aina, R., S. Sgorbati, A. Santagostino, M. Labra, A. Ghiani and S. Citterio (2004). "Specific hypomethylation of DNA is induced by heavy metals in white clover and industrial hemp." Physiologia Plantarum **121**(3): 472-480.

Ali, G. S. and A. S. Reddy (2008). "Regulation of alternative splicing of pre-mRNAs by stresses." Curr Top Microbiol Immunol **326**: 257-275.

Allen, E. and M. D. Howell (2010). "miRNAs in the biogenesis of trans-acting siRNAs in higher plants." Semin Cell Dev Biol **21**(8): 798-804.

Amiard, S., C. Charbonnel, E. Allain, A. Depeiges, C. I. White and M. E. Gallego (2010). "Distinct roles of the ATR kinase and the Mre11-Rad50-Nbs1 complex in the maintenance of chromosomal stability in Arabidopsis." Plant Cell **22**(9): 3020-3033.

Anders, S. and W. Huber (2010). "Differential expression analysis for sequence count data." Genome Biol **11**(10): R106.

Arnould, S., P. Chames, C. Perez, E. Lacroix, A. Duclert, J. C. Epinat, F. Stricher, A. S. Petit, A. Patin, S. Guillier, S. Rolland, J. Prieto, F. J. Blanco, J. Bravo, G. Montoya, L. Serrano, P. Duchateau and F. Paques (2006). "Engineering of large numbers of highly specific homing endonucleases that induce recombination on novel DNA targets." J Mol Biol **355**(3): 443-458.

Axtell, M. J. (2013). "Classification and Comparison of Small RNAs from Plants." Annu Rev Plant Biol.

Baek, D., J. Jiang, J. S. Chung, B. Wang, J. Chen, Z. Xin and H. Shi (2011). "Regulated AtHKT1 gene expression by a distal enhancer element and DNA methylation in the promoter plays an important role in salt tolerance." Plant Cell Physiol **52**(1): 149-161.

Bagheri, H., M. El-Soda, I. van Oorschot, C. Hanhart, G. Bonnema, T. Jansen-van den Bosch, R. Mank, J. J. Keurentjes, L. Meng, J. Wu, M. Koornneef and M. G. Aarts (2012). "Genetic analysis of morphological traits in a new, versatile, rapid-cycling Brassica rapa recombinant inbred line population." Front Plant Sci **3**: 183.

Bairoch, A. and R. Apweiler (2000). "The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000." Nucleic Acids Res **28**(1): 45-48.

Barampuram, S. and Z. J. Zhang (2011). "Recent advances in plant transformation." Methods Mol Biol **701**: 1-35.

Baroux, C., M. T. Raissig and U. Grossniklaus (2011). "Epigenetic regulation and reprogramming during gamete formation in plants." Curr Opin Genet Dev **21**(2): 124-133.

Basnakian, A. G. and S. J. James (1996). "Quantification of 3'OH DNA breaks by random oligonucleotide-primed synthesis (ROPS) assay." DNA Cell Biol **15**(3): 255-262.

Baubec, T., H. Q. Dinh, A. Pecinka, B. Rakic, W. Rozhon, B. Wohlrab, A. von Haeseler and O. M. Scheid (2010). "Cooperation of Multiple Chromatin Modifications Can Generate Unanticipated Stability of Epigenetic States in Arabidopsis." The Plant Cell Online **22**(1): 34-47.

Benediktsson, I., C. Spampinato and O. Schieder (1995). "Studies of the mechanism of transgene integration into plant protoplasts: improvement of the transformation rate." Euphytica **85**(1-3): 53-61.
Benjamini, Y. and Y. Hochberg (1995). "Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing." Journal of the Royal Statistical Society. Series B (Methodological) **57**(1): 289-300.
Berger, S. L. (2007). "The complex language of chromatin regulation during transcription." Nature **447**(7143): 407-412.
Berr, A., R. Menard, T. Heitz and W. H. Shen (2012). "Chromatin modification and remodelling: a regulatory landscape for the control of Arabidopsis defence responses upon pathogen attack." Cellular Microbiology **14**(6): 829-839.
Bertolini, L. R., M. Bertolini, E. A. Maga, K. R. Madden and J. D. Murray (2009). "Increased gene targeting in Ku70 and Xrcc4 transiently deficient human somatic cells." Mol Biotechnol **41**(2): 106-114.
Bezhani, S., C. Winter, S. Hershman, J. D. Wagner, J. F. Kennedy, C. S. Kwon, J. Pfluger, Y. Su and D. Wagner (2007). "Unique, shared, and redundant roles for the Arabidopsis SWI/SNF chromatin remodeling ATPases BRAHMA and SPLAYED." Plant Cell **19**(2): 403-416.
Bilichak, A., Y. Ilnystkyy, J. Hollunder and I. Kovalchuk (2012). "The progeny of Arabidopsis thaliana plants exposed to salt exhibit changes in DNA methylation, histone modifications and gene expression." PLoS One **7**(1): e30515.
Bird, A. (2007). "Perceptions of epigenetics." Nature **447**(7143): 396-398.
Bjedov, I., O. Tenaillon, B. Gerard, V. Souza, E. Denamur, M. Radman, F. Taddei and I. Matic (2003). "Stress-induced mutagenesis in bacteria." Science **300**(5624): 1404-1409.
Blevins, T., R. Rajeswaran, P. V. Shivaprasad, D. Beknazariants, A. Si-Ammour, H. S. Park, F. Vazquez, D. Robertson, F. Meins, Jr., T. Hohn and M. M. Pooggin (2006). "Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing." Nucleic Acids Res **34**(21): 6233-6246.
Blodner, C., C. Goebel, I. Feussner, C. Gatz and A. Polle (2007). "Warm and cold parental reproductive environments affect seed properties, fitness, and cold responsiveness in Arabidopsis thaliana progenies." Plant Cell Environ **30**(2): 165-175.
Bohmert, K., I. Camus, C. Bellini, D. Bouchez, M. Caboche and C. Benning (1998). "AGO1 defines a novel locus of Arabidopsis controlling leaf development." EMBO J **17**(1): 170-180.
Bonasio, R., S. Tu and D. Reinberg (2010). "Molecular signals of epigenetic states." Science **330**(6004): 612-616.
Bonisch, C. and S. B. Hake (2012). "Histone H2A variants in nucleosomes and chromatin: more or less stable?" Nucleic Acids Res.
Borsani, O., J. Zhu, P. E. Verslues, R. Sunkar and J. K. Zhu (2005). "Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis." Cell **123**(7): 1279-1291.
Bourc'his, D. and O. Voinnet (2010). "A small-RNA perspective on gametogenesis, fertilization, and early zygotic development." Science **330**(6004): 617-622.
Boyes, D. C., A. M. Zayed, R. Ascenzi, A. J. McCaskill, N. E. Hoffman, K. R. Davis and J. Gorlach (2001). "Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants." Plant Cell **13**(7): 1499-1510.

Boyko, A., T. Blevins, Y. Yao, A. Golubov, A. Bilichak, Y. Ilnytskyy, J. Hollunder, F. Meins, Jr. and I. Kovalchuk (2010). "Transgenerational adaptation of Arabidopsis to stress requires DNA methylation and the function of Dicer-like proteins." PLoS One **5**(3): e9514.
Boyko, A., A. Golubov, A. Bilichak and I. Kovalchuk (2010). "Chlorine ions but not sodium ions alter genome stability of Arabidopsis thaliana." Plant Cell Physiol **51**(6): 1066-1078.
Boyko, A., P. Kathiria, F. J. Zemp, Y. Yao, I. Pogribny and I. Kovalchuk (2007). "Transgenerational changes in the genome stability and methylation in pathogen-infected plants: (virus-induced plant genome instability)." Nucleic Acids Res **35**(5): 1714-1725.
Boyko, A. and I. Kovalchuk (2010). "Detection and quantification of DNA strand breaks using the ROPS (random oligonucleotide primed synthesis) assay." Methods Mol Biol **631**: 237-242.
Boyko, A. and I. Kovalchuk (2010). "Transgenerational response to stress in Arabidopsis thaliana." Plant Signal Behav **5**(8): 995-998.
Boyko, A. and I. Kovalchuk (2011). "Genome instability and epigenetic modification--heritable responses to environmental stress?" Curr Opin Plant Biol **14**(3): 260-266.
Boyko, A., A. Matsuoka and I. Kovalchuk (2009). "High frequency Agrobacterium tumefaciens-mediated plant transformation induced by ammonium nitrate." Plant Cell Rep **28**(5): 737-757.
Boyko, A., A. Matsuoka and I. Kovalchuk (2011). "Potassium chloride and rare earth elements improve plant growth and increase the frequency of the Agrobacterium tumefaciens-mediated plant transformation." Plant Cell Rep **30**(4): 505-518.
Bratzel, F., G. Lopez-Torrejon, M. Koch, J. C. Del Pozo and M. Calonje (2010). "Keeping cell identity in Arabidopsis requires PRC1 RING-finger homologs that catalyze H2A monoubiquitination." Curr Biol **20**(20): 1853-1859.
Britt, A. B. and G. D. May (2003). "Re-engineering plant gene targeting." Trends in Plant Science **8**(2): 90-95.
Brosnan, C. A., N. Mitter, M. Christie, N. A. Smith, P. M. Waterhouse and B. J. Carroll (2007). "Nuclear gene silencing directs reception of long-distance mRNA silencing in Arabidopsis." Proc Natl Acad Sci U S A **104**(37): 14741-14746.
Buchmann, R. C., S. Asad, J. N. Wolf, G. Mohannath and D. M. Bisaro (2009). "Geminivirus AL2 and L2 proteins suppress transcriptional gene silencing and cause genome-wide reductions in cytosine methylation." J Virol **83**(10): 5005-5013.
Buckley, B. A., K. B. Burkhart, S. G. Gu, G. Spracklin, A. Kershner, H. Fritz, J. Kimble, A. Fire and S. Kennedy (2012). "A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality." Nature **489**(7416): 447-451.
Buhtz, A., F. Springer, L. Chappell, D. C. Baulcombe and J. Kehr (2008). "Identification and characterization of small RNAs from the phloem of Brassica napus." Plant Journal **53**(5): 739-749.
Burch-Smith, T. M., M. Schiff, Y. Liu and S. P. Dinesh-Kumar (2006). "Efficient virus-induced gene silencing in Arabidopsis." Plant Physiol **142**(1): 21-27.
Burge, S. W., J. Daub, R. Eberhardt, J. Tate, L. Barquist, E. P. Nawrocki, S. R. Eddy, P. P. Gardner and A. Bateman (2012). "Rfam 11.0: 10 years of RNA families." Nucleic Acids Research.

Burgyan, J. and Z. Havelda (2011). "Viral suppressors of RNA silencing." Trends in Plant Science **16**(5): 265-272.
Calarco, J. P., F. Borges, M. T. Donoghue, F. Van Ex, P. E. Jullien, T. Lopes, R. Gardner, F. Berger, J. A. Feijo, J. D. Becker and R. A. Martienssen (2012). "Reprogramming of DNA Methylation in Pollen Guides Epigenetic Inheritance via Small RNA." Cell **151**(1): 194-205.
Camporeale, G., A. M. Oommen, J. B. Griffin, G. Sarath and J. Zempleni (2007). "K12-biotinylated histone H4 marks heterochromatin in human lymphoblastoma cells." J Nutr Biochem **18**(11): 760-768.
Cao, X. and S. E. Jacobsen (2002). "Role of the arabidopsis DRM methyltransferases in de novo DNA methylation and gene silencing." Curr Biol **12**(13): 1138-1144.
Carlsbecker, A., J. Y. Lee, C. J. Roberts, J. Dettmer, S. Lehesranta, J. Zhou, O. Lindgren, M. A. Moreno-Risueno, A. Vaten, S. Thitamadee, A. Campilho, J. Sebastian, J. L. Bowman, Y. Helariutta and P. N. Benfey (2010). "Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate." Nature **465**(7296): 316-321.
Carthew, R. W. and E. J. Sontheimer (2009). "Origins and Mechanisms of miRNAs and siRNAs." Cell **136**(4): 642-655.
Casati, P., M. Campi, F. Chu, N. Suzuki, D. Maltby, S. Guan, A. L. Burlingame and V. Walbot (2008). "Histone acetylation and chromatin remodeling are required for UV-B-dependent transcriptional activation of regulated genes in maize." Plant Cell **20**(4): 827-842.
Casson, S., M. Spencer, K. Walker and K. Lindsey (2005). "Laser capture microdissection for the analysis of gene expression during embryogenesis of Arabidopsis." Plant Journal **42**(1): 111-123.
Castel, S. E. and R. A. Martienssen (2013). "RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond." Nat Rev Genet **14**(2): 100-112.
Castresana, C., F. de Carvalho, G. Gheysen, M. Habets, D. Inzé and M. Van Montagu (1990). "Tissue-specific and pathogen-induced regulation of a Nicotiana plumbaginifolia beta-1,3-glucanase gene." The Plant Cell Online **2**(12): 1131-1143.
Cerda, S. and S. A. Weitzman (1997). "Influence of oxygen radical injury on DNA methylation." Mutat Res **386**(2): 141-152.
Cermak, T., E. L. Doyle, M. Christian, L. Wang, Y. Zhang, C. Schmidt, J. A. Baller, N. V. Somia, A. J. Bogdanove and D. F. Voytas (2011). "Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting." Nucleic Acids Res **39**(12): e82.
Charbonnel, C., M. E. Gallego and C. I. White (2010). "Xrcc1-dependent and Ku-dependent DNA double-strand break repair kinetics in Arabidopsis plants." The Plant Journal **64**(2): 280-290.
Chen, C. J., Q. liu, Y. C. Zhang, L. H. Qu, Y. Q. Chen and D. Gautheret (2011). "Genome-wide discovery and analysis of microRNAs and other small RNAs from rice embryogenic callus." RNA Biol **8**(3): 538-547.
Chen, M., S. Lv and Y. Meng (2010). "Epigenetic performers in plants." Dev Growth Differ **52**(6): 555-566.
Cheng, M., J. E. Fry, S. Pang, H. Zhou, C. M. Hironaka, D. R. Duncan, T. W. Conner and Y. Wan (1997). "Genetic Transformation of Wheat Mediated by Agrobacterium tumefaciens." Plant Physiol **115**(3): 971-980.

