DNA Methylation and Transgenerational Stress Memories in *Arabidopsis thaliana*

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I. Abstract

The long-standing proposition that plants may pass on a memory of stress events to the following generations is again gaining interest now that a plausible mechanism has been identified. Specifically, changes in DNA methylation, a dynamic epigenetic mark which regulates gene expression, can be inherited. Whilst indirect evidence indicates transgenerational stress memories may involve perturbations to the DNA methylome, we are still some way from identifying specific regions of the epigenome which can carry a memory of stress to the following generations. This research therefore sought to establish to what extent stress-induced changes in DNA methylation are inherited and what regions of the genome are epigenetically regulated in the progeny of stressed plants.

A novel stress memory was observed in the progeny of Arabidopsis thaliana plants subjected zinc stress. This stress memory was observed to be stable over an untreated generation and appears to be specific to zinc. RNA-Seq analysis suggests that the progeny of zinc stressed plants display an altered transcriptome relative to control progeny in the absence of stress. Genes involved in iron uptake in the roots, which are upregulated in zinc stress, show a reduced expression in the progeny of zinc stressed plants. Biochemical analysis identified alterations in iron uptake in the zinc stress progeny. The activity of the jasmonate signalling pathways also appears to be altered in the zinc stress progeny.

Wide-scale changes in DNA methylation were not observed during zinc stress or in the progeny of zinc stressed plants. Indeed, the DNA methylation profile of Arabidopsis thaliana was observed to be stable in response to a variety of stress conditions. Additionally, none of the other stressors tested resulted in a stress memory in the progeny. The results presented here suggest that transgenerational stress responses, such as the zinc stress memory identified, are rare in A.thaliana.
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VI. Author’s Declaration

The experiments and their analyses detailed herein were performed by the author under the supervision of Dr Louise Jones, except where specified in Chapter 2. Specifically, the RNA-Seq was performed by The University of Exeter Sequencing Service and fragment analysis and DNA sequencing analyses were performed by the University of York Technology Facility. No material contained herein has been previously published.
Chapter 1. General Introduction
As sessile organisms, plants react to changes in environmental conditions through a complex network of responses to adjust their development and cellular biochemistry (Raghavendra et al., 2010; Taj et al., 2010; Turner et al., 2002). In extreme environmental conditions, plant survival is dependent upon an appropriate stress response. For example, in response to osmotic stress, the initial stress response prevents or reduces damage, before homeostasis can be re-established in the stressful conditions (Jian-Kang Zhu, 2002). Following the adjustments in cellular biochemistry and systemic responses through hormonal signalling, plant growth is then resumed at a slower rate. Additionally, exposure to stressful environmental conditions can modify the response to subsequent stressors within the same generation, referred to as “stress imprinting” by Bruce et al (Bruce et al. 2007). Stress exposure can even modify the stress response of subsequent generations. When discussing heritable effects of stress, the terms “stress memory” and “transgenerational stress memory” are used to describe effects of parental stress that are observed only in the immediate progeny and those observed for multiple generations after the initial stress treatment. It should be noted at the outset that these “memories” are clearly quite separate from animal memories which rely upon a nervous system. Unfortunately, the study of transgenerational stress memories in plants is tainted by associations with Lamarckian theories on evolution and controversial scientists, including Trofim Lysenko, who believed cold-induced flowering (vernalisation) could be inherited and provided evidence for non-Darwinian evolution (Roll-Hansen, 1985). Previous attempts to explain observed intergenerational memories often evoked Lamarckian theories on evolution as no mechanisms were known which could encode such a memory (Gliboff, 2005; Vargas, 2009). We now know that modifications to the epigenome can affect gene expression. These chemical modifications include methylation of the genomic DNA and various chemical modifications to the tails of histones. With the discovery that some environmental conditions can induce heritable changes in DNA methylation, the hypothesis that transgenerational stress memories may be dependent upon changes in DNA methylation is gaining traction. However, much of the evidence is still circumstantial and, to date, there are no examples of transgenerational stress memories which have been shown to be wholly dependent upon a defined change(s) in DNA methylation.

This introduction will discuss in order, observations of stress imprints and transgenerational stress memories, the regulation, inheritance and function of DNA methylation, and the potential role for DNA methylation in transgenerational stress memories.
1.1 Stress imprinting

Stress imprints may be encoded in the short term by changes in the transcriptome or proteome, for which stability is determined by the rate of mRNA/protein turnover, or by chromatin modifications which have the potential to be maintained throughout the plant’s lifespan.

The molecular impacts of stress are usually short lived. For example, expression of the heat shock protein HSP70 is maintained for less than three hours after a heat shock treatment in *Oryza sativa* (Goswami *et al*., 2010). However, pre-treatment with arsenic increases the intensity of HSP70 expression during recovery and the duration of the recovery period, suggesting abiotic stressors interact to influence the duration of short term stress imprints (Goswami *et al*., 2010). Stress imprints may be observed lasting a few days after a stress treatment or hormone application. *A.thaliana* subjected to pre-treatment with *Pseudomonas syringae* (psm.) pv. *maculicola* or a salicylic acid (SA) analogue application display an enhanced transcriptional response to subsequent wounding by water infiltration 72 hours later (Jaskiewicz *et al*., 2011). Likewise, in response to repeated exposure to ABA, *A.thaliana* increases expression of ABA-responsive genes for at least 3 days after the end of exposure (Goh *et al*., 2003).

Longer lasting stress imprints have also been observed weeks or even months after the stress treatment. *Cakile maritima* plants exposed to salinity, drought, or cadmium followed by a 14 day recovery period display an altered response to subsequent NaCl stress, including a reduced synthesis of JA and a lower accumulation of hydrogen peroxide and malondialdehyde, indicative of a lower level of oxidative stress (Ellouzi *et al*., 2013). Stress imprints can even be maintained in the absence of much of the tissue which was exposed to the initial stress treatment. Drought treatment of *Arrhenatherum elatius* enhances tolerance to a subsequent drought treatment 3 months later, during which time the total aboveground biomass has been harvested and the plant regrown (Walter *et al*., 2011). The increased tolerance involves an improved photoprotection with correlated increase in plant biomass, although the mechanism by which the imprinting of the original drought treatment occurs has not been identified.

The classic examples of stress imprinting are systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Van Wees *et al*., 2008) which involve modification of the SA and JA response pathways respectively in response to biotic stress, and the production of volatile organic compounds (VOC) by herbivore challenged plants which signals to distal...
portions of the plant and even to neighbouring plants (Heil & Ton 2008). Alongside stress imprinting by endogenous plant signalling molecules such as SA, JA and VOCs, imprinting can also take place in response to beta-aminobutyric acid (BABA) -a plant xenobiotic compound - in an ABA-dependent manner (Ton & Mauch-Mani 2004). The mechanisms by which biotic stress imprinting enhances biotic tolerance includes the enhanced accumulation of inactive defence compounds and enhanced expression of MAP kinases and transcription factors (Conrath et al., 2006; Pastor et al., 2013). Stress imprints in response to biotic stress are associated with a cost to the plant since an enhanced response to one biotic stressor can reduce fitness in response to another biotic stressor (Conrath et al., 2006). Biotic stress imprinting can also influence abiotic stress tolerance, highlighting the overlap between biotic and abiotic stress response pathways. Pre-treatment with BABA, which induces resistance against pathogens (Jakab et al. 2001), also increases the speed of the stomatal closure response to drought and NaCl stressors with a concurrent increase in both drought and NaCl stress tolerance (Jakab et al. 2007).

### 1.2 Stress memories

So far we have considered responses to abiotic and biotic stressors which maintain an “imprint” of the stress in the treated generation. Heritable effects of stress treatment in plants have also been observed in response to a range of stress treatments, whereby the offspring appear to possess a “memory” of the parental stress.

Leading on from the biotic stress imprints, stress memories in response to biotic stress appear to be a common response across the plant kingdom (Rasmann et al., 2012b). *A.thaliana* and *Solanum lycopersicum* progeny originating from parents challenged with caterpillar herbivory or application of methyl jasmonate display a lower caterpillar growth when challenged themselves with *Pieris rapae* or *Helicoverpa zea* (Rasmann et al., 2012a). The *A.thaliana* progeny exhibit higher expression of jasmonate biosynthesis genes *LOX2* and *AOS* in response to *P.rapae* (Rasmann et al., 2012a), suggesting the increased tolerance in the following generation is likely to depend on alteration to the jasmonate signalling pathway. Additionally, the progeny of *A.thaliana* plants challenged with *Pst.* exhibit lower expression of the JA-responsive defence genes *PLANT DEFENSIN1.2 (PDF1.2)* and *VEGETATIVE STORAGE PROTEIN2 (VSP2)* in response to JA (Estrella Luna et al., 2012). This and other experiments suggest alteration of the JA-dependent biotic response is a common mechanism in biotic stress memories (Gális et al., 2009).
An increased stress tolerance is also observed in the progeny of cold and heat treated *A. thaliana*. The progeny of cold treated plants recover photosynthetic yield faster following freezing stress than control progeny, with associated changes in transcription factor expression levels (Blödner et al., 2007). However, the increased tolerance to freezing stress is accompanied by decreased fitness in favourable conditions, suggesting the tolerance comes with an attached cost to the plant in the absence of stress. Stress memories in *A. thaliana* in response to heat stress may be partially accession-dependent. Following 3 generations of heat stress, Sha-0 but not Col-0 plants display accelerated flowering in control conditions compared to the progeny of untreated plants (Suter and Widmer, 2013). Interestingly, the progeny of Sha-0 x Col-0 also display an increased salt stress tolerance when either parental plant has experienced 3 generations of salt stress, although the increased tolerance is not observed in either self-fertilised Sha-0 or Col-0, or in the reciprocal Col-0 x Sha-0 cross (Suter and Widmer, 2013). There are indications that hybrid vigour may involve 24nt siRNAs (Groszmann et al., 2011), which leads Suter & Widmer to suggest that the stress memory they observe depends on an interaction between the initial stress treatments and heterosis. Why this should be observed in the Sha-0 x Col-0 cross but not the reciprocal cross is unclear, however an imbalance has been observed between maternal and paternal 24nt siRNA in the seed (Mosher et al., 2009) which may explain why the stress memory is not observed in both crosses.

Evidence for a stress memory mechanism in response to salinity comes from the observation that the offspring of *Taraxacum officinale* (dandelion) plants exposed to NaCl stress show altered stress responses. Specifically, the progeny of NaCl stressed plants display a greater leaf length than control progeny under NaCl stress conditions (Verhoeven and Van Gurp, 2012). Additionally, Boyko et al observed an increase in NaCl stress tolerance in the immediate progeny of NaCl-treated *A. thaliana* (Boyko et al., 2010), suggesting stress memories in response to salinity may be a common response in plants. Stress tolerance was assessed by germination frequency and root growth, both of which were greater in the offspring of NaCl-treated plants (Boyko et al., 2010). However, this apparent memory of stress was unstable, being lost following a single untreated generation. It is unclear whether an increased germination frequency and root growth under high salinity can be considered beneficial to the progeny as germination under high salinity is usually inhibited to protect the seedling from developing in unfavourable conditions (J. Park et al., 2011) and modulation of root growth in high salinity may also be a protective response (F. Sun et al., 2008).
In addition to the above stress memory in response to salinity, *T. officinale* has previously been observed to pass on a memory of nutrient deficient conditions, as identified by a greater shoot:root ratio under nutrient-deficiency stress in the progeny of stressed plants relative to control progeny (Verhoeven and Van Gurp, 2012). However, this observation was not reproducible, suggesting the observation was either false positive, or else the plant responds to nutrient stress in interaction with other environmental conditions which were not sufficiently controlled. More convincing evidence for stress memories in response to nutrient stress comes from experiments with *Plantago lanceolata*. Parental effects on offspring traits are observed in the progeny of *P. lanceolata* plants grown under low nutrient conditions which display a shorter time to flowering under low nutrient conditions with concurrent increases in carbon storage and biomass compared to control progeny (Latzel *et al.*, 2013, 2010).

These observations allude to a possible common stress memory mechanism in plants in response to abiotic and biotic stress conditions which modifies growth traits in the offspring. However, the progeny of *A. thaliana* C24 ecotype plants subjected to NaCl stress produce hypertolerant progeny (Boyko *et al.*, 2010), whereas the progeny of *A. thaliana* Col-0 or Sha-0 do not (Suter and Widmer, 2013). This inconsistency between ecotype could be due to differences in stress treatment. Boyko *et al.* observed a stress memory in the C24 ecotype in response to 75 mM NaCl for 3 weeks from germination (Boyko *et al.*, 2010). In contrast, Suter and Widmer did not observe a stress memory in self fertilised Col-0 or Sha-0 ecotypes subjected to 50 mM NaCl for 4 weeks from germination for 3 successive generations (Suter and Widmer, 2013). Given the considerable variation in NaCl tolerance between *A. thaliana* ecotypes (Katori *et al.*, 2010), it’s difficult to assess whether these treatments would induce a similar level of NaCl stress, and hence whether the inconsistency in eliciting a stress memory response is due to ecotype specific stress memory mechanisms or due to differences in the stress treatment. Currently, the specific conditions required to elicit a stress memory response are far from clear. In the examples discussed, all the stress treatments were initiated in the vegetative growth stage, however, the duration of the treatment lasted from 3 days for the caterpillar herbivory treatment in *A. thaliana* and *S. lycopersicum* (Rasmann *et al.*, 2012a) to the entire lifespan for the nutrient stress treatment in *P. lanceolata* (Latzel *et al.*, 2013, 2010). As stress memories are observed in response to biotic stress and NaCl stress when the treatment is restricted to the vegetative state, it appears that the stress memory mechanism(s) does not depend upon the stress occurring during seed development. Further experiments are required to establish the specific conditions required to elicit a stress memory response. More detailed analyses are also required to establish to what extent the
stress memories are beneficial to the offspring and whether modifications of hormonal response pathways are a fundamental component of stress memories in general.

1.3 Transgenerational stress memories

Where a stress memory is observed to be stable over multiple generations in the absence of stress, the term “transgenerational stress memory” is used (Lang-Mladek et al., 2010; Molinier et al., 2006). While most stress memories do not persist following an untreated generation, there is increasing evidence that plants can transmit effects of stress through multiple generations, although there have been some difficulties reproducing observations between laboratories.

In plants, biotic and abiotic stress conditions have been observed to induce an increase in homologous recombination, which functions to repair double strand breaks (Boyko et al., 2006; Lebel et al., 1993; Lucht et al., 2002). Given that increased homologous recombination frequency (HRF) in response to stress has also been observed in mammals (Bhattacharyya et al., 1989; Boyko et al., 2006), it appears to be a general stress response mechanism. However, the hypothesis that increased HRF response to stress increases the genomic mutation rate as an adaptive response remains controversial (Rosenberg, 2001). Multiple groups have also identified increased HRF in the offspring of stressed plants.

Molinier et al. identified an increased HRF in the progeny of A.thaliana subjected to UV-C and flg22 treatments, which was still observed 4 generations after the UV-C treatment in which it was approximately 4-fold higher than in control progeny (Molinier et al., 2006). Surprisingly, gene expression was apparently unaffected in the UV-C-treated progeny (Molinier et al., 2006), raising the question of how HRF activity is differentially regulated in the progeny and whether the increase in HRF affects the transcriptome? It is still unclear whether increased HRF in response to UV-C is heritable for multiple untreated generations. Two attempts to reproduce this observation confirmed that UV-C increases HRF in the treated generation (Boyko et al., 2010; Pecinka et al., 2009). However, Pecinka et al observed no increase in HRF in the following generation (Pecinka et al., 2009), whilst Boyko et al observed an increase in the immediate progeny but this was much reduced following an untreated generation (Boyko et al., 2010). Boyko et al also reported that NaCl, heat and cold increased HRF in the following generation, whilst drought reduced HRF (Boyko et al., 2010). In contrast, Pecinka et al (2009) did not observe an increase in HRF in the progeny of NaCl, heat or cold treated plants and observed an increase in HRF in response to drought in the treated generation. These results highlight the difficulties in
reproducing observed transgenerational effects of stress between laboratories, even when measuring a restricted outcome such as HRF. A further concern is that the observed changes in HRF have all been observed using transgenic constructs consisting of two incomplete, overlapping regions of a reporter gene. HRF events are then inferred from the restoration of the functional reporter gene. As yet, the transgenerational effects on HRF observed in these transgenic lines has not been validated by an independent method.

Further stress conditions which have been reported to induce transgenerational modifications to HRF include treatments with heavy metals. Treatment with stressful concentrations of cadmium, nickel and copper all induce an increase in HRF in A.thaliana that can be inherited over untreated generations (Rahavi et al., 2011). Interestingly, the increase in HRF is also associated with an increased root growth under heavy metal stress conditions and an increased tolerance to NaCl and genotoxic stress in the immediate progeny. Although these results suggest stress-induced transgenerational changes in HRF may be linked to improved stress tolerance, the treatments described used concentrations of heavy metal ions approximately 1000-fold greater than usually applied to induce heavy metal stress (for comparable stress experiments see (Kim et al., 2006; Mei et al., 2009; Ou et al., 2012)) and would be expected to be lethal almost immediately. Presumably, the concentrations applied were actually considerably less than specified in the publication. Unfortunately, the stability of the increased root growth over an untreated generation was not assessed.

Transgenerational responses to heavy metals have been studied in monocotyledons. Treatment of O.sativa with 100 µM Hg²⁺ significantly increases the tolerance of the second generation progeny to 100-500 µM Hg²⁺ as measured by plant height, although no effect on plant biomass was observed (Ou et al., 2012). These two examples allude to a possible general transgenerational response to heavy metals across the plant kingdom, although further experiments are required to establish if the transgenerational stress memory in O.sativa improves plant fitness in stress and whether the increased HRF in A.thaliana for multiple generations following heavy metal stress is associated with increased stress tolerance.

Inheritance of transgenerational stress memories has been studied with transgene systems in which a reporter gene is repressed (Lang-Mladek et al., 2010; Pecinka et al., 2010; S.-H. Zhong et al., 2013). Transcriptional gene silencing (TGS) of a 35S::GUS A.thaliana line is released after heat (42 °C), freezing (-4°C) or harsh UVB treatment (Lang-Mladek et al., 2010). Small areas of GUS expression are still observed in the leaves of the progeny after two intervening untreated generations, indicating the treatments induce a transgenerational stress memory in the following generations that maintains the reduced TGS at the transgene
upon stress treatment. Interestingly, GUS derepression is lost during seed aging: GUS expression decreases following 3-6 months seed aging, and the derepression is completely reversed after 17 months of seed aging (Lang-Mladek et al., 2010). In a separate study, reduced TGS of a transgene was observed in response to heat stress but the derepressed state was not inherited (Pecinka et al., 2010). A likely explanation is that the derepressed state failed to penetrate into the meristem tissue as heat stress-induced nuclei decondensation was observed in leaf tissue but not meristem tissue, hence the failure to inherit the derepressed state through the germline. Although these results are based upon analysis of transgenic loci, they provide added evidence for an endogenous transgenerational stress memory response to abiotic stress in *A. thaliana* which is dependent upon stress-induced derepression penetrating into the meristem.

Further evidence for transgenerational responses to abiotic stress comes from a separate study of the progeny of heat-treated plants which appear to possess a memory of the environmental conditions of their progenitors. The progeny of *A. thaliana* which have been subjected to two successive generations of elevated temperature growth conditions (30 °C) followed by a generation of control conditions (23 °C) display an increased tolerance to heat compared to control progeny (Whittle et al., 2009). Interestingly, the increased tolerance is limited to seed production and does not extend to plant growth measurements. Thus, whilst the progeny of heat stressed plants displayed an increased fitness in heat stress, it does not appear the plants possessed an overall heat stress hypertolerance. No data were collected to investigate the mechanism underlying the stress memory but the authors reasonably postulate that epigenetic factors must play a role given that the increased fitness was observed after an intervening untreated generation.

Biotic stress conditions can also induce a transgenerational stress memory. The aforementioned stress memory in response to caterpillar herbivory or application of methyl jasmonate in *A. thaliana* and *Solanum lycopersicum* (Section 1.2) is still observed after an intervening untreated generation and the resistance is not specific to the parental herbivory treatment (Rasmann et al., 2012a). *A. thaliana* responds similarly to *Pseudomonas syringae* (*Pst*), with the second generation progeny of challenged plants exhibiting increased accumulation of SA-induced transcripts including *PATHOGENESIS-RELATED GENE 1* (*PRI*) in response to SA or avirulent *Pst* (Luna et al., 2012; Slaughter et al., 2012). This response appears to require BABA signalling as simulation of pathogen stress by BABA treatment also leads to an increase in pathogen tolerance in the second generation progeny, and the BABA-response mutant *ibs1* displays an abated transgenerational response (Slaughter et al., 2012).
In summary, both biotic and abiotic treatments may result in transgenerational responses. Observed molecular transgenerational effects include the release of TGS, increasing HRF and the increased accumulation of SA-induced transcripts upon induction. These molecular changes in the second generation progeny may also be associated with an increased stress tolerance or fitness under stress. We shall now consider the possible mechanism by which such transgenerational stress memories may be produced and inherited.

1.4 Possible mechanisms for transgenerational stress memories

Although there are now numerous examples of transgenerational stress memories in plants, the mechanism for these transgenerational effects of stress are still unclear. Where an increased stress tolerance is only observed in the following generation, it may be dependent upon changes in the seed composition, especially where the increased tolerance is observed early in the lifecycle of the progeny. For example, a shift in parental treatment from 25 °C to 15 °C is sufficient to significantly increase the content of fatty acids in the seed (Blödner et al., 2007). Fatty acids are generated by lipase catalysis of triacylglycerol in the first stage of oil breakdown upon initiation of germination (Theodoulou and Eastmond, 2012) and the fatty acid linoleic acid (18:2) has been shown to be essential for germination under high NaCl (Zhang et al., 2012). Hence, parental growth conditions could influence the germination of progeny under stress conditions through modification of seed content. Where the memory is observed two or more generations after the initial treatment, other mechanisms must exist to encode the transgenerational stress memory.

As transgenerational stress memories are also observed in animals, it is worth considering the mechanisms that have been identified. In mammals, a stress memory can be maintained through the maternal line via modification of the hypothalamo-pituitary-adrenal (HPA) axis. Exposure to stress during particular stages of pregnancy affects the set-point of HPA responses in the offspring, and hence their stress tolerance in a feedback loop that can maintain the altered HPA state for multiple generations (Matthews and Phillips, 2010). Although a similar mechanism obviously cannot be proposed for plants given the lack of a neuroendocrine system, modifications of hormonal responses may play a role in transgenerational stress memories in plants also by altering the response to endogenous hormones in the progeny. As discussed above, there are indications that alterations in JA and SA responses are important in transgenerational responses to biotic stressors in plants (Gális et al., 2009; Slaughter et al., 2012). However, the feedback loop in mammals involves successive modifications of hormonal stress responses in each generation. A similar
feedback loop cannot explain the observed stability of transgenerational stress memories over unstressed generations in plants where the intervening generation is not exposed to the stressor. In Caenorhabditis elegans, transgenerational stress memories can involve RNA interference (RNAi; Figure 1), in which mRNA is targeted for degradation by the RNA-induced silencing complex (RISC) through complimentary siRNAs, leading to post-transcriptional genes silencing (PTGS) (Gu et al., 2009; Lee et al., 2012). As C.elegans possess an RNA-dependent RNA polymerase (RdRP) they can sustain the presence of a stress-induced siRNA by continual amplification of the precursor double stranded RNA in the absence of stress, allowing a memory of stress to be maintained for multiple untreated generations (Alcazar et al., 2008; Buckley et al., 2012; Luteijn and Ketting, 2013; Shirayama et al., 2012). Although plants also possess RdRP enzymes, these have not been implicated in the perpetuation of transgenerational stress memories.

Instead, it is proposed that stress memories in plants are encoded by epigenetic changes (Akimoto et al., 2007; Bird, 2002; Gutzat and Mittelsten Scheid, 2012; Mirouze and Jerzy Paszkowski, 2011; Molinier et al., 2006; Thellier and Lüttge, 2013). The word epigenetics was coined by Conrad Waddington in 1942 to describe the complex developmental process by which gene products are regulated to produce the phenotype (Waddington, 2012). The Greek “epi-“ means “over” or “upon”, thus epigenetics originally referred to the network of developmental processes that exist between the genotype and phenotype. The use of the word epigenetic has changed considerably and it now commonly used to describe mitotically or meiotically heritable changes in chromatin and DNA methylation (Jablonka and Lamb, 2002). In this sense, epigenetics now refers to chemical changes “upon” DNA or associated proteins which are maintained through cell division and have the potential to influence transcriptional activity.

There are at least 8 different distinct types of modifications that can occur on histone tails, including acetylation, methylation and ubiquitination, with each modification capable of regulating transcription (Kouzarides, 2007; Li et al., 2007). In plants, the specific effects of these modifications are becoming clearer. For example, the distribution of histone 3 lysine 9 acetylation (H3K9ac) and H3K4 trimethylation (H3K4me3) are correlated and they are predominately located at the 5' region of genes and associated with high expression levels (Ha et al., 2011; Zhou et al., 2010). Further well characterised histone modifications include H3K9me which is biased towards coding regions of genes and associated with low gene expression (Zhou et al., 2010).
An additional epigenetic modification is the methylation of DNA at cytosine or adenosine residues. The functions of DNA methylation include DNA repair, preventing restriction digest and regulating transcription, depending on species and genomic loci (Berna\textit{tavichute et al.}, 2008; Cokus \textit{et al.}, 2008; Ratel \textit{et al.}, 2006a; Sheldon \textit{et al.}, 2008; Slotkin \textit{et al.}, 2009). In plants, the primary function of DNA methylation appears to be the transcriptional silencing of transposable elements (TEs) (Berna\textit{tavichute et al.}, 2008; Cokus \textit{et al.}, 2008; Suhua Feng \textit{et al.}, 2010a; R. Lister \textit{et al.}, 2008; Sheldon \textit{et al.}, 2008; Slotkin \textit{et al.}, 2009), although the silencing function also controls the expression of genes (Cao and Jacobsen, 2002a; Chan \textit{et al.}, 2004), and additional functions for DNA methylation in coding regions have been suggested (Takuno and Gaut, 2012; Widman \textit{et al.}, 2009).

Additional changes to chromatin structure such as the loss of nucleosome occupancy are sometimes referred to as epigenetic changes, however, these changes are observed to be transient and are therefore not heritable (Pecinka \textit{et al.}, 2010). When considering possible epigenetic mechanisms for transgenerational stress memories, it is crucial that changes in the epigenetic modification are not only mitotically heritable but also meiotically heritable. On current evidence it is suggested that while histone modifications are mitotically heritable (Kouskouti and Talianidis, 2005) they are not meiotically heritable (Kouzarides, 2007). Hence, it appears unlikely that changes in histone modifications could mediate a transgenerational stress memory. In contrast, changes in DNA methylation can be meiotically heritable in plants and are therefore considered a plausible mechanism by which transgenerational stress memories may be transmitted between generations, given their influence on transcription (Bossdorf \textit{et al.}, 2010; Johannes \textit{et al.}, 2009; Kinoshita \textit{et al.}, 2007; Mathieu \textit{et al.}, 2007; Rangwala \textit{et al.}, 2006; Verhoeven \textit{et al.}, 2010). Since the plant germline differentiates from meristem tissue, one would expect transgenerational stress memories to require a stress-induced epigenetic change which penetrates into the meristem, as suggested earlier in reference to nuclei decondensation in response to heat stress (Pecinka \textit{et al.}, 2010).

The remainder of this Chapter focuses on the pathways which regulate DNA methylation, the functions of DNA methylation, and the inheritance of DNA methylation, and finishes with a critical evaluation of the published evidence that changes in DNA methylation transmit transgenerational stress memories in plants.
1.5 DNA methylation

DNA methylation refers to the addition of a methyl group to cytosine or adenine residues. Initial studies failed to observe adenine methylation in multicellular eukaryotes above a detection limit of ~0.1% (Lawley et al., 1972). Although adenosine methylation has now been detected in plants (Ashapkin et al., 2002) and a mammalian DNA adenine methyltransferase has been identified (Ratel et al., 2006b), the presence of functional quantities of adenine DNA methylation in higher eukaryotes is still contentious (Ratel et al., 2006a; Vanyushin and Ashapkin, 2011). In contrast, cytosine DNA methylation is observed in most eukaryotes and serves various functions in plants including the silencing of transposons and repeat elements and TGS (Feng et al., 2010a; Glastad et al., 2011; M. M. Suzuki and Bird, 2008; Daniel Zilberman, 2008). Thus, the term “DNA methylation” usually refers solely to the presence of a methyl group on carbon 5 of a cytosine residue.