Cheng, M., B. Lowe, T. M. Spencer, X. Ye and C. Armstrong (2004). "Factors influencing Agrobacterium-mediated transformation of monocotyledonous species." In Vitro Cellular & Developmental Biology - Plant **40**(1): 31-45.
Cheung, W. L., K. Ajiro, K. Samejima, M. Kloc, P. Cheung, C. A. Mizzen, A. Beeser, L. D. Etkin, J. Chernoff, W. C. Earnshaw and C. D. Allis (2003). "Apoptotic phosphorylation of histone H2B is mediated by mammalian sterile twenty kinase." Cell **113**(4): 507-517.
Chi, X., Q. Yang, X. Chen, J. Wang, L. Pan, M. Chen, Z. Yang, Y. He, X. Liang and S. Yu (2011). "Identification and characterization of microRNAs from peanut (Arachis hypogaea L.) by high-throughput sequencing." PLoS One **6**(11): e27530.
Chilton, M.-D. M. and Q. Que (2003). "Targeted Integration of T-DNA into the Tobacco Genome at Double-Stranded Breaks: New Insights on the Mechanism of T-DNA Integration." Plant Physiol **133**(3): 956-965.
Chinnusamy, V. and J. K. Zhu (2009). "Epigenetic regulation of stress responses in plants." Curr Opin Plant Biol **12**(2): 133-139.
Chodavarapu, R. K., S. Feng, Y. V. Bernatavichute, P.-Y. Chen, H. Stroud, Y. Yu, J. A. Hetzel, F. Kuo, J. Kim, S. J. Cokus, D. Casero, M. Bernal, P. Huijser, A. T. Clark, U. Kramer, S. S. Merchant, X. Zhang, S. E. Jacobsen and M. Pellegrini (2010). "Relationship between nucleosome positioning and DNA methylation." Nature **466**(7304): 388-392.
Choi, C. S. and H. Sano (2007). "Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants." Mol Genet Genomics **277**(5): 589-600.
Cloix, C. and G. I. Jenkins (2008). "Interaction of the Arabidopsis UV-B-specific signaling component UVR8 with chromatin." Mol Plant **1**(1): 118-128.
Clough, S. J. and A. F. Bent (1998). "Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana." Plant Journal **16**(6): 735-743.
Cokus, S. J., S. Feng, X. Zhang, Z. Chen, B. Merriman, C. D. Haudenschild, S. Pradhan, S. F. Nelson, M. Pellegrini and S. E. Jacobsen (2008). "Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning." Nature **452**(7184): 215-219.
Collinge, D. B., H. J. Jorgensen, O. S. Lund and M. F. Lyngkjaer (2010). "Engineering pathogen resistance in crop plants: current trends and future prospects." Annu Rev Phytopathol **48**: 269-291.
Conesa, A., S. Gotz, J. M. Garcia-Gomez, J. Terol, M. Talon and M. Robles (2005). "Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research." Bioinformatics **21**(18): 3674-3676.
Covarrubias, A. A. and J. L. Reyes (2010). "Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs." Plant Cell Environ **33**(4): 481-489.
Crane, Y. M. and S. B. Gelvin (2007). "RNAi-mediated gene silencing reveals involvement of Arabidopsis chromatin-related genes in Agrobacterium-mediated root transformation." Proc Natl Acad Sci U S A **104**(38): 15156-15161.
Crooks, G. E., G. Hon, J. M. Chandonia and S. E. Brenner (2004). "WebLogo: a sequence logo generator." Genome Res **14**(6): 1188-1190.
Cropley, J. E. and D. I. Martin (2007). "Controlling elements are wild cards in the epigenomic deck." Proc Natl Acad Sci U S A **104**(48): 18879-18880.

Csaba, K. and S. Jeff (1986). "The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector." Molecular and General Genetics MGG.
Curtin, S. J., D. F. Voytas and R. M. Stupar (2012). "Genome Engineering of Crops with Designer Nucleases." Plant Gen. **5**(2): 42-50.
Curtin, S. J., J. M. Watson, N. A. Smith, A. L. Eamens, C. L. Blanchard and P. M. Waterhouse (2008). "The roles of plant dsRNA-binding proteins in RNAi-like pathways." FEBS Lett **582**(18): 2753-2760.
Curtin, S. J., F. Zhang, J. D. Sander, W. J. Haun, C. Starker, N. J. Baltes, D. Reyon, E. J. Dahlborg, M. J. Goodwin, A. P. Coffman, D. Dobbs, J. K. Joung, D. F. Voytas and R. M. Stupar (2011). "Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases." Plant Physiol **156**(2): 466-473.
Dai, X. and P. X. Zhao (2011). "psRNATarget: a plant small RNA target analysis server." Nucleic Acids Res **39**(Web Server issue): W155-159.
Daugherty, C. J. and M. E. Musgrave (1994). "Characterization of Populations of Rapid-Cycling Brassica-Rapa L Selected for Differential Waterlogging Tolerance." J Exp Bot **45**(272): 385-392.
De-La-Pena, C., A. Rangel-Cano and R. Alvarez-Venegas (2012). "Regulation of disease-responsive genes mediated by epigenetic factors: interaction of Arabidopsis-Pseudomonas." Mol Plant Pathol **13**(4): 388-398.
De Nadal, E., M. Zapater, P. M. Alepuz, L. Sumoy, G. Mas and F. Posas (2004). "The MAPK Hog1 recruits Rpd3 histone deacetylase to activate osmoresponsive genes." Nature **427**(6972): 370-374.
Deal, R. B., C. N. Topp, E. C. McKinney and R. B. Meagher (2007). "Repression of flowering in Arabidopsis requires activation of FLOWERING LOCUS C expression by the histone variant H2A.Z." Plant Cell **19**(1): 74-83.
DeBolt, S. (2010). "Copy number variation shapes genome diversity in Arabidopsis over immediate family generational scales." Genome Biol Evol **2**: 441-453.
Desfeux, C., S. J. Clough and A. F. Bent (2000). "Female reproductive tissues are the primary target of Agrobacterium-mediated transformation by the Arabidopsis floral-dip method." Plant Physiol **123**(3): 895-904.
Dhawan, R., H. Luo, A. M. Foerster, S. Abuqamar, H. N. Du, S. D. Briggs, O. Mittelsten Scheid and T. Mengiste (2009). "HISTONE MONOUBIQUITINATION1 interacts with a subunit of the mediator complex and regulates defense against necrotrophic fungal pathogens in Arabidopsis." Plant Cell **21**(3): 1000-1019.
Dickinson, H. G. and R. Grant-Downton (2009). "Bridging the generation gap: flowering plant gametophytes and animal germlines reveal unexpected similarities." Biol Rev Camb Philos Soc **84**(4): 589-615.
Ding, D., L. Zhang, H. Wang, Z. Liu, Z. Zhang and Y. Zheng (2009). "Differential expression of miRNAs in response to salt stress in maize roots." Ann Bot **103**(1): 29-38.
Ding, S. W. and O. Voinnet (2007). "Antiviral immunity directed by small RNAs." Cell **130**(3): 413-426.
Dinneny, J. R., T. A. Long, J. Y. Wang, J. W. Jung, D. Mace, S. Pointer, C. Barron, S. M. Brady, J. Schiefelbein and P. N. Benfey (2008). "Cell identity mediates the response of Arabidopsis roots to abiotic stress." Science **320**(5878): 942-945.

Dionisio-Sese, M. L. and S. Tobita (1998). "Antioxidant responses of rice seedlings to salinity stress." Plant Science **135**(1): 1-9.
Dong, J. Z., W. A. Keller, W. Yan and F. Georges (2004). "Gene expression at early stages of Brassica napus seed development as revealed by transcript profiling of seed-abundant cDNAs." Planta **218**(3): 483-491.
Dowen, R. H., M. Pelizzola, R. J. Schmitz, R. Lister, J. M. Dowen, J. R. Nery, J. E. Dixon and J. R. Ecker (2012). "Widespread dynamic DNA methylation in response to biotic stress." Proc Natl Acad Sci U S A **109**(32): E2183-2191.
Doyle, J. J. and E. E. Dickson (1987). "Preservation of Plant Samples for DNA Restriction Endonuclease Analysis." Taxon **36**(4): 715-722.
Doyle, M. R. and R. M. Amasino (2009). "A single amino acid change in the enhancer of zeste ortholog CURLY LEAF results in vernalization-independent, rapid flowering in Arabidopsis." Plant Physiol **151**(3): 1688-1697.
Drews, G. N. and A. M. Koltunow (2011). "The female gametophyte." Arabidopsis Book **9**: e0155.
Dunoyer, P., C. A. Brosnan, G. Schott, Y. Wang, F. Jay, A. Alioua, C. Himber and O. Voinnet (2010). "An endogenous, systemic RNAi pathway in plants." EMBO J **29**(10): 1699-1712.
Dunoyer, P., C. Himber and O. Voinnet (2006). "Induction, suppression and requirement of RNA silencing pathways in virulent Agrobacterium tumefaciens infections." Nat Genet **38**(2): 258-263.
Dunoyer, P., G. Schott, C. Himber, D. Meyer, A. Takeda, J. C. Carrington and O. Voinnet (2010). "Small RNA Duplexes Function as Mobile Silencing Signals Between Plant Cells." Science **328**(5980): 912-916.
Dyachenko, O. V., N. S. Zakharchenko, T. V. Shevchuk, H. J. Bohnert, J. C. Cushman and Y. I. Buryanov (2006). "Effect of hypermethylation of CCWGG sequences in DNA of Mesembryanthemum crystallinum plants on their adaptation to salt stress." Biochemistry (Mosc) **71**(4): 461-465.
Eamens, A., F. E. Vaistij and L. Jones (2008). "NRPD1a and NRPD1b are required to maintain post-transcriptional RNA silencing and RNA-directed DNA methylation in Arabidopsis." Plant Journal **55**(4): 596-606.
Eamens, A., M. B. Wang, N. A. Smith and P. M. Waterhouse (2008). "RNA silencing in plants: yesterday, today, and tomorrow." Plant Physiol **147**(2): 456-468.
Earley, K., M. Smith, R. Weber, B. Gregory and R. Poethig (2010). "An endogenous F-box protein regulates ARGONAUTE1 in Arabidopsis thaliana." Silence **1**(1): 15.
Ebbs, M. L. and J. Bender (2006). "Locus-specific control of DNA methylation by the Arabidopsis SUVH5 histone methyltransferase." Plant Cell **18**(5): 1166-1176.
Emde, A. K., M. Grunert, D. Weese, K. Reinert and S. R. Sperling (2010). "MicroRazerS: rapid alignment of small RNA reads." Bioinformatics **26**(1): 123-124.
Endo, M., Y. Ishikawa, K. Osakabe, S. Nakayama, H. Kaya, T. Araki, K. Shibahara, K. Abe, H. Ichikawa, L. Valentine, B. Hohn and S. Toki (2006). "Increased frequency of homologous recombination and T-DNA integration in Arabidopsis CAF-1 mutants." EMBO J **25**(23): 5579-5590.
Escobar, M. A. and A. M. Dandekar (2003). "Agrobacterium tumefaciens as an agent of disease." Trends in Plant Science **8**(8): 380-386.

Even-Faitelson, L., A. Samach, C. Melamed-Bessudo, N. Avivi-Ragolsky and A. A. Levy (2011). "Localized egg-cell expression of effector proteins for targeted modification of the Arabidopsis genome." Plant Journal **68**(5): 929-937.
Fahlgren, N., M. D. Howell, K. D. Kasschau, E. J. Chapman, C. M. Sullivan, J. S. Cumbie, S. A. Givan, T. F. Law, S. R. Grant, J. L. Dangl and J. C. Carrington (2007). "High-throughput sequencing of Arabidopsis microRNAs: evidence for frequent birth and death of MIRNA genes." PLoS One **2**(2): e219.
Fan, D., Y. Dai, X. Wang, Z. Wang, H. He, H. Yang, Y. Cao, X. W. Deng and L. Ma (2012). "IBM1, a JmjC domain-containing histone demethylase, is involved in the regulation of RNA-directed DNA methylation through the epigenetic control of RDR2 and DCL3 expression in Arabidopsis." Nucleic Acids Research **40**(18): 8905-8916.
Fauser, F., N. Roth, M. Pacher, G. Ilg, R. Sanchez-Fernandez, C. Biesgen and H. Puchta (2012). "In planta gene targeting." Proc Natl Acad Sci U S A **109**(19): 7535-7540.
Feng, S., S. J. Cokus, X. Zhang, P. Y. Chen, M. Bostick, M. G. Goll, J. Hetzel, J. Jain, S. H. Strauss, M. E. Halpern, C. Ukomadu, K. C. Sadler, S. Pradhan, M. Pellegrini and S. E. Jacobsen (2010). "Conservation and divergence of methylation patterning in plants and animals." Proc Natl Acad Sci U S A **107**(19): 8689-8694.
Feng, S. and S. E. Jacobsen (2011). "Epigenetic modifications in plants: an evolutionary perspective." Curr Opin Plant Biol **14**(2): 179-186.
Feng, S., S. E. Jacobsen and W. Reik (2010). "Epigenetic reprogramming in plant and animal development." Science **330**(6004): 622-627.
Filichkin, S. A., H. D. Priest, S. A. Givan, R. Shen, D. W. Bryant, S. E. Fox, W. K. Wong and T. C. Mockler (2010). "Genome-wide mapping of alternative splicing in Arabidopsis thaliana." Genome Res **20**(1): 45-58.
Foster, P. L. (2007). "Stress-induced mutagenesis in bacteria." Crit Rev Biochem Mol Biol **42**(5): 373-397.
Friesner, J. and A. B. Britt (2003). "Ku80- and DNA ligase IV-deficient plants are sensitive to ionizing radiation and defective in T-DNA integration." Plant Journal **34**(4): 427-440.
Galaud, J.-P., T. Gaspar and N. Boyer (1993). "Inhibition of internode growth due to mechanical stress in Bryonia dioica: relationship between changes in DNA methylation and ethylene metabolism." Physiologia Plantarum **87**(1): 25-30.
Gallego, M. E., J. Y. Bleuyard, S. Daoudal-Cotterell, N. Jallut and C. I. White (2003). "Ku80 plays a role in non-homologous recombination but is not required for T-DNA integration in Arabidopsis." The Plant Journal **35**(5): 557-565.
Galloway, L. and J. Etterson (2007). "Transgenerational plasticity is adaptive in the wild." Science (New York) **318**(5853): 1134-1136.
Gehring, M., K. L. Bubb and S. Henikoff (2009). "Extensive demethylation of repetitive elements during seed development underlies gene imprinting." Science **324**(5933): 1447-1451.
Gelvin, S. B. (2003). "Improving plant genetic engineering by manipulating the host." Trends Biotechnol **21**(3): 95-98.
Gelvin, S. B. (2009). "Agrobacterium in the genomics age." Plant Physiol **150**(4): 1665-1676.
Gelvin, S. B. (2010). "Plant proteins involved in Agrobacterium-mediated genetic transformation." Annu Rev Phytopathol **48**: 45-68.

Gendrel, A. V., Z. Lippman, C. Yordan, V. Colot and R. A. Martienssen (2002). "Dependence of heterochromatic histone H3 methylation patterns on the Arabidopsis gene DDM1." Science **297**(5588): 1871-1873.
Gohlke, J., C.-J. Scholz, S. Kneitz, D. Weber, J. Fuchs, R. Hedrich and R. Deeken (2013). "DNA Methylation Mediated Control of Gene Expression Is Critical for Development of Crown Gall Tumors." PLoS Genet **9**(2): e1003267.
Golden, T. A., S. E. Schauer, J. D. Lang, S. Pien, A. R. Mushegian, U. Grossniklaus, D. W. Meinke and A. Ray (2002). "Short Integuments1/suspensor1/carpel Factory, a Dicer Homolog, Is a Maternal Effect Gene Required for Embryo Development in Arabidopsis." Plant Physiol **130**(2): 808-822.
Grandbastien, M.-A. (1998). "Activation of plant retrotransposons under stress conditions." Trends in Plant Science **3**(5): 181-187.
Grant-Downton, R., S. Hafidh, D. Twell and H. G. Dickinson (2009). "Small RNA pathways are present and functional in the angiosperm male gametophyte." Mol Plant **2**(3): 500-512.
Grant-Downton, R., G. Le Trionnaire, R. Schmid, J. Rodriguez-Enriquez, S. Hafidh, S. Mehdi, D. Twell and H. Dickinson (2009). "MicroRNA and tasiRNA diversity in mature pollen of Arabidopsis thaliana." BMC Genomics **10**: 643.
Grant-Downton, R. and J. Rodriguez-Enriquez (2012). "Emerging Roles for Non-Coding RNAs in Male Reproductive Development in Flowering Plants." Biomolecules **2**(4): 608-621.
Grativol, C., A. S. Hemerly and P. C. Ferreira (2012). "Genetic and epigenetic regulation of stress responses in natural plant populations." Biochim Biophys Acta **1819**(2): 176-185.
Grawunder, U., M. Wilm, X. Wu, P. Kulesza, T. E. Wilson, M. Mann and M. R. Lieber (1997). "Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells." Nature **388**(6641): 492-495.
Guillemette, B., A. R. Bataille, N. Gevry, M. Adam, M. Blanchette, F. Robert and L. Gaudreau (2005). "Variant histone H2A.Z is globally localized to the promoters of inactive yeast genes and regulates nucleosome positioning." PLoS Biol **3**(12): 2100-2110.
Guleria, P., M. Mahajan, J. Bhardwaj and S. K. Yadav (2011). "Plant Small RNAs: Biogenesis, Mode of Action and Their Roles in Abiotic Stresses." Genomics, Proteomics & Bioinformatics **9**(6): 183-199.
Gurley, W. B. (2000). "HSP101: a key component for the acquisition of thermotolerance in plants." Plant Cell **12**(4): 457-460.
Gutzat, R. and O. Mittelsten Scheid (2012). "Epigenetic responses to stress: triple defense?" Curr Opin Plant Biol.
Haag, J. R. and C. S. Pikaard (2011). "Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing." Nat Rev Mol Cell Biol **12**(8): 483-492.
Hackenberg, M., P. J. Huang, C. Y. Huang, B. J. Shi, P. Gustafson and P. Langridge (2012). "A Comprehensive Expression Profile of MicroRNAs and Other Classes of Non-Coding Small RNAs in Barley Under Phosphorous-Deficient and -Sufficient Conditions." DNA Res.
Hall, A. E. (2001). Crop responses to environment. Boca Raton, CRC Press.

Hanin, M. and J. Paszkowski (2003). "Plant genome modification by homologous recombination." Curr Opin Plant Biol **6**(2): 157-162.
Harvey, J. J., M. G. Lewsey, K. Patel, J. Westwood, S. Heimstadt, J. P. Carr and D. C. Baulcombe (2011). "An antiviral defense role of AGO2 in plants." PLoS One **6**(1): e14639.
He, X. F., Y. Y. Fang, L. Feng and H. S. Guo (2008). "Characterization of conserved and novel microRNAs and their targets, including a TuMV-induced TIR-NBS-LRR class R gene-derived novel miRNA in Brassica." FEBS Lett **582**(16): 2445-2452.
Hedhly, A. (2011). "Sensitivity of flowering plant gametophytes to temperature fluctuations." Environmental and Experimental Botany **74**: 9-16.
Heil, M. and J. Ton (2008). "Long-distance signalling in plant defence." Trends in Plant Science **13**(6): 264-272.
Henderson, I. R. and S. E. Jacobsen (2007). "Epigenetic inheritance in plants." Nature **447**(7143): 418-424.
Herrera-Estrella, L., J. Simpson and M. Martinez-Trujillo (2005). "Transgenic plants: an historical perspective." Methods Mol Biol **286**: 3-32.
Hiei, Y., S. Ohta, T. Komari and T. Kumashiro (1994). "Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA." Plant Journal **6**(2): 271-282.
Himanen, K., M. Woloszynska, T. M. Boccardi, S. De Groeve, H. Nelissen, L. Bruno, M. Vuylsteke and M. Van Lijsebettens (2012). "Histone H2B monoubiquitination is required to reach maximal transcript levels of circadian clock genes in Arabidopsis." The Plant Journal **72**(2): 249-260.
Hohn, T. and F. Vazquez (2011). "RNA silencing pathways of plants: silencing and its suppression by plant DNA viruses." Biochim Biophys Acta **1809**(11-12): 588-600.
Holeski, L. M., G. Jander and A. A. Agrawal (2012). "Transgenerational defense induction and epigenetic inheritance in plants." Trends Ecol Evol **27**(11): 618-626.
Hood, E. E., R. T. Fraley and M.-D. Chilton (1987). "Virulence of Agrobacterium tumefaciens Strain A281 on Legumes." Plant Physiol **83**(3): 529-534.
Houben, A., D. Demidov, A. D. Caperta, R. Karimi, F. Agueci and L. Vlasenko (2007). "Phosphorylation of histone H3 in plants--a dynamic affair." Biochim Biophys Acta **1769**(5-6): 308-315.
Hsieh, L. C., S. I. Lin, A. C. Shih, J. W. Chen, W. Y. Lin, C. Y. Tseng, W. H. Li and T. J. Chiou (2009). "Uncovering small RNA-mediated responses to phosphate deficiency in Arabidopsis by deep sequencing." Plant Physiol **151**(4): 2120-2132.
Hsieh, T. F., C. A. Ibarra, P. Silva, A. Zemach, L. Eshed-Williams, R. L. Fischer and D. Zilberman (2009). "Genome-wide demethylation of Arabidopsis endosperm." Science **324**(5933): 1451-1454.
Hu, Z. H., J. Q. Chen, G. T. Wu, W. Jin, C. X. Lang, R. Z. Huang, F. L. Wang, Z. H. Liu and X. Y. Chen (2005). "Highly efficient transformation and plant regeneration of tall fescue mediated by Agrobacterium tumefaciens." Zhi Wu Sheng Li Yu Fen Zi Sheng Wu Xue Xue Bao **31**(2): 149-159.
Huettel, B., T. Kanno, L. Daxinger, W. Aufsatz, A. J. Matzke and M. Matzke (2006). "Endogenous targets of RNA-directed DNA methylation and Pol IV in Arabidopsis." EMBO J **25**(12): 2828-2836.

Husaini, A. M., Z. Rashid, R. U. Mir and B. Aquil (2011). "Approaches for gene targeting and targeted gene expression in plants." GM Crops **2**(3): 150-162.
Ibarra, C. A., X. Feng, V. K. Schoft, T. F. Hsieh, R. Uzawa, J. A. Rodrigues, A. Zemach, N. Chumak, A. Machlicova, T. Nishimura, D. Rojas, R. L. Fischer, H. Tamaru and D. Zilberman (2012). "Active DNA demethylation in plant companion cells reinforces transposon methylation in gametes." Science **337**(6100): 1360-1364.
Iizuka, M. and M. M. Smith (2003). "Functional consequences of histone modifications." Curr Opin Genet Dev **13**(2): 154-160.
Ilnytskyy, Y., A. Boyko and I. Kovalchuk (2004). "Luciferase-based transgenic recombination assay is more sensitive than beta-glucoronidase-based." Mutation Research-Genetic Toxicology and Environmental Mutagenesis **559**(1-2): 189-197.
Ilnytskyy, Y., A. Boyko and I. Kovalchuk (2004). "Luciferase-based transgenic recombination assay is more sensitive than beta-glucoronidase-based." Mutat Res **559**(1-2): 189-197.
Ishida, Y., Y. Hiei and T. Komari (2007). "Agrobacterium-mediated transformation of maize." Nat Protoc **2**(7): 1614-1621.
Ishida, Y., H. Saito, S. Ohta, Y. Hiei, T. Komari and T. Kumashiro (1996). "High efficiency transformation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens." Nat Biotechnol **14**(6): 745-750.
Ito, H., H. Gaubert, E. Bucher, M. Mirouze, I. Vaillant and J. Paszkowski (2011). "An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress." Nature **472**(7341): 115-119.
Iyer-Pascuzzi, A. S., T. Jackson, H. Cui, J. J. Petricka, W. Busch, H. Tsukagoshi and P. N. Benfey (2011). "Cell identity regulators link development and stress responses in the Arabidopsis root." Dev Cell **21**(4): 770-782.
Jablonka, E. and G. Raz (2009). "Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution." Q Rev Biol **84**(2): 131-176.
Jackson, J. P., L. Johnson, Z. Jasencakova, X. Zhang, L. PerezBurgos, P. B. Singh, X. Cheng, I. Schubert, T. Jenuwein and S. E. Jacobsen (2004). "Dimethylation of histone H3 lysine 9 is a critical mark for DNA methylation and gene silencing in Arabidopsis thaliana." Chromosoma **112**(6): 308-315.
Jaskiewicz, M., U. Conrath and C. Peterhansel (2011). "Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response." EMBO Rep **12**(1): 50-55.
Jaubert, M., S. Bhattacharjee, A. F. Mello, K. L. Perry and P. Moffett (2011). "ARGONAUTE2 mediates RNA-silencing antiviral defenses against Potato virus X in Arabidopsis." Plant Physiol **156**(3): 1556-1564.
Jeddeloh, J. A., T. L. Stokes and E. J. Richards (1999). "Maintenance of genomic methylation requires a SWI2/SNF2-like protein." Nat Genet **22**(1): 94-97.
Jia, Q., P. Bundock, P. J. J. Hooykaas and S. d. Pater (2012). "Agrobacterium tumefaciens T-DNA Integration and Gene Targeting in Arabidopsis thaliana Non-Homologous End-Joining Mutants." Journal of Botany **2012**.
Jia, X., V. Mendu and G. Tang (2010). "An array platform for identification of stress-responsive microRNAs in plants." Methods Mol Biol **639**: 253-269.

Jia, X., W. X. Wang, L. Ren, Q. J. Chen, V. Mendu, B. Willcut, R. Dinkins, X. Tang and G. Tang (2009). "Differential and dynamic regulation of miR398 in response to ABA and salt stress in Populus tremula and Arabidopsis thaliana." Plant Mol Biol **71**(1-2): 51-59.
Johnson-Brousseau, S. A. and S. McCormick (2004). "A compendium of methods useful for characterizing Arabidopsis pollen mutants and gametophytically-expressed genes." Plant J **39**(5): 761-775.
Johnson, L., X. Cao and S. Jacobsen (2002). "Interplay between two epigenetic marks. DNA methylation and histone H3 lysine 9 methylation." Curr Biol **12**(16): 1360-1367.
Johnson, R. A., R. P. Hellens and D. R. Love (2011). "A transient assay for recombination demonstrates that Arabidopsis SNM1 and XRCC3 enhance non-homologous recombination." Genet Mol Res **10**(3): 2104-2132.
Johnson, T. B. and R. D. Coghill (1925). "RESEARCHES ON PYRIMIDINES. C111. THE DISCOVERY OF 5-METHYL-CYTOSINE IN TUBERCULINIC ACID, THE NUCLEIC ACID OF THE TUBERCLE BACILLUS1." Journal of the American Chemical Society **47**(11): 2838-2844.
Jones, H. D., A. Doherty and H. Wu (2005). "Review of methodologies and a protocol for the Agrobacterium-mediated transformation of wheat." Plant Methods **1**(1): 5.
Jullien, P. E., D. Susaki, R. Yelagandula, T. Higashiyama and F. Berger (2012). "DNA methylation dynamics during sexual reproduction in Arabidopsis thaliana." Curr Biol **22**(19): 1825-1830.
Jung, H. W., T. J. Tschaplinski, L. Wang, J. Glazebrook and J. T. Greenberg (2009). "Priming in systemic plant immunity." Science **324**(5923): 89-91.
Kathiria, P., C. Sidler, A. Golubov, M. Kalischuk, L. M. Kawchuk and I. Kovalchuk (2010). "Tobacco mosaic virus infection results in an increase in recombination frequency and resistance to viral, bacterial, and fungal pathogens in the progeny of infected tobacco plants." Plant Physiol **153**(4): 1859-1870.
Katiyar-Agarwal, S., R. Morgan, D. Dahlbeck, O. Borsani, A. Villegas, Jr., J. K. Zhu, B. J. Staskawicz and H. Jin (2006). "A pathogen-inducible endogenous siRNA in plant immunity." Proc Natl Acad Sci U S A **103**(47): 18002-18007.
Khorasanizadeh, S. (2004). "The nucleosome: from genomic organization to genomic regulation." Cell **116**(2): 259-272.
Khraiwesh, B., J. K. Zhu and J. Zhu (2012). "Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants." Biochim Biophys Acta **1819**(2): 137-148.
Kidner, C. A. and R. A. Martienssen (2004). "Spatially restricted microRNA directs leaf polarity through ARGONAUTE1." Nature **428**(6978): 81-84.
Kim, D. H. and S. Sung (2012). "Environmentally coordinated epigenetic silencing of FLC by protein and long noncoding RNA components." Curr Opin Plant Biol **15**(1): 51-56.
Kim, J. M., T. K. To, J. Ishida, T. Morosawa, M. Kawashima, A. Matsui, T. Toyoda, H. Kimura, K. Shinozaki and M. Seki (2008). "Alterations of lysine modifications on the histone H3 N-tail under drought stress conditions in Arabidopsis thaliana." Plant Cell Physiol **49**(10): 1580-1588.
Kim, J. Y., K. J. Kwak, H. J. Jung, H. J. Lee and H. Kang (2010). "MicroRNA402 affects seed germination of Arabidopsis thaliana under stress conditions via targeting DEMETER-LIKE Protein3 mRNA." Plant Cell Physiol **51**(6): 1079-1083.