In mammals, DNA methylation is almost completely limited to CG dinucleotides, although, non-CG methylation has recently been observed in embryonic stem cells (Lister et al., 2009). In plants, DNA methylation is more diverse and occurs frequently in CG, CHG and CHH contexts (where H = A, T or C) (Lister et al., 2008; Xiaoyu Zhang et al., 2006). In the model plant species, A.thaliana, 55% of methylated cytosines reside in CG sites, with CHG and CHH sites accounting for 23% and 22% of methylated cytosines respectively (Lister et al., 2008). In contrast to vertebrates where methylation is observed across the genome except in CpG islands (Suzuki and Bird, 2008), in A.thaliana a mosaic DNA methylation pattern is observed across the genome, with TEs, pseudogenes and repeat elements representing the majority of methylation sites and methylation observed to a lesser extent in gene bodies and promoters (Cokus et al., 2008; Suhua Feng et al., 2010a; Lister et al., 2008; Xiaoyu Zhang et al., 2006). Methylation of TEs involves extensive CG, CHG and CHH methylation (Bernatavichute et al., 2008; S. J. Cokus et al., 2008; Sheldon et al., 2008; Slotkin et al., 2009). DNA methylation of coding regions is almost entirely within the CG context, with CHG and CHH methylation enriched at the 3’ and 5’ ends of genes and in promoter regions (Cokus et al., 2008).

The DNA methylation pathways of A.thaliana have been studied extensively with methylation in each sequence context found to be controlled by distinct but overlapping pathways (Mathieu et al., 2007; Singh et al., 2008). At the simplest level, the three sequence contexts may be considered to be regulated by three specific methyltransferase: METHYLTRANSFERASE 1 (MET1) - a homolog of mammalian DNA
METHYLTRANSFERASE 1 (DNMT1) - catalyses methylation of CG dinucleotides (Finnegan and Dennis, 1993), the plant specific methyltransferase, CHROMOMETHYLASE 3 (CMT3) controls methylation at CHG sites (Lindroth et al., 2001), while DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), a homolog of mammalian DNMT3 is primarily responsible for CHH methylation via the RNA-directed DNA methylation pathway (RdDM) (Cao and Jacobsen, 2002b). However, CG, CHG and CHH methylation are all redundantly controlled by MET1, CMT3 and DRM2 to some extent (Chan et al., 2004; Henikoff and Comai, 1998; Mirouze et al., 2009; Singh et al., 2008; Zilberman et al., 2003) as described in the following section.

1.5.1 Maintenance of DNA methylation

In A.thaliana, CG methylation at any one site is typically 80-100%, whereas CHG and CHH methylation is typically lower (30-80% and 20-50%, respectively) (Cokus et al., 2008; R. Lister et al., 2008). Since the above figures relate to methylome analysis on mixed tissue samples, the higher level of CG methylation may reflect more consistent CG methylation patterns between different tissues types. However, the observed relationship between sequence context and degree of methylation may also reflect the differing mechanisms for maintaining CG, CHG and CHH DNA methylation patterns.

The symmetry of CG methylation allows faithful maintenance of DNA methylation patterns after DNA replication. In mammals, DNMT1 is localised to the replication fork and displays a preference for activity at hemi-methylated DNA (Ooi and Bestor, 2008). Maintenance of mammalian CG methylation is partly dependent upon UBIQUITIN-LIKE, CONTAINING PHD AND RING FINGER DOMAINS 1 (UHRFI) which binds hemi-methylated DNA and directly interacts with DNMT1 (Bostick et al., 2007). The A.thaliana UHRFI homolog, VARIANT IN METHYLATION 1 (VIM1), is required for heterochromatin CG methylation (Woo et al., 2007), suggesting a conserved mechanism for CG methylation maintenance. In addition, VIM2 and VIM3 can compensate for the loss of VIM1 in the vim1 mutant ( Woo et al., 2008), indicating the VIM proteins act redundantly.

The symmetrical nature of CHG methylation could also enable faithful maintenance through the binding of proteins to hemi-methylated CHG sites, however, there is no evidence that such a mechanism exists. Instead, it appears CHG methylation is maintained at loci such as the retrotransposon Ta3, through a feedback loop involving CMT3 and KRYPTONITE (KYP) (Cao and Jacobsen, 2002a; Chan et al., 2006; Jackson et al., 2002; Johnson et al., 2007): CMT3-mediated CHG methylation is required for recruitment or activation of KYP
(Johnson et al., 2007), in turn KYP catalyses methylation of histone H3 lysine 9 (H3K9me2), enabling binding of CMT3 via its chromodomain (Malagnac et al., 2002). As such, approximately 90% of CHG methylation overlaps with H3K9me2 enriched regions (Bernatavichute et al., 2008).

Non-CG methylation patterns can also be maintained by the RdDM pathway (discussed below) through continuous de novo methylation by DRM2. For example, FWA is regulated by methylation of repeat elements around the transcription start site (Kinoshita et al., 2007) and requires RdDM to maintain CHG and CHH methylation (Chan et al., 2004). The specific requirement for particular methyltransferases to maintain methylation is dependent upon the loci. Unlike FWA, non-CG methylation at AtSN1, SUP and MEA-ISR is reduced in both cmt3 and drm2 single mutants (Zilberman et al., 2003), indicating that methyltransferases can function cooperatively at some loci. Further evidence for redundancy in the activities of CMT3 and DRM2 comes from their joint requirement for DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1 (DRD1) activity (Kanno et al., 2004), and the observation that the cmt3 drm2 drm1 (cdd) triple mutant shows a pleiotropic suite of developmental abnormalities (Cao and Jacobsen, 2002a) whilst the cmt3 mutant and drm2 drm1 double mutant show no such abnormalities (Cao and Jacobsen, 2002b; Lindroth et al., 2001). However, some CHH methylation still remains in the cdd mutant (S. J. Cokus et al., 2008), suggesting CHH methylation may also be laid down by MET1. This is supported by the observation that CHH methylation at a retrotransposon is lost only in the met1 drm2 double mutant and not in either single mutant (Mirouze et al., 2009).

Methylation at the transgenic RPS locus is decreased in all sequence contexts in met1, cmt3 and drm2 single mutants, with non-CG methylation reduced drastically in all three single mutants, hinting at a possible loci-specific cooperative activity between the three methyltransferases (Singh et al., 2008). Interestingly, initiation of methylation at the RPS loci is also independent of RdDM (Gentry and Meyer, 2013; Singh et al., 2008), and appears to be dependent upon the stem-loop formation potential of a RPS sequence (Gentry and Meyer, 2013), suggesting secondary DNA secondary structure may regulate the recognition of methylation targets by methyltransferases, as has been suggested in animals also (Bestor, 1987).

### 1.5.2 RNA-dependent DNA methylation

DRM2-dependent DNA methylation is guided by 24-nt small interfering RNAs (siRNAs) via the RNA-dependent DNA methylation pathway which was first discovered in tobacco
plants challenged with viral cDNA (Wassenegger et al., 1994). This TGS pathway shares protein functions with the PTGS RNAi pathway including the involvement of ARGONAUTE and DICER-LIKE proteins (Huettel et al., 2007) Figure 1). The RdDM pathway is detailed here as it is most widely described (M. Matzke et al., 2009).

Production of siRNAs for RdDM can occur via multiple routes, including RNA POLYMERASE II (POLII) transcription of double-stranded RNAs from endogenous inverted repeats (IR) which subsequently form secondary structures (Dunoyer et al., 2010), or transcription by the plant specific RNA POLYMERASE IV (POL IV) (Ream et al., 2009; Yokthongwattana et al., 2010). POL IV appears to be physically coupled to downstream components (Law et al., 2011), including RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) which generates double stranded RNA from the POL IV transcript (Jia et al., 2009). Double stranded RNA from IRs or POL IV/RDR2 activity is processed by DICER-LIKE 3 (DCL3) to generate 24-nt siRNAs (Liu et al., 2009) which are methylated by HUA ENHANCER 1 (HEN1) (Li et al., 2005; B. Yu et al., 2010). Subsequently, the siRNAs are loaded onto ARGONAUTE 4 (AGO4) (He et al., 2009; Zilberman et al., 2003). NRPE1 (previously named NRPD1b), the largest subunit of POLV then binds the AGO4-siRNA, and the complex moves towards the genomic region of homology. AGO4-siRNA interacts with the C-terminal domain (CTD) of NRPE1 (Shami et al., 2007) and the nascent POLV transcript scaffold (Wierzbicki et al., 2008), recruiting DRM2 which methylates the region of DNA homologous to the siRNA, in all sequence contexts (Cao and Jacobsen, 2002a; I. R. Henderson et al., 2010; M. Matzke et al., 2007). This process depends upon a multitude of accessory proteins, including DRM3 and KTF1 (Gao et al., 2010; I. R. Henderson et al., 2010; L. M. Johnson et al., 2008; J. a Law et al., 2011, 2010; Rowley et al., 2011).

A considerable degree of redundancy exists within the RdDM pathway. Two additional argonaute proteins, AGO6 and AGO9 can both function in place of AGO4 (Eun et al., 2011; Havecker et al., 2010). Further redundancy is observed in the dicer proteins, of which there are four in A. thaliana (DCL1-4) (Gasciolli et al., 2005). In the absence of DCL3, both DCL2 and DCL4 can produce RDR2-dependent siRNAs for the RdDM pathway (Gasciolli et al., 2005). This redundancy prevents dcl2 and dcl3 single mutants displaying a phenotype, whereas the dcl2 dcl3 double mutant shows a stochastic developmental phenotype after three generations (Gasciolli et al., 2005). Further evidence for the interaction between PTGS and TGS pathways comes from the competition between siRNAs and miRNAs for HEN1-mediated 3’ methylation, the identification of miRNA genes which also produce siRNAs (Chellappan et al., 2010), and the association of POLV-dependent siRNAs with the essential miRNA argonaute, AGO1(Wang et al., 2011). Recent reports indicate that splicing factors
may also be involved in RdDM during DCL3 and POLV activity (Ausiń et al., 2012; Dou et al., 2013). However, there is still debate as to whether the general splicing machinery is involved in RdDM or only specific factors are involved (Zhang et al., 2013).

The major targets of RdDM in the genome are TEs and repeat elements (Huettel et al., 2006; Kanno et al., 2010, 2008, 2004), although the promoter regions of some genes contain elements targeted by RdDM, such that their expression is correlated with upstream RdDM-mediated DNA methylation (Huettel et al., 2006). Analysing the genome, methylome, 24-nt siRNA and gene expression data sets for *A. thaliana*, Baev *et al* (2010) identified over 1700 genes with promoter regions which appear to be methylated by the RdDM pathway. Interestingly, 100 of these genes have previously been annotated as biotic or abiotic stress responsive. In agreement with these findings, a recent ChIP-Seq analysis of NRPE1 (POLV) binding sites revealed an overrepresentation of promoters and observed that promoters overlapping POLV binding sites contained much higher levels of DNA methylation and 24-nt siRNAs, which was suggested to be due to the increased prevalence of transposons in the promoters bound by POLV (Zhong et al., 2012).

In summary, RdDM involves the production of 24nt siRNAs which target genomic sequences for DNA methylation by DRM2. The production of siRNAs occurs through multiple routes and is dependent on one or more of three RNA polymerases. Redundancy exists in the downstream DICER-LIKE proteins and in the ARGONAUTES which bring siRNAs to POLV. RdDM and miRNA pathways interact, via the competition for bi-functional proteins such as HEN1, and the redundancy between siRNA and miRNA pathway components. The major function of RdDM appears to be repressing the expression of TEs and repeat elements (discussed in 1.5.4.1); although there is increasing evidence that RdDM may regulate gene expression also. The dynamics of methylation mediated by the RdDM pathway are discussed in section 1.5.5.1.
RNA interference (RNAi) and RNA-directed DNA methylation (RdDM): Shared protein functionalities.

For simplicity most of the pathway components are not shown. RNAi: Single stranded RNA (ssRNA) from POL II transcription of a micro RNA (miRNA) gene folds to form a pre-miRNA. Dicer cleavage activity produces a miRNA which is bound by an argonaute protein and brought into the RNA-induced silencing complex (RISC). miRNA:mRNA homology targets the mRNA for degradation in post-transcriptional gene silencing (PTGS). RdDM shares many similarities with RNAi. An RNA-dependent RNA polymerase produces doubles stranded RNA (dsRNA) from a POL IV transcript. Dicer activity produces siRNAs which are bound by an argonaute. siRNA homology with a nascent POL V transcript targets the genomic region for DNA methylation by DRM2. dsRNAs from POL II transcription of inverted repeats and viral replication intermediates can also enter the RdDM pathway. Adapted from Verdel et al. (2009).
Demethylation can occur both actively and passively. Passive loss of DNA methylation occurs when DNA methylation patterns are not faithfully maintained following cell division or methyl-C spontaneously deaminates to thymine (Frederico et al., 1990). Active DNA methylation requires the activity of one of four demethylases in *A. thaliana*: REPRESSOR OF SILENCING 1 (*ROS1*) (Gong et al., 2002; Ponferrada-Marín et al., 2009; Zhu et al., 2007), DEMETER (*DME*) (Choi et al., 2002; Mok et al., 2010; Penterman et al., 2007) and DEMETER-LIKE 1 & 2 (*DML1*, *DML2*) (Choi et al., 2002). *DME* is predominantly expressed in the central cell of the female gametophyte where it functions to imprint genes by demethylating the maternal genome (Choi et al., 2002; Jullien et al., 2006). In contrast, *ROS1*, *DML1* and *DML2* demethylate genic regions, including those targeted by RdDM (Lister et al., 2008; Penterman et al., 2007). Hypermethylation in the *ros1 dml1 dml2* triple mutant is primarily at the 5’ and 3’ ends of genes and variable across the genome, suggesting the demethylation may reduce the spread of DNA methylation into specific genic regions which could affect transcription (Lister et al., 2008; Ponferrada-Marín et al., 2009; Zhu et al., 2007). The mechanism of action of *ROS1* has been studied most extensively of the four demethylases. In the current model, *ROS1* binds DNA independently of DNA methylation to perform a searching function (Ponferrada-Marín et al., 2010), followed by formation of a transient ‘interrogation complex’ to extrude normal and damaged bases for inspection (Ponferrada-Marín et al., 2010) before conversion to an ‘excision complex’ upon recognition of the methylated DNA. *ROS1* and *DME* share a hitherto unique discontinuous glycosylase domain (Ponferrada-Marín et al., 2010), suggesting the mechanism of action of these two demethylases may be conserved. Following cleavage of the phosphodiester backbone at the 5me-C, the 3’ phosphate is removed by ZINC FINGER DNA 3’ PHOSPHOESTERASE (ZDP) (Martínez-Macías et al., 2012), and the gap filled by an undefined polymerase before ligation to complete the conversion from methyl-cytosine to cytosine.

Many second site suppressors of *ros-1* induced hypermethylation are RdDM components (He et al., 2009; Penterman et al., 2007; Zheng et al., 2007), suggesting interplay between these two pathways. Currently, it is not known how demethylases are targeted to specific sequences, however, *ROS1* activity is dependent upon the siRNA binding protein *ROS3* (Zheng et al., 2008), suggesting it may be targeted to genomic regions by siRNAs. The counteracting DNA methylation and demethylation pathways could enable plants to modulate DNA methylation at specific loci in response to internal developmental cues and external environmental factors, and there is increasing evidence that this is an important function of DNA methylation (Baek et al., 2010). However, the major role of DNA
methylation in plants appears to be transcriptional silencing of “parasitic” DNA elements. We shall now consider molecular functions of DNA methylation, starting with its role in genome defence.

1.5.4 Functions of DNA methylation in plants

The function of cytosine DNA methylation in prokaryotes appears to be limited to protecting DNA from restriction digest, whilst adenosine methylation functions in DNA repair, transposition and expression (Ratel et al., 2006a). In plants, cytosine DNA methylation functions in genomic defence through transcriptional silencing of transposable elements (Bernatavichute et al., 2008; S. J. Cokus et al., 2008; Sheldon et al., 2008; Slotkin et al., 2009). The main evidence for this comes from the observation that hypomethylation directly results in increased transposition (Jia et al., 2009; Lippman et al., 2004, 2003; Miura et al., 2001). This is in contrast with humans, where hypomethylation is not observed to result in increased transposition (Wilson et al., 2007) and DNA methylation is thought to predominantly exert its function at genes (Suzuki and Bird, 2008). TEs and other repeat elements have now been shown to represent the majority of methylation sites across the genome in plants (Feng et al., 2010a; R. Lister et al., 2008), suggesting the major function of DNA methylation is to suppress the activity of TEs and repeat elements in order to protect the genome from these parasitic elements. As we shall see later, this silencing function at TEs and repeat elements has been co-opted to control the expression of genes with repeat elements in their promoter regions (Cao and Jacobsen, 2002a; Chan et al., 2004), and CG methylation in coding regions likely functions entirely separately from these TGS functions (Takuno and Gaut, 2012; Widman et al., 2009).

The molecular mechanism by which DNA methylation regulates transcription involves the inhibition of protein binding by bringing about secondary changes to the chromatin structure that render it inaccessible (Bell and Felsenfeld, 2000). In A.thaliana, the loss of CG methylation is associated with a reduction in the heterochromatic mark H3K9me (Tariq et al., 2003), and the percentage of DNA contained within the densely packed heterochromatic chromocentres decreases in the met1 mutant (Vaillant et al., 2008). Plant and animal methyl-binding proteins (MBPs) frequently either repress transcription through histone modifications or are themselves histone-modifying enzymes (Ballestar and Wolffe, 2001; Feng and Zhang, 2001; Johnson et al., 2007; Yaish et al., 2009; Zemach and Grafi, 2007). For example, the human MBP METHYL CPG BINDING PROTEIN 2 (MECP2) binds to methylated promoter regions and depressed gene expression by recruiting histone deacetylases (Xu and Pozzo-Miller, 2013). In agreement with a model in which DNA
methylations brings in MBPs to form heterochromatin in *A.thaliana*, MBPs are observed to colocalise to the highly methylated chromocentres, and the colocalisation is disrupted in the DNA methylation deficient mutants *met1* and *ddm1* (Zemach *et al.*, 2005).

1.5.4.1 DNA methylation of transposons and repeat elements

The first evidence for the importance of DNA methylation in controlling TE activity came from studies in maize. Inactivation of the *Activator (Ac)* element was observed to correlate with the extent of DNA methylation, with active Ac elements showing hypomethylation in their transposase promoter sequences (Brutnell *et al.*, 1997; Chomet *et al.*, 1987; Wang *et al.*, 1996). The generality of this mechanism in maize was established through studies of the *Mutator (Mu)* and Mu-family elements in maize which also show a correlation between DNA methylation and transposon activity (Banks *et al.*, 1988; Hershberger *et al.*, 1991). Further studies in *A.thaliana* identified transcriptional reactivation of TEs in the DNA-methylation defective *met1* and *ddm1* single mutants (Lippman *et al.*, 2004, 2003; Miura *et al.*, 2001).

TEs are typically associated with MET1 activity, H3K9me2 and matching siRNAs, leading to CG, CHG and CHH methylation (Bernatavichute *et al.*, 2008; Cokus *et al.*, 2008; Sheldon *et al.*, 2008; Slotkin *et al.*, 2009). However, detailed studies highlight the variability in DNA methylation between different TE loci, and the differing requirements to maintain transcriptional repression. For example, gypsy class retrotransposon ATPG3 elements are transcribed and mobilised in the *ddm1* single mutant and the *met1 cmt3* double mutant, both of which are depleted in CG and non-CG methylation, but not in the *met1* and *cmt3* single mutants (Tsukahara *et al.*, 2009). Similarly, the endogenous DNA-type transposon CACTA is remobilised only in the *met1 cmt3* double mutant, although transcription is derepressed in both *met1* and *cmt3* single mutants (Kato *et al.*, 2003). By contrast, Evadé (*EVD*), a copia-like retrotransposon, is mobilized in *met1* concurrent with a loss of CG methylation, but not in *cmt3* or *kyp*, despite loss of CHG methylation (Mirouze *et al.*, 2009). In the *met1 nrpe2a* double mutant, only *EVD* is mobilised, with other DNA transposons remaining inactivated, highlighting the selectivity in transposon control (Mirouze *et al.*, 2009). Interestingly, mobilisation of *EVD* was much more pronounced in the *met1 kyp* double mutant than the *met1* single mutant although *EVD* transcript levels remained the same, and no increase in *EVD* transcripts was observed in the *kyp* single mutant, suggesting *kyp* may restrain mobilisation of *EVD* at the post-transcriptional level. This requirement for post-transcriptional control by *kyp* to silence a transposon shares similarities with the silencing of the LTR-retrotransposon MAGGY in the fungus Magnaporthe grisea which occurs post-
transcriptionally and does not involve DNA methylation (Nakayashiki et al., 2001).
Unfortunately, H3K9me2 marks laid down by KYP are closely associated with CHG methylation in A. thaliana (Bernatavichute et al., 2008), which is a common mark in TEs (Cokus et al., 2008; Tompa et al., 2002), making inference of the respective contributions of H3K9me2 and CHG methylation to TE silencing difficult. Interestingly, the met1 cmt3 double mutant does not show the drastic EVD mobilisation of the met1 kyp double mutant (Mirouze et al., 2009), suggesting KYP and CMT3 may act independently to repress transposition at some loci. It is clear that other histone modifications also play a role in silencing TEs in concert with the DNA methylation pathways. For example the histone deacetylase mutant, hda6, shows activation of several classes of TEs with an associated DNA hypomethylation at the loci (Lippman et al., 2003). As both the silencing and hypomethylation were reversible upon backcrossing with wild type plants, this suggests HAD6 is involved in de novo TGS at these loci.

The relative contribution of RdDM to TE repression is unclear. In A. thaliana, met1 and ddm1 display a widespread reactivation of TEs (Lippman et al., 2004; R. Lister et al., 2008; Xiaoyu Zhang et al., 2006; Daniel Zilberman et al., 2007), whilst release of TE silencing is restricted to a small number of specific loci in RdDM mutants (Huettel et al., 2006; Kanno et al., 2010, 2008, 2004), and this silencing is typically more prominent with the addition of the met1 mutation (Mathieu et al., 2007; Mirouze et al., 2009). Additionally, the previously described increase in EVD mobilisation in the met1 nrpe2a double mutant is not observed in the met1 drm2 double mutant, which suggests POLIV and or POLV may operate independently of RdDM to suppress some transposons (Mirouze et al., 2009). However, there is convincing evidence that RdDM is required for complete transposon silencing at some loci. Following backcrossing of the methylation deficient ddm1 mutant, a progressive remethylation is observed at many transposons, and the transcriptional activity of a transposon 5 generations after the backcrossing was observed to correlate with its capacity to be remethylated (Teixeira et al., 2009). Crucially, this remethylation was severely impaired when ddm1 was crossed with rdr2, indicating the remethylation is RdDM-dependent. Furthermore, a maize rdr2 mutant displays increased expression of DNA TEs, although transposition rates in wild type and rdr2 were not established (Jia et al., 2009).

1.5.4.2 Promoter DNA methylation

The silencing of transposons by methylation appears to have been co-opted to control the expression of genes with repeat elements in the promoter regions. Consistent with this,
RdDM is targeted towards promoters containing repeat regions (Baev et al., 2010) and differential gene expression is observed in RdDM-deficient maize (Jia et al., 2009).

Specific examples of promoter DNA methylation regulating gene expression include the FWA and SDC loci, both of which have repeat elements in their promoters (Henderson and Jacobsen, 2008; Kinoshita et al., 2007; Lippman et al., 2004). Methylation at the FWA gene is restricted to the promoter and 5’ untranslated region which contains siRNA-generating tandem repeats (Kinoshita et al., 2007; Lippman et al., 2004). This methylation is correlated with transcriptional silencing which is released following demethylation of the maternal allele in the endosperm (Kinoshita et al., 2007). Endogenous FWA is demethylated and reactivated in ddm1 and met1 (Johannes et al., 2009; Kakutani, 1997; Kinoshita et al., 2007), indicating CG methylation is required for complete silencing of endogenous FWA. Similar to FWA, SDC contains tandem repeats in its promoter region which are targeted by RdDM for non-CG methylation, leading to transcriptional silencing (Henderson and Jacobsen, 2008). In contrast to FWA silencing which is dependent upon met 1 and RdDM, methylation at SDC appears to be maintained by continuous de novo methylation as silencing is immediately restored in the F1 progeny of drm1drm2cmt3 plants upon outcrossing (Henderson and Jacobsen, 2008), or transformation with DRM2 or CMT3 transgenes (Chan et al., 2006).

Higher levels of promoter methylation are not always correlated with low levels of gene expression. In some specific cases, promoter DNA methylation has also been correlated with increased gene expression. The leucine-rich repeat gene Pib is upregulated in O.sativa in response to the blast-causing fungal pathogen Magnaporthe grisea and abiotic stressors (Wang et al., 1999). Two regions of the promoter were examined by bisulphite sequencing (~1200 bp in total) in response to treatment with 5-azacytidine (5-azaC), a chemical homolog of cytosine which reduces the activity of methyltransferases, resulting in hypomethylation (Li et al., 2011). Surprisingly, demethylation of the heavily methylated promoter of Pib by 5-azaC treatment was correlated with a decrease in gene expression. However, the regulation of Pib expression in response to M.grisea does not involve demethylation at the two regions examined. As the 5-azaC treatment causes genome wide hypomethylation, it is not possible to establish whether the decreased Pib expression is actually a direct effect of promoter demethylation, or a secondary effect of demethylation of a trans-acting repressive factor. The analysis of Pib highlights the difficulty in establishing the effect of promoter DNA methylation on gene expression with treatments such as 5-azaC which affect DNA methylation across the whole genome. Additionally, there are difficulties in analysing the DNA methylation status of promoters by bisulphite sequencing only a portion of the promoter, as the regulatory elements are often unknown. Indeed, Pib contains
a transposon 2.4 kb upstream whose methylation status was not examined. Although this is more than 1 kb upstream of the region that was examined, promoters are frequently defined as up to 3 kb upstream of the start site and siRNA targeted regions have been shown capable of regulating gene expression from this far upstream, as highlighted by the example below.

In a more targeted approach to examine the role of promoter DNA methylation Baek et al. (2010) removed a putative siRNA targeted region from the promoter of the salt stress induced gene \textit{HKT1}. Although this region is located 2.6 kb upstream of the start site, their results established that methylation of the siRNA region is crucial for suppressing expression of \textit{HKT1} in the aerial portion of the plant. In the \textit{rdr2} mutant, promoter non-CG methylation is reduced, with a correlated increase in the expression of \textit{HKT1} in the aerial portion. Plants lacking the siRNA region were also salt hypersensitive, suggesting correct spatial expression of \textit{HKT1} by RDR2-dependent methylation of the upstream region is required for salt tolerance in Arabidopsis.

The above examples indicates that promoter DNA methylation controls gene expression at specific loci, however, the genome-wide correlation between promoter DNA methylation and gene expression is poor. For example, changes in promoter methylation and gene expression under drought stress do not show a significant correlation in \textit{A.thaliana} (Colaneri and Jones, 2013). Furthermore, there is evidence that in humans, promoter DNA methylation may be responsive to gene expression rather than regulating it. Following the recent ENCODE publications of an integrated map of DNA elements in the human genome, a genome wide assessment of the interaction between DNA methylation and TFs has been performed for the first time (Bernstein et al., 2012; Thurman et al., 2012). Although this established that >98% of sites bound by TF are in DNAaseI hotspots, i.e. euchromatin devoid of DNA methylation, a negative correlation was observed between the expression of an individual TF and the methylation status of its binding sites across the genome (Thurman et al., 2012). It was suggested that this correlation is not easily explained by DNA methylation inhibition of TF binding and is more reasonably explained by a model in which the DNA methylation machinery passively methylates vacant TF binding sites (Thurman et al., 2012). It is possible then that in mammals, promoter DNA methylation prevents the binding of TFs at specific loci but that the major function of DNA methylation is to reinforce transcriptional silencing of inactive genes, as suggested by Bird (2002). Further studies in \textit{A.thaliana} and other plant species are required to establish if this is the case for plants also.
So far, the functions of DNA methylation which have been discussed are genome defence against parasitic DNA, and the control of gene expression by promoter DNA methylation. DNA methylation is also observed in coding regions in most eukaryotes (Feng et al., 2010a) and may yet be found to be as important as promoter DNA methylation in regulating the transcriptome.

1.5.4.3 Gene body DNA methylation

The function of gene body DNA methylation in plants is currently unclear, with the regulation of alternative splicing and suppression of cryptic promoters being the most commonly postulated functions (Chodavarapu et al., 2010; Lister et al., 2008; Maunakea et al., 2010; Zhang et al., 2006). Consideration has also been given to the suggestion that gene-body DNA methylation may simply be a by-product of transcription (Teixeira and Colot, 2009). This is supported by the observation that genic DNA methylation is variable between individuals (Vaughn et al., 2007). However, two genome-wide analyses by Widman et al. and Takuno and Gaut indicate genic DNA methylation is likely to be functional in Arabidopsis (Takuno and Gaut, 2012; Widman et al., 2009). Widman et al. analysed the conservation of all nucleotides including methyl-cytosine in duplicated genes and established that methylated cytosines were more highly conserved than any other nucleotide, strongly suggesting a function for the methyl group. Takuno and Gaut (2012) took an alternative approach, hypothesising that if gene body DNA methylation played a role in either suppressing cryptic promoters or enhancing splicing, as previously suggested (Chodavarapu et al., 2010; Maunakea et al., 2010), it should be enriched in essential genes and/or long genes. Analysis of existing data sets confirmed that methylated genes tend to be larger and knock-out mutants of these genes are more likely to have a phenotype, suggesting methylated genes are more “essential” (Takuno and Gaut, 2012). However, previous analyses indicate that the level of DNA methylation for pseudogenes, transposons and repeats also increases with the length of the genomic unit (Cokus et al., 2008), suggesting the observed correlation between length and gene body DNA methylation may not be informative. Although their analysis did not provide evidence for the exact function of genic DNA methylation, it does suggest a functional role for DNA methylation. In A.thaliana, gene body methylation does not show a linear correlation with gene expression, with methylated genes possessing a moderate level of gene expression (Zilberman et al., 2007). Interestingly, loss of genic DNA methylation in A.thaliana leads to a slight increase in gene expression, which may indicate that DNA methylation interferes with transcription elongation (Zilberman et al., 2007), as occurs in the fungus Neurospora crassa (Rountree and Selker, 1997) and mammals (Lorincz et al., 2004). In vitro experiments have
established that DNA methylation alters the force required to separate the DNA strands, in a sequence specific manner (Severin et al., 2011), suggesting inhibition of elongation may be due a change in stacking energies of the cytosines as a result of methylation. However, given the relationship between genic DNA methylation and gene expression, the major function of gene-body DNA methylation in A.thaliana does not appear to be to suppress gene expression.