Kim, S., J. Y. Yang, J. Xu, I. C. Jang, M. J. Prigge and N. H. Chua (2008). "Two cap-binding proteins CBP20 and CBP80 are involved in processing primary MicroRNAs." Plant Cell Physiol **49**(11): 1634-1644.
Kim, S. W., Z. Li, P. S. Moore, A. P. Monaghan, Y. Chang, M. Nichols and B. John (2010). "A sensitive non-radioactive northern blot method to detect small RNAs." Nucleic Acids Res **38**(7): e98.
Kinoshita, N., H. Wang, H. Kasahara, J. Liu, C. Macpherson, Y. Machida, Y. Kamiya, M. A. Hannah and N. H. Chua (2012). "IAA-Ala Resistant3, an evolutionarily conserved target of miR167, mediates Arabidopsis root architecture changes during high osmotic stress." Plant Cell **24**(9): 3590-3602.
Kirik, A., A. Pecinka, E. Wendeler and B. Reiss (2006). "The Chromatin Assembly Factor Subunit FASCIATA1 Is Involved in Homologous Recombination in Plants." The Plant Cell Online **18**(10): 2431-2442.
Kishore, J., I. Ahmad, R. Kaur and K. M. P (2008). "Beliefs and perceptions about cancers among patients attending radiotherapy OPD in Delhi, India." Asian Pac J Cancer Prev **9**(1): 155-158.
Kobor, M. S., S. Venkatasubrahmanyam, M. D. Meneghini, J. W. Gin, J. L. Jennings, A. J. Link, H. D. Madhani and J. Rine (2004). "A protein complex containing the conserved Swi2/Snf2-related ATPase Swr1p deposits histone variant H2A.Z into euchromatin." PLoS Biol **2**(5): E131.
Komari, T. (1989). "Transformation of callus cultures of nine plant species mediated by agrobacterium." Plant Science **60**(2): 223-229.
Korbes, A. P., R. D. Machado, F. Guzman, M. P. Almerao, L. F. de Oliveira, G. Loss-Morais, A. C. Turchetto-Zolet, A. Cagliari, F. dos Santos Maraschin, M. Margis-Pinheiro and R. Margis (2012). "Identifying conserved and novel microRNAs in developing seeds of Brassica napus using deep sequencing." PLoS One **7**(11): e50663.
Kotak, S., J. Larkindale, U. Lee, P. von Koskull-Doring, E. Vierling and K. D. Scharf (2007). "Complexity of the heat stress response in plants." Curr Opin Plant Biol **10**(3): 310-316.
Koturbash, I., M. Baker, J. Loree, K. Kutanzi, D. Hudson, I. Pogribny, O. Sedelnikova, W. Bonner and O. Kovalchuk (2006). "Epigenetic dysregulation underlies radiation-induced transgenerational genome instability in vivo." Int J Radiat Oncol Biol Phys **66**(2): 327-330.
Kouzarides, T. (2007). "Chromatin modifications and their function." Cell **128**(4): 693-705.
Kovalchuk, I., V. Abramov, I. Pogribny and O. Kovalchuk (2004). "Molecular aspects of plant adaptation to life in the Chernobyl zone." Plant Physiol **135**(1): 357-363.
Kovalchuk, I. and O. Kovalchuk (2012). Epigenetics in health and disease. Upper Saddle River, N.J., FT Press.
Kovalchuk, O., P. Burke, A. Arkhipov, N. Kuchma, S. J. James, I. Kovalchuk and I. Pogribny (2003). "Genome hypermethylation in Pinus silvestris of Chernobyl--a mechanism for radiation adaptation?" Mutat Res **529**(1-2): 13-20.
Kovarˇik, A., B. Koukalová, M. Bezdeˇk and Z. Opatrn´ (1997). "Hypermethylation of tobacco heterochromatic loci in response to osmotic stress." TAG Theoretical and Applied Genetics **95**(1): 301-306.

Krasnikova, M. S., I. A. Milyutina, V. K. Bobrova, L. V. Ozerova, A. V. Troitsky, A. G. Solovyev and S. Y. Morozov (2009). "Novel miR390-dependent transacting siRNA precursors in plants revealed by a PCR-based experimental approach and database analysis." J Biomed Biotechnol **2009**: 952304.
Kreps, J. A., Y. Wu, H. S. Chang, T. Zhu, X. Wang and J. F. Harper (2002). "Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress." Plant Physiol **130**(4): 2129-2141.
Krupovic, M., J. J. Ravantti and D. H. Bamford (2009). "Geminiviruses: a tale of a plasmid becoming a virus." BMC Evol Biol **9**: 112.
Kruszka, K., M. Pieczynski, D. Windels, D. Bielewicz, A. Jarmolowski, Z. Szweykowska-Kulinska and F. Vazquez (2012). "Role of microRNAs and other sRNAs of plants in their changing environments." Journal of Plant Physiology **169**(16): 1664-1672.
Kultz, D. (2005). "Molecular and evolutionary basis of the cellular stress response." Annu Rev Physiol **67**: 225-257.
Kumar, A. and J. L. Bennetzen (1999). "Plant retrotransposons." Annu Rev Genet **33**: 479-532.
Kumar, S. V. and P. A. Wigge (2010). "H2A.Z-containing nucleosomes mediate the thermosensory response in Arabidopsis." Cell **140**(1): 136-147.
Kurihara, Y., E. Kaminuma, A. Matsui, M. Kawashima, M. Tanaka, T. Morosawa, J. Ishida, Y. Mochizuki, K. Shinozaki, T. Toyoda and M. Seki (2009). "Transcriptome analyses revealed diverse expression changes in ago1 and hyl1 Arabidopsis mutants." Plant Cell Physiol **50**(9): 1715-1720.
Kwon, C. S. and D. Wagner (2007). "Unwinding chromatin for development and growth: a few genes at a time." Trends Genet **23**(8): 403-412.
Lacroix, B. and V. Citovsky (2009). "Agrobacterium aiming for the host chromatin: Host and bacterial proteins involved in interactions between T-DNA and plant nucleosomes." Commun Integr Biol **2**(1): 42-45.
Lang-Mladek, C., O. Popova, K. Kiok, M. Berlinger, B. Rakic, W. Aufsatz, C. Jonak, M. T. Hauser and C. Luschnig (2010). "Transgenerational inheritance and resetting of stress-induced loss of epigenetic gene silencing in Arabidopsis." Mol Plant **3**(3): 594-602.
Lang, J., O. Smetana, L. Sanchez-Calderon, F. Lincker, J. Genestier, A.-C. Schmit, G. Houlné and M.-E. Chabouté (2012). "Plant γH2AX foci are required for proper DNA DSB repair responses and colocalize with E2F factors." New Phytologist **194**(2): 353-363.
Langmead, B., C. Trapnell, M. Pop and S. L. Salzberg (2009). "Ultrafast and memory-efficient alignment of short DNA sequences to the human genome." Genome Biol **10**(3): R25.
Lario, L. D., E. Ramirez-Parra, C. Gutierrez, P. Casati and C. P. Spampinato (2011). "Regulation of plant MSH2 and MSH6 genes in the UV-B-induced DNA damage response." J Exp Bot **62**(8): 2925-2937.
Larkindale, J., J. D. Hall, M. R. Knight and E. Vierling (2005). "Heat stress phenotypes of arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance." Plant Physiol **138**(2): 882-897.

Larkindale, J. and B. Huang (2004). "Thermotolerance and antioxidant systems in Agrostis stolonifera: Involvement of salicylic acid, abscisic acid, calcium, hydrogen peroxide, and ethylene." Journal of Plant Physiology **161**(4): 405-413.
Larkindale, J., M. Mishkind and E. Vierling (2007). Plant Responses to High Temperature. Plant Abiotic Stress, Blackwell Publishing Ltd**:** 100-144.
Larsen, F., G. Gundersen, R. Lopez and H. Prydz (1992). "CpG islands as gene markers in the human genome." Genomics **13**(4): 1095-1107.
Laubinger, S., T. Sachsenberg, G. Zeller, W. Busch, J. U. Lohmann, G. Ratsch and D. Weigel (2008). "Dual roles of the nuclear cap-binding complex and SERRATE in pre-mRNA splicing and microRNA processing in Arabidopsis thaliana." Proc Natl Acad Sci U S A **105**(25): 8795-8800.
Laurent, L., E. Wong, G. Li, T. Huynh, A. Tsirigos, C. T. Ong, H. M. Low, K. W. Kin Sung, I. Rigoutsos, J. Loring and C. L. Wei (2010). "Dynamic changes in the human methylome during differentiation." Genome Res **20**(3): 320-331.
Lauria, M. and V. Rossi (2011). "Epigenetic control of gene regulation in plants." Biochim Biophys Acta **1809**(8): 369-378.
Law, J. A., I. Ausin, L. M. Johnson, A. A. Vashisht, J. K. Zhu, J. A. Wohlschlegel and S. E. Jacobsen (2010). "A protein complex required for polymerase V transcripts and RNA-directed DNA methylation in Arabidopsis." Curr Biol **20**(10): 951-956.
Law, J. A. and S. E. Jacobsen (2010). "Establishing, maintaining and modifying DNA methylation patterns in plants and animals." Nat Rev Genet **11**(3): 204-220.
Le, B. H., C. Cheng, A. Q. Bui, J. A. Wagmaister, K. F. Henry, J. Pelletier, L. Kwong, M. Belmonte, R. Kirkbride, S. Horvath, G. N. Drews, R. L. Fischer, J. K. Okamuro, J. J. Harada and R. B. Goldberg (2010). "Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors." Proc Natl Acad Sci U S A **107**(18): 8063-8070.
Lee, H. C., A. P. Aalto, Q. Yang, S. S. Chang, G. Huang, D. Fisher, J. Cha, M. M. Poranen, D. H. Bamford and Y. Liu (2010). "The DNA/RNA-dependent RNA polymerase QDE-1 generates aberrant RNA and dsRNA for RNAi in a process requiring replication protein A and a DNA helicase." PLoS Biol **8**(10).
Lee, J.-Y. and D.-H. Lee (2003). "Use of Serial Analysis of Gene Expression Technology to Reveal Changes in Gene Expression in Arabidopsis Pollen Undergoing Cold Stress." Plant Physiol **132**(2): 517-529.
Leelavathi, S., V. G. Sunnichan, R. Kumria, G. P. Vijaykanth, R. K. Bhatnagar and V. S. Reddy (2004). "A simple and rapid Agrobacterium-mediated transformation protocol for cotton (Gossypium hirsutum L.): embryogenic calli as a source to generate large numbers of transgenic plants." Plant Cell Rep **22**(7): 465-470.
Li, J., M. Vaidya, C. White, A. Vainstein, V. Citovsky and T. Tzfira (2005). "Involvement of KU80 in T-DNA integration in plant cells." Proc Natl Acad Sci U S A **102**(52): 19231-19236.
Li, T., B. Liu, M. H. Spalding, D. P. Weeks and B. Yang (2012). "High-efficiency TALEN-based gene editing produces disease-resistant rice." Nat Biotechnol **30**(5): 390-392.
Li, W., X. Cui, Z. Meng, X. Huang, Q. Xie, H. Wu, H. Jin, D. Zhang and W. Liang (2012). "Transcriptional regulation of Arabidopsis MIR168a and argonaute1 homeostasis in abscisic acid and abiotic stress responses." Plant Physiol **158**(3): 1279-1292.

Li, W. X., Y. Oono, J. Zhu, X. J. He, J. M. Wu, K. Iida, X. Y. Lu, X. Cui, H. Jin and J. K. Zhu (2008). "The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance." Plant Cell **20**(8): 2238-2251.
Li, X., X. Wang, K. He, Y. Ma, N. Su, H. He, V. Stolc, W. Tongprasit, W. Jin, J. Jiang, W. Terzaghi, S. Li and X. W. Deng (2008). "High-resolution mapping of epigenetic modifications of the rice genome uncovers interplay between DNA methylation, histone methylation, and gene expression." Plant Cell **20**(2): 259-276.
Lin, S. I. and T. J. Chiou (2008). "Long-distance movement and differential targeting of microRNA399s." Plant Signal Behav **3**(9): 730-732.
Lippman, Z., A. V. Gendrel, M. Black, M. W. Vaughn, N. Dedhia, W. R. McCombie, K. Lavine, V. Mittal, B. May, K. D. Kasschau, J. C. Carrington, R. W. Doerge, V. Colot and R. Martienssen (2004). "Role of transposable elements in heterochromatin and epigenetic control." Nature **430**(6998): 471-476.
Lippman, Z., B. May, C. Yordan, T. Singer and R. Martienssen (2003). "Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification." PLoS Biol **1**(3): E67.
Lister, R., R. C. O'Malley, J. Tonti-Filippini, B. D. Gregory, C. C. Berry, A. H. Millar and J. R. Ecker (2008). "Highly integrated single-base resolution maps of the epigenome in Arabidopsis." Cell **133**(3): 523-536.
Liu, C., F. Lu, X. Cui and X. Cao (2010). "Histone methylation in higher plants." Annu Rev Plant Biol **61**: 395-420.
Liu, H., Y. Fu, B. Li, X. Yu, J. Xie, J. Cheng, S. A. Ghabrial, G. Li, X. Yi and D. Jiang (2011). "Widespread horizontal gene transfer from circular single-stranded DNA viruses to eukaryotic genomes." BMC Evol Biol **11**: 276.
Liu, H. T., D. Y. Sun and R. G. Zhou (2005). "Ca2+ and AtCaM3 are involved in the expression of heat shock protein gene in Arabidopsis." Plant Cell and Environment **28**(10): 1276-1284.
Liu, Y., M. Schiff, R. Marathe and S. P. Dinesh-Kumar (2002). "Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus." Plant Journal **30**(4): 415-429.
Lobbes, D., G. Rallapalli, D. D. Schmidt, C. Martin and J. Clarke (2006). "SERRATE: a new player on the plant microRNA scene." EMBO Rep **7**(10): 1052-1058.
Loss-Morais, G., P. M. Waterhouse and R. Margis (2013). "Description of plant tRNA-derived RNA fragments (tRFs) associated with argonaute and identification of their putative targets." Biol Direct **8**: 6.
Loury, R. and P. Sassone-Corsi (2004). "Analysis of histone phosphorylation: coupling intracellular signaling to chromatin remodeling." Methods Enzymol **377**: 197-212.
Luna, E., T. J. Bruce, M. R. Roberts, V. Flors and J. Ton (2012). "Next-generation systemic acquired resistance." Plant Physiol **158**(2): 844-853.
Luo, M., X. Liu, P. Singh, Y. Cui, L. Zimmerli and K. Wu (2012). "Chromatin modifications and remodeling in plant abiotic stress responses." Biochim Biophys Acta **1819**(2): 129-136.
Ma, S. and H. J. Bohnert (2007). "Integration of Arabidopsis thaliana stress-related transcript profiles, promoter structures, and cell-specific expression." Genome Biol **8**(4): R49.

Ma, Z., C. Coruh and M. J. Axtell (2010). "Arabidopsis lyrata small RNAs: transient MIRNA and small interfering RNA loci within the Arabidopsis genus." Plant Cell **22**(4): 1090-1103.
Magori, S. and V. Citovsky (2011). "Epigenetic control of Agrobacterium T-DNA integration." Biochim Biophys Acta **1809**(8): 388-394.
Mallory, A. and H. Vaucheret (2010). "Form, function, and regulation of ARGONAUTE proteins." Plant Cell **22**(12): 3879-3889.
Mallory, A. C., D. P. Bartel and B. Bartel (2005). "MicroRNA-directed regulation of Arabidopsis AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes." Plant Cell **17**(5): 1360-1375.
Mallory, A. C., A. Hinze, M. R. Tucker, N. Bouche, V. Gasciolli, T. Elmayan, D. Lauressergues, V. Jauvion, H. Vaucheret and T. Laux (2009). "Redundant and specific roles of the ARGONAUTE proteins AGO1 and ZLL in development and small RNA-directed gene silencing." PLoS Genet **5**(9): e1000646.
Mallory, A. C. and H. Vaucheret (2009). "ARGONAUTE 1 homeostasis invokes the coordinate action of the microRNA and siRNA pathways." EMBO Rep **10**(5): 521-526.
March-Diaz, R., M. Garcia-Dominguez, J. Lozano-Juste, J. Leon, F. J. Florencio and J. C. Reyes (2008). "Histone H2A.Z and homologues of components of the SWR1 complex are required to control immunity in Arabidopsis." Plant Journal **53**(3): 475-487.
Marin, E., V. Jouannet, A. Herz, A. S. Lokerse, D. Weijers, H. Vaucheret, L. Nussaume, M. D. Crespi and A. Maizel (2010). "miR390, Arabidopsis TAS3 tasiRNAs, and Their AUXIN RESPONSE FACTOR Targets Define an Autoregulatory Network Quantitatively Regulating Lateral Root Growth." The Plant Cell Online **22**(4): 1104-1117.
Marques, M., L. Laflamme, A. L. Gervais and L. Gaudreau (2010). "Reconciling the positive and negative roles of histone H2A.Z in gene transcription." Epigenetics **5**(4): 267-272.
Martin-Hernandez, A. M. and D. C. Baulcombe (2008). "Tobacco rattle virus 16-kilodalton protein encodes a suppressor of RNA silencing that allows transient viral entry in meristems." J Virol **82**(8): 4064-4071.
Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads.
Martínez-Priego, L., L. Donaire, D. Barajas and C. Llave (2008). "Silencing suppressor activity of the Tobacco rattle virus-encoded 16-kDa protein and interference with endogenous small RNA-guided regulatory pathways." Virology **376**(2): 346-356.
Martinez de Alba, A. E., V. Jauvion, A. C. Mallory, N. Bouteiller and H. Vaucheret (2011). "The miRNA pathway limits AGO1 availability during siRNA-mediated PTGS defense against exogenous RNA." Nucleic Acids Res **39**(21): 9339-9344.
Marton, I., A. Zuker, E. Shklarman, V. Zeevi, A. Tovkach, S. Roffe, M. Ovadis, T. Tzfira and A. Vainstein (2010). "Nontransgenic genome modification in plant cells." Plant Physiol **154**(3): 1079-1087.
Mason, G., P. Caciagli, G. P. Accotto and E. Noris (2008). "Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in Bemisia tabaci." J Virol Methods **147**(2): 282-289.
Mason, G., E. Noris, S. Lanteri, A. Acquadro, G. Accotto and E. Portis (2008). "Potentiality of Methylation-sensitive Amplification Polymorphism (MSAP) in

Identifying Genes Involved in Tomato Response to Tomato Yellow Leaf Curl Sardinia Virus." Plant Molecular Biology Reporter **26**(3): 156-173.
Mathieu, O., J. Reinders, M. Caikovski, C. Smathajitt and J. Paszkowski (2007). "Transgenerational stability of the Arabidopsis epigenome is coordinated by CG methylation." Cell **130**(5): 851-862.
McCue, A. D., S. Nuthikattu, S. H. Reeder and R. K. Slotkin (2012). "Gene expression and stress response mediated by the epigenetic regulation of a transposable element small RNA." PLoS Genet **8**(2): e1002474.
Melamed-Bessudo, C. and A. A. Levy (2012). "Deficiency in DNA methylation increases meiotic crossover rates in euchromatic but not in heterochromatic regions in Arabidopsis." Proc Natl Acad Sci U S A **109**(16): E981-988.
Meyers, B. C., S. Kaushik and R. S. Nandety (2005). "Evolving disease resistance genes." Curr Opin Plant Biol **8**(2): 129-134.
Mi, S., T. Cai, Y. Hu, Y. Chen, E. Hodges, F. Ni, L. Wu, S. Li, H. Zhou, C. Long, S. Chen, G. J. Hannon and Y. Qi (2008). "Sorting of Small RNAs into Arabidopsis Argonaute Complexes Is Directed by the 52 Terminal Nucleotide." Cell **133**(1): 116-127.
Miller, G., K. Schlauch, R. Tam, D. Cortes, M. A. Torres, V. Shulaev, J. L. Dangl and R. Mittler (2009). "The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli." Sci Signal **2**(84): ra45.
Mirouze, M., M. Lieberman-Lazarovich, R. Aversano, E. Bucher, J. Nicolet, J. Reinders and J. Paszkowski (2012). "Loss of DNA methylation affects the recombination landscape in Arabidopsis." Proceedings of the National Academy of Sciences **109**(15): 5880-5885.
Mirouze, M. and J. Paszkowski (2011). "Epigenetic contribution to stress adaptation in plants." Curr Opin Plant Biol **14**(3): 267-274.
Mladenov, E. and G. Iliakis (2011). "Induction and repair of DNA double strand breaks: the increasing spectrum of non-homologous end joining pathways." Mutat Res **711**(1-2): 61-72.
Moissiard, G. and O. Voinnet (2006). "RNA silencing of host transcripts by cauliflower mosaic virus requires coordinated action of the four Arabidopsis Dicer-like proteins." Proc Natl Acad Sci U S A **103**(51): 19593-19598.
Molinier, J., G. Ries, C. Zipfel and B. Hohn (2006). "Transgeneration memory of stress in plants." Nature **442**(7106): 1046-1049.
Molnar, A., C. W. Melnyk, A. Bassett, T. J. Hardcastle, R. Dunn and D. C. Baulcombe (2010). "Small Silencing RNAs in Plants Are Mobile and Direct Epigenetic Modification in Recipient Cells." Science **328**(5980): 872-875.
Montgomery, T. A., M. D. Howell, J. T. Cuperus, D. Li, J. E. Hansen, A. L. Alexander, E. J. Chapman, N. Fahlgren, E. Allen and J. C. Carrington (2008). "Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation." Cell **133**(1): 128-141.
Morel, J. B., C. Godon, P. Mourrain, C. Beclin, S. Boutet, F. Feuerbach, F. Proux and H. Vaucheret (2002). "Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance." Plant Cell **14**(3): 629-639.
Mosher, R. A., W. E. Durrant, D. Wang, J. Song and X. Dong (2006). "A comprehensive structure-function analysis of Arabidopsis SNI1 defines essential regions and transcriptional repressor activity." Plant Cell **18**(7): 1750-1765.

Mosher, R. A. and C. W. Melnyk (2010). "siRNAs and DNA methylation: seedy epigenetics." Trends in Plant Science **15**(4): 204-210.
Mosher, R. A., C. W. Melnyk, K. A. Kelly, R. M. Dunn, D. J. Studholme and D. C. Baulcombe (2009). "Uniparental expression of PolIV-dependent siRNAs in developing endosperm of Arabidopsis." Nature **460**(7252): 283-286.
Mosher, R. A., F. Schwach, D. Studholme and D. C. Baulcombe (2008). "PolIVb influences RNA-directed DNA methylation independently of its role in siRNA biogenesis." Proc Natl Acad Sci U S A **105**(8): 3145-3150.
Moxon, S., R. Jing, G. Szittya, F. Schwach, R. L. Rusholme Pilcher, V. Moulton and T. Dalmay (2008). "Deep sequencing of tomato short RNAs identifies microRNAs targeting genes involved in fruit ripening." Genome Res **18**(10): 1602-1609.
Mysore, K. S., J. Nam and S. B. Gelvin (2000). "An Arabidopsis histone H2A mutant is deficient in Agrobacterium T-DNA integration." Proceedings of the National Academy of Sciences **97**(2): 948-953.
Nam, J., K. S. Mysore, C. Zheng, M. K. Knue, A. G. Matthysse and S. B. Gelvin (1999). "Identification of T-DNA tagged Arabidopsis mutants that are resistant to transformation by Agrobacterium." Molecular and General Genetics MGG **261**(3): 429-438.
Naumann, K., A. Fischer, I. Hofmann, V. Krauss, S. Phalke, K. Irmler, G. Hause, A. C. Aurich, R. Dorn, T. Jenuwein and G. Reuter (2005). "Pivotal role of AtSUVH2 in heterochromatic histone methylation and gene silencing in Arabidopsis." EMBO J **24**(7): 1418-1429.
Navarro, L., F. Jay, K. Nomura, S. Y. He and O. Voinnet (2008). "Suppression of the microRNA pathway by bacterial effector proteins." Science **321**(5891): 964-967.
Nishizawa-Yokoi, A., S. Nonaka, H. Saika, Y. I. Kwon, K. Osakabe and S. Toki (2012). "Suppression of Ku70/80 or Lig4 leads to decreased stable transformation and enhanced homologous recombination in rice." New Phytol **196**(4): 1048-1059.
Nobuta, K., C. Lu, R. Shrivastava, M. Pillay, E. De Paoli, M. Accerbi, M. Arteaga-Vazquez, L. Sidorenko, D. H. Jeong, Y. Yen, P. J. Green, V. L. Chandler and B. C. Meyers (2008). "Distinct size distribution of endogeneous siRNAs in maize: Evidence from deep sequencing in the mop1-1 mutant." Proc Natl Acad Sci U S A **105**(39): 14958-14963.
Nodine, M. D. and D. P. Bartel (2010). "MicroRNAs prevent precocious gene expression and enable pattern formation during plant embryogenesis." Genes Dev **24**(23): 2678-2692.
Nolin, S. L., W. T. Brown, A. Glicksman, G. E. Houck, Jr., A. D. Gargano, A. Sullivan, V. Biancalana, K. Brondum-Nielsen, H. Hjalgrim, E. Holinski-Feder, F. Kooy, J. Longshore, J. Macpherson, J. L. Mandel, G. Matthijs, F. Rousseau, P. Steinbach, M. L. Vaisanen, H. von Koskull and S. L. Sherman (2003). "Expansion of the fragile X CGG repeat in females with premutation or intermediate alleles." Am J Hum Genet **72**(2): 454-464.
Numa, H., J. M. Kim, A. Matsui, Y. Kurihara, T. Morosawa, J. Ishida, Y. Mochizuki, H. Kimura, K. Shinozaki, T. Toyoda, M. Seki, M. Yoshikawa and Y. Habu (2010). "Transduction of RNA-directed DNA methylation signals to repressive histone marks in Arabidopsis thaliana." EMBO J **29**(2): 352-362.