An enrichment of DNA methylation in exons is observed across a variety of plants and animals, suggesting this enrichment may be an ancestral condition (Feng et al., 2010a). The exon enrichment for genic DNA methylation in Arabidopsis is thought to be driven by nucleosome enrichment in exons over introns and postulated to play a role in exon definition and alternative splicing regulation (Chodavarapu et al., 2010). DNA methylation has been studied in honey bee (Apis mellifera) where it is observed at very low levels (<1%) and almost exclusively in the CG context in exons (Feng et al., 2010a). Disruption of CG methylation in A.mellifera by RNAi knockdown of DNMT3 causes widespread and diverse changes in alternative splicing which are significantly correlated with changes in DNA methylation (Li-Byarlay et al., 2013). Additionally, genic DNA methylation in mammals has been shown to regulate alternative promoters in a tissue and cell-type specific manner (Maunakea et al., 2010) and to reduce transcriptional noise (Huh et al., 2013). To the author’s knowledge, similar such observations linking DNA methylation to the regulation of alternative splicing or cell-type specific promoters have not been made in plants, although a shared functionality would not be unexpected given the shared enrichment in exons.

Interestingly, in the absence of MET1 activity in A.thaliana, CHG methylation is increased in the transcribed region of genes (Lister et al., 2008), suggesting either CHG methylation may compensate for the lack of CG methylation in met1, or else CG methylation represses genic CHG methylation.

On current evidence, exonic DNA methylation appears to be common across the plant and animal kingdom and predominantly functions to regulate alternative splicing (Alexander and Beggs, 2010; Schor and Kornblihtt, 2010), with additional functions suppressing cryptic promoters (Li-Byarlay et al., 2013) and regulating transcript elongation (Lorincz et al., 2004; Rountree and Selker, 1997; Zilberman et al., 2007).

An additional role for genic DNA methylation has been proposed based on the observation that siRNAs are produced from introns in rice and observed to drive RdDM-dependent cis-methylation (Chen et al., 2011). The authors hypothesise this methylation functions to homeostatically self-regulate transcription, however further analysis is required to establish whether these siRNAs and the intron methylation they facilitate are indeed functional or are
predominantly due to incidental and non-beneficial incorporation of intronic RNA into the RdDM pathway.

In summary, DNA methylation has multiple molecular functions. The primary function of DNA methylation in plants is to silence “parasitic” DNA, however, methylation at promoter regions has also been observed to regulate gene expression and gene-body DNA methylation seems likely to have multiple functions, including the regulation of splicing.

1.5.5 Dynamics of DNA methylation in A.thaliana

As described in section 1.5.3, the apparent possible dual requirement of RdDM and ROS1-dependent DNA demethylation for siRNAs may allow the two opposing pathways to target the same genomic regions (He et al., 2009; Penterman et al., 2007; Zheng et al., 2007). Given that plants possess mechanisms for both active DNA methylation and demethylation, and DNA methylation can influence the transcriptome, it is unsurprising that DNA methylation is to some extent dynamic through development (Gehring and Henikoff, 2007; Vining et al., 2012). Perhaps the most striking examples are the dynamic DNA methylomes of male and female gametes (Gutierrez-Marcos and Dickinson, 2012), including the demethylation of the A.thaliana endosperm which is required for the endosperm-specific gene expression profile (Choi et al., 2002; Gehring et al., 2009; Hsieh et al., 2009). Clearly, regulation of expression for proteins involved in the DNA methylation/demethylation pathways could lead to genome wide changes in DNA methylation. Indeed the promoter region of DRM2 is observed to be heavily methylated (Ashapkin et al., 2002), and ROS1 expression is regulated by RdDM (Huettel et al., 2006; Li et al., 2012), suggesting feedback mechanisms exists to regulate levels of DNA methylation at the genome wide level. However, how such genome wide changes in DNA methylation could result in specific changes in the transcriptome as part of the plant response to stress stimuli is more difficult to envisage. The RdDM pathway would appear to be a likely candidate mechanism for such directed DNA methylation changes as expression of a particular component in response to particular developmental or environmental stimuli could drive specific changes to the methylome. For example, in rice, the expression of an archesporial-specific argonaute family protein MEIOSIS ARRESTED AT LEPTOTENE1 (MEL1) is essential for the completion of normal sporogenesis and meiosis in both male and female organs (Nonomura et al., 2007). It is reasonable to believe MEL1 drives changes in gene expression required for sporogenesis by changing the active pool of argonaute-bound small RNAs. This could occur by sequestering specific small RNA species, as occurs with AGO10 and mi165/166 in A.thaliana (Zhu et al., 2011), or alternatively by binding small RNAs that are not normally
bound by an argonaute. Currently, it is not clear whether the change in MEL1 expression drives a change in RdDM activity.

This section will discuss mechanisms for dynamic DNA methylation which show potential for specific changes in gene expression that could be utilised by plants in response to external stimuli. Following this, the inheritance of DNA methylation patterns will be discussed.

1.5.5.1 De novo RdDM at transposons and repeat elements

The aforementioned competing activity of RdDM and ROS1/DML1/DML2 at the same loci suggests plants may be capable of fine tuning DNA methylation at some RdDM targeted loci. Furthermore, RdDM has been shown to be required for de novo methylation of unmethylated sequences, suggesting it may be involved in methylating sites in response to stress. As described in section 1.5.4.1, remethylation of transposons in backcrossed ddm1 plants is dependent upon rdr2 (Teixeira et al., 2009). Further evidence for RdDM-dependent de novo DNA methylation comes from studies in which additional copies of two genes with repeat elements in their promoter, FLOWERING WAGENINGEN (FWA) and SUPPRESSOR OF DRM2, DRM1, CMT3 (SDC) were introduced into A.thaliana (Aisin et al., 2009; Cao and Jacobsen, 2002a; Chan et al., 2004). Transgenic copies of FWA are targeted for methylation at their promoter by RdDM and become transcriptionally silenced (Aisin et al., 2009; Cao and Jacobsen, 2002a; Chan et al., 2004). The requirement for RdDM for de novo DNA methylation and silencing of FWA transgenes is demonstrated with the RdDM mutants rdr2, dcl3, drm2 drm1, ago4, idn1 and idn2 which all show depleted non-CG methylation at a newly introduced FWA transgene and a non-FWA silenced phenotype, in contrast with cmt3 and kyp which display the wild-type phenotype (Aisin et al., 2009; Chan et al., 2004). Furthermore, a stable non silenced endogenous fwa epiallele can be reverted to the silenced FWA epiallele by generating a large quantity of siRNAs from FWA transgenes to direct RdDM (Kinoshita et al., 2007), demonstrating that RdDM is required to establish DNA methylation and silencing at this locus. Similarly, de novo methylation of tandem repeats in the promoter of transgenic SDC loci is reduced in the RdDM mutants drm2 drm1, idn1 and idn2 (Aisin et al., 2009; Henderson and Jacobsen, 2008), suggesting RdDM may be the general mechanism by which de novo DNA methylation is initiated in A.thaliana. RdDM-dependent, MET1-independent, de novo methylation has also been observed at a transgenic 35S promoter following targeting of the promoter sequence by infection with plant RNA viruses modified to carry a portion of the 35S promoter which generates homologous siRNAs (Jones et al., 2001). On current evidence it appears that de novo DNA methylation
of transposons and repeat elements is dependent upon the RdDM pathway and does not involve MET1 or CMT3.

The observation that de novo DNA methylation can also be directed towards specified loci by generating homologous siRNAs suggests that plants may be able to modify their DNA methylation pattern by expressing stress-induced siRNAs, or suppressing specific siRNAs in response to stress. This is further supported by a recent observation that induced expression of the inverted repeat IR71 leads to production of IR71-derived siRNAs which in turn drives DNA methylation in trans and cis (Dunoyer et al., 2010). Given the role of DNA methylation at promoter regions (discussed in 1.5.4.2), *A. thaliana* could potentially modify the siRNA pool in response to stress to regulate gene expression. Furthermore, since TGS induced at the 35S promoter can be maintained in the following generations in the absence of the initial RdDM trigger (Jones et al., 2001), it’s possible that stress induced changes in the siRNA pool could lead to heritable changes in DNA methylation and their associated downstream effects on gene expression.

### 1.5.5.2 Dynamics of gene body DNA methylation

As described in section 1.5.4.3, DNA methylation in coding regions is predominantly observed in the CG context and siRNAs are depleted in the gene body (Cokus et al., 2008). Gene body DNA methylation looks to involve two processes: CG methylation of coding regions and restriction of CHG methylation in coding regions. Restriction of CHG methylation appears to be dependent upon the activity of the H3K9 demethylase IBM1, as the *imb1* mutant displays increased genic CHG methylation without affecting CHG methylation of transposons (Miura et al., 2009). As previously described in section 1.5.1, CMT3 activity and H3K9 methylation are closely associated. Thus, it seems IBM1 restricts genic CHG methylation by demethylating H3K9. Based on this observation, Teixeira and Colot (2009) proposes a model for gene body DNA methylation in which POL II activity is coupled to MET1 activity (by an unknown mechanism) and IBM1 activity which demethylates H3K9, preventing its recognition by the chromodomain-containing, CHG methyltransferase CMT3. Given that genic CHG methylation occurs in *met1* (Lister et al., 2008) and *met1drm1drm2* (Cokus et al., 2008) mutant plants, it appears that genic CG methylation may be required to recruit IBM1. This model implies that genic CG DNA methylation at an individual loci may be dynamic in response to changes in gene expression and could therefore be responsive to changes in environmental conditions which necessitate changes in the transcriptome. Thus, changes in genic DNA methylation in response to environmental stimuli could affect alternative splicing events or regulate transcription from
alternative start sites, given the postulated functions of genic DNA methylation (1.5.4.3). If these changes in DNA methylation persisted into the following generation they could then maintain a memory of the transcriptome changes induced by the stress event. The obvious contradiction in this model is that it requires genic DNA methylation to be dynamic in response to the stress-induced transcriptome changes but at least partially maintained when the transcriptome changes are reversed after the stress event. For such a model to exist, additional mechanisms are required which more permanently fix the genic DNA methylation state, perhaps by modifying additional chromatin markers.

1.5.6  Inheritance of parental DNA methylation profile

Mammals set down their germline in early development and reprogram DNA methylation through genome-wide erasure of DNA methylation in primordial germ cells and in the early stages of embryo development, before immediate re-methylation by the de novo methyltransferases DNMT3A and DNMT3B (Feng et al., 2010b; Hackett and Surani, 2013; Hemberger et al., 2009; Reik, 2007). In contrast, plants germ cells differentiate from somatic cells in developing flowers and their methylation pattern reflects the DNA methylation profile of the somatic cell from which they derive (Boavida et al., 2005). Thus, mammals possess a barrier to epigenetic inheritance which is not present in plants (Paszkowski and Grossniklaus, 2011). However, Calarco et al (2012) recently demonstrated that the inheritance of DNA methylation patterns in plants differs for the three sequence contexts. Whereas CG and CHG methylation are faithfully maintained in both the male and female germlines, CHH methylation is almost completely abolished in the sperm cell of the pollen grain. The associated loss of DRM2 expression suggests the loss of CHH methylation is due to a limited capacity for RdDM in sperm cells. As the CHH methylation is restored in the embryo when RdDM has been observed to be active (Jullien et al., 2012) it is thought RdDM restores the DNA methylation. POLIV-dependent 24-nt siRNAs in the seed have previously been shown to be predominantly maternal (Mosher et al., 2009). Thus, the CHH methylation pattern set down in the embryo will likely reflect the maternal epigenetic status. Given that CG methylation patterns are faithfully maintained through the germlines, whereas CHH methylation undergoes a resetting process which likely involves maternally-driven de novo methylation, it is not surprising that CG methylation has previously been postulated to be the coordinating epigenetic mark (Mathieu et al., 2007).

Differential methylation statuses of a single gene are referred to as epialleles. In some cases epialleles have been observed to be heritable over multiple generations and can confer phenotypes (Richards, 2006). For example, hypomethylation of the FWA promoter in ddm1
correlates with FWA expression and time to flowering (Kinoshita et al., 2007). This hypomethylation is variable and in the most highly demethylated lines, a stable fwa epiallele is generated which remains unsilenced after outcrossing and is not observed to spontaneously revert to the wild-type silenced FWA epiallele (Johannes et al., 2009), indicating stable inheritance of the altered DNA methylation state at this locus. Naturally occurring epialleles have also been observed. An interesting example is the naturally occurring mutant of Linaria vulgaris in which flower symmetry switches from bilateral to radial, a mutant first described by Linnaeus over 250 years ago. This mutant phenotype is wholly dependent on the methylation status of LCYC which controls dorsoventral asymmetry and is silenced by DNA methylation (Cubas et al., 1999). The hypermethylated lcyc epiallele and its associated radial flower symmetry co-segregate, with occasional reversion to the unmethylated LCYC epiallele (Cubas et al., 1999). A further naturally occurring epiallele which co-segregates with a phenotype is the hypermethylated epiallele of COLOURLESS NON-RIPENING (CNR), which prevents ripening in tomatoes (Manning et al., 2006).

Induced changes in DNA methylation have also been shown to be heritable. 5-azA induces gene expression changes (Jones, 1985) with associated developmental phenotypes including early flowering and dwarfism in Perilla frutescens (Kondo et al., 2010, 2006), and early flowering in vernalisation-requiring A.thaliana ecotypes (Burn et al., 1993). This 5-azA-induced dwarfism in P.frutescens is inherited (Kondo et al., 2010), in line with similar observations of 5-azA-induced heritable dwarfism phenotypes in rice (Sano et al., 1990) and maize (Sano et al., 1989). Taking an alternative approach to study inheritance of DNA methylation and the effect of DNA methylation on plant phenotypes, two groups independently generated epigenetic Recombinant Inbred Lines (epiRILs) that possess almost identical DNA sequences, but contrasting DNA methylation patterns (Johannes et al., 2009; Reinders et al., 2009). In both cases, a wild type A.thaliana Col-0 plant was crossed with a hypomethylation mutant (met1 or ddm1), before propagation through single seed descent for at least 6 generations to produce lines with varied stable DNA methylation patterns. Considerable phenotypic variation was observed between the epiRIL lines, as measured by flowering time, NaCl stress tolerance, biomass, Pseudomonas resistance and plant height (Johannes et al., 2009; Reinders et al., 2009), suggesting the variability in inherited DNA methylation induced impacted on plant phenotypes. This was further supported by DNA methylation analysis of the ddm1/Col-0 epiRILs, in which late-flowering time outliers were all observed to display low expression levels for FWA, and associated high DNA methylation of the promoter region (Johannes et al., 2009). A further study of the ddm1/Col-0 epiRILs observed that trait plasticities in drought stress were higher within the epiRIL
lines than the control lines, suggesting that the variation in DNA methylation between the epiRILs results in variability of stress responses (Zhang et al., 2013).

The studies outlined above provide further evidence for the hypothesis that DNA methylation may transmit transgenerational stress memories. Altered DNA methylation patterns may be faithfully inherited in plants, and these DNA methylation states are observed to co-segregate with particular phenotypes, including stress tolerance. The remainder of the introduction will discuss dynamic DNA methylation in stress responses, before considering the possible role for DNA methylation in transgenerational stress memories.

1.5.7 DNA methylation as a stress response.

The group of Hiroshi Sano at the Nara Institute of Science and Technology, Japan, were instrumental in providing early evidence for changes in DNA methylation in response to stress in plants (Steward et al., 2002; Wada et al., 2004). Their initial observations of decreasing global DNA methylation in the root tissues of maize (Zea mays L. cv. Golden Arrow) plants subjected to cold stress, along with a more detailed analysis of demethylation of CG and CHG loci in a long terminal repeat suggested that DNA methylation may regulate expression in response to stress (Steward et al., 2002). Unfortunately, this initial observation was somewhat hindered by the severity of the treatment which prevented recovery and ultimately killed the plants. As such, it is difficult to assess whether the response observed truly related to the cold stress or was the result of cell death. Their follow-up studies in tobacco plants (Nicotiana tabacum cv. Xanthi nc) investigated the expression of genes differentially expressed in a transgenic tobacco line in which DNA methylation was suppressed by anti-sense NtMET1 and hence, whose expression was thought to be regulated by DNA methylation (Choi and Sano, 2007; Wada et al., 2004). Infection with tobacco mosaic virus induced accumulation of NtA1ixl, a known pathogen response gene, and was associated with NtA1ixl gene-body methylation changes (Wada et al., 2004). However, the expression changes preceded the changes in DNA methylation CCGG loci by 24 hours, indicating that changes in DNA methylation were not responsible for triggering a stress responsive change in expression for this gene. In a separate study, they observed that aluminium, cold and salt stress induced expression of NtGPDL and gene-body demethylation (Choi and Sano, 2007). This expression change was thought to be in response to oxidative stress as a similar expression change was observed with paraquat treatment but not pathogen infection. Demethylation was observed to precede the expression change by 5 hours, implying the expression change may be been due to the change in DNA methylation
for this particular gene. Another early indication that DNA methylation may be important in plant stress responses came from a screen for mutants with deregulated expression of a luciferase transgene under the control of an ABA-responsive promoter (Gong et al., 2002). This screen identified the DNA Glycosylase/Lyase gene, ROS1 which functions to demethylate RdDM targets. Mutants of ROS1 were observed to be hypersensitive to genotoxic stress and hydrogen peroxide, suggesting the demethylase function of ROS1 may be important in response to some abiotic stressors (Gong et al., 2002).

For this section, DNA methylation change in response to different stressors will be discussed separately with most of the examples given being experiments with the model plant species A.thaliana.

DNA methylation changes have been observed in response to NaCl stress in A.thaliana (Baek et al., 2010; Boyko et al., 2010), tobacco (Choi and Sano, 2007), maize (Tan, 2010), cotton (Cao et al., 2011), rice (Karan et al., 2012) and crystalline iceplant (Dyachenko et al., 2006), suggesting alterations in DNA methylation may be a common NaCl stress response across the plant kingdom. However, studying different rice genotypes, Karan et al noted that there did not appear to be a consistent methylation change between rice genotypes and no correlation was observed between DNA methylation and gene expression (Karan et al., 2012), indicating that the observed DNA methylation changes in response to NaCl stress do not necessarily represent an adaptive response. Although there is evidence that NaCl stress preferentially leads to differential methylation of stress responsive genes (Choi and Sano, 2007; Tan, 2010), this observation has rarely been followed by a detailed analysis to establish if the DNA methylation drives functional gene expression change. One example where DNA methylation has been shown to be important for NaCl stress tolerance is the aforementioned regulation of the Na\textsuperscript{+}-selective uniporter HKT1 in A.thaliana. HKT1 gene expression appears to be regulated by MET1 and RdDM at a small RNA binding region 2.6 kb upstream of the start site as this region is hypomethylated in the met1 and rdr2 mutants, and removal of this region lowers NaCl stress tolerance so that it resembles met1 (Baek et al., 2010). The regulation of HKT1 by DNA methylation in response to NaCl stress clearly demonstrates that DNA methylation can play a role in stress tolerance by modifying gene expression.

Osmotic stress, which is physiologically similar to NaCl stress (Verslues et al., 2006), also alters DNA methylation in maize (Tan, 2010), rice (Zhang et al., 2013) and Arabidopsis (Colaneri and Jones, 2013). Two genes which function in the biosynthesis of proline which accumulates in response to osmotic stress (Yoshiba et al., 1995), PYRROLINE-5-
CARBOXYLATE SYNTHETASE (P5CS) and ORNITHINE-DELTA-AMINOTRANSFERASE (δ-OAT) (Roosens et al., 1998; Székely et al., 2008), are demethylated and upregulated in rice in response to polyethylene glycol (PEG) treatment (Zhang et al., 2013). Using a genome-wide approach to examine DNA methylation changes in response to PEG in *A. thaliana*, Colaneri and Jones (2013) established that differentially methylated regions centre around the transcription start site, suggesting that DNA methylation changes in response to PEG may affect gene expression. These differentially methylated regions are predominantly hypermethylated and membrane transport proteins were enriched within the genes affected. Interestingly, no correlation was observed between transcription and differential methylation in promoter regions. The authors suggest this was due to threshold selections used and detection limitations, as some known stress responsive genes were not identified as PEG stress-responsive by RNA-Seq (Colaneri and Jones, 2013).

Elucidation of the possible role of DNA methylation in NaCl and osmotic stress responses in plants is only just beginning. On current evidence, it appears that osmotic stress induces wide scale changes in DNA methylation which seem to be targeted to stress responsive genes. Which DNA methylation/demethylation mechanisms are involved is currently unknown, although the CG hypermethylation around transcription start sites and changes in CG methylation at *P5CS* and *delta-OAT* loci suggests a role for MET1 and/or DRM2. Whether the observed modified DNA methylation patterns trigger functional transcriptome changes in response to NaCl or osmotic stress has not yet been determined.

Heavy metals stressors can also cause changes in DNA methylation (Ou et al., 2012). Rice DNA methylation patterns are altered in response to both excesses of the essential heavy metal ion Cu^{2+} and also in response to lower concentrations of the non-essential heavy metal ions Cd^{2+}, Cr^{3+} and Hg^{2+} (Ou et al., 2012). These methylation changes are detected for transposable elements and genes involved in heavy metal stress response, implying a broad change in DNA methylation occurs during heavy metal stress. Interestingly, all the observed changes were at CHG loci, suggesting the involvement of CMT3 in the stress response. Again, the effect of the changes in DNA methylation on the expression of these genes was not determined.

In low relative humidity (LRH) *A. thaliana* reduces stomatal index (SI) and induces CG and CHH methylation of the promoters of two stomatal developmental genes (*SPEECHLESS* and *FAMA*) (Tricker et al., 2012). Both the SI and *SPCH* and *FAMA* expression are unaffected by LRH in the methyltransferase mutants *drm2drm1* and *met1*, whereas *cmt3* displays a wild type response, indicating CG and CHG methylation changes at these loci are
required for the LRH response. Furthermore, increased production of siRNAs mapping to regions upstream of *SPCH* and intergenic to *FAMA* are detected upon LRH treatment and the RdDM mutants, *rdr2* and *dcl3* fail to respond to LRH, firmly implicating the RdDM pathway in the response (Tricker et al., 2012).

The RdDM pathway has also been implicated in restricting retrotransposition triggered by environmental stress. The retrotransposon *ONSEN* is transcriptionally and transpositionally activated in response to heat stress in *A. thaliana* and hyperactivated in RdDM mutants in heat stress, indicating activation of *ONSEN* is repressed by RdDM (Ito et al., 2011). Interestingly, the new *ONSEN* insertions occur predominantly in genes, and can confer heat stress-responsive characteristics on nearby genes, suggesting stress events could lead to genome rearrangements with potential associated benefits or costs of causing novel gene heat-sensitivity (Ito et al., 2011). Intriguingly, siRNAs have recently been shown to be capable of moving across grafts (Dunoyer et al., 2010; Molnar et al., 2010), suggesting the existence of a systemic silencing pathways which could enable plants to regulate DNA methylation in response to stress in tissues distal to the tissue perceiving the stress. Further experiments are required to establish whether this does indeed occur.

In addition to the abiotic stressors described so far, many biotic stressors have been observed to cause changes in DNA methylation in plants. In the example given earlier, tobacco mosaic virus infection associated changes in DNA methylation at the pathogen response gene *NtAlax1* occurred after the increase in gene expression (Wada et al., 2004). Since this publication there have been a number of observations which indicate DNA methylation is important in some pathogen stress responses. Firstly, DNA methylation can inhibit the expression of defence genes. Chemically induced abolition of DNA methylation at the promoter of the disease resistance gene *Xa21G* increases expression of *Xa21G* and confers an increased tolerance to *Xanthomonas oryzae* pv. *Oryzae* (Akimoto et al., 2007). In contrast, demethylation of the *Pib* promoter in *O. sativa* by 5aza-C treatment is correlated with a decrease in gene expression and compromised blast resistance (Wang et al., 1999). Secondly, defence responses include demethylation of endogenous loci (Dowen et al., 2012; A. Yu et al., 2013). The pathogen-derived signature flg22 triggers a derepression of RdDM targets including *AtGP1*, *AtSN1* and the *Onsen* long terminal repeats, concurrent with a downregulation of AGO4, AGO6, POL IV and POLV subunits, IDN2 and MET1 (Yu et al., 2013), suggesting a general repression of the TGS pathways. Activation of *AtSN1* in response to flg22 relies upon ROS1-dependent CHH demethylation (Yu et al., 2013), indicating the depression requires active demethylation in addition to the passive demethylation by TGS repression. The requirement for active demethylation also extends to
the defence gene RMG1 whose promoter is targeted by RdDM and whose activation in response to flg22 is ROS1-dependent (Yu et al., 2013). Growth of the bacterial pathogen Pseudomonas syringae pv. tomato strain DC3000 (Pst, DC) is increased in rosi and inhibited in RdDM mutants, indicating that the identified regulation of defence genes by RdDM repression and ROS1 activity is required for the pathogen response (Yu et al., 2013).

In response to Pst, A. thaliana differentially methylates small regions of the genome, with an enrichment for genic regions (Dowen et al., 2012). This occurs predominately at CG and CHH loci with a significant underrepresentation of CHG, in contrast with the methylation response to heavy metals discussed above which occurred solely at CHG loci (Ou et al., 2012). Given that gene body methylation does not correlate with gene expression (Zilberman et al., 2007), is it interesting to note that gene-body hypomethylation in response to Pst is associated with increased expression, indicating the methylation changes are correlated with transcriptome changes in response to Pst (Dowen et al., 2012). Mutants impaired in CG and CHH methylation display misregulation of these genes in the absence or presence of Pst, over and above the background misregulation (Dowen et al., 2012), further indicating the expression of these genes is regulated by DNA methylation.

The list of stress conditions in which have DNA methylation changes have been observed in plants is now considerable, and includes osmotic (Colaneri and Jones, 2013; Tan, 2010; Zhang et al., 2013), NaCl (Baek et al., 2010; Cao et al., 2011; Choi and Sano, 2007; Dyachenko et al., 2006; Karan et al., 2012; Tan, 2010), temperature (Boyko et al., 2010; Hashida et al., 2006; Steward et al., 2002), nitrogen deficiency (Kou et al., 2011), low humidity (Tricker et al., 2012) heavy metal (Ou et al., 2012; Rahavi et al., 2011) stressors, and a range of biotic stressors (Dowen et al., 2012; Wada et al., 2004, Yu et al., 2013). However, as promoters may be passively methylated at vacant transcription factor binding sites (1.5.4.2) and gene-body DNA methylation is thought to be coupled to transcription (1.5.5.2), it appears that DNA methylation can reflect as well as regulate gene expression.

As we saw above, DNA methylation changes may occur before or after gene expression changes in response to stress, and there have been further observations of DNA methylation changes occurring as a secondary effect of stress-responsive expression changes (Hashida et al., 2006). Therefore, the question of whether changes DNA methylation represent an actual stress response with downstream effects on transcript abundance or are themselves secondary effects caused by changes in gene expression in response to stress requires further investigation in each instance.

In summary, DNA methylation is observed to be dynamic in response to various stress conditions in plants. The response to individual stressors appears to involve particular DNA
methylation/demethylation components as different sequence contexts are affected. Whether these changes in DNA methylation actually regulate the expression of stress responsive genes needs to be determined in each individual case by monitoring DNA methylation and gene expression over time following stress initiation. While examples exist where DNA methylation changes precede gene expression changes, in the majority of cases, the cause and effect relationship between the DNA methylation and gene expression changes has not been established. The importance of DNA methylation in stress responses is suggested by the biotic stress hypersensitivity of plants in which DNA methylation has been chemically reduced. However, such chemical treatments induce widespread demethylation of DNA, preventing analysis of which stress response pathways might be controlled by stress-responsive dynamic DNA methylation.

1.6 DNA methylation in stress imprinting, stress memories and transgenerational stress memories.

Beyond the role for DNA methylation in responding transiently to stress conditions, it’s becoming increasingly clear that modifications to the DNA methylome can be maintained through the plant’s lifespan and into the following generation(s) (Bossdorf et al., 2010; Johannes et al., 2009; Kinoshita et al., 2007; Mathieu et al., 2007; Rangwala et al., 2006; Verhoeven et al., 2010), giving them the potential to encode stress imprints and transgenerational stress memories.