Nylander, M., J. Svensson, E. T. Palva and B. V. Welin (2001). "Stress-induced accumulation and tissue-specific localization of dehydrins in Arabidopsis thaliana." Plant Mol Biol **45**(3): 263-279.
Oliveros, J. "VENNY. An interactive tool for comparing lists with Venn diagrams."
Olmedo-Monfil, V., N. Duran-Figueroa, M. Arteaga-Vazquez, E. Demesa-Arevalo, D. Autran, D. Grimanelli, R. K. Slotkin, R. A. Martienssen and J. P. Vielle-Calzada (2010). "Control of female gamete formation by a small RNA pathway in Arabidopsis." Nature **464**(7288): 628-632.
Onodera, Y., J. R. Haag, T. Ream, P. Costa Nunes, O. Pontes and C. S. Pikaard (2005). "Plant nuclear RNA polymerase IV mediates siRNA and DNA methylation-dependent heterochromatin formation." Cell **120**(5): 613-622.
Ou, X., Y. Zhang, C. Xu, X. Lin, Q. Zang, T. Zhuang, L. Jiang, D. von Wettstein and B. Liu (2012). "Transgenerational Inheritance of Modified DNA Methylation Patterns and Enhanced Tolerance Induced by Heavy Metal Stress in Rice (Oryza sativa L.)." PLoS One **7**(9): e41143.
Păcurar, D. I., H. Thordal-Christensen, M. L. Păcurar, D. Pamfil, C. Botez and C. Bellini (2011). "Agrobacterium tumefaciens: From crown gall tumors to genetic transformation." Physiological and Molecular Plant Pathology **76**(2): 76-81.
Palma, K., S. Thorgrimsen, F. G. Malinovsky, B. K. Fiil, H. B. Nielsen, P. Brodersen, D. Hofius, M. Petersen and J. Mundy (2010). "Autoimmunity in Arabidopsis acd11 is mediated by epigenetic regulation of an immune receptor." PLoS Pathog **6**(10): e1001137.
Palmgren, G., O. Mattson and F. T. Okkels (1993). "Treatment of Agrobacterium or leaf disks with 5-azacytidine increases transgene expression in tobacco." Plant Mol Biol **21**(3): 429-435.
Panella, M., R. Aina, M. Renna, A. Santagostino and L. Palin (2004). "A study of air pollutants and acute asthma exacerbations in urban areas: status report." Environ Pollut **128**(3): 303; author reply 305.
Pant, B. D., A. Buhtz, J. Kehr and W. R. Scheible (2008). "MicroRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis." Plant Journal **53**(5): 731-738.
Paprotka, T., K. Deuschle, V. Metzler and H. Jeske (2011). "Conformation-selective methylation of geminivirus DNA." J Virol **85**(22): 12001-12012.
Paszkowski, J. and U. Grossniklaus (2011). "Selected aspects of transgenerational epigenetic inheritance and resetting in plants." Curr Opin Plant Biol **14**(2): 195-203.
Pavet, V., C. Quintero, N. M. Cecchini, A. L. Rosa and M. E. Alvarez (2006). "Arabidopsis displays centromeric DNA hypomethylation and cytological alterations of heterochromatin upon attack by pseudomonas syringae." Mol Plant Microbe Interact **19**(6): 577-587.
Pecinka, A., H. Q. Dinh, T. Baubec, M. Rosa, N. Lettner and O. Mittelsten Scheid (2010). "Epigenetic regulation of repetitive elements is attenuated by prolonged heat stress in Arabidopsis." Plant Cell **22**(9): 3118-3129.
Pecinka, A. and O. Mittelsten Scheid (2012). "Stress-induced chromatin changes: a critical view on their heritability." Plant Cell Physiol **53**(5): 801-808.

Pecinka, A., M. Rosa, A. Schikora, M. Berlinger, H. Hirt, C. Luschnig and O. Mittelsten Scheid (2009). "Transgenerational stress memory is not a general response in Arabidopsis." PLoS One **4**(4): e5202.
Pecinka, A., M. Rosa, A. Schikora, M. Berlinger, H. Hirt, C. Luschnig and O. M. Scheid (2009). "Transgenerational Stress Memory Is Not a General Response in Arabidopsis." PLoS One **4**(4): e5202.
Pembrey, M. E., L. O. Bygren, G. Kaati, S. Edvinsson, K. Northstone, M. Sjostrom and J. Golding (2006). "Sex-specific, male-line transgenerational responses in humans." Eur J Hum Genet **14**(2): 159-166.
Pereira, V. (2004). "Insertion bias and purifying selection of retrotransposons in the Arabidopsis thaliana genome." Genome Biol **5**(10): R79.
Pflieger, S., S. Blanchet, L. Camborde, G. Drugeon, A. Rousseau, M. Noizet, S. Planchais and I. Jupin (2008). "Efficient virus-induced gene silencing in Arabidopsis using a 'one-step' TYMV-derived vector." Plant Journal **56**(4): 678-690.
Pina, C., F. Pinto, J. A. Feijo and J. D. Becker (2005). "Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation." Plant Physiol **138**(2): 744-756.
Pogribny, I., P. Yi and S. J. James (1999). "A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands." Biochem Biophys Res Commun **262**(3): 624-628.
Ponferrada-Marin, M. I., M. I. Martinez-Macias, T. Morales-Ruiz, T. Roldan-Arjona and R. R. Ariza (2010). "Methylation-independent DNA binding modulates specificity of Repressor of Silencing 1 (ROS1) and facilitates demethylation in long substrates." J Biol Chem **285**(30): 23032-23039.
Prandl, R., E. Kloske and F. Schoffl (1995). "Developmental regulation and tissue-specific differences of heat shock gene expression in transgenic tobacco and Arabidopsis plants." Plant Mol Biol **28**(1): 73-82.
Puchta, H., B. Dujon and B. Hohn (1996). "Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination." Proceedings of the National Academy of Sciences **93**(10): 5055-5060.
Purkayastha, A. and I. Dasgupta (2009). "Virus-induced gene silencing: a versatile tool for discovery of gene functions in plants." Plant Physiol Biochem **47**(11-12): 967-976.
Qi, J., S. Yu, F. Zhang, X. Shen, X. Zhao, Y. Yu and D. Zhang (2010). "Reference Gene Selection for Real-Time Quantitative Polymerase Chain Reaction of mRNA Transcript Levels in Chinese Cabbage (Brassica rapa L. ssp. pekinensis)." Plant Molecular Biology Reporter **28**(4): 597-604.
Rahavi, M. R., Z. Migicovsky, V. Titov and I. Kovalchuk (2011). "Transgenerational adaptation to heavy metal salts in Arabidopsis." Front Plant Sci **2**: 91.
Raja, P., B. C. Sanville, R. C. Buchmann and D. M. Bisaro (2008). "Viral Genome Methylation as an Epigenetic Defense against Geminiviruses." J Virol **82**(18): 8997-9007.
Rajagopalan, R., H. Vaucheret, J. Trejo and D. P. Bartel (2006). "A diverse and evolutionarily fluid set of microRNAs in Arabidopsis thaliana." Genes Dev **20**(24): 3407-3425.
Ramachandran, V. and X. Chen (2008). "Small RNA metabolism in Arabidopsis." Trends in Plant Science **13**(7): 368-374.

Rasmann, S., M. De Vos, C. L. Casteel, D. Tian, R. Halitschke, J. Y. Sun, A. A. Agrawal, G. W. Felton and G. Jander (2012). "Herbivory in the previous generation primes plants for enhanced insect resistance." Plant Physiol **158**(2): 854-863.
Ray, A. and M. Langer (2002). "Homologous recombination: ends as the means." Trends in Plant Science **7**(10): 435-440.
Redon, C., D. Pilch, E. Rogakou, O. Sedelnikova, K. Newrock and W. Bonner (2002). "Histone H2A variants H2AX and H2AZ." Curr Opin Genet Dev **12**(2): 162-169.
Rivera, A. L., M. Gomez-Lim, F. Fernandez and A. M. Loske (2012). "Physical methods for genetic plant transformation." Phys Life Rev **9**(3): 308-345.
Rodriguez-Negrete, E. A., J. Carrillo-Tripp and R. F. Rivera-Bustamante (2009). "RNA silencing against geminivirus: complementary action of posttranscriptional gene silencing and transcriptional gene silencing in host recovery." J Virol **83**(3): 1332-1340.
Roudier, F., I. Ahmed, C. Berard, A. Sarazin, T. Mary-Huard, S. Cortijo, D. Bouyer, E. Caillieux, E. Duvernois-Berthet, L. Al-Shikhley, L. Giraut, B. Despres, S. Drevensek, F. Barneche, S. Derozier, V. Brunaud, S. Aubourg, A. Schnittger, C. Bowler, M. L. Martin-Magniette, S. Robin, M. Caboche and V. Colot (2011). "Integrative epigenomic mapping defines four main chromatin states in Arabidopsis." EMBO J **30**(10): 1928-1938.
Rouget, C., C. Papin, A. Boureux, A. C. Meunier, B. Franco, N. Robine, E. C. Lai, A. Pelisson and M. Simonelig (2010). "Maternal mRNA deadenylation and decay by the piRNA pathway in the early Drosophila embryo." Nature **467**(7319): 1128-1132.
Ruiz-Ferrer, V. and O. Voinnet (2009). "Roles of plant small RNAs in biotic stress responses." Annu Rev Plant Biol **60**: 485-510.
Saez, A., A. Rodrigues, J. Santiago, S. Rubio and P. L. Rodriguez (2008). "HAB1–SWI3B Interaction Reveals a Link between Abscisic Acid Signaling and Putative SWI/SNF Chromatin-Remodeling Complexes in Arabidopsis." The Plant Cell Online **20**(11): 2972-2988.
Saleh, A., R. Alvarez-Venegas and Z. Avramova (2008). "An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in Arabidopsis plants." Nat Protoc **3**(6): 1018-1025.
Salomon, S. and H. Puchta (1998). "Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells." EMBO J **17**(20): 6086-6095.
Sambrook, J. and D. W. Russell (2006). The condensed protocols from Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.
Saze, H. (2008). "Epigenetic memory transmission through mitosis and meiosis in plants." Semin Cell Dev Biol **19**(6): 527-536.
Saze, H., K. Tsugane, T. Kanno and T. Nishimura (2012). "DNA methylation in plants: relationship to small RNAs and histone modifications, and functions in transposon inactivation." Plant Cell Physiol **53**(5): 766-784.
Schilling, E. and M. Rehli (2007). "Global, comparative analysis of tissue-specific promoter CpG methylation." Genomics **90**(3): 314-323.
Schmidt, A., S. E. Wuest, K. Vijverberg, C. Baroux, D. Kleen and U. Grossniklaus (2011). "Transcriptome analysis of the Arabidopsis megaspore mother cell uncovers the importance of RNA helicases for plant germline development." PLoS Biol **9**(9): e1001155.

Scholthof, H. B., V. Y. Alvarado, J. C. Vega-Arreguin, J. Ciomperlik, D. Odokonyero, C. Brosseau, M. Jaubert, A. Zamora and P. Moffett (2011). "Identification of an ARGONAUTE for antiviral RNA silencing in Nicotiana benthamiana." Plant Physiol **156**(3): 1548-1555.
Schonrock, N., V. Exner, A. Probst, W. Gruissem and L. Hennig (2006). "Functional genomic analysis of CAF-1 mutants in Arabidopsis thaliana." J Biol Chem **281**(14): 9560-9568.
Schwab, R., A. Maizel, V. Ruiz-Ferrer, D. Garcia, M. Bayer, M. Crespi, O. Voinnet and R. A. Martienssen (2009). "Endogenous TasiRNAs mediate non-cell autonomous effects on gene regulation in Arabidopsis thaliana." PLoS One **4**(6): e5980.
Seki, M., M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carninci, Y. Hayashizaki and K. Shinozaki (2001). "Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray." Plant Cell **13**(1): 61-72.
Seong, K. H., D. Li, H. Shimizu, R. Nakamura and S. Ishii (2011). "Inheritance of stress-induced, ATF-2-dependent epigenetic change." Cell **145**(7): 1049-1061.
Sha, A. H., X. H. Lin, J. B. Huang and D. P. Zhang (2005). "Analysis of DNA methylation related to rice adult plant resistance to bacterial blight based on methylation-sensitive AFLP (MSAP) analysis." Mol Genet Genomics **273**(6): 484-490.
Shah, J. M. and K. Veluthambi (2010). "DIANTHIN, a negative selection marker in tobacco, is non-toxic in transgenic rice and confers sheath blight resistance." Biologia Plantarum **54**(3): 443-450.
Shaked, H., C. Melamed-Bessudo and A. A. Levy (2005). "High-frequency gene targeting in Arabidopsis plants expressing the yeast RAD54 gene." Proc Natl Acad Sci U S A **102**(34): 12265-12269.
Silhavy, D., A. Molnar, A. Lucioli, G. Szittya, C. Hornyik, M. Tavazza and J. Burgyan (2002). "A viral protein suppresses RNA silencing and binds silencing-generated, 21- to 25-nucleotide double-stranded RNAs." EMBO J **21**(12): 3070-3080.
Singer, K., Y. M. Shiboleth, J. Li and T. Tzfira (2012). "Formation of complex extrachromosomal T-DNA structures in Agrobacterium tumefaciens-infected plants." Plant Physiol **160**(1): 511-522.
Slaughter, A., X. Daniel, V. Flors, E. Luna, B. Hohn and B. Mauch-Mani (2012). "Descendants of primed Arabidopsis plants exhibit resistance to biotic stress." Plant Physiol **158**(2): 835-843.
Slotkin, R. K. and R. Martienssen (2007). "Transposable elements and the epigenetic regulation of the genome." Nat Rev Genet **8**(4): 272-285.
Slotkin, R. K., M. Vaughn, F. Borges, M. Tanurdzic, J. D. Becker, J. A. Feijo and R. A. Martienssen (2009). "Epigenetic reprogramming and small RNA silencing of transposable elements in pollen." Cell **136**(3): 461-472.
Smith, A. P., A. Jain, R. B. Deal, V. K. Nagarajan, M. D. Poling, K. G. Raghothama and R. B. Meagher (2010). "Histone H2A.Z Regulates the Expression of Several Classes of Phosphate Starvation Response Genes But Not as a Transcriptional Activator." Plant Physiol **152**(1): 217-225.
Smith, M. R., M. R. Willmann, G. Wu, T. Z. Berardini, B. Moller, D. Weijers and R. S. Poethig (2009). "Cyclophilin 40 is required for microRNA activity in Arabidopsis." Proc Natl Acad Sci U S A **106**(13): 5424-5429.