Given that DNA methylation is frequently altered in response to stress, is mitotically heritable, and is linked to transcriptional regulation, there is considerable scope for DNA methylation to encode some of the longer term stress imprints discussed earlier. Unfortunately, many observations of stress-dependent changes in DNA methylation do not include a temporal element. As such, little is known about the persistence of the changes after stress and what contribution DNA methylation makes to stress imprints.

The discovery that DNA methylation patterns can also be inherited meiotically in plants has led to the hypothesis that DNA methylation may act as a epigenetic mark which carries a memory of the stress into the following generation (Boyko and Kovalchuk, 2011; Thellier and Lüttge, 2013). As discussed earlier, chemical alterations to the DNA methylome can be inherited, along with any associated phenotype, the classic example being the inheritance of 5-azaC-induced dwarfism (Kondo et al., 2010). This is in contrast to the observed “resetting” of DNA methylation patterns in mammalian germline development (Feng et al.,
Taraxacum officinale (Dandelion) exposed to low nutrient growth conditions, salt stress, jasmonic acid (JA) application, or SA application all displayed an altered DNA methylation pattern that is largely inherited by the progeny via asexual reproduction (74-92% of changes inherited) (Verhoeven et al., 2010). In some of the stress memories and transgenerational stress memories discussed earlier, DNA methylation changes were also observed in the hypertolerant progeny.

A. thaliana appears to transmit stress memories through alteration in its DNA methylation profile. NaCl stress increases HRF and NaCl tolerance in the progeny with an associated DNA hypermethylation (Boyko et al., 2010). The DNA hypermethylation seems essential for the stress memory as treatment with 5-azaC eliminates the hypermethylation in the progeny of NaCl treated plants and concurrently erases the stress memory (Boyko et al., 2010). NaCl-induced hypermethylation in the progeny is observed in both gene-body and promoter DNA methylation and is enriched at genes involved in regulating chromatin structure (Bilichak et al., 2012). The altered DNA methylation pattern inherited by the progeny seems likely to encode the stress memory as DNA methylation changes broadly correlate with changes in H3K9ac and HK9me2 and gene expression differences between the progeny of NaCl stressed plants and control progeny. Interestingly, the stress memory is dependent upon DCL3, suggesting a role for siRNAs and RdDM (Boyko et al., 2010). Furthermore, a follow up experiment in which salt stressed plants were crossed with untreated plants showed that increased HRF is only observed in progeny in which the maternal plant was NaCl treated (Boyko and Kovalchuk, 2010). Unfortunately, the salt tolerance and DNA methylation of the crosses were not assessed, although it is expected that the increased HRF, increased salt tolerance and DNA hypermethylation will co-segregate. Given that RdDM is responsible for CHH methylation (Matzke et al., 2009) and CHH methylation is lost in the paternal germline shortly before fertilisation (Calarco et al., 2012), a reasonable postulation would be that the NaCl stress memory involves DCL3-dependent CHH hypermethylation which is maintained in the female gamete and passed onto the following generation.

RdDM has also been implicated in transgenerational responses to biotic stress in A. thaliana. Unlike wild type plants, nrpd2a,b and dcl2,3,4 knock-out mutants do not display an increased tolerance in the progeny of plants subjected to herbivory or MeJA application (Rasmann et al., 2012a), suggesting RdDM is required to lay down a memory of the parental JA response. Additionally, in the absence of parental stress, the A. thaliana cmt3 drm2 drm1 (cdd) mutant displays an increased tolerance to Pst. relative to wild-type which appears similar to the previously described transgenerational stress memory in response to Pst.
challenge in *A. thaliana* (Luna et al., 2012). As the increased SA-induced PR-1 expression in the progeny of *Pst.* challenged plants is also observed in the *cdd* mutant in the absence of parental stress (Luna et al., 2012), DNA hypomethylation in *cdd* is proposed to mimic the DNA methylome of *Pst.* challenged progeny.

Nitrogen deficiency stress in *O. sativa* induces DNA methylation changes which are inherited by approximately 50% of the progeny (Kou et al., 2011). This new DNA methylation pattern then becomes stable, being inherited by 100% of the following generation. This occurs with modifications to cytosine methylation in CG, CHG and CHH contexts, a surprising observation given that CHH methylation requires *de novo* methylation of the daughter strand following cell division (Cao and Jacobsen, 2002b) and CHH methylation is considered to be less meiotically heritable (Calarco et al., 2012). Crucially, the altered DNA methylome two generations after the stress treatment is correlated with an increased tolerance to nitrogen deficiency, with only those plants displaying the modified DNA methylation pattern also displaying an increased tolerance (Kou et al., 2011).

Although the regions of the *O. sativa* DNA methylome modified by nitrogen deficiency have not yet been determined, these results strongly indicate that the modified DNA methylome induced by nitrogen deficiency encodes a transgenerational stress memory. The previously described transgenerational stress memory in response to heavy metal stress in *O. sativa* is also observed to be concurrent with DNA methylation changes in the progeny (Ou et al., 2012), suggesting DNA methylation may be a general mechanism by which *O. sativa* encodes stress memories.

In summary, a few studies have attempted to understand the underlying biochemical basis for the increased tolerance in the progeny of stressed plants by identifying genomic regions which are differentially methylated in the progeny of stress plants. There is now a considerable body of evidence that DNA methylation patterns are responsive to stress and stress-modified methylomes can be inherited. Furthermore, RdDM components have been implicated in some stress memories (Boyko et al., 2010) and transgenerational stress memories (Luna et al., 2012; Rasmann et al., 2012a) and nitrogen-deficiency in rice is associated with a stress memory two generations later in which stress tolerant and non-stress tolerant fall into two distinct cohorts with regard to their DNA methylation pattern (Kou et al., 2011). Unfortunately, DNA methylation does not show a simple linear relationship with gene expression (Colaneri and Jones, 2013; Zilberman et al., 2007). Thus, in many instances, differences in DNA methylation are observed in the progeny but it is not possible to infer the downstream effects on gene expression and ultimately which changes in DNA methylation (if any) the increased stress tolerance is dependent upon. As such, whilst some
in the field are of the firm belief that plants do pass on a memory of stress to the following generation via epigenetic mechanisms (Boyko and Kovalchuk, 2011), there are also those who argue that further detailed and well-designed experiments examining such mechanisms are required to “[move] from interesting observations towards robust evidence” (Pecinka and Mittelsten Scheid, 2012).

1.7 How could inherited changes in DNA methylation increase stress tolerance in the progeny?

As all the molecular functions of DNA methylation identified to date involve regulation of the transcriptome, it is reasonable to expect that if changes in DNA methylation do indeed transmit a stress memory, they will enhance stress tolerance by modifying the transcriptome.

One could postulate two possible routes by which DNA methylation could enhance stress tolerance of the progeny by modifying the transcriptome (Figure 2). Both mechanisms involve at least partial inheritance of the stress-induced changes in DNA methylation through to G2 plants in which the altered epigenome changes the transcriptome. However, the mechanisms differ in how the altered epigenome exerts its stress tolerance benefits. In mechanism 1, the basal transcriptome is altered in G2 such that the plants are “stress adjusted”, whereby they display an altered expression of stress responsive genes in non-stress conditions, reducing the initial shock of stress and reducing stress sensitivity. In mechanism 2, the G2 plants are “stress primed” such that the transcriptional response to stress is altered, enabling a more rapid and/or more effective response to stress. These models are not mutually exclusive and the expression of some loci may be affected in the G2 plants both in the absence of stress and upon subsequent stress treatment.

Currently, there have been few studies characterising the transcriptome component of a transgenerational stress memory from which we can assess the relative contribution of these two mechanisms. In most published transgenerational stress memories, the hypertolerant progeny do not display alter growth traits in non-stress conditions, suggesting mechanism 2 is more likely. However, the second generation progeny of P.lanceolata plants grown under low nutrient conditions do display altered plant growth traits under control conditions (Case et al., 1996), and the progeny of cold treated A.thaliana show a reduced fitness in favourable conditions (Blödner et al., 2007), both of which may be due to the parental treatment modifying the basal transcriptome of the offspring. Providing more direct evidence for mechanism 1, it appears gene expression is affected in the progeny of NaCl-treated
A. thaliana under control conditions and involves the hypermethylation and reduced expression of chromatin modifying genes (Bilichak et al., 2012) although the inheritance of these transcriptome alterations over an untreated generation was not examined. On the other hand, biotic stress memories appear to involve increased expression of defence genes in response to stress (Pastor et al., 2013; Slaughter et al., 2012), in line with mechanism 2. Transgenerational responses to biotic stress appears to involve a similar stress priming, as the second generation progeny of PstDC3000 challenged plants display an increased expression of PR-1 in response to SA, with no change in basal expression (Luna et al., 2012).
Mechanisms for an epigenetic transgenerational stress memory

Figure 2. Possible mechanism by which stress-induced changes in DNA methylation could enhance stress tolerance in plants two generations after the stress treatment. 

$G_0$ plants subjected to stress treatment which induces modification of the DNA methylome. The DNA methylome is then inherited by the following generations, with the stress-induced changes at least partially maintained. In the $G_2$ plants, two mechanisms are proposed. Mechanism 1 invokes a stress primed state for the progeny of stress plants, in which the inherited changes in the DNA methylome produce a mild stress-like transcriptome in the absence of stress, reducing the shock of a subsequent stress treatment and/or reducing sensitivity to the stress treatment.

Mechanism 2 proposes that the altered DNA methylome manifests itself in an altered transcriptional response to the stress, increasing the speed of response and/or responding to the stress in a more effective manner. The two mechanisms are not mutually exclusive.
1.8 Concluding remarks

It is becoming increasingly clear that plants possess mechanisms to enhance the tolerance of their progeny in response to various stressful environmental conditions. Where this stress memory is only observed in the immediate progeny, it is conceivable that this involves loading the seed with transcripts, lipids or metabolites to enhance stress tolerance. However, transgenerational stress memories observed two or more generations after the original stress treatment requires information flow between the generations which can be stably maintained.

Evidence outlined in this Chapter leads to the hypothesis that transgenerational stress memories in plants are encoded by modified DNA methylation patterns. DNA methylation is dynamic in response to stress, it can be maintained through mitosis and meiosis, and it can exert transcriptional control over nearby genomic regions and perhaps regulate alternative splicing.

As discussed above, although an increasing body of evidence supports the hypothesis that DNA methylation transmits transgenerational stress memories, there are no published experiments which directly link a stress memory to a particular DNA methylation event(s). Until a stress memory is shown to be dependent upon DNA methylation modifications at specific genomic region(s), and the downstream effects of these specific changes in DNA methylation established, the hypothesis that DNA methylation can encode stress memories in plants will remain unproven.

1.9 Research aims

The aims of this research project were to further our understanding of how transgenerational stress memories can enhance stress tolerances, and to establish whether changes in DNA methylation can transmit stress memories in the model plant species, *Arabidopsis thaliana*. The hypothesis was that transgenerational stress memories are dependent upon stress-induced changes in DNA methylation that are inherited into the following generations. This altered DNA methylome was expected to increase stress tolerance in the second generation progeny by modifying the transcriptome as discussed in section 1.7.

Two parallel approaches were taken (Figure 3). One approach was to start by analysing DNA methylation changes during stress and then to establish whether the altered DNA
methylome was inherited by the progeny. The results of this approach are discussed in Chapter 3.

In a second approach, stress conditions were screened to identify conditions which generate an increased stress tolerance in the following generations. The results from the second approach, including the identification and characterisation of a novel transgenerational stress memory are discussed in Chapter 4. For both approaches, a wide range of stress conditions were examined, including conditions that had previously been shown to generate either a change in DNA methylation, or a transgenerational stress memory.

Ideally one of these approaches would have identified conditions which resulted in a transgenerational stress memory with an associated change in DNA methylation. The inheritance of DNA methylation could then be examined to establish if all stress-induced changes in DNA methylation were inherited and whether the inheritance was variable between individually treated plants. Following this, a transcriptome analysis could then be utilised to identify difference in the transcriptomes of the second generation progeny of stressed plants. This would enable interrogation of the relative contribution of the two mechanisms proposed in section 1.7, as well as identifying genomic regions for further DNA methylation analysis.

Unfortunately, no stress treatment was observed to generate a transgenerational stress memory with an associated change in DNA methylation. Therefore, the transcriptome analysis was performed on the progeny of plants displaying the novel transgenerational stress memory detailed in Chapter 4. The transcriptomics analysis and follow-up biochemical analysis of the transgenerational stress memory progeny are discussed in Chapter 5.

Finally, Chapter 6 provides an overall discussion of the results and identifies possible future directions. The methods employed in Chapters 3-5 are detailed in Chapter 2.
Figure 3. **Experimental plan by two parallel approaches.** Both approaches start with a screen of suitable stress conditions to identify either heritable DNA methylation changes or a transgenerational stress memory. If either approach is successful the final stage is a transcriptomic analysis of the hypertolerant G₂ plants.
Chapter 2. Methods
2.1 Plant cultivation and husbandry

The general *A. thaliana* cultivation, husbandry and seed collections methods are detailed below. Details of stress treatments and stress tolerance experiments are in section 2.6. *A. thaliana* was cultivated in soil, on agar-plates and in hydroponics. Unless otherwise specified the ecotype used in all experiments was Col-0. Col-0 seeds were obtained from The Nottingham Arabidopsis Stock Centre (NASC). All experiments were conducted on seeds derived from a single self-fertilised plant to ensure experimental plants were isogenic. Fresh Col-0 seeds were sown every 6 months to maintain a collection of fresh isogenic seeds for experimentation.

2.1.1 Soil

Cultivation of *A. thaliana* in soil was carried out in line with the protocols outlined by The European Arabidopsis Stock Centre (http://arabidopsis.info/). Unik Paks with 5, 15, or 0 cavities were filled with a 5:2:1 mixture of potting soil, vermiculite and perlite and treated with Intercept™ (Bayer) according to the manufacturer’s instructions. Three seeds were sown in the centre of each cavity before stratification (4 °C in the dark for 48 hours), after which trays were placed in a growth room or glass house. Growth room relative humidity was kept between 0-30%, with temperature between 18-22 °C and light (~ 80 µmol) supplied from above for 16 hours a day. Green house temperature was maintained between 17-24 °C, with light levels maintained at a minimum of 100 µmol for 16 hours a day. Soil moisture was maintained by watering trays from below twice daily. After 7-10 days, plants were thinned to 1 per cavity plug by removing the outermost seedlings.

Two methods were used to collect seeds from soil cultivated plants. The first method involved tying inflorescence stems loosely to vertical supports approximately 2 weeks after initiation of bolting. Upon observation of the first fully dried silique, the above ground portion of the plant was contained within a glassine bag and water was restricted once approximately 90% of siliques were fully dried. Glassine bags containing dried plant material were excised from the plant and massaged to shatter siliques for seed collection (section 2.2).

The second method utilised microperforated cellophane (360 holes/inch², Focus Packaging & Design) tubes (48 mm in diameter, 300 mm in length, sealed at on end) which were taped to a plastic plant label and placed over the plant 21 days post stratification (dps). Upon
observation of the first fully dried silique, the tube was tied approximately 50 mm above the base of the inflorescence stem. Water was restricted once approximately 90% of siliques were fully dried. Once all siliques were fully dried, the inflorescence material was excised from the plant and the seeds collected as described above.

### 2.1.2 Agar-plate

Seeds were surface sterilised by vapour phase sterilisation as described by Clough and Bent (Clough and Bent, 1998). Briefly, seeds were placed into racked 1.5 ml microcentrifuge tubes which were left open and placed inside a sealable container in a fume hood. Concentrated HCl (3 ml) was added to 100 ml commercial bleach in a beaker placed inside the container which was immediately sealed. Seeds were incubated in the resulting chlorine gas for 3 hours before the container was opened and the microcentrifuge tubes immediately sealed. Sterilised seeds were sown directly onto square petri plates containing ATS media (section 2.8.1) (Lincoln et al., 1990) solidified with 1% BacTo™ Agar in a sterile environment and sealed with micropore tape. Plates were held vertical in racks and stratified (4 °C in the dark for 48 hours) before being placed a growth room or glasshouse as described above.

### 2.1.3 Hydroponics

Hydroponic tanks were constructed consisting of 1 L plastic boxes made opaque with commercial black tape into which 10 equally spaced 7 mm holes were punched into the lid. For the cultivation of plants in hydroponics, plants were sown onto agar-plates as described above. Fourteen dps (approximately 13 days after germination) seedlings were transferred into hydroponic tanks containing 900 ml ATS. More plants than required in a particular experiment were transferred to hydroponic tanks (10 % excess) so that plants which displayed signs of wilting within 48 hours could be removed and replaced with healthy plants. No obvious relationship between plant growth traits at transfer and the likelihood to display signs of wilting after transfer to hydroponics were observed. The ATS was replenished every 7 days. Siliques were removed once they became dry and they were stored in glassine bags. Once sufficient quantities of siliques were collected, seeds were separated from the siliques by gentle disruption and sieving before air drying and storage as detailed below.
2.2 Seed storage

Seeds collected from both soil and hydroponic cultivated plants were separated from the dry reproductive material by sieving. For storage, seeds were transferred to 1.75 ml glass vials and left at room temperature to air-dry for 14 days, after which the cap was screwed on. Where sufficient quantities of seed were collected (i.e. for identification of stress memories), the moisture content of seeds was measured before seed storage. Approximately 100 mg of seeds were weighed in a 1.75 ml glass vial and heated to 85 °C for 24 hours. Weight post-heating was recorded and used to calculate the moisture content of the seeds using the formula:

\[
% \text{ Moisture Content} = 100 \times \frac{\text{(wet weight} - \text{dry weight})}{\text{wet weight}}
\]

The measurement was repeated twice for each treatment group. Where moisture content exceeded 6%, seeds were air-dried for a further 7 days and the moisture content re-measured. Labelled seed vials were stored in the dark at room temperature.

2.3 General molecular biology

2.3.1 Nucleic acid extractions and analyses

2.3.1.1 DNA extraction

DNA was extracted with a DNeasy Plant Mini Kit according to the manufacturer’s protocol. Briefly, plant tissue (rosettes, roots or whole seedlings) weighing approximately 200 µg was ground to a fine powder in liquid nitrogen in a 1.5 ml microcentrifuge tube, to which extraction buffer and RNase A were added. Successive column-based steps to remove detergent, proteins, and polysaccharides from the lysate were followed by isopropanol precipitation of the DNA onto a silica column from which it was eluted with deionised water. All DNA samples were analysed by agarose gel electrophoresis to inspect DNA for degradation and analysed on a NanoDrop 2000/8000 (Thermo Scientific) to establish the concentration of the sample. Samples were stored at 4 °C (<1 month) or at -20 °C (> 1 month).
1.1.1.1 RNA extraction

RNA extraction was based on the Trizol method (Chomczynski and Sacchi, 2006). Approximately 100 mg of plant tissue was ground to a fine powder in liquid nitrogen in a microcentrifuge tube, taking care not to allow the tissue to thaw, before addition of 1 ml Trizol reagent and vigorous mixing. After 3 minutes incubation, 0.2 ml chloroform was added and the tube gently mixed and inverted for 3 minutes. Samples were then centrifuged for 5 min at ≥ 20 000 g relative centrifugal force (RCF). The upper aqueous layer (~500 µl) was removed to a fresh microcentrifuge tube and 500 µl isopropanol and 100 µl 3M NaOAc added. Precipitation of RNA was carried out by incubation at room temperature for 5 minutes before centrifugation at ≥ 20 000 g for 20 minutes. The supernatant was removed and the pellet washed with 1 ml 70 % ethanol and air dried for approximately 5 minutes. RNA was dissolved in 50 µl of sterile water. All RNA samples were analysed by agarose gel electrophoresis to inspect for degradation, and analysed on a NanoDrop 2000/8000 to establish the concentration of the sample. Samples were stored at -20 °C (> 1 month) or -80 °C (>1 month). After grinding of plant material, the extraction was performed on ice where possible and centrifugation steps were performed at 4 °C.

2.4 Generation of promoter::GUS lines

8 genes were selected for promoter activity analysis: DRM2 (AT2G33830), DCL3 (AT3G43920), RDR2 (AT4G11130), NRPD1 (AT1G63020), NRPE1 (AT2G40030), CMT3 (AT1G69770), MET1 (AT5G49160) and ROS1 (AT2G36490). Promoters were defined as the region upstream of the gene transcription start site up to the translated region of the next upstream gene, up a maximum of 3 kb. Promoter regions were amplified from genomic DNA by PCR with PfuUltra™ II fusion which is optimised for amplifying long sequences with a high fidelity. PCR primers were designed with Primer3 (http://primer3.ut.ee/).

PCR was performed by addition of the following to 0.5 µl Col-0 DNA template: 1 units PfuUltra DNA polymerase, 5 µM dNTPs, 10 nM 3’ primer, 10 nM 5’ primer and sterile water to 20 µl, with 1 X PfuUltra buffer. Cycling parameters were dependent upon primer annealing temperatures (see Table 13). The PCR program was:
Chapter 2 Methods

- 94 °C - 1 min
- 94°C - 30 s
- Annealing temperature (See Table 13) - 30 s
- 72 °C - 30 s
- 72°C - 5 min
- 4 °C – Hold

Cycle 40 times

PCR products were purified from a 1 % agarose gel using the Gel Extraction Kit (GenoCruz™) and sequenced to confirm successful amplification of target promoter sequences. Attempts were made to clone all promoter fragments into the Gateway® pENTR™ entry vector (see below). Where this was unsuccessful, promoter fragments were cloned into the pCXGUS-P binary vector (section 2.4.3).

2.4.1 Biochemistry

2.4.1.1 Ferric reductase activity assay

Root ferric reductase activity was quantified using the method described by Yi and Guerinot (1996). Seeds were sown onto ATS-agar and transferred at 7 dps onto ATS-supplemented with varying containing concentrations of zinc. At 14 dps, root material from a whole plate of seedlings (30 plants) was pooled together, weighed and added to 1 ml assay solution comprising 100 µM Fe(III)-EDTA, 300 µM FerroZine in distilled water. Following 20 minutes incubation in the dark, the root material was removed and the absorbance at 562 nm measured with a Ultraspec 2000 Spectrophotomoter (Pharma Biotech) zeroed at 750 nm. An aliquot of assay solution containing no plants acted as a blank. The concentration of reduced iron (Fe(II)-EDTA) was determined using the molecular extinction coefficient of 28.6 mM/cm. Testing for statistical significance was carried out in SPSS using a 2-way ANOVA.

2.4.1.2 Chloroform extraction

Chloroform extraction was performed based on the method described by Porra et al (1989) using the aerial portion of the same seedlings from which root material was removed for the ferric reductase assay above. Aerial tissue was submerged in 4 ml DMF and incubated overnight in the dark at 4 °C. The DMF was then removed and stored in the dark at 4 °C whilst a second extraction in 4 ml DMF for 6 hours was performed. The two 5 ml portions
were then pooled and absorbance determined at 647 nm and 664 nm with an Ultraspec 2000 Spectrophotometer (Pharma Biotech) zeroed at 750 nm and DMF-resistant UV cuvettes. Total chlorophyll (A & B) content was calculated using the equation: Chlorophyll = 17.67 A\textsuperscript{647} + 7.12 A\textsuperscript{664}. Testing for statistical significance was carried out in SPSS using a 2-way ANOVA.

2.4.2 Cloning with the Gateway\textsuperscript{®} system

To enable directional cloning into the pENTR™ entry vector, the forward PCR primers contained the sequence, CACC, at the 5' end of the primer which then base pair with the overhang sequence, GTGG, in the pENTR™ vector. The following promoters were cloned into the pENTR™ entry vector using the pENTR™/SD/DD-TOPO® cloning kit according to the manufacturer’s instruction: pNRPE1(POLV), pCMT3, pDRM2 and pRDR2.

A transformation mixture was prepared consisting of 0.5 µl gel purified PCR product, 0.5 µl salt solution , 0.5 µl sterile deionised water and 0.5 µl vector and incubated on ice for 10 minutes. The transformation mixture was added to a vial of One Shot\textsuperscript{®} TOP10 E. coli cells and incubated for 30 min on ice. Transformation was achieved by heat-shocking the cells for 30 seconds at 42°C without shaking, before immediately transferring to ice. The cells were then diluted in Super Optimal Broth (S.O.C) medium (Hanahan, 1983) and incubated at 37°C for 1 hour with shaking. Cells were then spread on Lysogeny Broth (LB) (Bertani, 1951) - agar plates containing 50 µg/ml kanamycin, and incubated overnight at 37°C.

Colonies were examined by PCR to identify transformants containing the insert in the correct orientation. A pipette tip was used to pick colonies and add them to a reaction mixture containing 1 units Taq DNA polymerase, 5 µM dNTPs, 10 nM M13 forward primer , 10 nM 5' primer and sterile water to 20 µl, with 1 X Standard Taq buffer. Cycling parameters were dependent upon primer annealing temperatures (see Table 13). The PCR program was:

- 94 °C - 1 min
- 94° C - 30 s
- Annealing temperature (see Table 13) - 30 s
- 72 °C - 30 s
- 72° C - 5 min
- 4 °C – Hold

Cycle 30 times
Positive colonies were then picked and cultured overnight in 3 ml LB medium containing 50 µg/ml kanamycin before isolation of plasmid by QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer’s instructions. Plasmids were then analysed by PCR as above with 0.5 µl of plasmid preparation used as template and analysed by restriction digest to confirm the presence and orientation of the insert.

Inserts were transferred to the Gateway™ binary vector pBGWFS7 which contains the GUS gene (Xiao et al., 2010) by performing a recombination reaction with the Gateway® LR Clonase™ II Enzyme Mix kit (Invitrogen) according to the manufacturer’s instructions. 50 µl of Library Efficiency® DH5α™ competent cells was incubated with the LR reaction mixture and heat-shock transformed as described above. After incubation at 37 °C for 1 hour, the cells were spread onto LB-agar plates containing 100 µg/ml spectinomycin. Positive colonies were identified by colony PCR as above, before culturing in 3ml LB medium containing 100 µg/ml spectinomycin for isolation of plasmid by QIAprep Spin Miniprep Kit according to the manufacturer’s instructions.

2.4.3 Amplification of promoter sequences and cloning into pCXGUS-P

Promoter sequences for pNRPD1(POLIV), pROS1 and pMET1 were incorporated into a pCXGUS-P binary T-vector according to the method described by Chen (2009). Briefly, a ligation mixture was prepared composed of 50 ng of the T-vector and the corresponding concentration of A-tailed PCR product to give a 6:1 molar ratio of insert:vector, with 20 units T4 DNA ligase in 10 µl total volume. The ligation mixture was transformed into 50 µl Library Efficiency® DH5α™ competent cells by the heat shock method described above. After incubation at 37 °C for 1 h, the cells were spread onto LB-agar containing 50 µg/µl kanamycin. Positive colonies were identified by colony PCR as described above, before isolation of plasmid by QIAprep Spin Miniprep Kit and restriction digest and plasmid sequencing to confirm insertion and direction of insert.

2.4.4 Transformation of Agrobacterium tumefaciens

Regardless of cloning procedure, successful cloning into the binary vector (pBGWFS7 or pCXGUS-P) was followed by transformation into Agrobacterium tumefaciens strain GV3101. Competent A. tumefaciens were prepared as described by Höfgen and Willmitzer (1988). Transformation was achieved by electroporation as described by Weigel and Glazebrook (2006). Briefly, pre-prepared frozen 500 µl aliquots of competent A. tumefaciens were thawed on ice, mixed with 1 µl binary vector and incubated on ice for 5
min. The cells and binary vector were then transferred to an electroporation cuvette and electroporated in a Gene Pulser apparatus (Bio-Rad) at 25 mF and 2500 V with resistance set to 200 ohms. The cells were then diluted in 1 ml LB medium, transferred to a 1.5 ml microcentrifuge tube and incubated at 28 °C for 2 h. Cells were spread onto LB containing 100 µg/ml spectinomycin (pBGWFS7) or 50 µg/ml kanamycin (pCXGUS-P) for selection. Positive colonies were identified by colony PCR as described above.

### 2.4.5 Transformation Arabidopsis thaliana

*Arabidopsis thaliana* was transformed with the positive colonies by the *Agrobacterium tumefaciens*-mediated floral dip method described by Clough and Bent (1998). Briefly, GV3101 harbouring a binary vector was cultured overnight in 3 ml LB with selective antibiotics at 28 ºC with shaking. An aliquot of 100 µl was removed and added to 500 ml LB with selective antibiotics and incubated at 28 ºC with shaking overnight. An infiltration mixture was prepared by harvesting cells by centrifugation at 5 000 g for 10 min before resuspending in 500 ml 5% sucrose, 0.05% Silwet L-77 in a beaker. The aerial portion of *A.thaliana* plants with primary bolts clipped and secondary bolts of approximately 2-10 cm were submerged in the infiltration mixture with gentle agitation for approximately 5 s. Dipped plants were covered to maintain humidity and left in a low light location overnight before being uncovered and returned to the greenhouse. Seeds were harvested by the glassine method (section 2.1.1).