Smyth, D. R., J. L. Bowman and E. M. Meyerowitz (1990). "Early flower development in Arabidopsis." Plant Cell **2**(8): 755-767.
Sokol, A., A. Kwiatkowska, A. Jerzmanowski and M. Prymakowska-Bosak (2007). "Up-regulation of stress-inducible genes in tobacco and Arabidopsis cells in response to abiotic stresses and ABA treatment correlates with dynamic changes in histone H3 and H4 modifications." Planta **227**(1): 245-254.
Song, C., C. Wang, C. Zhang, N. K. Korir, H. Yu, Z. Ma and J. Fang (2010). "Deep sequencing discovery of novel and conserved microRNAs in trifoliate orange (Citrus trifoliata)." BMC Genomics **11**: 431.
Sood, P., A. Bhattacharya and A. Sood (2011). "Problems and possibilities of monocot transformation." Biologia Plantarum **55**(1): 1-15.
Sridha, S. and K. Wu (2006). "Identification of AtHD2C as a novel regulator of abscisic acid responses in Arabidopsis." The Plant Journal **46**(1): 124-133.
Sridhar, V. V., A. Kapoor, K. Zhang, J. Zhu, T. Zhou, P. M. Hasegawa, R. A. Bressan and J. K. Zhu (2007). "Control of DNA methylation and heterochromatic silencing by histone H2B deubiquitination." Nature **447**(7145): 735-738.
Steward, N., M. Ito, Y. Yamaguchi, N. Koizumi and H. Sano (2002). "Periodic DNA methylation in maize nucleosomes and demethylation by environmental stress." J Biol Chem **277**(40): 37741-37746.
Stocks, M. B., S. Moxon, D. Mapleson, H. C. Woolfenden, I. Mohorianu, L. Folkes, F. Schwach, T. Dalmay and V. Moulton (2012). "The UEA sRNA workbench: a suite of tools for analysing and visualizing next generation sequencing microRNA and small RNA datasets." Bioinformatics **28**(15): 2059-2061.
Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-45.
Sun, K., K. Hunt and B. A. Hauser (2004). "Ovule abortion in Arabidopsis triggered by stress." Plant Physiol **135**(4): 2358-2367.
Sunkar, R., V. Chinnusamy, J. Zhu and J.-K. Zhu (2007). "Small RNAs as big players in plant abiotic stress responses and nutrient deprivation." Trends in Plant Science **12**(7): 301-309.
Sunkar, R., A. Kapoor and J. K. Zhu (2006). "Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in Arabidopsis is mediated by downregulation of miR398 and important for oxidative stress tolerance." Plant Cell **18**(8): 2051-2065.
Sunkar, R., Y. F. Li and G. Jagadeeswaran (2012). "Functions of microRNAs in plant stress responses." Trends in Plant Science **17**(4): 196-203.
Sunkar, R. and J. K. Zhu (2004). "Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis." Plant Cell **16**(8): 2001-2019.
Swoboda, P., S. Gal, B. Hohn and H. Puchta (1994). "Intrachromosomal homologous recombination in whole plants." EMBO J **13**(2): 484-489.
Szittya, G., S. Moxon, D. M. Santos, R. Jing, M. P. Fevereiro, V. Moulton and T. Dalmay (2008). "High-throughput sequencing of Medicago truncatula short RNAs identifies eight new miRNA families." BMC Genomics **9**: 593.
Tam, P. P., I. H. Barrette-Ng, D. M. Simon, M. W. Tam, A. L. Ang and D. G. Muench (2010). "The Puf family of RNA-binding proteins in plants: phylogeny, structural modeling, activity and subcellular localization." BMC Plant Biol **10**: 44.

Tamaru, H. and E. U. Selker (2001). "A histone H3 methyltransferase controls DNA methylation in Neurospora crassa." Nature **414**(6861): 277-283.
Tan, T. L., R. Kanaar and C. Wyman (2003). "Rad54, a Jack of all trades in homologous recombination." DNA Repair (Amst) **2**(7): 787-794.
Tang, Z., L. Zhang, C. Xu, S. Yuan, F. Zhang, Y. Zheng and C. Zhao (2012). "Uncovering small RNA-mediated responses to cold stress in a wheat thermosensitive genic male-sterile line by deep sequencing." Plant Physiol **159**(2): 721-738.
Tariq, M. and J. Paszkowski (2004). "DNA and histone methylation in plants." Trends Genet **20**(6): 244-251.
Teixeira, F. K., F. Heredia, A. Sarazin, F. Roudier, M. Boccara, C. Ciaudo, C. Cruaud, J. Poulain, M. Berdasco, M. F. Fraga, O. Voinnet, P. Wincker, M. Esteller and V. Colot (2009). "A role for RNAi in the selective correction of DNA methylation defects." Science **323**(5921): 1600-1604.
Tel-Zur, N. and I. L. Goldman (2007). "Analysis of sub-populations of rapid-cycling Brassica rapa following recurrent bi-directional selection for cotyledon size." Plant Breeding **126**(1): 62-66.
Tenea, G. N., J. Spantzel, L.-Y. Lee, Y. Zhu, K. Lin, S. J. Johnson and S. B. Gelvin (2009). "Overexpression of Several Arabidopsis Histone Genes Increases Agrobacterium-Mediated Transformation and Transgene Expression in Plants." The Plant Cell Online **21**(10): 3350-3367.
Thiriet, C. and J. J. Hayes (2005). "Chromatin in need of a fix: phosphorylation of H2AX connects chromatin to DNA repair." Mol Cell **18**(6): 617-622.
Tillett, R. L., A. Ergul, R. L. Albion, K. A. Schlauch, G. R. Cramer and J. C. Cushman (2011). "Identification of tissue-specific, abiotic stress-responsive gene expression patterns in wine grape (Vitis vinifera L.) based on curation and mining of large-scale EST data sets." BMC Plant Biol **11**: 86.
Tingay, S., D. McElroy, R. Kalla, S. Fieg, M. Wang, S. Thornton and R. Brettell (1997). "Agrobacterium tumefaciens-mediated barley transformation." The Plant Journal **11**(6): 1369-1376.
Tittel-Elmer, M., E. Bucher, L. Broger, O. Mathieu, J. Paszkowski and I. Vaillant (2010). "Stress-induced activation of heterochromatic transcription." PLoS Genet **6**(10): e1001175.
Tiwari, S. B., G. Hagen and T. Guilfoyle (2003). "The roles of auxin response factor domains in auxin-responsive transcription." Plant Cell **15**(2): 533-543.
Toedling, J., O. Skylar, T. Krueger, J. J. Fischer, S. Sperling and W. Huber (2007). "Ringo--an R/Bioconductor package for analyzing ChIP-chip readouts." BMC Bioinformatics **8**: 221.
Trapnell, C., L. Pachter and S. L. Salzberg (2009). "TopHat: discovering splice junctions with RNA-Seq." Bioinformatics **25**(9): 1105-1111.
Trapnell, C., A. Roberts, L. Goff, G. Pertea, D. Kim, D. R. Kelley, H. Pimentel, S. L. Salzberg, J. L. Rinn and L. Pachter (2012). "Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks." Nat Protoc **7**(3): 562-578.
Tricker, P. J., J. G. Gibbings, C. M. Rodriguez Lopez, P. Hadley and M. J. Wilkinson (2012). "Low relative humidity triggers RNA-directed de novo DNA methylation and suppression of genes controlling stomatal development." J Exp Bot **63**(10): 3799-3813.

Tsaftaris, A. S., A. N. Polidoros, A. Kapazoglou, E. Tani and N. M. Kovačević (2008). Epigenetics and Plant Breeding. Plant Breeding Reviews, John Wiley & Sons, Inc.**:** 49-177.
Turunen, M. and K. Latola (2005). "UV-B radiation and acclimation in timberline plants." Environ Pollut **137**(3): 390-403.
Tzfira, T. and V. Citovsky (2006). "Agrobacterium-mediated genetic transformation of plants: biology and biotechnology." Curr Opin Biotechnol **17**(2): 147-154.
Tzfira, T., L. R. Frankman, M. Vaidya and V. Citovsky (2003). "Site-Specific Integration of Agrobacterium tumefaciens T-DNA via Double-Stranded Intermediates." Plant Physiol **133**(3): 1011-1023.
Urano, K., Y. Kurihara, M. Seki and K. Shinozaki (2010). "'Omics' analyses of regulatory networks in plant abiotic stress responses." Curr Opin Plant Biol **13**(2): 132-138.
Vaghchhipawala, Z. E., B. Vasudevan, S. Lee, M. R. Morsy and K. S. Mysore (2012). "Agrobacterium May Delay Plant Nonhomologous End-Joining DNA Repair via XRCC4 to Favor T-DNA Integration." Plant Cell **24**(10): 4110-4123.
Vaillant, I. and J. Paszkowski (2007). "Role of histone and DNA methylation in gene regulation." Curr Opin Plant Biol **10**(5): 528-533.
Vainstein, A., I. Marton, A. Zuker, M. Danziger and T. Tzfira (2011). "Permanent genome modifications in plant cells by transient viral vectors." Trends Biotechnol **29**(8): 363-369.
van Attikum, H., P. Bundock and P. J. Hooykaas (2001). "Non-homologous end-joining proteins are required for Agrobacterium T-DNA integration." EMBO J **20**(22): 6550-6558.
van Attikum, H., P. Bundock, R. M. Overmeer, L. Y. Lee, S. B. Gelvin and P. J. J. Hooykaas (2003). "The Arabidopsis AtLIG4 gene is required for the repair of DNA damage, but not for the integration of Agrobacterium T-DNA." Nucleic Acids Research **31**(14): 4247-4255.
van Attikum, H. and P. J. Hooykaas (2003). "Genetic requirements for the targeted integration of Agrobacterium T-DNA in Saccharomyces cerevisiae." Nucleic Acids Res **31**(3): 826-832.
van den Burg, H. A. and F. L. W. Takken (2009). "Does chromatin remodeling mark systemic acquired resistance?" Trends in Plant Science **14**(5): 286-294.
Van Loon, L. C. (1997). "Induced resistance in plants and the role of pathogenesis-related proteins." European Journal of Plant Pathology **103**(9): 753-765.
Varallyay, E., A. Valoczi, A. Agyi, J. Burgyan and Z. Havelda (2010). "Plant virus-mediated induction of miR168 is associated with repression of ARGONAUTE1 accumulation." EMBO J **29**(20): 3507-3519.
Vaucheret, H. (2008). "Plant ARGONAUTES." Trends in Plant Science **13**(7): 350-358.
Vaucheret, H., A. C. Mallory and D. P. Bartel (2006). "AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1." Mol Cell **22**(1): 129-136.
Vaucheret, H., F. Vazquez, P. Crete and D. P. Bartel (2004). "The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development." Genes Dev **18**(10): 1187-1197.
Vaughn, M. W., M. Tanurdzic, Z. Lippman, H. Jiang, R. Carrasquillo, P. D. Rabinowicz, N. Dedhia, W. R. McCombie, N. Agier, A. Bulski, V. Colot, R. W. Doerge and R. A.

Martienssen (2007). "Epigenetic natural variation in Arabidopsis thaliana." PLoS Biol **5**(7): e174.
Vazquez, F. (2006). "Arabidopsis endogenous small RNAs: highways and byways." Trends in Plant Science **11**(9): 460-468.
Vazquez, F., H. Vaucheret, R. Rajagopalan, C. Lepers, V. Gasciolli, A. C. Mallory, J. L. Hilbert, D. P. Bartel and P. Crete (2004). "Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs." Mol Cell **16**(1): 69-79.
Verhoeven, K. J., J. J. Jansen, P. J. van Dijk and A. Biere (2010). "Stress-induced DNA methylation changes and their heritability in asexual dandelions." New Phytol **185**(4): 1108-1118.
Voinnet, O. (2001). "RNA silencing as a plant immune system against viruses." Trends in Genetics **17**(8): 449-459.
Voinnet, O., S. Rivas, P. Mestre and D. Baulcombe (2003). "An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus." Plant Journal **33**(5): 949-956.
Wada, Y., H. Ohya, Y. Yamaguchi, N. Koizumi and H. Sano (2003). "Preferential de novo methylation of cytosine residues in non-CpG sequences by a domains rearranged DNA methyltransferase from tobacco plants." J Biol Chem **278**(43): 42386-42393.
Walley, J. W. and K. Dehesh (2010). "Molecular mechanisms regulating rapid stress signaling networks in Arabidopsis." J Integr Plant Biol **52**(4): 354-359.
Walley, J. W., H. C. Rowe, Y. Xiao, E. W. Chehab, D. J. Kliebenstein, D. Wagner and K. Dehesh (2008). "The chromatin remodeler SPLAYED regulates specific stress signaling pathways." PLoS Pathog **4**(12): e1000237.
Wang, C., X. Cai, X. Wang and Z. Zheng (2006). "Optimisation of tobacco rattle virus-induced gene silencing in Arabidopsis." Functional Plant Biology **33**(4): 347-355.
Wang, D., S. A. MacFarlane and A. J. Maule (1997). "Viral determinants of pea early browning virus seed transmission in pea." Virology **234**(1): 112-117.
Wang, F., L. Li, L. Liu, H. Li, Y. Zhang, Y. Yao, Z. Ni and J. Gao (2012). "High-throughput sequencing discovery of conserved and novel microRNAs in Chinese cabbage (Brassica rapa L. ssp. pekinensis)." Mol Genet Genomics **287**(7): 555-563.
Wang, W. S., Y. J. Pan, X. Q. Zhao, D. Dwivedi, L. H. Zhu, J. Ali, B. Y. Fu and Z. K. Li (2011). "Drought-induced site-specific DNA methylation and its association with drought tolerance in rice (Oryza sativa L.)." J Exp Bot **62**(6): 1951-1960.
Wang, X.-B., J. Jovel, P. Udomporn, Y. Wang, Q. Wu, W.-X. Li, V. Gasciolli, H. Vaucheret and S.-W. Ding (2011). "The 21-Nucleotide, but Not 22-Nucleotide, Viral Secondary Small Interfering RNAs Direct Potent Antiviral Defense by Two Cooperative Argonautes in Arabidopsis thaliana." The Plant Cell Online **23**(4): 1625-1638.
Wang, X., A. A. Elling, X. Li, N. Li, Z. Peng, G. He, H. Sun, Y. Qi, X. S. Liu and X. W. Deng (2009). "Genome-wide and organ-specific landscapes of epigenetic modifications and their relationships to mRNA and small RNA transcriptomes in maize." Plant Cell **21**(4): 1053-1069.
Wang, X., H. Wang, J. Wang, R. Sun, J. Wu, S. Liu, Y. Bai, J. H. Mun, I. Bancroft, F. Cheng, S. Huang, X. Li, W. Hua, M. Freeling, J. C. Pires, A. H. Paterson, B. Chalhoub, B. Wang, A. Hayward, A. G. Sharpe, B. S. Park, B. Weisshaar, B. Liu, B. Li, C. Tong, C. Song, C. Duran, C. Peng, C. Geng, C. Koh, C. Lin, D. Edwards, D. Mu, D. Shen, E. Soumpourou, F. Li, F. Fraser, G. Conant, G. Lassalle, G. J. King, G. Bonnema, H. Tang,

H. Belcram, H. Zhou, H. Hirakawa, H. Abe, H. Guo, H. Jin, I. A. Parkin, J. Batley, J. S. Kim, J. Just, J. Li, J. Xu, J. Deng, J. A. Kim, J. Yu, J. Meng, J. Min, J. Poulain, K. Hatakeyama, K. Wu, L. Wang, L. Fang, M. Trick, M. G. Links, M. Zhao, M. Jin, N. Ramchiary, N. Drou, P. J. Berkman, Q. Cai, Q. Huang, R. Li, S. Tabata, S. Cheng, S. Zhang, S. Sato, S. Sun, S. J. Kwon, S. R. Choi, T. H. Lee, W. Fan, X. Zhao, X. Tan, X. Xu, Y. Wang, Y. Qiu, Y. Yin, Y. Li, Y. Du, Y. Liao, Y. Lim, Y. Narusaka, Z. Wang, Z. Li, Z. Xiong and Z. Zhang (2011). "The genome of the mesopolyploid crop species Brassica rapa." Nat Genet **43**(10): 1035-1039.

Wang, Z., C. Zang, J. A. Rosenfeld, D. E. Schones, A. Barski, S. Cuddapah, K. Cui, T. Y. Roh, W. Peng, M. Q. Zhang and K. Zhao (2008). "Combinatorial patterns of histone acetylations and methylations in the human genome." Nat Genet **40**(7): 897-903.

Wassenegger, M., S. Heimes, L. Riedel and H. L. Sanger (1994). "RNA-directed de novo methylation of genomic sequences in plants." Cell **76**(3): 567-576.

Waters, A. J., I. Makarevitch, S. R. Eichten, R. A. Swanson-Wagner, C. T. Yeh, W. Xu, P. S. Schnable, M. W. Vaughn, M. Gehring and N. M. Springer (2011). "Parent-of-origin effects on gene expression and DNA methylation in the maize endosperm." Plant Cell **23**(12): 4221-4233.

Weake, V. M. and J. L. Workman (2008). "Histone ubiquitination: triggering gene activity." Mol Cell **29**(6): 653-663.

Wei, W., Z. Ba, M. Gao, Y. Wu, Y. Ma, S. Amiard, Charles I. White, Jannie M. Rendtlew Danielsen, Y.-G. Yang and Y. Qi (2012). "A Role for Small RNAs in DNA Double-Strand Break Repair." Cell **149**(1): 101-112.

Weigel, D. and J. Glazebrook (2006). "Glufosinate ammonium selection of transformed Arabidopsis." CSH Protoc **2006**(7).

Weinthal, D., A. Tovkach, V. Zeevi and T. Tzfira (2010). "Genome editing in plant cells by zinc finger nucleases." Trends in Plant Science **15**(6): 308-321.

Wierzbicki, A. T., R. Cocklin, A. Mayampurath, R. Lister, M. J. Rowley, B. D. Gregory, J. R. Ecker, H. Tang and C. S. Pikaard (2012). "Spatial and functional relationships among Pol V-associated loci, Pol IV-dependent siRNAs, and cytosine methylation in the Arabidopsis epigenome." Genes Dev **26**(16): 1825-1836.

Williams, P. H. and C. B. Hill (1986). "Rapid-Cycling Populations of Brassica." Science **232**(4756): 1385-1389.

Willmann, M. R., M. W. Endres, R. T. Cook and B. D. Gregory (2011). "The Functions of RNA-Dependent RNA Polymerases in Arabidopsis." Arabidopsis Book **9**: e0146.

Windels, P., S. Buck and A. Depicker (2008). Agrobacterium Tumefaciens-Mediated Transformation: Patterns of T-Dna Integration Into the Host Genome. Agrobacterium: From Biology to Biotechnology. T. Tzfira and V. Citovsky, Springer New York**:** 441-481.

Wright, D. A., J. A. Townsend, R. J. Winfrey, Jr., P. A. Irwin, J. Rajagopal, P. M. Lonosky, B. D. Hall, M. D. Jondle and D. F. Voytas (2005). "High-frequency homologous recombination in plants mediated by zinc-finger nucleases." Plant Journal **44**(4): 693-705.

Wu, K., L. Zhang, C. Zhou, C.-W. Yu and V. Chaikam (2008). "HDA6 is required for jasmonate response, senescence and flowering in Arabidopsis." J Exp Bot **59**(2): 225-234.

Wu, L., H. Zhou, Q. Zhang, J. Zhang, F. Ni, C. Liu and Y. Qi (2010). "DNA methylation mediated by a microRNA pathway." Mol Cell **38**(3): 465-475.
Wuest, S. E., K. Vijverberg, A. Schmidt, M. Weiss, J. Gheyselinck, M. Lohr, F. Wellmer, J. Rahnenfuhrer, C. von Mering and U. Grossniklaus (2010). "Arabidopsis female gametophyte gene expression map reveals similarities between plant and animal gametes." Curr Biol **20**(6): 506-512.
Xie, Z., K. Khanna and S. Ruan (2010). "Expression of microRNAs and its regulation in plants." Semin Cell Dev Biol **21**(8): 790-797.
Xie, Z. X., L. K. Johansen, A. M. Gustafson, K. D. Kasschau, A. D. Lellis, D. Zilberman, S. E. Jacobsen and J. C. Carrington (2004). "Genetic and functional diversification of small RNA pathways in plants." PLoS Biol **2**(5): 642-652.
Xu, P., Y. Zhang, L. Kang, M. J. Roossinck and K. S. Mysore (2006). "Computational Estimation and Experimental Verification of Off-Target Silencing during Posttranscriptional Gene Silencing in Plants." Plant Physiol **142**(2): 429-440.
Yang, X. and L. Li (2011). "miRDeep-P: a computational tool for analyzing the microRNA transcriptome in plants." Bioinformatics **27**(18): 2614-2615.
Yao, Y. and I. Kovalchuk (2011). "Abiotic stress leads to somatic and heritable changes in homologous recombination frequency, point mutation frequency and microsatellite stability in Arabidopsis plants." Mutat Res **707**(1-2): 61-66.
Ye, G. N., D. Stone, S. Z. Pang, W. Creely, K. Gonzalez and M. Hinchee (1999). "Arabidopsis ovule is the target for Agrobacterium in planta vacuum infiltration transformation." Plant Journal **19**(3): 249-257.
Yoon, E. K., J. H. Yang, J. Lim, S. H. Kim, S. K. Kim and W. S. Lee (2010). "Auxin regulation of the microRNA390-dependent transacting small interfering RNA pathway in Arabidopsis lateral root development." Nucleic Acids Res **38**(4): 1382-1391.
Young, L. W., R. W. Wilen and P. C. Bonham-Smith (2004). "High temperature stress of Brassica napus during flowering reduces micro- and megagametophyte fertility, induces fruit abortion, and disrupts seed production." J Exp Bot **55**(396): 485-495.
Yu, X., H. Wang, Y. Lu, M. de Ruiter, M. Cariaso, M. Prins, A. van Tunen and Y. He (2012). "Identification of conserved and novel microRNAs that are responsive to heat stress in Brassica rapa." J Exp Bot **63**(2): 1025-1038.
Zaratiegui, M., D. V. Irvine and R. A. Martienssen (2007). "Noncoding RNAs and gene silencing." Cell **128**(4): 763-776.
Zemach, A., M. Y. Kim, P. Silva, J. A. Rodrigues, B. Dotson, M. D. Brooks and D. Zilberman (2010). "Local DNA hypomethylation activates genes in rice endosperm." Proc Natl Acad Sci U S A **107**(43): 18729-18734.
Zhang, F., M. L. Maeder, E. Unger-Wallace, J. P. Hoshaw, D. Reyon, M. Christian, X. Li, C. J. Pierick, D. Dobbs, T. Peterson, J. K. Joung and D. F. Voytas (2010). "High frequency targeted mutagenesis in Arabidopsis thaliana using zinc finger nucleases." Proceedings of the National Academy of Sciences **107**(26): 12028-12033.
Zhang, K., V. V. Sridhar, J. Zhu, A. Kapoor and J. K. Zhu (2007). "Distinctive core histone post-translational modification patterns in Arabidopsis thaliana." PLoS One **2**(11): e1210.
Zhang, M., J. N. Kimatu, K. Xu and B. Liu (2010). "DNA cytosine methylation in plant development." J Genet Genomics **37**(1): 1-12.

Zhang, X., O. Clarenz, S. Cokus, Y. V. Bernatavichute, M. Pellegrini, J. Goodrich and S. E. Jacobsen (2007). "Whole-genome analysis of histone H3 lysine 27 trimethylation in Arabidopsis." PLoS Biol **5**(5): e129.
Zhang, X., I. R. Henderson, C. Lu, P. J. Green and S. E. Jacobsen (2007). "Role of RNA polymerase IV in plant small RNA metabolism." Proc Natl Acad Sci U S A **104**(11): 4536-4541.
Zhang, X., J. Singh, D. Li and F. Qu (2012). "Temperature-dependent survival of Turnip crinkle virus-infected arabidopsis plants relies on an RNA silencing-based defense that requires dcl2, AGO2, and HEN1." J Virol **86**(12): 6847-6854.
Zhang, X., J. Xia, Y. E. Lii, B. E. Barrera-Figueroa, X. Zhou, S. Gao, L. Lu, D. Niu, Z. Chen, C. Leung, T. Wong, H. Zhang, J. Guo, Y. Li, R. Liu, W. Liang, J. K. Zhu, W. Zhang and H. Jin (2012). "Genome-wide analysis of plant nat-siRNAs reveals insights into their distribution, biogenesis and function." Genome Biol **13**(3): R20.
Zhang, X., D. Yao, Q. Wang, W. Xu, Q. Wei, C. Wang, C. Liu, C. Zhang, H. Yan, Y. Ling, Z. Su and F. Li (2013). "mRNA-seq Analysis of the Gossypium arboreum transcriptome Reveals Tissue Selective Signaling in Response to Water Stress during Seedling Stage." PLoS One **8**(1): e54762.
Zhang, X., J. Yazaki, A. Sundaresan, S. Cokus, S. W. Chan, H. Chen, I. R. Henderson, P. Shinn, M. Pellegrini, S. E. Jacobsen and J. R. Ecker (2006). "Genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis." Cell **126**(6): 1189-1201.
Zhang, Y., F. Zhang, X. Li, J. A. Baller, Y. Qi, C. G. Starker, A. J. Bogdanove and D. F. Voytas (2012). "TALENs enable efficient plant genome engineering." Plant Physiol.
Zhang, Z., H. Chen, X. Huang, R. Xia, Q. Zhao, J. Lai, K. Teng, Y. Li, L. Liang, Q. Du, X. Zhou, H. Guo and Q. Xie (2011). "BSCTV C2 Attenuates the Degradation of SAMDC1 to Suppress DNA Methylation-Mediated Gene Silencing in Arabidopsis." The Plant Cell Online **23**(1): 273-288.
Zhao, Y. T., M. Wang, S. X. Fu, W. C. Yang, C. K. Qi and X. J. Wang (2012). "Small RNA profiling in two Brassica napus cultivars identifies microRNAs with oil production-and development-correlated expression and new small RNA classes." Plant Physiol **158**(2): 813-823.
Zheng, X., O. Pontes, J. Zhu, D. Miki, F. Zhang, W.-X. Li, K. Iida, A. Kapoor, C. S. Pikaard and J.-K. Zhu (2008). "ROS3 is an RNA-binding protein required for DNA demethylation in Arabidopsis." Nature **455**(7217): 1259-1262.
Zheng, Y., X. W. He, Y. H. Ying, J. F. Lu, S. B. Gelvin and H. X. Shou (2009). "Expression of the Arabidopsis thaliana histone gene AtHTA1 enhances rice transformation efficiency." Mol Plant **2**(4): 832-837.
Zhou, C., L. Zhang, J. Duan, B. Miki and K. Wu (2005). "HISTONE DEACETYLASE19 Is Involved in Jasmonic Acid and Ethylene Signaling of Pathogen Response in Arabidopsis." The Plant Cell Online **17**(4): 1196-1204.
Zhou, J., X. Wang, K. He, J. B. Charron, A. A. Elling and X. W. Deng (2010). "Genome-wide profiling of histone H3 lysine 9 acetylation and dimethylation in Arabidopsis reveals correlation between multiple histone marks and gene expression." Plant Mol Biol **72**(6): 585-595.

Zhou, L., Y. Liu, Z. Liu, D. Kong, M. Duan and L. Luo (2010). "Genome-wide identification and analysis of drought-responsive microRNAs in Oryza sativa." J Exp Bot **61**(15): 4157-4168.
Zhou, Y., Y. Lu and W. Tian (2012). "Epigenetic features are significantly associated with alternative splicing." BMC Genomics **13**(1): 123.
Zhu, J. K. (2008). "Reconstituting plant miRNA biogenesis." Proc Natl Acad Sci U S A **105**(29): 9851-9852.
Zhu, J. K. (2009). "Active DNA demethylation mediated by DNA glycosylases." Annu Rev Genet **43**: 143-166.
Zhu, Y., J. Nam, J. M. Humara, K. S. Mysore, L. Y. Lee, H. Cao, L. Valentine, J. Li, A. D. Kaiser, A. L. Kopecky, H. H. Hwang, S. Bhattacharjee, P. K. Rao, T. Tzfira, J. Rajagopal, H. Yi, Veena, B. S. Yadav, Y. M. Crane, K. Lin, Y. Larcher, M. J. Gelvin, M. Knue, C. Ramos, X. Zhao, S. J. Davis, S. I. Kim, C. T. Ranjith-Kumar, Y. J. Choi, V. K. Hallan, S. Chattopadhyay, X. Sui, A. Ziemienowicz, A. G. Matthysse, V. Citovsky, B. Hohn and S. B. Gelvin (2003). "Identification of Arabidopsis rat mutants." Plant Physiol **132**(2): 494-505.
Zhu, Z., K. W. Hughes and L. Huang (1991). "Effects of 5-Azacytidine on Transformation and Gene Expression in Nicotiana tabacum." In Vitro Cellular & Developmental Biology. Plant **27P**(2): 77-83.
Ziemienowicz, A., Y.-S. Shim, A. Matsuoka, F. Eudes and I. Kovalchuk (2012). "A Novel Method of Transgene Delivery into Triticale Plants Using the Agrobacterium Transferred DNA-Derived Nano-Complex." Plant Physiol **158**(4): 1503-1513.
Zilberman, D., D. Coleman-Derr, T. Ballinger and S. Henikoff (2008). "Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks." Nature **456**(7218): 125-129.
Zilberman, D., M. Gehring, R. K. Tran, T. Ballinger and S. Henikoff (2007). "Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription." Nat Genet **39**(1): 61-69.
Zinn, K. E., M. Tunc-Ozdemir and J. F. Harper (2010). "Temperature stress and plant sexual reproduction: uncovering the weakest links." J Exp Bot **61**(7): 1959-1968.
Zvereva, A. S. and M. M. Pooggin (2012). "Silencing and innate immunity in plant defense against viral and non-viral pathogens." Viruses **4**(11): 2578-2597.