### 2.4.6 Selection of representative lines

Seeds were collected from dipped plants and grown under selective conditions to identify progeny containing the insertion. Selection of plants transformed with the Gateway® expression vector was carried out by sowing seeds in soil at a density of approximately 0.1-1 per mm² across a seed tray insert. After 7 days, trays were sprayed with Basta (phosphinothricin) solution 120 mg/L three times over a period of 7 days. Plants which survived the Basta treatments and appeared healthy were then potted on and self-fertilised before seeds collection. Segregation analysis was carried out in the following generation to identify plants which produced progeny with a 3:1 ratio of Basta resistant:Basta sensitive as these will only contain the resistance gene on one chromosome and are therefore more likely to possess a single insertion. Segregation analysis was performed by Basta to identify lines segregating 3:1 for resistance (Exact Binomial Test conducted in R). Where the null hypothesis that the line segregated 3:1 was not rejected ($p>0.05$), the plants were self-fertilised to identify offspring which were homozygous for the Basta resistance trait. A
minimum of 6 independent transformants were examined by GUS staining (see section 2.4.7) before a single transformant was selected as possessing a representative expression of GUS. This line was then used for all experiments to investigate promoter activity. Selection of representative transformants from plants transformed with the pCXGUS-P vector was carried out in an identical manner except that hygromycin selection was used. Hygromycin selection was performed by sowing sterilised seeds on ATS-agar supplemented with 50 µg/ml hygromycin. At14 dps, plants displaying healthy root growth were transferred to ATS-agar and cultivated until sufficiently large to transfer to soil for self-fertilisation and seed collection.

2.4.7 GUS staining protocol

The method for visually inspecting GUS activity was adapted from the method described by Kobayashi (2007). Plant tissue (rosette, root or whole seedlings) were fixed in 3.7% formaldehyde in pH 7.0 phosphate buffer (0.1 M NaH$_2$PO$_4$, 0.1 M Na$_2$HPO$_4$) for 30 min before washing in several changes of ice cold phosphate buffer for 30 min. Tissues were then submerged in 1.5 ml GUS staining solution (10 mM EDTA, 0.1 % Triton-X-100, 1 mM K$_3$Fe(CN)$_6$, 2.0 mM X-Gluc, 0.1 M NaPO$_4$, pH 7.0 made to volume with sterile deionised water) in a 2 ml microcentrifuge tube and vacuum infiltrated for 5 min. Vacuum infiltration was repeated for tissue samples that did not sink. Samples were then incubated at 37 °C for 24 h to allow the GUS protein to convert colourless X-Gluc (5-bromo-4-chloro-3-indolyl β-Dglucuronide) to 5-br-4-Cl-3-indolyl which undergoes subsequent oxidation to form an insoluble indigo final product. GUS stained plant tissue was washed in distilled water and taken through a series of ethanol washes by incubation in 20%, 35% and 50% ethanol for 30 min each. Following this, tissues were fixed for 30 min in FAA (50% Ethanol, 3.7% Formaldehyde, 5% acetic acid, sterile water to volume) for 30 min at room temperature. A second ethanol series followed involving incubation in 70% (overnight), then 80% and 90% (1 h each) ethanol, before a final overnight clearance in 1.5 ml 5:2 (w:v) chloral hydrate, glycerol solution. Chloral hydrate cleared tissues were then mounted on slides for microscope imaging. A 35S::GUS line (donated by Leyser, O) was used as a positive control and WT Col-0 used as a negative control for each GUS staining procedure. The exception to this protocol was the GUS staining of the pMET:GUS line which was performed with a 1 h incubation step as the activity of the promoter was far in excess of the other promoters examined.
2.5 DNA methylation analysis

DNA methylation was analysed by bisulphite sequencing and Methylation-Sensitive Amplified Polymorphism (MSAP).

2.5.1 Methylation Sensitive Amplified Polymorphism

MSAP protocols were based previously published methods (Cervera et al., 2002; Kageyama et al., 2008). Two protocols were developed based on the AseI and HpaII/MspI and AseI and MboI/Sau3AI/DpnII restriction endonuclease combinations.

2.5.1.1 AseI and HpaII/MspI MSAP

DNA samples were digested with AseI and HpaII, and AseI and MspI in parallel. Digestion and ligation were carried out simultaneously having established that sequential and simultaneous digestion and ligation steps produced the same band profile. The following were added to 100 ng DNA: 10 units AseI, 10 units HpaII or MspI, 20 units T4 DNA ligase, 62.5 pM HpaII/MspI oligonucleotide adapter, 12.5 pM AseI oligonucleotide adapter and sterile water to 20 µl, with 1 X T4 DNA ligase buffer. The oligonucleotide adapters were prepared by adding equal quantities of 100 µM adapter oligonucleotides 1 and 2 (Table 13) and heating to 90 ºC for 1 minute before bringing back down to 25 ºC over 15 min. Digestion and ligation were carried out at 37 ºC for 6 hours before denaturation of enzymes at 65 ºC for 10 min. Ligated samples were then diluted by addition of 180 µl sterile water. Pre-selective PCR was performed by addition of the following to 1 µl diluted ligation sample: 1 units Taq DNA polymerase, 5 µM dNTPs, 15 nM AseI +1 primer, 15 nM HpaII/MspI +1 primer and sterile water to 20 µl, with 1 X Standard Taq buffer. Cycling parameters were dependent upon primer annealing temperatures (See Table 13). Pre-selective program was:

- 72 ºC - 2 min
- 94 ºC - 1 min
- 94 ºC - 30 s
- Annealing temperature (see Table 13) - 30 s
- 72 ºC - 30 s
- 72ºC - 5 min
- 4 ºC - Hold

Cycle 20 times
Pre-selective PCR samples were analysed by agarose gel electrophoresis to examine amplification before dilution by addition of 180 µl sterile water. Selective PCR was performed by addition of the following to 1 µl pre-selective PCR sample: 1 units Taq DNA polymerase, 5 µM each dNTPs, 5 nM AseI +2 primer, 15 nM HpaII/MspI +3 primer, and sterile water to 20 µl with 1 X standard taq buffer. AseI +2 primers were labelled with ARD700 or FAM for polyacrylamide gel electrophoresis (PGE) or capillary electrophoresis (CE) analysis respectively. Cycling parameters were dependent upon primer annealing temperatures (see Table 13). Selective program was:

- 94 °C – 60 s
- 94°C - 30 s
- (Annealing temperature +10 °C) – 0.5 °C/cycle - 30 s
- 72 °C - 30 s
- 94°C - 30 s
- Annealing temperature - 30 s
- 72 °C - 30 s
- 72°C - 5 min

Selective PCR samples were analysed by agarose gel electrophoresis to examine amplification before 1:10 dilution with sterile water.

PGE was performed with a Li-COR 4200L according to the manufacturer’s instructions. Polyacrylamide gels (6%) were prepared by addition of 200 µl ammonium persulfate to 5 ml of SequaFLOWGel Complete Buffer and 20 ml of SequaFLOWGel XR Monomer Solution which was mixed and immediately dispensed between glass plates held between rail assemblies. Running buffer comprised 0.8 x TBE (section 2.8.4). A buffer tank was attached to the top of the polyacrylamide gel cassette and loading buffer added to the tanks above and below the gel. A pre-run was carried out for each gel to optimise the focal plane of the detection microscope. Loading buffer (2.8.5) was then thawed on ice and added to diluted samples (10-fold dilution in sterile water) which were heated to 90 °C for 5 min to denature them. Loading buffer was removed from the top tank to below the level of the gel. Ficoll (1.5 ml) was then added to the top of the gel and the samples comb-loaded onto the gel, before gentle replacement of the running buffer. Samples were run for 1 min before removal of the comb. Samples were then run for 2 h and fragments detected by excitation of the ARD700-labelled AseI +2 primer as the sample passed an excitation laser. IRDye700 50-700 Sizing Standard samples were run at either end of the gel. Fragment
presence/absence scores were obtained by visual inspection of the gel images and tabulated accordingly.

Capillary electrophoresis analysis was performed with an ABI 3130XL. 0.3 µl of GeneScan™ 600 Liz Size standard was added to 0.7µl of 20-fold diluted sample before loading on a 96-well plate and analysis on the ABI 3130XL according to the manufacturer’s instructions. Fragments were detected by excitation of the FAM-labelled AseI +2 primer. Data analysis was performed in GeneMapper® Software v3.7. The peak sizes of each sample were automatically determined based on a 2nd-order least squares size calling curve using the retention times for the size standard peaks. Total peak intensities for each individual sample were normalised across the experiment.

2.5.1.2 AseI and MboI/DpnII/Sau3AI MSAP

The AseI and MboI MSAP assay was performed in an identical manner to the 1.3.1.1 AseI and HpaII/MspI MSAP assay described above with the following exceptions. As ligation of adapter oligos onto MboI, DpnII and Sau3AI reconstitutes the cleavage sites, digestion and ligation steps were performed separately. The following were added to 100 ng DNA: 10 units AseI, 10 units MboI or DpnII or Sau3AI, and sterile water to 10 µl, with 1 X T4 DNA ligase buffer, with incubation at 37 °C for 6 h before denaturation of enzymes at 65 °C for 10 min. Ligation was achieved by addition of 20 units T4 DNA ligase, 62.5 pM MboI/DpnII/Sau3AI oligonucleotide adapter, 12.5 pM AseI oligonucleotide adapter and sterile water to 10 µl, with 1 X T4 DNA ligase buffer. Ligation was carried out at 4°C overnight before denaturation of enzymes at 65 °C for 10 min. Ligated samples were then amplified as described above except that HpaII/MspI primers were replaced with MboI/DpnII/Sau3AI primers.

2.5.2 Bisulphite sequencing

Bisulphite sequencing to detect DNA methylation involves conversion of unmethylated cytosine to uracil in the presence of the bisulphite ion (HSO\textsuperscript{3}⁻), with methylated cytosine remaining unconverted. Unmethylated cytosines are displayed as thymines in the resulting amplified sequence of the sense strand whereas methylated cytosine are displayed as cytosines.
2.5.2.1 Bisulphite conversion

To prepare genomic DNA for bisulphite conversion, a restriction digestion was performed to reduce the complexity of the genomic DNA. DNA was digested overnight with ApaI due to the absence of its recognition sites (GGGCCC) from the regions which were sequenced. Bisulphite conversion was performed with the EZ DNA Methylation-Lightning™ Kit (Zymo Research) according to the manufacturer’s instructions. Briefly, 130 µl of Lightning Conversion Reagent was added to 100 ng of DNA sample in a PCR tube and mixed. Samples were heated to 98°C for 8 min, followed by 54°C for 60 min. Samples were then bound to silica-based column and washed to remove reagents before DNA desulphonated on-column for 20 min at room temperature, followed by a further wash step to remove reagents. Samples were eluted in 20 µl sterile deionised water and stored at -20 °C.

2.5.2.2 Bisulphite PCR

Primers for bisulphite sequencing were manually designed to amplify the sense strand with minimal incorporation of cytosine into the 3’ primer and guanine into the 5’ primer. To ensure that full conversion of cytosines had occurred, bisulphite sequencing was performed on a 157 bp sequence of the chloroplast PsaA promoter which is not methylated and therefore all cytosine within this region should be converted to thymines (Finn et al., 2011). For bisulphite PCR reactions, the following were added to 0.5 µl of bisulphite treated DNA sample: 1 unit Taq DNA polymerase, 5 µM dNTPs, 10 nM 3’ primer, 10 nM 5’ primer and sterile water to 20 µl, with 1 X Standard Taq buffer. Cycling parameters were dependent upon primer annealing temperatures (See Table 13 below). PCR program:

- 94 °C - 1 min
- 94°C - 30 s
- Annealing temperature (see Table 13) - 30 s
- 72 °C - 30 s
- 72°C - 5 min
- 4 °C - Hold

Cycle 40 times

Where no product of the expected size was observed, the PCR was repeated with 0.5 µl of the first PCR reaction used as the template for an identical second reaction. Sequence reads for the PsaA fragment indicated that 100% (21/21) of cytosines in this region were converted, indicating that the bisulphite treatment was successful.
2.5.2.3 Sequencing

Bisulphite PCR samples were sequenced by the Genomics Laboratory within the Technology facility at the University of York. Samples were analysed on a ABI Prism 3130XL. Resulting Seq files were inspected in Chromas. The underlying methylation status of each cytosine within the region amplified was determined by visually comparing the cytosine and thymine peak height ratio (C:T) resulting from the incorporation of these nucleotides: 

- C:T > 3:1 = unmethylated,
- C:T < 1:3 = methylated,
- 3:1 < C:T > 1:3 = partially methylated

2.6 Stress treatments

Stress treatments were used for two purposes: Screening for conditions which generated either a change in DNA methylation or a transgenerational stress memory and examining stress tolerance in the progeny of plants subjected to stress treatments. All experiments were conducted with *A. thaliana* ecotype Col-0 unless otherwise specified. Stress treatments were either initiated from germination onwards or at a set number of days after the end of stratification. Where stress treatments were conducted to examine stress tolerance in the progeny, parental plants self-fertilised and seeds were collected from individual plants separately. A summary of the stress treatments and F1 generation stress tolerance testing is given in Table 1.

2.6.1 Stress treatments for screening

2.6.1.1 Screening stress conditions for DNA methylation changes

NaCl, drought, PEG, sorbitol and temperature stress treatments were performed to examine DNA methylation in response to stress. These treatments are detailed below. The DNA methylation profile of zinc stressed plants was also examined. The zinc stress treatment was performed as part of the screen for transgenerational stress memories. This experiment in detailed in section 2.6.2.4.
### Table 1. Summary of the stress treatments and the stress tolerance carried out in the following generation

For each F0 treatment, the conditions tested are described, including the cultivation method, the number of plants in each treatment group, and the developmental stage during which the stress treatment occurred. The F1 stress tolerance experiments were identical for all F1 progeny from each F0 treatment group. The statistical tests employed to identify significant difference between control and stress progeny are listed.

<table>
<thead>
<tr>
<th>F0 treatment</th>
<th>Cultivation</th>
<th>Number of plants</th>
<th>Developmental stage</th>
<th>F1 stress tolerance testing (for all F1 progeny in F0 treatment group)</th>
<th>Statistical test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nitrogen deficiency</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 mM NO₃ (Control)</td>
<td>Agar</td>
<td>40</td>
<td>0-21 dps</td>
<td>Fresh weight: 10 plants on agar: 9 mM or 1.8 mM NO₃ from 0-14 dps (x 6 repeats). Seed weight: 10 plants in hydroponics. Transferred to tanks containing 9 mM, 1.8 mM or 0.9 mM NO₃ from 21 dps (x 6 repeats).</td>
<td>2-way ANOVA</td>
</tr>
<tr>
<td>1.8 mM NO₃</td>
<td>Agar</td>
<td>40</td>
<td>0-21 dps</td>
<td>Seed weight: 10 plants in hydroponics.</td>
<td>2-way ANOVA</td>
</tr>
<tr>
<td>9 mM NO₃ (Control)</td>
<td>Hydroponics</td>
<td>30</td>
<td>&gt;21 dps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.8 mM NO₃</td>
<td>Hydroponics</td>
<td>30</td>
<td>&gt;21 dps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9 mM NO₃</td>
<td>Hydroponics</td>
<td>20</td>
<td>&gt;21 dps</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Temperature extremes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 °C</td>
<td>Soil</td>
<td>40</td>
<td>&gt;21 dps</td>
<td>Fresh weight: 20 plants in soil at 12, 22 or 34 °C for 0-21 dps (x 4 repeats). Seed weight: 20 plants in soil at 12, 22 or 34 °C from germination (x 4 repeats).</td>
<td>2-way ANOVA</td>
</tr>
<tr>
<td>22 °C (Control)</td>
<td>Soil</td>
<td>40</td>
<td>&gt;21 dps</td>
<td></td>
<td>2-way ANOVA</td>
</tr>
<tr>
<td>24 °C</td>
<td>Soil</td>
<td>40</td>
<td>&gt;21 dps</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Elevated NaCl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM NaCl (Control)</td>
<td>Agar</td>
<td>40</td>
<td>0-21 dps</td>
<td>Germination: 100 seeds on agar: 0, 50, 100, 150, 200 mM NaCl for 0-14 dps (x 4 repeats). Root growth: 30 plants transferred at 7 days to 0, 150, 175 mM NaCl. Growth measured 5 days.</td>
<td>Student's t-test with multiple testing correction and G-test. NA</td>
</tr>
<tr>
<td>100 mM NaCl</td>
<td>Agar</td>
<td>40</td>
<td>0-21 dps</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Low water potential</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control + 15% PEG</td>
<td>Agar</td>
<td>40</td>
<td>0-21 dps</td>
<td>Germination: 50 seeds on agar: 5, 10, 15, 20, 25% PEG for 0-14 dps (x 4 repeats).</td>
<td>Student's t-test with multiple testing correction and G-test. NA</td>
</tr>
<tr>
<td><strong>Elevated Zinc</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM ZnSO₄ (Control)</td>
<td>Agar</td>
<td>40</td>
<td>0-10 dps</td>
<td>Fresh weight: 50 seeds on agar: 1 µM, 25 µM or 500 µM ZnSO₄ from 0-10 dps (x 4 repeats).</td>
<td>2-way ANOVA</td>
</tr>
<tr>
<td>500 µM ZnSO₄</td>
<td>Agar</td>
<td>40</td>
<td>0-10 dps</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>1 mM ZnSO₄</td>
<td>Agar</td>
<td>40</td>
<td>0-10 dps</td>
<td>Developmental progression: 30 plants on agar: 1µM, 50 µM, 250 µM, 500 µM ZnSO₄ from 3 leaf stage for 13 days.</td>
<td>2-way ANOVA</td>
</tr>
<tr>
<td><strong>Elevated Zinc (Repeat experiment)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 µM ZnSO₄ (Control)</td>
<td>Agar</td>
<td>80</td>
<td>0-10 dps</td>
<td>Developmental progression: 30 plants on agar: supplemented with 50-500 µM, 500 µM Cu or 100 mM NaCl from 0-14 days.</td>
<td>Randomised block design ANOVA</td>
</tr>
<tr>
<td>500 µM ZnSO₄</td>
<td>Agar</td>
<td>80</td>
<td>0-10 dps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM ZnSO₄</td>
<td>Agar</td>
<td>80</td>
<td>0-10 dps</td>
<td>Fresh weight: 10 plants in hydroponics. 1 µM, 50 µM or 500 µM ZnSO₄ from 21-35 days (x 4 repeats).</td>
<td></td>
</tr>
</tbody>
</table>

For each F0 treatment, the conditions tested are described, including the cultivation method, the number of plants in each treatment group, and the developmental stage during which the stress treatment occurred. The F1 stress tolerance experiments were identical for all F1 progeny from each F0 treatment group. The statistical tests employed to identify significant difference between control and stress progeny are listed.
2.6.1.1.1  **NaCl and drought in soil**

Twenty one dps, watering was ceased for 4 trays of 40 plants each. Three trays were then watered from below with 25, 50 or 100 mM NaCl with the 4th tray remaining unwatered to affect drought stress. As water retention was greater in cavities in the middle of the tray, 1-5ml of sterile water was added daily to the outermost cavities to maintain more equal soil moisture across the tray for the drought stress. An additional tray of plants were watered throughout to provide a control group. At 35 dps, rosette tissue was excised from 10 random plants in each tray and snap frozen in liquid nitrogen for extraction of DNA (section 2.3.1.1) for MSAP analysis (section 2.5.1).

2.6.1.1.2  **NaCl, PEG and sorbitol in hydroponics**

Plants were transferred to hydroponics at 14 dps and cultivated on liquid ATS media as outlined in 2.1.3. Seven days later, ATS was removed and replaced with ATS supplemented with 150 or 200 mM NaCl or 5%, 10% or 15% PEG for 1 tank each (10 plants). One tank was supplied with ATS not supplemented with NaCl or PEG as a control group. The maximum quantum yield of photosystem II (Fv/Fm) was measured daily with a Pocket PEA portable chlorophyll fluorimeter as a measure of stress. Treatments proceeded until a decrease in Fv/Fm of stress plants was observed relative to control plants. NaCl treatments lasted for 48 hours, whilst PEG treatments lasted for 7 days. Rosette and root tissues were separately pooled for each treatment group and snap frozen for DNA extraction (section 2.3.1.1) for MSAP analysis (section 2.5.1). For MSAP analysis of the roots of individual plants subjected to 5% PEG stress, the above 5% PEG stress treatment protocol was carried out and DNA extracted from root tissue from each plant separately after 7 days. For the comparison of osmotic stress induced by PEG, sorbitol and sterilised PEG stress, the above 5% PEG stress treatment was performed with 2 additional tanks. One tank was treated with ATS supplemented with sufficient sorbitol to induce an identical osmotic stress (as measured by osmometer measurements of the solutions). The second tank was supplied with a sterile solution of ATS supplemented with 5% PEG.

2.6.1.1.3  **Temperature stress**

Three trays of 40 plants each were grown as described in 2.1.1. At 21 dps, the trays were placed in Sanyo MLR-352 growth cabinets set at 12 °C (cold), 22 °C (control) or 34 °C (hot). Light intensity for all cabinets was set at ~ 120 μmol, with a 16 hour photoperiod. At 35 dps, Fv/Fm was measured with a portable chlorophyll fluorimeter and the rosette
diameter was measured for 10 random plants in each tray. Rosette tissue was then excised from 20 random plants in each tray and snap frozen in liquid nitrogen for extraction of DNA (section 2.3.1.1) for MSAP analysis (section 2.5.1).

2.6.1.2 Screening stress conditions for induction of a transgenerational stress memory

2.6.1.2.1 Nitrogen deficiency stress

Plants were subjected to nitrogen deficiency stress on agar-plates (2.1.2) and hydroponics cultivation (2.1.3). For nitrogen deficiency stress on agar-plates, 40 seeds each were sown onto ATS-agar with nitrogen content in the media altered to 9 mM NO$_3$ (control) or 1.8 mM NO$_3$ (nitrogen deficient) (see section 2.8). At 21 dps, seedlings were transferred to soil and cultivated as described in 2.1.1. with seeds collected by the glassine bag method. Seeds were collected separately from the 80 plants.

For nitrogen deficiency stress in hydroponics, plants were cultivated as described in (2.1.3). 21 dps, ATS was removed and replaced with ATS with nitrogen content adjusted to 9 mM NO$_3$ (control), 1.8 mM or 0.9 mM NO$_3$ (nitrogen deficiency) for one tank each (10 plants). ATS with adjusted nitrogen content was refreshed every 7 days and siliques collected as described in (2.1.3). Seeds were collected separately from the 30 plants.

2.6.1.2.2 Temperature stress

Plants were cultivated as described in 2.6.1.1.3 except rosette tissue was not removed and plants were maintained in the control (23 °C), cold (12 °C) and hot (34 °C) temperature conditions through to seed collection by the cellophane method (2.1.1).

2.6.1.2.3 NaCl stress

Plants were subjected to NaCl stress on ATS-agar (2.1.2). Forty seeds were sown onto ATS-agar supplemented with 100 mM NaCl, with an additional 40 seeds sown onto ATS-agar as a control group. At 21 dps, seedlings were transferred to soil and cultivated as described in 2.1.1. with seeds collected by the glassine bag method. Seeds were collected separately from the 80 lines and labelled as C 1-40 (control) and N 1-40 (NaCl). A single seed from each of the 40 NaCl lines was subjected to a NaCl stress and control treatment as described above, with collected seeds denoted as NN 1-40 (NaCl, NaCl) or NC 1-40 (NaCl, control). A single
seed from each of the 40 control lines was taken through a second control treatment, with collected seeds labelled as CC 1-40 (control, control). An additional experiment was conducted with the C24 ecotypes following the treatment outlined by Boyko et al (2010). Briefly, the above experimental plan was followed with \( \frac{1}{2} \) Murashige & Skoog (MS) media (see section 2.8) (Murashige and Skoog, 1962) used in place of ATS and NaCl stress induced by addition of 75 mM NaCl, with 80 plants in each treatment group. Twenty one dps, rosette tissue was excised from 10 random plants in each tray and snap frozen in liquid nitrogen for extraction of DNA (section 2.3.1.1) for bisulphite sequencing (section 2.5.2).

Seeds were collected separately from the 160 lines. An equal portion of the seeds from the two treatment groups were pooled and sown onto soil (section 2.1.1). Twenty one dps, rosette tissue was excised from 10 random plants in each tray and snap frozen in liquid nitrogen for extraction of DNA (section 2.3.1.1) for bisulphite sequencing (section 2.5.2).

2.6.1.2.4  Drought stress

Drought stress was simulated by addition of PEG to ATS-agar to reduce water potential. PEG-infused ATS-agar plates were prepared as described by Verslues (2006). Briefly, 40 ml ATS-agar in a square petri plate was overlaid with 40 ml ATS containing 30% PEG 6000 and placed in the dark at 4 °C to allow the PEG to equilibrate between the solidified and liquid ATS. After 48 h the ATS-PEG solution was removed. Water potential measurement of the ATS-PEG solution before and after incubation on top of the ATS-agar confirmed that equilibrium was reached within this time period. 40 seeds were sown onto ATS-agar-PEG plates. At 21 dps, plants were transferred to soil and cultivated as described in 2.1.1, with seeds collected by the glassine bag method. Seeds were collected separately from the 40 lines and labelled P 1-40 (PEG). The initial PEG and NaCl stress experiments were conducted at the same time, therefore the C 1-40 seed lines acted as control for both NaCl and PEG stress experiments.

2.6.1.2.5  Zinc stress

Plants were subjected to zinc stress in ATS-agar cultivation (2.1.2). Forty seeds each were sown onto ATS-agar supplemented with 500 \( \mu \)M ZnSO\(_{4}\) or 1mM ZnSO\(_{4}\). Forty seeds were sown onto ATS-agar without supplemented zinc (ATS contains 1 \( \mu \)M ZnSO\(_{4}\)) as a control. At 10 dps, seedlings from both treatment groups were transferred onto ATS-agar and at 21 dps, seedlings were transferred into soil and placed in a growth room and seeds collected by the glassine method. Seeds were collected separately from the 120 lines and labelled as Zn(1) 1-40 (control), Zn(500) 1-40 (500 \( \mu \)M Zn\(^{2+}\)) or Zn(1000) 1-40 (1 mM Zn\(^{2+}\)). A single
seed from each of the 40 Zn(500) lines was subjected to each of 500 μM Zn$^{2+}$ stress and control treatment as described above, with collected seeds denoted as ZZ 1-40 (zinc, zinc) or ZC 1-40 (zinc, control). A single seed from each of the 40 control lines was taken through a second control treatment, with collected seeds labelled as Zn(1)CC 1-40 (control, control). As a repeat of the initial stress treatment, 80 seeds each were sown onto ATS-agar supplemented with 500 μM ZnSO$_4$ and ATS-agar (control). Seedlings were transferred at 10 dps to trays of soil and placed in a glasshouse. Seeds were collected separately from the 160 lines by the cellophane method and labelled Zn(500)$^2$ 1-80 and Control$^2$ 1-80.

### 2.6.2 Stress treatments for examination of stress tolerance

Unless otherwise specified, stress treatments for the examination of stress tolerance were conducted on pooled seeds. These were generated by pooling an equal quantity of seeds from each plant for each treatment group. Statistical tests applied are specified for each stress treatment in Table 1.

#### 2.6.2.1 Nitrogen deficiency stress

The progeny of plants subjected to nitrogen deficiency stress were sown onto ATS-agar with nitrogen adjusted to 9 mM NO$_3$ or 0.9 mM NO$_3$ as described in 2.6.1.2.1. Ten plants per parental treatment were sown onto a plate of 9 mM NO$_3$ (high) or 0.9 mM NO$_3$ (low) conditions. Fourteen dps, the fresh weight of the 10 seedlings was recorded. The progeny were also cultivated hydroponically (2.1.3) on liquid ATS media with nitrogen adjusted to 9 mM (high) NO$_3$, 1.8 mM NO$_3$ (medium) or 0.9 mM NO$_3$ (low) as described in 2.6.1.2.1. Inflorescence stems were covered with a glassine bag once the first silique had dried, and the total seed produced by each plant weighed once production of siliques had ceased. Average seed weight per plant was then calculated for each parental treatment at each concentration of NO$_3$. Both experiments were repeated 6 times.

#### 2.6.2.2 Temperature stress

Forty seeds each from the progeny of temperature stressed plants were sown onto soil and cultivated in growth cabinets set at 12 °C, 22°C and 34°C as described in section 2.6.1.1.3. At 21 dps, the fresh aerial weight of 20 plants was recorded per parental treatment per growth condition. Seeds were collected from each plant individually by the cellophane method and weighed. This experiment was repeated 4 times. Drought stress...
Fifty seeds from the progeny of drought stressed and non-stressed plants were sown onto ATS-agar supplemented with 4-16% PEG as described in section 2.6.1.2.4. Germination frequency was recorded at least once every 24 hours for 14 days by noting radicle emergence. The experiment was repeated 4 times.

2.6.2.3 NaCl stress

One hundred seeds from the progeny of NaCl stressed and non-stressed Col-0 plants were sown onto ATS-agar supplemented with 50-200 mM NaCl. Germination frequency was monitored as described above, with 4 repeat experiments. Thirty seeds from the progeny of NaCl stressed and non-stressed Col-0 plants were also sown onto ATS-agar and transferred 7 dps onto ATS-agar supplemented with 150 mM or 175 mM NaCl or control ATS-agar. Root growth was recorded 48 h later. The germination of NaCl stressed progeny of the C24 ecotype was monitored as described above for the Col-0 ecotype; 50 seeds from both treatment groups were sown onto ATS supplemented with 0-150 mM NaCl, with 4 repeat experiments.

2.6.2.4 Zinc stress

The stress tolerance of the progeny of zinc stressed plants was examined by measuring plant fresh weight, root growth and developmental progression in ATS agar, and by measuring plant fresh weight in hydroponics. Plant fresh weight under zinc stress in ATS-agar was examined by sowing Zn(500), Zn(1000) and Control seed (50 each) onto ATS-agar supplemented with 0µM, 250 µM and 500 µM Zn²⁺. Ten dps, the fresh weight of the seedlings was recorded. The experiment was repeated 6 times.

For the examination of stress tolerance in the second generation, 50 seeds each of CC, ZC and ZZ were sown onto ATS-agar supplemented with 0-500 µM Zn²⁺ and the seedling weight recorded 10 dps, with 5 repeat experiments. For the examination of stress tolerance in the individual CC and ZC lines, 34 seeds each from 17 CC and 17 ZC lines were sown onto ATS-agar supplemented with 500 µM Zn²⁺. Plant weight was measured 14 dps.

For the examination of stress tolerance in the progeny of plants subjected to the repeat zinc stress, 100 seeds each of Zn(500)² and control² were sown onto ATS-agar supplemented with 0-1000 µM Zn²⁺ and the fresh weight measured 10 dps.
Chapter 2 Methods

As a screen for possible general heavy metal stress tolerance of the progeny of zinc stressed plants, 50 Zn(500) and Control seeds were each sown onto ATS-agar supplemented with 50-500 mM of NiSO₄, CuSO₄, CoSO₄ or CsCl and the seedling weight recorded 10 dps. As plant weight was higher for Zn(500) in 50, 100 and 500 µM Ni, 500 µM Co and 100 mM NaCl, the experiment was then repeated 3 times with ATS-media supplemented with these concentrations of Ni, Co and NaCl.

Zinc stress tolerance was also examined in the Zn(500)² and Control² plants in hydroponics. Forty plants each of Zn(500)² and Control² were cultivated by hydroponics (8 tanks of 10 plants each, section2.1.3). At 21 dps, ATS was replaced with ATS supplemented with 0, 50, 250 or 500 µM Zn²⁺. Thirty five dps, root and aerial root portions from each tank were weighed. As considerable variation was observed in the plant weight between repeat experiment the experiment was repeated 9 times. To examine the general heavy stress tolerance of Zn(500)², the hydroponics stress tolerance experiment was repeated with ATS-agar supplemented with 250 µM Zn²⁺, 100 µM Ni, 50 µM Cd, 50 µM Co.

To measure root growth under zinc stress, 120 Zn(500), Zn(1000) and Control seeds were sown onto ATS-agar and at 7 dps the seedlings were transferred onto ATS-agar supplemented with 0, 50, 250 and 500 µM Zn²⁺. Root growth was measured 7 days later. The experiment was repeated 3 times.

To monitor developmental progression under zinc stress, Zn(500), Zn(1000) and Control seeds were sown onto ATS-agar and the seedlings transferred to ATS-agar supplemented with 0, 50, 250 and 500 µM Zn²⁺ upon emergence of the first two rosette leaves. The developmental stage of the seedlings was then recorded for the next 13 days by counting rosette leaves. For each parental treatment and growth condition 3 plates of 10 plants each were monitored.

2.6.3 Statistical tests applied to identify parental effects

The statistical tests that were applied to test for effects of parental stress treatment on the stress tolerance in the F1 progeny are listed in Table 1. In all cases, the null hypothesis was that parental stress treatment had no effect on stress tolerance measurements in the progeny. The alternative hypothesis was that parental treatment does affect the stress tolerance in the progeny. All statistical tests were designed to identify significant changes in either direction, i.e. 2-tailed. In most cases a two-way analysis of variance (2-way ANOVA) with Tukey post hoc testing was conducted in SPSS, with the categorical variables being the F0 parental
treatment and F1 stress tolerance conditions, and the dependent variable being the measurement of stress tolerance, e.g. plant fresh weight. Where significant contributions of the parental treatment were observed (p<0.05) these are reported.

As considerable inter-experimental variation was observed when measuring fresh weight in plants subjected to zinc stress from 21-35 dps, an alternative ANOVA test was applied. Here, statistical testing was conducted using a randomised block design ANOVA to enable testing for a parental effect on fresh weight against a background of experimental-to-experiment variation (Lew, 2007).

To identify significant effects of parental stress on F1 progeny germination under stress, two statistical tests were applied. To identify significant effects of parental treatment on the number of germinated seeds after 14 days in stress conditions, the results were analysed with a G-test with a Holm-Bonferroni correction for multiple testing. The G-test is similar to a chi-squared test in that it is a likelihood ratio test suitable for testing for significant deviations from the expected frequency, however it is preferable to the widely used chi-squared test as the chi-squared test involves approximations to avoid the calculation of log-likelihood values and is therefore less precise. Germination frequency was recorded at least once every 24 hours. Hence it was also possible to compare germination rates. A 5-parameter logistics curve of the equation \( F(x) = A + \frac{D}{1+(X/C)^B}^E \) was fitted to the germination frequency data with the online ReaderFit tool (ReaderFit.com) where A is the minimum asymptote, B is the Hill slope, C is the inflection point, D is the maximum asymptote and E is the asymmetry factor. The time taken for 50% of final germination frequency to be reached (G50) was calculated by extracting parameters from the curve and entering them into the formula:

\[
G50 = C((2^{(1/E)} - 1)^{(1/B)})
\]

Germination rates were compared for the parental treatment groups by student’s t-test, with a Holm-Bonferroni correction applied to correct for the multiple conditions tested in the F1 progeny.
2.7 RNA-Seq and Quantitative reverse transcriptase-PCR

2.7.1 RNA extraction for RNA-Seq and qRT-PCR

RNA extraction for RNA-Seq and qRT-PCR was performed with the RNeasy Plant Mini Kit according to the supplier instructions to purify RNAs >200 nucleotides in length. Briefly, approximately 100 µg of plant tissue was placed in a microcentrifuge tube and ground to a fine powder in liquid nitrogen. Lysis was achieved with a highly denaturing guanidine thiocyanate and β-Mercaptoethanol-based buffer before homogenisation of the material via a shredder column. RNA was precipitated and bound to a silica-based column membrane followed by DNase treatment of the RNA, multiple wash steps and, finally, elution in 50 µl sterile water. Samples were analysed and stored as described in 1.1.1.1.

2.7.2 RNA-Seq

RNA samples for RNA-Seq were prepared from seedlings (CC and ZC; section 2.6.1.2.5) 10 dps following germination on ATS-agar supplemented with 0 or 500 µM Zn²⁺ (Control and 500 µM Zn). RNA-Seq was performed with a single replicate for each parental treatment grown under each condition. For each RNA-Seq sample, RNA was separately extracted from 3 batches of seedlings (Control = 37-40 plants per batch, 500 µM Zn = 100-113 plants per batch) and pooled in equal measure. RNA-Seq samples are referred to as Mock(C), Mock(Z), Zinc(C) and Zinc(Z) to denote whether they received a zinc stress treatment in their parental treatment (Mock = CC, Zinc = ZC) and the conditions the G2 progeny were grown under for transcriptome analysis (C = control, Z = zinc stress). 5µg of each RNA-Seq sample was sent to the University of Exeter Sequencing Service for analysis by Illumina HiSeq 2500. RNA samples were examined in house for contamination by proteins and salts by nanodrop 2000/8000, and by analysis with a 2100 Bioanalyser (Agilent) at the University of Exeter. Library preparation from polyA mRNA-enriched cDNA was carried out by the University of Exeter with the ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre). Fragments in the range of 150-350 were sequenced. All 4 samples were analysed in a single lane of an Illumina HiSeq 2500 for 100 bp paired-end sequencing.

2.7.3 Bioinformatics

Filtering and trimming of reads was conducted by the University of Exeter using the FastqMcf sequence quality filter, clipper and processor to remove low quality reads. Reads
were trimmed from the 3’ end to remove bases with Phred scores below 20 (<99% base calling accuracy). After trimming, reads less than 20 bp were discarded. Reads with average Phred scores below 25 were also removed.

The remainder of the bioinformatics was conducted by the author via the web-based Galaxy server using the Tuxedo suite (Trapnell et al., 2012). Arabidopsis thaliana reference genome and gene annotations (TAIR10) were downloaded from illumina’s iGenomes website (http://support.illumina.com/sequencing/sequencing_software/igenome.ilmn). For each sample, filtered reads were mapped onto the reference genome with the Tophat tool using the default parameters except where specified below. A description of the parameter is included in brackets:

- -r (mean inner distance between mate pairs) 150
- --mate-std-dev (standard distribution for the distribution on inner distance between paired reads) 200
- -a (minimum anchor length) 20
- -i (minimum intron length) 50
- -I (maximum anchor length) 50000
- --library-type (specifies the RNA-Seq protocol) fr-unstranded
- --no-coverage-search (disables the coverage based search for exons)
- -G (Supply Tophat with a set of gene model annotation as a .GTF file)
- --no-novel-juncs (only look for reads across junctions in the supplied .GTF file)

Quantification of transcript abundance was performed with Cuffdiff using the “accepted hits” .bam files from Tophat and the reference annotation .GTF file. Cuffdiff analysis was performed with default parameters except as specified below:

- -N (Normalises by the upper quartile of the number of fragments mapping to individual loci instead of the total number of sequenced fragments. This can improve robustness of differential expression calls for less abundant genes and transcripts (Bullard et al., 2010; Dillies et al., 2012))

Transcript abundances were outputted as “Fragments Per Kilobase exon model per Million mapped fragment” (FPKM) although this is somewhat misleading given the parameters used here. As the denominator in the normalisation was the upper quartile of the number of fragments mapping to an individual loci rather than the total number of fragments mapped to
either the genome or the gene annotations, the correct unit for transcript abundance would be “Fragments Per Kilobase exon model per upper quartile of fragments mapping to an individual loci”. As the upper quartile of the number of fragments mapping to an individual loci will be less than the total number of mapped fragments, the denominator for normalisation is lower, hence the FPKM values are higher than if normalisation was performed against the total number of fragments.

2.7.4 Identification of differentially regulated genes

As RNA-Seq was not performed with repeats, variance of transcript abundance could not be estimated. Therefore to identify genes with differential expression between two samples an alternative approach was taken. The fold-differences between gene expression in the control samples (Mock(C) and Zinc(C)) was calculated for all genes to estimate the distribution of gene expression differences between the two samples. Relationships were observed between the expression and length of a gene and the absolute value of the fold difference between the two control samples; on average shorter or lowly expressed a gene displayed a higher absolute fold difference. As this was assumed to be due to a relationship between the accuracy of transcript abundance quantification and the length and expression of a gene, lowly expressed or short genes were removed from the analysis. Thresholds for inclusion were set as FPKM values greater than 0.01 in both samples and an average FPKM greater than 1, and length in excess of 256 bp. Genes with a low expression value (14501) were removed from the analysis. Essentially, the abundance cannot be accurately quantified for these genes as it is close to 0. A further 80 short genes were removed, leaving 18697 remaining genes. To remove these relationships entirely, curves were fitted to the correlations and the curve parameters used to adjust the fold difference of each individual gene towards the mean based on its length and average expression across the control samples. After these adjustments, no correlation existed between the length or expression of a gene and the fold difference between the control samples.

For each gene the null hypothesis is that its expression should not be different between the two control samples. It was expected that this null hypothesis would be correct for the vast majority of genes. The distribution of log$_2$ fold-differences for the control samples therefore represents the biological variation, analytical random error and inaccuracies in the quantification of transcript abundance. Genes which are statistically outside of this distribution will likely be differentially regulated. Post adjustment, the distribution of fold differences between the two samples was observed to be normal ($p>0.05$, Two-sample Kolmogorov-Smirnov test). The average log$_2$ fold-difference between the two control
samples was calculated as -0.046, with a standard deviation of 0.077. Z-scores were then calculated for each gene, based on the formula: \( z = (x - \mu) / \sigma \), where \( x \) is the fold difference value for an individual gene, \( \mu \) is the mean of the distribution and \( \sigma \) is the standard deviation of the distribution.

To calculate Z-scores for the genes in other pairwise comparisons of samples, e.g. Mock(C) and Mock(Z), the same adjustment was performed to remove the relationship between gene expression or length and absolute log₂ fold difference between the samples. The mean of the distribution of log₂ fold differences was then calculated. Z-scores were calculated with \( \mu \) as the mean of the distribution of log₂ fold differences for the samples being compared, and the \( \sigma \) being the standard deviation calculated above in the comparison of the two control samples. This was because a \( \sigma \) value calculated from the distribution of log₂ fold difference between two samples where large numbers of genes are expected to change expression would be inflated by these gene expression changes and would no longer only represent the variation occurring from the sources mentioned above.

To select genes which were differentially expressed between the two control samples, a conservative Z-score threshold of +/- 5 was applied, equivalent to a p-value of <5.8 x 10⁻⁷. For comparison, a Bonferroni correction (Abdi, 2007) with an \( \alpha \) value of 0.05 and 18697 comparisons gives a corrected \( \alpha \) of 2.7 x 10⁻⁶.

### 2.7.5 Gene Ontology enrichment analysis

Gene ontology analysis was performed with Gene Ontologizer (http://compbio.charite.de/contao/index.php/ontologizer2.html). Gene ontology terms and terms relationships were downloaded from TAIR (ftp://ftp.arabidopsis.org/home/tair/Ontologies/). The frequency of ontology terms within a gene set was compared to frequencies in the genome population to identify enriched terms. Model-based Gene Set Analysis (MGSA) analysis (Bauer et al., 2010) within Gene Ontologizer was employed to analyse all enriched terms and identify the likely active terms by probabilistic inference. The advantage of this approach over term-for-term analysis is that it returns a much smaller set of active categories which explain the enriched terms. A marginal posterior probability cut off of 0.5 was employed for identification of active categories, as recommended by Bauer (Bauer et al., 2010).
RNA-Seq samples used for quantitative Reverse-Transcriptase PCR (qRT-PCR) were prepared as described in 2.7.1. For the validation of the RNA-Seq results, the same RNA samples were used, such that for each parental treatment in each F2 growth condition, 3 biological replicate RNA samples were analysed. Generation of complimentary DNA (cDNA) was carried out with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. To 200 ng RNA sample, 500 µg Oligo(dT)$_{12,18}$ and 1 µM 10 mM dNTP were added in a total volume of 12 µl made up with sterile deionised water. The mixture was heated to 65 °C for 5 min and chilled on ice before addition of 2 µl 0.1 M DTT and 4 µl 5X buffer and incubation at 42 °C for 2 min. 1 µl Superscript RT was then added with gentle mixing by pipette and incubation at 42 °C for 50 min. Inactivation of Superscript RT was achieved by heating the reaction to 70 °C for 15 min.

qRT-PCR primers for were designed using Primer Express® v3.0.1 (Applied Biosystems) with the supplier’s recommended primer parameters for SYBR green qRT-PCR: Theoretical melting temperature (Tm) between 58-60 °C, 20-80 % GC content, optimal length of 20 nucleotides and the 5’ end not to contain greater than 2 G/Cs. In addition amplicons were also designed to span at least one intron to avoid amplification of genomic DNA.

qRT-PCR was performed on an ABI Prism 7000 Sequence Detection Systems using 50 ng cDNA, 12.5 µl SYBR® Green I PCR Master Mix, 1 µl each of 3’ and 5’ primers (10 µM) and sterile deionised water to 5 µl. All primers were first tested on a pooled sample of cDNA in a dilution series to test for linearity between the cycle at which the threshold of fluorescence was reached and the cDNA concentration. This was followed by a melting curve to confirm that only one amplicon was produced. Where primers failed to show linearity and a single product, new primers were designed for the target mRNA.

qRT-PCT was performed using the triplicate cDNA samples that were pooled for RNA-Seq, with 3 technical replicates each. For all samples, 3 normalisation genes were analysed on the same plate. 2 normalisation genes were selected based on their previously observed stability with increased metal concentrations (Remans et al., 2008) (AT5G15710, AT2G28390) and an inspection of the RNA-Seq data which confirmed that three genes are stable over the zinc stress treatment. The third gene (AT5G14030) was chosen based on its apparent stability in the RNA-Seq data and its stability across the perturbations available in Genevestigator (https://www.genevestigator.com/gv/).
For each amplicon, the fluorescence threshold was set automatically and the quantification carried out in Excel. The number of cycles taken to reach the threshold (Ct) for a particular gene in a particular sample was transformed into dCT by subtracting the average Ct for the 3 normalisation genes in that sample. The ddCT was then calculated by subtracting the dCT value from the control sample (Mock(C)) for the gene. Final results were either displayed as the fold difference in expression relative to control (ddCT) or by quantification of the expression by calculating $100 \times (2^{\text{dCT}})$.

## 2.8 Recipes

### 2.8.1 ATS medium

ATS medium was prepared as described by Lincoln et al (1990):

- 5 mM KNO$_3$
- 2.5 mM KPO buffer (adjusted to pH 5.5)
- 2 mM MgSO$_4$
- 2 mM Ca(NO$_3$)$_2$
- 50 µM Fe(III)-EDTA
- 70 µM H$_3$BO$_3$
- 14 µM MnCl$_2$
- 0.5 µM CuSO$_4$
- 1 µM ZnSO$_4$
- 0.2 µM Na$_2$MoO$_4$
- 10 µM NaCl
- 0.01 µM CoCl$_2$

Adjustment of nitrogen content in ATS media required alterations to the media. Specifically, KNO$_3$ and Ca(NO$_3$)$_2$ were reduced 5-fold or 10-fold as required with the potassium and calcium replaced by addition of KCl and CaCl$_2$.

### 2.8.2 MS medium

MS medium was prepared using MS salts (PhytoTechnology Laboratories). Full strength MS media contained the following salts and was adjusted to pH 5.8.
• 19 mM KNO₃
• 21 mM NH₄NO₃
• 100 µM H₃BO₃
• 3 mM CaCl
• 0.1 µM CoCl₂
• 0.1 µM CuSO₄
• 100 µM NaEDTA
• 100 µM Fe(II)SO₄
• 1.5 mM MgSO₄
• 100 µM MnSO₄
• 1 µM Na₂MoO₄
• 5 µM KI
• 1.25 mM KPO₄ monobasic
• 30 µM ZnSO₄

2.8.3 Agarose gel electrophoresis loading buffer

Samples analysed by agarose gel electrophoresis were diluted 1:6 in 6X loading buffer comprising:

• 30 % glycerol
• 0.25 % Bromophenol blue
• 0.25 % Xylene blue
• 300 µl/L 0.5 M EDTA

2.8.4 Agarose gel electrophoresis running buffer

Agarose gels were run in 1X TBE buffer. 5X TBE stock comprised:

• 54 g/L Tris base (C₉H₁₇NO₃)
• 27.5 g/L boric acid (H₃BO₃)
• 20 ml/L 0.5 M EDTA (pH 8.0)
2.8.5 Polyacrylamide gel electrophoresis loading buffer

Polyacrylamide gel electrophoresis analysed samples were analysed in 1X loading buffer. 5X loading buffer comprised:

- 10 mM EDTA, pH8.0
- 240 mg/L bromophenol blue
- Made to volume with deionised formamide

5X buffer was stored in 500 µl aliquots at -20 °C.
Chapter 3. DNA methylation as a stress response
As discussed in the introduction there is an increasing amount of interest in transgenerational stress memories in plants, and the possible role of DNA methylation in acting as the mechanism for transgenerational information flow. DNA methylation can regulate gene expression (Baek et al., 2010; Cao and Jacobsen, 2002a; Chan et al., 2004; Johannes et al., 2009; Kakutani, 1997; Kinoshita et al., 2007; Li-Byarlay et al., 2013; Wang et al., 1999) and has been observed to be dynamic in response to stress (Baek et al., 2010; Boyko et al., 2010; Cao et al., 2011; Choi and Sano, 2007; Colaneri and Jones, 2013; Dowen et al., 2012; Dyachenko et al., 2006; Hashida et al., 2006; Karan et al., 2012; Kondo et al., 2010; Kou et al., 2011; Ou et al., 2012; Rahavi et al., 2011; Steward et al., 2002; Tan, 2010; Tricker et al., 2012; Wada et al., 2004; Yu et al., 2013; Zhang et al., 2013). Furthermore, changes to the DNA methylome have been shown to be heritable in plants (Bossdorf et al., 2010; Johannes et al., 2009; Kinoshita et al., 2007; Mathieu et al., 2007; Rangwala et al., 2006; Verhoeven et al., 2010). However, to date, only a few observations of stress memories or transgenerational stress memories have been found to be concurrent with DNA methylation changes in the progeny (Boyko et al., 2010; Ou et al., 2012), and even fewer have linked changes in DNA methylation to transcriptome differences which could enhance the stress tolerance of the progeny (Bilichak et al., 2012). Further questions also remain surrounding which methylation/demethylation pathways are involved in generating the inherited changes in DNA methylation, how stable these changes are across multiple generations and whether their stability can be enhanced by successive generations of stress treatment.

This Chapter will focus on the direct analysis of DNA methylation changes during stress, starting with the development of techniques for examining DNA methylation, and moving on to present the results obtained by utilising these techniques to study a range of stress responses.

3.1 Developing methods to investigate the mechanism for stress-dependent changes in DNA methylation

Bisulphite sequencing is considered the gold standard method for DNA methylation analysis as it provides single base pair resolution. However, it remains time-consuming and requires the experimenter to specify a genomic region of interest. For the analysis of multiple samples and where a genomic region of interest has not been defined, methylation sensitive amplified polymorphism (MSAP) is a more suitable alternative. This assay is based on amplified fragment length polymorphism (AFLP) (Vos et al., 1995) and utilises methylation-sensitive restriction endonucleases to enable analysis of a small fraction of
cytosines across the whole genome. For the purposes of examining DNA methylation changes in response to stress, MSAP is more appropriate than bisulphite sequencing as multiple conditions and individual plants and/or tissues may be analysed within a single gel or capillary electrophoresis run. MSAP was therefore utilised here to screen multiple conditions for changes in DNA methylation, to establish the reproducibility of these changes across individual plants, and to investigate the mechanism of the DNA methylation changes by comparing wild type and single gene knock-out lines. For these purposes, the MSAP assay was first modified as described below to improve the value of the assay output.

Another approach taken to investigate the mechanism of stress-dependent changes in DNA methylation was to generate GUS reporter lines under the control of promoters for genes which may be involved in the DNA methylation changes. Analysing the activities of these promoters in stress conditions will help interrogate the possible role for these genes in the stress-responsive changes in DNA methylation.

### 3.1.1 Methylation sensitive amplified polymorphism assays

MSAP is frequently utilised to identify changes in DNA methylation as a result of stress (Baránek et al., 2009; Karan et al., 2012; Kondo et al., 2010; X. Ou et al., 2012; Tan, 2010; L. Zhong et al., 2009). Commonly, the methylation-sensitive restriction endonucleases, HpaII and MspI are paired with the methylation-insensitive EcoRI (Reyna-López et al., 1997) for MSAP analysis of DNA methylation in plants. The comparison of HpaII and MspI band profiles yields information on both CG and CHG methylation as MspI cannot cleave CCGG sites where the external cytosine is methylated, whereas HpaII cannot cleave CCGG sites where the internal cytosine is methylated but is capable of cleaving CCGG sites where the external cytosine is hemimethylated (McClelland et al., 1994) (Figure 4 and Figure 5).

Frequently, the methylation status of anonymous loci is inferred from the presence/absence scores of bands in the HpaII and MspI profiles (Ou et al., 2012; Tan, 2010). This inference is flawed because the loss or gain of a band may be due to either a methylation or a demethylation event (Figure 5). The implications of this may be considerable. If Figure 5 represented a methylation change in response to stress, the assumed methylation event could lead one to investigate the role of CMT3 and DRM2-dependent methylation in the stress response, whereas the assumed demethylation event could lead one to investigate the role of DNA demethylases. Additionally, EcoRI cleavage of the GAATTC site is blocked by methylation of the cytosine (Brennan et al., 1986), further complicating the inference of methylation status from band presence/absence scores in the EcoRI & HpaII/MspI MSAP assay.
Chapter 3 DNA methylation as a stress response

Figure 4. **Methylation-sensitive amplified polymorphism assay (MSAP).** Genomic DNA is digested by EcoRI & HpaII or EcoRI & MspI, before ligation of adapter oligonucleotides. Two amplification steps (+1 PCR and +3 PCR) produce a subpopulation of fragments which can be analysed by gel electrophoresis or capillary electrophoresis. Band presence or absence will be dependent on restriction site methylation status (Figure 5).

Figure 5. **HpaII and MspI methylation sensitivities and inferring DNA methylation status from MSAP band presence or absence using the isoschizomers.** Top: Isoschizomers methylation sensitivities. MspI will cleave in the absence of DNA methylation or where the internal cytosine is methylated. HpaII will cleave in the absence of DNA methylation or where the external cytosine is hemimethylated. Bottom: Inferring methylation status. Each change in EcoRI & HpaII and MspI & EcoRI band profiles may represent a methylation or demethylation event, depending on whether the band represents a “simple” or “complex” fragment. In the example given, the difference observed between the two samples may be due to a CHG methylation event which prevents MspI cleavage, or a demethylation event which allows MspI cleavage.
To improve the MSAP assay, two approaches were taken; firstly, the original HpaII/MspI assay was modified to enable inference of methylation status based on band presence/absence scores. Secondly, the MSAP assay was completely redeveloped with the intention that the new MSAP assay would enable analysis of CG, CHG and CHH methylation in isolation. The two approaches will be discussed in turn.

3.1.1.1 Modifying the existing HpaII/MspI MSAP assay

Three modifications were proposed for the HpaII/MspI MSAP protocol; firstly, replace EcoRI with AseI, secondly, repeat the final amplification with only the AseI primer and thirdly, generate an additional methylation free template with a whole genome amplification (WGA) step (Figure 6). Replacing EcoRI with AseI ensures only methylation changes at the HpaII/MspI site will affect fragment presence as the AseI site does not contain any cytosines. The “AseI only” amplification identifies bands which have AseI sites at both ends which can therefore be removed from the analysis. When analysed by MSAP, the methylation-free genomic template generated by WGA should produce a band profile equivalent to a genomic DNA sample in which all sites are demethylated. Since the “complex fragments” with internal restriction sites will not appear in the profile of the unmethylated WGA sample, the analysis can focus on only the “simple fragments” by analysing only those fragments which are also present in the WGA sample. Having removed the “AseI only” fragments and restricted the analysis to only the simple fragments, assigning the methylation status based on presence/absence in the HpaII and MspI profiles should be straightforward as each fragment will have only one HpaII/MspI restriction site. In most instances the methylation status of both cytosines can be inferred, the only exception is fragments which are present in the WGA sample but absent from the AseI & HpaII and AseI & MspI profiles (Figure 6). In these fragments the external cytosine is expected to be fully methylated but the methylation status of the internal cytosine cannot be determined.

As a first step in establishing the modified HpaII/MspI MSAP assay, the fragment profiles of 32 primer pairs were analysed by capillary electrophoresis to identify the most suitable primer pairs. An initial analysis at a single annealing temperature (55 ºC) analysed the number of peaks (fragments), the distribution of the peaks across the size range analysed and the number of peaks that were hard to resolve for each primer pair (Table 2). Ideally a primer pair should produce a large number of peaks spread evenly across the size range analysed (50-700 bp), with each peak easily resolved from the surrounding peaks. Shoulder peaks may be difficult to score as their separation from the main peak can change from sample to sample, and are therefore best avoided.
Figure 6. A modified MSAP assay workflow. The original assay workflow is shown in the grey box, with EcoRI having been replaced by AseI. An additional sample is generated by whole genome amplification (WGA) of genomic DNA which is then taken through the digestion and amplification steps. For fragment analysis, bands which are present when performing the final amplification step (+3 PCR) with only the AseI primer are removed. Combining the fragment presence/absence data from the WGA sample with the AseI & MspI and AseI & HpaII profiles, loci methylation status can be precisely assigned in most instances.

The number of peaks which could easily be scored varied considerably between the primer pairs, with the best primer pairs producing 91 peaks, and the worst 21, and two primer pairs failing to amplify any fragments. Primer pairs with the AseI primer “GG” more consistently produced a suitable fragment profile. However, primer pairs with 3 different AseI primers were selected for further analysis as the other AseI primers may have higher optimal annealing temperatures, making them appear less suitable at the initial annealing temperature. In total, 4 primer pairs were taken forward for optimisation of annealing temperature (Table 3). From this a single annealing temperature was chosen for each primer.
pair. Ideally, the primer pair analysis would have also examined the number of peaks which overlapped with WGA peaks as these represent fragments for which methylation status can be inferred. However, MSAP analysis of a WGA sample generated with the GenomePlex® WGA kit was unsatisfactory, with the final amplification step prone to producing an inconsistent fragment profile, and few fragments aligning between the WGA and genomic samples (Figure 7). Successful whole genome amplification was not achieved until the redesigned MSAP had been developed (1.1.1.2), after which DNA methylation analysis was performed with the redesigned MSAP assay. Therefore, the application of the WGA step will be discussed alongside the redesigned MSAP assay.
Table 2. **MSAP primer pair analysis.** 32 primer pairs (4 AseI x 8 HpaII/MspI primers) were examined at a single annealing temperature (55 °C). The name of the primer reflects the selective 3’ nucleotides. The number of peaks >100 relative florescence units (rfu) were recorded, along with the number of bands under and over 200 base pairs (bp), and the number of peaks which were hard to score (shoulder peaks, stutter peaks and peaks with poor separation). Primer pairs selected for further analyse highlighted in yellow.
### Table 3. MSAP primer pair annealing temperature analysis

4 primer pairs selected from Table 2 and analysed to determine the most suitable annealing temperature. Fragment profile details recorded as per Table 2 with number of peaks >500 rfu also recorded. Selected annealing temperatures highlighted in yellow.

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<th>Aoel Primer</th>
<th>Hipall Primer</th>
<th>Annealing temperature</th>
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<th>&gt;200</th>
<th>% &gt;200</th>
<th>Poor resolution (shoulder/stutter/little separation)</th>
<th>Nº peaks - poor resolution</th>
<th>No. of peaks (&gt;500 rfu)</th>
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Chapter 3 DNA methylation as a stress response

3.1.1.2 Redoing the MSAP assay for improved methylation analysis

In addition to modifying the existing HpaII/MspI MSAP assay, the MSAP was redeveloped in the belief that this would enable analysis of CG, CHG and CHH methylation in isolation. HpaII/MspI were replaced with Sau3AI/MboI, isoschizomers whose cleavage site (GATC) contains a single cytosine at the 3’ end. Sau3AI is sensitive to methylation of this cytosine, whereas MboI and DpnII are not (Hermann and Jeltsch, 2003). In the final amplification step, the additional 5’ nucleotides of the Sau3AI/MboI primer specify the context in which this 3’ cytosine resides. It was erroneously believed that the presence of all fragments from a single amplification would therefore depend on the methylation status of this cytosine in the context specified by the Sau3AI/MboI primer, such that CG, CHG and CHH methylation could be examined in isolation from a single digested DNA sample. Upon later

Figure 7. Alignment of whole genome amplified (WGA) sample and genomic sample in MSAP assay analysed by polyacrylamide gel electrophoresis. Left: A single genomic analysed by Asel & HpaII MSAP with the digestion step repeated 4 times. Right: The same genomic sample subjected to WGA and analysed by Asel & HpaII MSAP with the WGA step repeated 4 times. Black arrows indicate fragments aligning between the genomic and WGA MSAP profiles.

In addition to modifying the existing HpaII/MspI MSAP assay, the MSAP was redeveloped in the belief that this would enable analysis of CG, CHG and CHH methylation in isolation. HpaII/MspI were replaced with Sau3AI/MboI, isoschizomers whose cleavage site (GATC) contains a single cytosine at the 3’ end. Sau3AI is sensitive to methylation of this cytosine, whereas MboI and DpnII are not (Hermann and Jeltsch, 2003). In the final amplification step, the additional 5’ nucleotides of the Sau3AI/MboI primer specify the context in which this 3’ cytosine resides. It was erroneously believed that the presence of all fragments from a single amplification would therefore depend on the methylation status of this cytosine in the context specified by the Sau3AI/MboI primer, such that CG, CHG and CHH methylation could be examined in isolation from a single digested DNA sample. Upon later
examination, it is clear that this is not the case. Whilst the sequence context of one cytosine within the restriction site can indeed be specified by the 5’ nucleotides of the Sau3AI/MboI primer, the sequence context of the other cytosine remains unknown (Figure 8). As methylation of either cytosine will prevent cleavage by a methylation-sensitive restriction endonuclease, it is not possible to determine the sequence context of the methylated cytosine. Unfortunately, this was not realised until the assay had been developed and employed extensively in place of the HpaII/MspI assay as it was believed the new MSAP represented an improvement upon the HpaII/MspI assay. Hence, the results below include results obtained using the new AseI & Sau3AI/MboI assay. Whilst this assay will still examine changes in DNA methylation, and will allow determination of loci methylation status, it is not possible to determine the sequence context of the cytosines. 11 Sau3AI/MboI primers were tested with the “GG” AseI primer as per the development of the AseI & HpaII/MspI MSAP assay. Most primers were observed to produce suitable fragment profiles following annealing temperature optimisation, with the “GTC” and “AGC” Sau3AI/MboI primers producing the greatest number of fragments.

![Diagram of MSAP assay](Image)

**Figure 8. Redeveloped MSAP assay. HpaII/MspI replaced with Sau3AI/MboI which cut at the GATC site.** 3’ nucleotides on Sau3AI/MboI (+3) primer specify the sequence context in which one of the cytosines resides for all the fragments amplified. The sequence context of the other cytosine is unknown.

The GATC restriction site was originally chosen because there are a multitude of isoschizomers for this site, one of which, MboI has been previously shown to be methylation insensitive (Hermann and Jeltsch, 2003). A comparison of fragments produced by parallel AseI & Sau3AI and AseI & MboI MSAP assays would therefore allow direct inference of
methylation status in a similar manner to the comparison of AseI & HpaII/MspI and WGA fragments. Unfortunately, fragment profiles from MSAP with AseI and MboI, SauAI or DpnII were identical (Figure 9). Furthermore, differences in band profiles were observed between the two genetically identical samples, indicating that all three enzymes were sensitive to DNA methylation in the conditions tested. As the MSAP assay protocol is simplified by digestion in T4 DNA ligase buffer and buffer sensitivity was lowest for MboI, this restriction endonuclease was used in all further MSAP assay. To enable inference of loci methylation status by MboI digest alone, a WGA step was introduced to generate a band profile from unmethylated DNA.

The whole genome amplification was initially tested during the modification of the AseI & HpaII/MspI MSAP assay and was performed through a process of genome fragmentation, adapter ligation to produce a library and library amplification. As mentioned in 3.1.1.1, MSAP analysis of the WGA sample was inconsistent, with the fragment profile varying considerably between independent WGA samples. Therefore, an alternative WGA process was tested utilising Phi29. This enzyme is capable of strand displacement amplification and generates fragments up to 10 Kb long. Although the Phi29-based WGA is relatively unbiased (Hosono et al., 2003), the AseI & MboI MSAP band profile is not completely consistent between independently amplified samples (Figure 10). Therefore, 6 WGA samples were always run alongside the genomic AseI & MboI MSAP samples, with analysis of genomic bands only performed if the band was observed in a minimum of 4 of the WGA samples. As we will see later, WGA band profiles for the AseI & MboI MSAP assay are not identical to the genomic samples, confirming the previous conclusion that MboI is sensitive to DNA methylation. Thus, a comparison of WGA and MboI fragments allows inference of loci methylation status. As the WGA step was not introduced immediately, some figures do not include a WGA sample. In these cases, no inference is drawn on the nature of the DNA methylation changes observed.
Figure 9. **A comparison of the MSAP band profiles using AseI in combination with Mbol, Sau3AI or DpnII.** Two genetically identical *A. thaliana* samples were digested with AseI in combination with the three GATC isoschizomers and the resulting fragments amplified by a single primer pair and analysed by gel electrophoresis.
3.2 DNA methylation changes in stress

3.2.1 DNA methylation in stress conditions

In order to investigate the mechanisms responsible for DNA methylation changes in stress responses, and analyse the possible inheritance of these changes, it was first necessary to establish conditions which generated reproducible changes in DNA methylation. Therefore, DNA methylation was examined in low-water potential conditions that have previously been shown to produce changes in DNA methylation in plants, namely, osmotic stress (NaCl (Karan et al., 2012; Tan, 2010; Verhoeven et al., 2010), drought and PEG (Tan, 2010)), and
cold stress (Hashida et al., 2006; Steward et al., 2002). In addition, elevated temperature was
examined as it has previously been shown to increase stress tolerance of the progeny
(Whittle et al., 2009), presumably through an epigenetic mechanism. Initially, the DNA
methylation of a pooled sample of plants was analysed with the intention to re-examine the
DNA methylation of individual plants if changes were observed in the pooled stress sample.

An initial examination of DNA methylation in aerial tissues of A.thaliana grown in soil with
NaCl and drought stress from day 21-35 failed to identify any changes across 239 AseI &
HpaII/MspI fragments (data not shown). Since methylation changes may be limited to the
root tissues, osmotic stress conditions were reapplied under hydroponics culture to allow
DNA extraction from the root tissue. 14 day old A.thaliana were transferred from
tissue culture to hydroponics culture and supplied with ATS media. On day 21, 150 or 200 mM
NaCl or 5-15%PEG was added to the hydroponics media to produce an osmotic stress.
Aerial and root DNA were extracted and analysed for DNA methylation by MSAP after 2
days (NaCl) or 7 days (PEG). Additional plants were grown in soil at 23 °C for 3 weeks and
transferred to elevated (34 °C) or reduced (10 °C) temperatures, with DNA extracted from
aerial tissues for MSAP analysis 3 weeks later. Stress was measured by determining the
maximum quantum yield of photosystem II (Fv/Fm) and measuring leaf diameter (Figure
11). Whilst 150 mM and 200 mM NaCl were both observed to significantly decrease Fv/Fm
after 24h and 48 h respectively, a significant reduction in Fv/FM in response to PEG was
only observed after 168h. The increases in osmotic pressure (Mpa) upon addition of 5-15%
PEG 6000 were observed to be 0.05 ± 0.01, 0.10 ± 0.01 and 0.21 ± 0.02 respectively, in
broad agreement with previous measurements (Money, 1989).

Of the conditions tested, only PEG stress was observed to cause a change in DNA
methylation (Figure 12). Furthermore, this change in DNA methylation in response to PEG
stress was observed exclusively in the roots, and appeared to be largely independent of the
severity of treatment. Zinc stress also failed to alter DNA methylation in either the aerial or
root tissues. These data are presented in the following section (4.1.3).
Figure 11. **Plant stress measurements in response to PEG, NaCl and temperature extremes.**

**A.** Fv/Fm in response to PEG and NaCl. Fv/Fm measured for 10 plants at timepoints indicated. Asterisks indicate significant difference compared to control (Student’s t-test, Benjamini-Hochberg correction) * = *p* < 0.05, ** *p* < 0.005.

**B.** Effect of elevated or reduced temperature on growth and photosynthetic capacity. Plants grown under normal growth conditions (22 °C) for 3 weeks before transfer to elevated (34 °C) or reduced (10 °C) temperature. Rosette diameter and Fv/Fm of 10 plants measured at 5 weeks, error bars = SD.
Chapter 3 DNA methylation as a stress response

3.2.2 DNA methylation in response to PEG stress

Since the severity of PEG-dependent changes in DNA methylation pattern was largely unaffected by the concentration of PEG used and did not occur with NaCl treatment, and the reduction in Fv/Fm was only slightly greater in 15% PEG relative to 5% PEG although the osmotic pressure of 15% PEG was 4-fold greater, it was apparent that the DNA methylation change could be due to non-osmotic stress. Addition of PEG to hydroponic media reduces availability of oxygen and can lead to hypoxia (Verslues et al., 1998), which can in turn encourage saprophytic microorganisms (Asao, 2012). Experiments were therefore performed to examine the possibility that hypoxia explained the observed DNA methylation changes. The possible contribution of hypoxia was examined by repeating the 5% PEG treatment.
under various aeration regimes and by testing a line which overexpresses RAP2.2 whose expression is hypoxia-responsive and expression levels observed to correlate with hypoxia survival rates (Hinz et al., 2010).

During the previous PEG treatments, the hydroponics solution was replaced twice and aerated for 20 minutes every 2 hours, using an air pump. Altering either the extent of aeration or maintaining the roots in the same ATS media + 5% PEG solution for the duration of the treatment did not affect the severity of DNA methylation change (Figure 13), suggesting the DNA methylation changes observed were not due to hypoxia. To further confirm that hypoxia was not contributing to the changes in DNA methylation, the response of a hypoxia-tolerant RAP2.2 overexpressor line (Hinz et al., 2010) to the 5% PEG treatment was examined. Although the gel resolution is extremely poor, a visual assessment confirmed that DNA methylation changes occurred in the hypoxia stress-tolerant line.

In order to verify that the DNA methylation changes were indeed due to osmotic stress, an equivalent osmotic pressure (0.05 Mpa) was induced with sorbitol. Additionally, alongside the comparison of PEG and sorbitol treatments, an additional 5% PEG treatment using a pre-sterilised PEG solution was tested to further confirm the DNA methylation changes were not due to stress induced by microorganisms introduced with the PEG. Although the DNA methylation patterns are not identical between the two osmotic stressors, sorbitol did produce some of the same fragment profile changes (Figure 14), indicating that, at least in part, the DNA methylation changes in response to 5% PEG relate to the reduced water potential of the hydroponics solution. The band profiles obtained from treatment with unsterilised and sterilised PEG were not identical, with both additional bands and missing bands in sterilised PEG compared to unsterilised PEG or sorbitol, suggesting either that the sterilisation had an effect on the stress experienced by the plants, or else that the response to PEG is inconsistent. To examine the consistency of the DNA methylation changes in response to 5% PEG, the root DNA methylation profiles of 5 individual plants was analysed. The PEG stress DNA methylation response was observed to be consistent between the individual plants (Figure 15). The previously observed independence of the response to the severity of PEG treatment was also confirmed.

The observed DNA methylation changes detected by AseI & MboI and AseI & HpaII/MspI MSAP indicate genome-wide DNA methylation changes occur in response to PEG stress. To examine the contribution of the DNA methylation and demethylation pathways and proteins, the methylation profile was analysed for T-DNA knockout lines met1, cmt3, drm2, dcl3, rdr2 and ros1 in the Col-0 background in control and PEG stress conditions and compared to
wild type (WT). Additionally, a WGA sample was also run alongside the genomic samples to enable inference of the DNA methylation change in response to PEG stress. Looking at the band profiles for Col-0, it is apparent that the majority of the methylation changes are DNA hypermethylation events as most changes in band presence/absence are either WGA bands present in the control root samples but not the PEG root sample, or non-WGA bands which are absent in the control root sample but present in the PEG root sample (Figure 16). Restricting the analysis to only those bands present in the WGA sample, we can observe that 37 out of 41 (90.2%) methylation differences observed between the control and PEG root samples in the primer pairs shown in Figure 16 are DNA hypermethylation events. Over the 7 primer pairs tested, hypermethylation events represent 86% of methylation changes in PEG stress (Table 4).

When comparing band profiles for the T-DNA mutants under PEG stress to WT, it is important to consider that differences may be due to changes in DNA methylation under control conditions. Therefore analysis for each mutant was restricted to bands whose presence/absence score changed between control and PEG conditions in Col-0 but was the same in Col-0 and mutant profiles in control conditions. Inspecting the band profiles in PEG stress conditions, it appears that many of the methylation changes are maintained in the absence of a single DNA methyltransferase, component of the RdDM pathway or demethylase. However, 27% of PEG stress-dependent DNA methylation changes are dependent upon DRM2 and in some cases the RdDM pathway components RDR2 and DCL3 also (12%). Less than 10% of methylation changes were observed to be dependent upon MET1. A multidimensional scaling approach (MDS) was taken to visualise the similarities and differences between the single T-DNA knockout lines in PEG stress. Fragment presence/absence scores (1/0) were converted into a distance matrix. Again, where the fragment presence/absence for a mutant line in control conditions was not identical to WT, the fragment was not included in the analysis. WT and knockout lines were then plotted in 2 dimensions to approximate the true distances between them. As we can see in Figure 17, drm2 was most dissimilar to WT in PEG stress, with the other RdDM mutants, dcl3 and rdr2 lying somewhere between WT and drm2 on the first dimension. Compared to the RdDM mutants, met1, ros1 and cmt3 were more similar to WT. In summary, PEG stress results in DNA hypermethylation in the root tissues which is largely maintained in single mutant backgrounds, although it appears that the drm2, dcl3 and rdr2 mutants are impaired in their ability to modify DNA methylation in response to PEG stress.
Chapter 3 DNA methylation as a stress response

Figure 13. The effect of aeration on DNA methylation changes in response to PEG stress. A single primer pair is shown for the AseI & Hpall and Asel & Mspl digests of DNA sample from 10 plants. Treatments = Control (C) and 5% PEG (P). Aerations = Constant (C), Intermittent – 20 minutes every 2 hours (I) and No aeration (N). Fresh media was either supplied every 3 days (Y) or not supplied (N).
Figure 14. A comparison of DNA methylation changes in response to 5% PEG, 5% PEG post-sterilisation and an equivalent sorbitol concentration. In all instances, 10 plants were treated at day 21 in hydroponics culture and DNA extracted at day 28. A single CG and CHG primer pair is shown for the AseI & MboI MSAP assay. Arrows indicating example methylation changes are colour coded to indicate the treatments in which the change is observed.
Figure 15. The consistency of DNA methylation changes in response to PEG stress. Plants were treated at day 21 in hydroponics culture and DNA extracted at day 28. Where specified, DNA was extracted from the roots of a single plant. Otherwise, DNA was extracted from 10 plants. Two primer pairs are shown for the Asel & Mbol MSAP assay.
Figure 16. Effect of single knock out mutations on DNA methylation changes in response to PEG stress. In all instances 10 plants were subjected to 5% PEG on day 21 in hydroponics culture and DNA extracted on day 28. Root Asel & MboI MSAP profiles with two primer pairs shown for wild type (Col-0) and 6 T-DNA knockout mutants. Col-0 whole genome amplification (WGA) samples shown alongside. Arrows indicate band profile changes between WT control and WT PEG samples. Red = PEG-dependent changes maintained in all mutants. Black = PEG-dependent changes which are not observed in at least one mutant.
Figure 17. Multidimensional scaling analysis of DNA methylation pattern in Arabidopsis thaliana roots in response to PEG stress. Fragment presence/absence scores for WT (Col-0) and knockout lines were converted into a distance matrix. WT and knockout lines were then plotted in 2 dimensions to approximate the true distances between them. The closer two points are, the more similar their respective methylation patterns.

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<td>3</td>
<td>1</td>
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<th>GG+AGC</th>
<th>GG+ACC</th>
<th>GG+ACG</th>
<th>GC+ACG</th>
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<td>Total hypermethylation events</td>
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<td>Total hypomethylation events</td>
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<td>86</td>
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Table 4. Methylation changes in PEG stress in Col-0. The number of changes in band profiles (either gain or loss of a band) across all 7 primer pairs used are presented. For those bands which align with a band in the WGA it is possible to assign a change in the methylation status of a locus based on the loss or gain of the band in the PEG sample. Of the changes in band profile where methylation status could be assigned, 14% were hypomethylation events and 86 were hypomethylation events (demethylation).
3.3 Promoter activity in response to PEG stress

3.3.1 Promoter::GUS reporter lines

As discussed in the introduction, DNA methylation changes in response to stress are frequently observed in plants. Currently, very little is known about the mechanism by which this change in DNA methylation occurs. For instance, are the demethylation events observed due to increased activity of a demethylase such as ROS1, the decreased activity of a methyltransferase such as MET1, or a change in the siRNA profile directing RdDM? To examine the mechanism of stress-dependent changes in DNA methylation in A.thaliana, transgenic promoter::GUS (β-GLUCORONIDASE) reporter lines were generated. These reporter lines contain the β-glucoronidase gene under the control of the promoter sequence of a gene of interest. As β-glucoronidase catalyses the production of a coloured product from a colourless substrate, it is possible to examine the expression and tissue localisation of the β-glucoronidase gene under the control of the specified promoter sequence. Five promoter::GUS lines were generated to examine promoter activity in response to stress for the CG methyltransferase MET1, the RdDM pathway components NRPD1, RDR2, DCL3 and the demethylase ROS1. Promoters were defined as the region between the gene of interest and the next upstream gene, up to a maximum of 3kb. A construct containing a promoter upstream of GUS was stably transformed into A.thaliana by Agrobacterium-mediated transformation. To increase the likelihood of selecting plants with a single insertion, multiple independent lines in which the transgene was segregating with a ratio of 3:1 were taken forward. Homozygous progeny were tested to identify lines with representative GUS staining. These representative lines were then utilised to study the promoter activities of the 5 promoters. The intention had been to generate further lines to examine the activity of the NRPE1, DRM2 and CMT3 promoters also, however, the number of successfully transformed lines was insufficient to establish a representative transgenic line for these promoters.

Before presenting the results from the transgenic line, it is important to consider the limitations of using a reporter gene approach to study the expression localisation of a gene. Firstly, the promoters of these genes have not been well defined. Therefore, the promoters used in the reporter assay may not include regulatory elements that control expression of the gene of interest. Although up to 3kb upstream of the transcription start site was taken to define the promoter region, this may still miss long-distance enhancer elements, or elements that reside between the transcription and translation start sites. Secondly, the reporter gene
Chapter 3 DNA methylation as a stress response

expression has not been quantified here but rather visually assessed. As such, the assay is not sensitive to small changes in expression.

3.3.2 Promoter activity

As described above, consistent DNA methylation changes were observed in response to PEG stress. To investigate the mechanism behind this response, the promoter::GUS lines were utilised. The advantage of this approach over gene expression analysis by techniques such as qRT-PCR is that the promoter::GUS lines enable visualisation of expression localisation throughout the plant rather than providing a value for the whole tissue or part thereof. Plants were sown onto ATS-agar plates and transferred to hydroponics culture on day 14. On day 21, PEG stress was initiated by addition of 5% PEG to hydroponics growth media. On day 28 aerial and root tissues were taken for GUS staining. The promoter activity for the 4 genes involved in methylating DNA (MET1, NRPD1, RDR2 and DCL3) decreased slightly in PEG stress, whilst ROS1 promoter activity was largely unaffected (Figure 18). No differences were observed for the aerial tissues in control and PEG stress conditions (images not shown). In control conditions, NRPD1 promoter activity was visible throughout the roots, with GUS expression highest in the stele, root tips and root hairs of young lateral roots. When exposed to PEG stress, NRPD1 promoter activity was observed to decrease slightly and GUS expression was no longer visible in the root hairs, although expression was still observed in the primary root, lateral roots and root tips. In control conditions, RDR2 promoter activity was visible throughout the roots, with GUS expression strongest in the primary root, and the stele and root tip of lateral roots. In PEG stress conditions, RDR2 promoter activity was slightly reduced, although the localisation of expression was unchanged. In control conditions, root DCL3 promoter activity was weak with a low level of GUS expression observed in the upper-most portion of the primary root and a small number of lateral roots. In PEG stress, DCL3 promoter activity was insufficient for GUS expression to be observed anywhere in the roots. MET1 promoter activity was far in excess of the other promoters, such that substrate incubation had to be reduced from 24 hours to 1 hour. In control conditions, expression of GUS under the control of the MET1 promoter was observed most prominently in the lateral roots and root tips, with expression in the primary root limited to the portion closest to the aerial tissues. When exposed to PEG stress, GUS expression in the pMET1:GUS line was slightly reduced in all portions of the root tissues. GUS expression under the control of the ROS1 promoter was observed to be weak in control conditions and limited to the upper-most section of the primary root and a small number of lateral roots. ROS1 promoter activity did not appear to respond to PEG stress. In summary, promoter activity for MET1 and the RdDM genes POL IV, RDR2 and DCL3 deceased
slightly in response to PEG stress. Localisation of expression was only observed to change in PEG stress in the pPOLIV:GUS line which showed loss of GUS expression in the root hairs. GUS expression under the control of the ROS1 promoter was lower than under the control of the other promoters examined in control conditions, and was unaffected by PEG stress.
Figure 18. **Promoter activity in PEG stress.** Promoter::GUS lines subjected to PEG stress on day 21 in hydroponics culture by addition of 5% PEG. Root tissue harvested on day 28 and stained for GUS expression. All lines except pMET1:GUS were incubated with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) for 24 hours, pMET1:GUS was incubated with X-Gluc for 1 hour. The results presented here are representative of multiple independent lines. The following lines were used above: pPOLV:GUS_3_1, pRDR2:GUS_17_2, pMET1:GUS_5_7, pDCL3:GUS_6_1 and pROS1:GUS_17_3.
3.4 Discussion

This Chapter has detailed the development of methods for examining the mechanism underlying DNA methylation changes in response to stress and the results obtained with these techniques. DNA methylation is frequently implicated in the stress response of plants by MSAP analysis (Cao et al., 2011; Karan et al., 2012; Ou et al., 2012; Tan, 2010; Verhoeven et al., 2010; Zhong et al., 2009). However, flaws were identified here with the interpretation of MSAP output. Specifically, current MSAP analyses do not allow inference of a discrete methylation status of loci, although this is commonplace (Cao et al., 2011; Karan et al., 2012; Tan, 2010; L. Zhong et al., 2009). Direct inference of methylation status from MSAP analysis is not possible because, without sequencing fragments, it is not possible to know whether they have restriction sites only at their terminals, a prerequisite to infer methylation status from fragment presence or absence. The modifications described above were therefore carried out on the widely used EcoRI & HpaII/MspI MSAP assay to restrict the analysis to only those bands with a single CCGG site. Analysis of a Phi29 WGA sample by AseI & MboI MSAP assay clearly demonstrated that the WGA sample aligned well with the genomic samples, enabling inference of methylation status. A similar alignment of Phi29 WGA MSAP fragment profile with AseI & HpaII/MspI genomic fragment profiles would enable inference of the methylation status of cytosines in the CCGG site. To the author’s knowledge, this is the first use of WGA in MSAP to allow direct inference of methylation status. A modified MSAP to generate a fragment profile in the absence of methylation was proposed previously by Baurens et al (Baurens et al., 2003) to enable identification of loci with a fully methylated external cytosine which in a standard HpaII/MspI MSAP analyses would remain undetected. Their proposed solution was to digest genomic DNA with EcoRI, ligate adapters and amplify the fragments before passing the EcoRI sample through the complete MSAP assay, a modification previously described for AFLP analysis and named secondary digest AFLP (SD-AFLP) (Baurens et al., 2003). However, it appears they did not consider the possibility that EcoRI & HpaII/MspI fragments could contain internal restriction sites as their hypotheses for why only 60% of HpaII/MspI fragments were observed in the SD-AFLP focused on the amplification steps. Regardless, SD-AFLP may be seen as a direct alternative to WGA to generate a methylation-free template to enable identification of bands without internal restriction sites. The WGA modification proposed here has two major advantages over SD-AFLP: Firstly, taq PCR amplification of EcoRI fragments will be inefficient for long fragments, whereas Phi29 is highly efficient and has a low error rate (Blanco et al., 1989; Hutchison et al., 2005),
secondly, EcoRI is not methylation insensitive (Brennan et al., 1986). Thus, the WGA modification demonstrated here represents a valuable improvement on current practice.

In addition to modifying the existing EcoRI & HpaII/MspI MSAP assay, a new AseI & MboI/Sau3AI MSAP assay was developed to examine DNA methylation at GATC sites. Previous reports suggest that MboI is cytosine methylation insensitive, whereas Sau3AI is sensitive to cytosine methylation (Brooks and Roberts, 1982; Hermann and Jeltsch, 2003). However, when digesting in T4 DNA ligase buffer it appears both enzymes are methylation sensitive. Previous reports have also indicated that MboI may be impaired by cytosine methylation (L. Huang et al., 1982). The apparent methylation sensitivity of MboI reported here could also explain a previous report that the promoter of DRM2 is resistant to MboI cleavage which was postulated to be due to adenosine methylation as a small number of DpnII sites were identified (GA\textsuperscript{me}TC) (Ashapkin et al., 2002). However, there was no correlation between lines displaying DpnII sensitivity and those displaying MboI insensitivity. As far as the author is aware, this is the only report of adenine methylation to date. It is possible then that the resistance to MboI digestion was instead due to the cytosine methylation which was reported in the same paper. Given the contrasting reports of methylation sensitivity for MboI (Brooks and R. J. Roberts, 1982; Hermann and Jeltsch, 2003; L. Huang et al., 1982) it is clear that further experiments are required to fully elucidate the sequences at which it will cleave. Parallel AseI & MboI MSAP with digestion performed in MboI or T4 DNA ligase buffer was carried out here and yielded identical final fragment profiles (data not shown). Thus, it appear MboI is methylation sensitive to an identical extent as Sau3AI in the conditions tested here, and hence the combination of these two enzymes would not yield any more information on methylation state than either in isolation. Therefore, to generate a fragment profile which was unaffected by restriction site methylation status from which the direction of methylation change could be determined, a WGA step was introduced as per the AseI & HpaII/MspI MSAP assay.

The innovation of using the GATC restriction site was to use the amplification steps to specify the sequence context of a cytosine within a restriction site, rather than to simply amplify a subpopulation of fragments, in the belief that this would allow analysis of CG, CHG and CHH methylation in isolation. Unfortunately, the sequence context of the other cytosine was not considered. Since methylation of either cytosine will prevent cleavage by a methylation-sensitive restriction endonuclease, specifying the sequence context of a single cytosine within the restriction site is insufficient to enable examination of CG, CHG or CHH methylation in isolation. As this was not realised when the assay was developed, priority was given to the development of the AseI & MboI assay over the AseI & HpaII/MspI assay.
Hence, when whole genome amplification was achieved with Phi29, the WGA step was used with the AseI & MboI assay for the continued examination of root-specific DNA methylation changes in response to PEG stress. Although the AseI & MboI MSAP assay will still provide evidence for changes in DNA methylation, and the direction of change can be determined by comparing sample fragment profiles with the WGA fragment profile, the AseI & HpaII/MspI MSAP assay would have been more suitable as it would have enabled analysis of both CG and CCG methylation. Unfortunately, this was not realised during the experimentation and therefore all later experiments analysing DNA methylation changes were performed by AseI & MboI MSAP only.

A variety of stressors likely to result in DNA methylation changes based upon previous publications were examined by MSAP analysis, including osmotic stress (NaCl (Karan et al., 2012; Tan, 2010; Verhoeven et al., 2010), drought and PEG (Tan, 2010)), cold stress (Hashida et al., 2006; Steward et al., 2002) and heat stress (Whittle et al., 2009). Across the stress conditions examined, DNA methylation changes were only observed in response to PEG stress, and these changes were restricted to the roots. The observation that DNA methylation changes in response to PEG stress occur predominantly in the roots fits with previous observations of DNA methylation changes in response to alkali and NaCl stress in Gossypium hirsutum (Cao et al., 2011), NaCl and heavy metal stress in Oryza sativa L. (Karan et al., 2012; Ou et al., 2012). Upon first observing the DNA methylation response to PEG stress there were a number of reasons to believe the response may not be due to osmotic stress. Firstly, no response was observed to NaCl which will also result in osmotic stress. Secondly, the severity of the response was not correlated with the severity of the treatment. These observations led to a concern that the DNA methylation response may be due to hypoxia as PEG has been observed to cause hypoxia in hydroponics culture (Verslues et al., 1998). It appears hypoxia was not responsible for the observed DNA methylation changes in response to PEG as this response was still observed in a hypoxia tolerant line and was not affected by changes to the aeration regime. Furthermore, a second osmoticum, sorbitol, was also observed to induce DNA methylation changes in A.thaliana roots, although the changes were not identical to PEG. Given that sorbitol is synthesised by some plant species (Teo et al., 2006) and can be taken up and broken down by A.thaliana (Aguayo et al., 2013), whereas PEG is xenobiotic and more biologically inert, it is not surprising the changes in DNA methylation in response to sorbitol and PEG are not identical. Aligning the genomic and WGA MSAP fragment profiles, it’s clear that the majority of methylation changes are hypermethylation events. These changes are highly reproducible, with individual plants showing almost identical changes in root DNA methylation, suggesting a highly targeted hypermethylation in A.thaliana roots in response
to PEG stress. Interestingly, a previous examination of DNA methylation changes in response to PEG stress in *A. thaliana* also found the majority of CG methylation changes were hypermethylation events (Colaneri and Jones, 2013). Colaneri & Jones detected 10,862 differentially methylated CG sites across the *A. thaliana* genome in response to PEG stress, of which 9,898 (91%) were hypermethylation events, in line with the observation here that 86% of the DNA methylation changes detected were hypermethylation events. From the study of single knockout mutants it appears that this hypermethylation is partly dependent on the RdDM pathway. However, even in a DRM2 knockout line which should be incapable of RdDM-mediated DNA methylation, the majority of DNA hypermethylation events were maintained in response to PEG stress. This suggests the previously observed redundancy at some loci between *CMT3*, *MET1* and *DRM2* (Cao and Jacobsen, 2002a; Xiao et al., 2006) may partially maintain the PEG stress hypermethylation response in the absence of a single methyltransferase of RdDM component. Unfortunately, the sequence context of the cytosines examined here cannot be determined from the AseI & MboI MSAP assay, however, since DRM2 is capable of methylation cytosine in any context, it’s likely the hypermethylation is not restricted to the previously observed CG hypermethylation (Colaneri and Jones, 2013). Given that RdDM has been observed here to function in the PEG stress response, it is perhaps surprising that the activity of the *DRM2*, *DCL3* and *POL IV* promoters in root tissues appears to decrease slightly during PEG stress and no change in localisation of expression was observed. An exception was the *POL IV* promoter, whose activity was no longer observed in the root hairs during PEG stress. It’s possible this slight decrease in activity relates to the reduced growth rate of the roots during PEG stress -which may explain why MET1 promoter activity slightly decreased also - and the function of RdDM in PEG stress does not require transcriptional regulation of these or other components of the pathway. Quantitative reverse-transcriptase PCR analysis of transcript abundance in the root tissues for components of the RdDM pathway could help establish if the slightly decreased promoter activity observed here was indeed a reflection of reduced growth rate, or whether these genes are truly downregulated during PEG stress. Either way, the role of RdDM in the PEG stress response should be examined by analysis of siRNA in the root tissues in response to PEG. This could identify further regions of the genome that are targeted for hypermethylation by RdDM in the roots during PEG stress. A previous analysis of functional groups affected by CG hypermethylation indicated that genes annotated with terms relating to membrane transport were enriched (Colaneri and Jones, 2013). If a similar enrichment was identified for siRNAs generated during PEG stress, this would provide firm evidence that RdDM has a biological function in the PEG stress response.
Interestingly, a broadly similar change in DNA methylation in response to PEG stress was observed across a range of PEG concentrations. The poor correlation between severity of treatment and severity of response could indicate a gating of the PEG stress DNA methylation response, such that upon sufficient osmotic stress being received, the root DNA becomes hypermethylated to an identical extent regardless of the severity of the osmotic stress experienced. How such an apparently binary response to PEG stress could be established is unclear although presumably this would require some form of gating to restrict the observed DNA methylation changes to only those stress conditions which cross a threshold. Before an investigation of the hypothesised gating can begin, it would be beneficial to test a wider range of PEG concentrations to establish if the PEG response is indeed binary, or whether a correlation between PEG concentration and extent of hypermethylation can be observed at lower concentrations of PEG.

The original aim of analysing DNA methylation in response to stress was to identify conditions which could lead to changes in DNA methylation that could be inherited by the progeny in the hope that these would correlate with an enhanced stress tolerance. As the only changes in DNA methylation observed here were restricted to the root tissues, analysis of inherited DNA methylation changes and their hypothesised function in plant stress memories could not be investigated through this approach. The lack of DNA methylation changes in response to the other stressors tested suggests abiotic stress responses in *A. thaliana* do not usually include wide scale regulation of DNA methylation.

In summary, the modifications proposed for the EcoRI & HpaII/MspI MSAP assay will significantly improve the value of the data output, enabling inference of the discrete methylation status of cytosines at CCGG which is currently not possible. Replacing EcoRI with AseI and introducing a final amplification with only the AseI primer are both straightforward modifications that have been carried out here. The alignment of a Phi29 WGA MSAP fragment profile with genomic MSAP fragment profiles has been demonstrated here with another MSAP assay and could easily be incorporated into an AseI & HpaII/MspI MSAP workflow. Indeed, the WGA step could be incorporated into any MSAP which uses a combination of a methylation-sensitive and a non-sensitive restriction endonuclease to enable inference of the direction of methylation change. The attempt to develop a MSAP assay which could examine CG, CHG and CHH methylation in isolation relied upon flawed reasoning. However, the AseI & MboI MSAP assay could be employed in parallel with AseI & HpaII/MspI MSAP to ensure observed methylation changes are not limited to a particular restriction site. Additionally, promoter::GUS lines have been
generated which enable examination of promoter activities in response to stress for 5 genes involved in DNA methylation/demethylation.

Unexpectedly, DNA methylation changes were not observed in the majority of stress conditions examined here. Instead, only osmotic stress generated by addition of PEG or sorbitol to the growth media was found to result in DNA methylation changes. Furthermore, these changes were limited to the root tissues. The majority DNA methylation changes in response to PEG stress were observed to be hypermethylation events which are not dependent on a single methyltransferase, RdDM component or demethylase, suggesting the methyltransferases act redundantly in the PEG stress hypermethylation. However, there were some DNA methylation changes which were DRM2/RdDM-dependent. The activity of the MET1, NRPD1, DCL3 and RDR2 promoters were all slightly decreased in PEG stress, although, with the exception of a loss of pNRPD1 activity in the root hairs in PEG stress, localisation changes were not observed. This is taken to indicate that the RdDM-dependent DNA hypermethylation is brought about by changes in the siRNA profile or post-transcriptional control of RdDM proteins. However, given that many RdDM components were not analysed here, it is also possible that the DNA hypermethylation depends on the regulation of another RdDM gene in response to PEG stress.

Although the aim to investigate the role of DNA methylation in transgenerational stress memories was not achieved by the approach detailed above, the results do provide further evidence for the role of DNA methylation in low water potential stress in A. thaliana. The next two Chapters focus on an alternative approach to studying transgenerational stress memories, starting from a screen of stressors to identity suitable conditions to generate a transgenerational stress memory, and going on to present a transcriptomics analysis of the stress tolerant progeny.
Chapter 4. Identification and characterisation of a novel transgenerational stress memory
Chapter 4. Identification and characterisation of a novel transgenerational stress memory

4.1 Identifying conditions that produce a transgenerational stress memory in Arabidopsis thaliana

As described in the introduction, stress memories and transgenerational stress memories have been observed in the progeny of plants subjected to a variety of stress conditions (Boyko and Kovalchuk, 2010; Boyko et al., 2010; Ito et al., 2011; Lang-Mladek et al., 2010; Luna et al., 2012; Molinier et al., 2006; Ou et al., 2012; Rahavi et al., 2011; Rasmann et al., 2012a; Slaughter et al., 2012; Whittle et al., 2009) although independent attempts to reproduce results are often not successful (Boyko et al., 2010; Pecinka et al., 2009).

Therefore, to investigate the stress memory mechanism in Arabidopsis thaliana, a number of stress conditions were first examined. In all instances, the immediate progeny (F1) were tested for an increased stress tolerance. Where observed, stress tolerant progeny were taken through a second generation of non-stress conditions and the progeny (F2) re-tested to provide evidence for a transgenerational stress memory. For all stress conditions tested, 40 isogenic Col-0 plants were given the stress treatment, with an additional 40 plants receiving no treatment as a control. Seeds were collected from individual treated plants and pooled in equal quantities for use in stress tolerance testing to ensure the examination of the F1 generation was not biased towards plants producing a greater number of seeds. Stress tolerance testing in the F1 generation involved measuring germination frequency and rate, plant fresh weight, seed production or root growth as specified. In all instances the data are plotted with the conditions tested in the F1 generation along the x-axis and the parental treatments defined in the legends.

4.1.1 Stress tolerance in the progeny of stress treated A.thaliana

Plants were subjected to a range of conditions and examined in the following generation to establish whether the progeny displayed an increased stress tolerance. Conditions examined were chosen based on previously observed stress memories and comprised: nitrogen deficiency, elevated and reduced temperature, excess NaCl, drought, and excess zinc (Figure 19). Where possible, the stress treatments used here emulate the previous publications with regards to the defined treatment and the developmental stage at which treatment occurs. Stress tolerance testing in the F1 generation also reflects the published stress memories.
4.1.1.1 Nitrogen deficiency

Rice (*Oryza sativa L.*) plants grown under nitrogen deficient conditions produce progeny with an increased tolerance to nitrogen deficiency, as measured by plant weight, and concurrent local and global changes in DNA methylation (Kou *et al*., 2011). In order to establish whether a similar transgenerational stress memory mechanism existed in *Arabidopsis thaliana*, and whether the mechanism is sensitive to the developmental stage at which stress is introduced, nitrogen deficiency was initiated at two developmental stages: plants were either subjected to nitrogen deficiency from day 21 through to seed production in a hydroponics set up similar to the previously published transgenerational stress memory in rice (Kou *et al*., 2011), or subjected to nitrogen deficient conditions from germination for 21 days. The progeny were examined for a stress memory by measuring fresh weight and seed production under nitrogen deficient conditions.

As expected, fresh weight and seed production in F1 plants was negatively affected by reducing nitrogen concentration. However, an increased stress tolerance was not observed in the progeny of nitrogen deficiency treated plants. No difference in fresh weight was observed for the progeny of stressed plants compared to control progeny when grown under nitrogen deficient conditions (Figure 20A). Likewise, seed production under nitrogen deficient conditions was unaffected by parental treatment (Figure 20B).

4.1.1.2 Elevated and reduced temperature

Subjecting *Arabidopsis thaliana* to successive generations to mild heat stress (30 °C) has previously been observed to result in an increased heat tolerance in the progeny relative to the progeny of control plants (Whittle *et al*., 2009). However, of the many fitness parameters tested in the progeny under heat stress, only seed production was found to be significantly improved. Interestingly, mild cold (16 °C) was not observed to result in an increased tolerance to reduced temperature in the progeny. To increase the likelihood of detecting a stress memory after a single treated generation, a less mild treatment was performed here; 3 week old *Arabidopsis thaliana* plants were subjected to elevated (34 °C) or reduced (10 °C) temperature through to seed production. In the following generation, the tolerance of the progeny was assessed by recording fresh weight and seed production under elevated or reduced temperature.

Both stress treatments were observed to reduce rosette diameter and decrease the photosynthetic capacity of the F0 plants as measured by Fv/Fm, indicating both elevated and
reduced temperatures were stressful (Figure 11B). However, attempts to reproduce the published transgenerational stress memory (Whittle et al., 2009) in response to elevated temperature were unsuccessful. No difference was observed in the stress tolerance of the progeny of heat or cold-treated plants as measured by aerial fresh weight or seed production (Figure 21A, B).
Germination on stress plates

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<tr>
<td>NO₃ deficiency</td>
<td>ABA (0.001 M ABA)</td>
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<tr>
<td>PEG</td>
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Stress in hydroponics

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Temperature stress

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<td>Cold</td>
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</table>

Figure 19. Schematic of stress conditions examined. *Arabidopsis thaliana* subjected to stress either from germination on media-agar plates (top), from day 21 in hydroponics (middle) or from day 21 in soil (bottom).
Figure 20. **Biomass accumulation and seed production in the F1 progeny of plants grown under nitrogen deficient conditions.** Plants subjected to nitrogen deficiency from day 0-21 or from day 21 onwards. Sufficient nitrogen = 9 mM NO₃, nitrogen deficiency = 1.8 mM NO₃ or 0.9 mM NO₃. Growth conditions on X-axis. Parental treatment colour coded as indicated in legend. A. Progeny sown onto ATS-agar with 9 mM NO₃ or 0.9 mM NO₃. Whole seedlings fresh weight recorded after 28 days. Data presented as average of 10 plants, 4-6 repeat experiments, Error bars = SE. B. Progeny grown hydroponically with 9 mM, 1.8 mM or 0.9 mM NO₃. Seed weight recorded once the plant had ceased producing siliques. Data presented as average of 5 plants, 6 repeat experiments. Error bars = SE.
4.1.1.3 Drought

Low water potential stressors have been employed previously to examine transgenerational memories of stress in plants with varying success (Boyko et al., 2010; Pecinka et al., 2009). Here, PEG was used to reduce the water potential and simulate drought stress. As osmotic stress treatment in early developmental stages has previously been shown to generate a stress memory (Boyko et al., 2010), treatment was initiated from germination for 14 days by supplementing growth media with 15% PEG 6000, after which all plants were grown on in non-stress conditions through to seed collection. In the following generation, germination frequency and rate, and biomass under low water potential (up to 16% PEG) were recorded. Germination frequency and rate of germination were both lower when PEG was increased to
12%. However, both germination frequency and germination rate were observed to be unaffected by parental treatment (Figure 22). Likewise, plant biomass was reduced by PEG, with no observed hypertolerance in the progeny of PEG-treated plants (data not shown).

**Figure 22.** Effect of parental treatment on germination frequency and germination rate in F1 progeny under low water-potential stress conditions. Growth conditions on X-axis. Parental treatment colour coded as indicated in legend. **A.** Germination (radicle emergence) recorded at day 14 for 50 plants, 4 repeat experiments. **B.** Time (hours) for half of final number of germinated seeds to germinate (G50). Error bars = SE.
4.1.1.4 NaCl

NaCl treatment of *A. thaliana* (acc. C24) has previously been reported to increase the germination frequency and root growth of the progeny under high NaCl concentrations (Boyko *et al.*, 2010). To examine whether NaCl treatment could also generate a stress memory in the Col-0 accession, plants were subjected to 100 mM NaCl for 3 weeks from germination, in line with the published treatment. In the following generation (F1), germination frequency and rate and root growth were measured under high NaCl. Germination frequency at 150 mM NaCl was significantly increased in the progeny of NaCl stressed plants (*p*<0.05, G-test), although the increase was much smaller than previously reported (7% compared to ~40%; Figure 23A). The rate of germination (G50) and root growth under high NaCl were unaffected by parental treatment (Figure 23B, C).

To investigate if the increased NaCl tolerance could be inherited over an untreated generation, and if it could be enhanced by treating sequential generations with NaCl, the progeny of NaCl treated plants were taken through an untreated generation or subjected to a second NaCl stress treatment. An identical germination experiment was performed to examine the stress tolerance of the F2 progeny, referred to here as CC, NC and NN to denote the previous two parental treatments, where C=control and N=100 mM NaCl. Germination frequency under high NaCl was observed to be unaffected by parental treatment (Figure 24). Rate of germination was also unaffected (data not shown).

4.1.1.5 Reproducing a published stress memory

As mentioned above, Boyko, *A et al* (2010) previously observed that the progeny of *A. thaliana* (C24) plants subjected to 3 weeks of 25 or 75 mM NaCl displayed a ~40% increase in germination frequency under 125-150 mM NaCl compared to the progeny of control plants. Having failed to reproduce such a considerable increase using the Col-0 accession, the NaCl stress treatment was repeated following the original publication with regards to accession, defined treatment and stress tolerance testing in the following generation (Boyko *et al.*, 2010). Again, the result could not be reproduced here: repeating their 75 mM NaCl treatment of C24 *A. thaliana* did not result in an increased germination frequency or germination rate in the progeny under any concentration of NaCl (Figure 25A & B).
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Figure 23. Effect of parental treatment on NaCl stress tolerance. *A. thaliana* exposed to 100 mM NaCl for 3 weeks from germination. NaCl stress tolerance in Control and NaCl progeny examined: A. Germination frequency. *=*p<0.05 (G-test) 100 plants, 4 repeat experiments. Error bars = SE. B. Germination rate. G50 = Time in hours for germination to reach 50% of maxima. 100 plants. 3 repeat experiments. Error bars = SE. C. 7 day old plants transferred onto NaCl stress plates. Root growth measured after 48 hours. Average of 30 plants, error bars = SD.
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Figure 24. Effect of two sequential treated generations on germination under high NaCl. Parental treatments colour coded as indicated in legend. C=Control, N=NaCl, i.e NC denotes NaCl stress in F0 and control growth conditions in F1. Stress tolerance testing conducted on F2 progeny. Germination frequency of 50 plants measured. 4 repeat experiments. Error bars = SE.

A

B

Figure 25. Reproducing a NaCl-dependent stress memory. Germination frequency (A) and rate (B) of the F1 progeny of NaCl-treated A.thaliana (acc. C24) under high NaCl compared to Control progeny. 50 plants, 8 repeat experiments. Error bars = SE.
4.1.2 Zinc stress results in transgenerational stress memory

Unlike the stress treatments described so far, elevating zinc in the media to a stressful excess does result in an increased zinc stress tolerance in the progeny. Furthermore, the stress tolerance appears to be specific to zinc, is maintained in the progeny after an intervening non-stress generation, and is reproducible.

4.1.2.1 The progeny of zinc stressed plants are more tolerant to elevated zinc

The progeny of *A. thaliana* and *O. sativa* treated with heavy metals, have previously been reported to display an enhanced tolerance (Ou *et al.*, 2012; Rahavi *et al.*, 2011). As discussed previously, Rahavi *et al* (2011) reportedly grew *A. thaliana* on media supplemented with 50-100 mM cadmium, nickel or copper (Rahavi *et al.*, 2011). The reported stress memory in *O. sativa* involved parental treatment with 50-1000 µM mercury and was correlated with inheritance of hypomethylated CHG loci (Ou *et al.*, 2012). To date, there have been no reports of a transgenerational stress memory in any plant species after parental treatment with zinc.

To investigate if zinc stress results in a stress memory in *A. thaliana*, 40 plants were subjected to 14 days of elevated Zn⁡⁺ by germinating seeds directly on ATS-agar (containing 1 µM ZnSO₄) supplemented with 500 µM or 1 mM ZnSO₄. The intention had been to subject plants to 21 days of elevated Zn⁡⁺, however, extreme chlorosis was observed with the 1mM Zn²⁺ treatment from day 10. Both stress treatments were therefore stopped on day 14 with all plants transferred to control conditions for the remainder of their lifespan. The progeny will here be referred to as “Zn(500)”, “Zn(1000)” and “control”.

Zinc stress tolerance in the progeny was examined by measuring fresh weight and root growth. Seeds germinated on ATS-agar supplemented with 0, 250 or 500 µM ZnSO₄ were weighed 10 days after the end of stratification. Parental treatment had a significant effect on zinc tolerance in the progeny: Zn(500) and Zn(1000) displayed a 23% and 35% increase respectively in plant fresh weight compared to progeny of control plants when germinated on 250 µM Zn²⁺, and Zn(1000) was observed to be significantly heavier than control at 500 µM Zn (*p*<0.05, ANOVA; Figure 26A). There was an apparent increase in fresh weight under non-stress conditions for Zn(1000), however, this was not significant (*p*>0.05, ANOVA).
Root growth was measured by transferring 7 day old seedlings to ATS-agar supplemented with 0-500 µM Zn\(^{2+}\) and measuring additional root growth at day 14. Although the mean root length was higher in Zn(500) and Zn(1000) compared to control at 250 µM Zn\(^{2+}\), this difference was not significant (Figure 26B).

4.1.2.2 The increased weight is not due to an increased rate of development in zinc stress

In the experiments quantifying plant weight under zinc stress, a reduced developmental progression was observed above 250 µM zinc. To investigate if the increased plant weight in Zn(500) relative to control progeny in zinc stress was correlated with an increased rate of development, seedlings at the 2 leaf stage were transferred to ATS-agar plates supplemented with 0, 50, 250 or 500 µM Zn and rosette developmental progression recorded for 13 days. As expected, 250 µM and 500 µM zinc reduced the rate of development, however, no difference was observed between the progenies under any condition (Figure 27).

4.1.2.3 Zinc stress reproducibly results in an increased zinc stress tolerance in the progeny

As previously discussed, reported stress memories can show poor reproducibility. To confirm that the stress memory observed was at least reproducible within the same laboratory, 60 isogenic Col-0 seeds were sown onto ATS-agar supplemented with 500 µM ZnSO\(_4\) and transferred to control conditions on day 10. As a control, 60 seeds were sown onto ATS-agar without supplemented zinc and transferred to ATS-agar on day 10. The progeny will be referred to here as Zn(500)\(^2\) and control\(^2\) to denote the parental treatment and their being the progeny of the repeat experiment. Previously, the increased tolerance in the progeny was observed when seeds were sown onto ATS-agar supplemented with high Zn\(^{2+}\) and also when 14 day old seedlings were transferred to hydroponics and subjected to zinc stress from day 21-28 (data not shown, single experiment with 10 plants each). Unexpectedly, Zn(500)\(^2\) plants were not more tolerant of zinc stress as determined by fresh weight 9 days after germination on 50-1000 µM Zn\(^{2+}\) (Figure 28). However, an increased tolerance to zinc was observed when zinc stress tolerance was tested at day 21 (Figure 29A & B). The contribution of experiment-to-experiment variation to the noise in the data required that this variation be accounted for in the statistical testing (Figure 29C). A randomized block design ANOVA was therefore employed to test for a significant effect of parental treatment on plant aerial and root weights under zinc stress. This approach allows
detection of a significant effect of parental treatment against a background of experiment-to-experiment variation (Lew, 2007). A significant effect of parental treatment on both aerial and root weights was observed at 250 µM Zn$^{2+}$ (p = 0.001 & 0.047, respectively). A significant effect of experimental data set was also observed on aerial and root weights at 250 µM Zn$^{2+}$ (p= 0.000 & 0.043, respectively). In summary, the progeny of zinc stressed plants reproducibly show an increased tolerance to zinc relative to control progeny, although the developmental stage at which the increased tolerance may be observed is inconsistent.

4.1.2.4 The stress memory is stable over one untreated generation

To examine the stability of the stress memory over one untreated generation and whether it could be enhanced by multiple treatments, all 20 individual Zn(500) lines were subjected to an additional stress treatment or control treatment and all control lines were progressed through an additional control treatment. The progeny will here be referred to as “ZZ”, “ZC” and “CC” to denote the nature of the two successive parental treatments, where” Z” denotes a zinc stress treatment and “C” a control treatment. Parental treatment had a significant effect on plant weight at 500 µM zinc (p<0.01, ANOVA), but did not significantly affect plant weight at 250 µM zinc or in control conditions (Figure 30). The stress memory appeared stable over one untreated generation with a 20% increase in plant fresh weight observed for ZC compared to CC when germinated on 500 µM Zn (p<0.05, ANOVA, Tukey HSD post hoc). Although the average weight of ZC plants was lower than ZZ at both concentrations of Zn$^{2+}$ tested, the difference was not significant (p>0.05, ANOVA, Tukey HSD post hoc).

4.1.2.5 The increased tolerance is not limited to a hypertolerant subset of seed lines

As seeds were collected from treated plants individually, it was possible to establish whether the observed increase in fresh weight was limited to the progeny of a subset of plants, i.e. was the transgenerational stress memory stochastic with regards to the individually treated plants. Seeds from 17 individual CC and ZC lines were sown onto ATS-agar supplemented with 500 µM Zn$^{2+}$ and weighed after 14 days. As expected, ZC lines were on average heavier than CC lines. However, the variance in plant weight between the individual lines was almost identical (CC variance = 1.2, ZC = 1.29; Figure 31), indicating that the increased tolerance was not limited to a few hypertolerant seed lines.
The heavy metal stress tolerance appears to be specific to zinc

Increased tolerance to zinc stress may be expected to correlate with increased tolerance to other heavy/transitional metal as there is a considerable degree of overlap between the stress responses to excess heavy metals (Maestri et al., 2010). As a screen to identify possible cross-tolerance with other heavy metals, Zn(500) and control seeds were germinated on varying concentrations of nickel, copper, cobalt and caesium (this data is not shown as no replicates were conducted). Fresh weight was higher in Zn(500) compared to control at only 200 µM Ni and 500 µM Cu, results which were not verified upon repetition (Figure 32A). Furthermore, Zn(500) plants were not observed to be hypertolerant of NaCl compared to control plants. Additionally, Zn(500)$^2$ plants were subjected to nickel, cadmium and cobalt stress at day 28. Again, Zn(500)$^2$ plants were not observed to be more tolerant of metals other than zinc (Figure 32B).

The transgenerational stress memory appears to “reset” during seed aging

Previous investigations of transgenerational phenomena have indicated that after 3-17 months of seed storage, a parental stress-dependent transgenerational release of silencing may be lost (Lang-Mladek et al., 2010). To examine if a similar resetting occurred during seed storage for the observed zinc transgenerational stress memory, 12 month old CC, ZC and ZZ seeds were sown onto agar plates supplemented with 500 µM Zn$^{2+}$. Previously, ZC and ZZ seedlings were observed to be approximately 20% heavier than CC seedlings (Figure 30). After 16 months seed storage no difference was observed between the 3 parental treatments, suggesting the memory had been reset (Figure 33).
Figure 26. **Effect of parental treatment on zinc stress tolerance in F1 progeny.** Parental treatment denoted by bar colour where Zn(500) = 500 µM ZnSO₄ from stratification for 10 days A. Fresh weight (FW) of plants sown onto media supplemented with zinc. Weight recorded 10 days after the end of stratification. Average of 46-50 plants, 6 repeat experiments. Error bars = SE. *= p<0.05 relative to Control (ANOVA, Tukey post hoc). B. Root growth. Plants transferred 7 days after the end of stratification and onto media supplemented with zinc and additional root growth measured after 7 days. Average of 25-30 plants, 3 repeat experiments. Error bars = SE.

Figure 27. **Effect of parental treatment on F1 progeny development in zinc stress.** Parental treatment indicated by line type. Seedlings transferred at the 2-leaf stage onto ATS-agar plates supplemented with 0-500 µM Zn²⁺ and the number of additional leaves recorded daily for 13 days. Data presented as average of 30 plants per parental treatment and zinc concentration.
Figure 28. Effect of parental treatment on zinc stress tolerance in the F1 progeny assessed at an early developmental stage. Parental treatment denoted by bar colour as indicated in legend, where Zn(500) = 500 µM ZnSO₄ from stratification for 10 days. The superscript 2 is used to indicate that these F1 plants are the progeny of the repeat zinc stress experiment. Fresh weight (FW) of plants sown onto media supplemented with zinc. Weight recorded 10 days after the end of stratification. Average of 100 plants, 10 repeat experiments. Error bars = SE.
Figure 29. Effect of parental treatment on zinc stress tolerance in the F1 progeny assessed at a later developmental stage. Parental treatment denoted by bar colour as indicated in legend, where Zn(500) = 500 µM ZnSO₄ from stratification for 10 days. The superscript 2 is used to indicate that these F1 plants are the progeny of the repeat zinc stress experiment. F1 progeny subjected to zinc stress from day 21. A. & B. Aerial and root portions weighed on day 35. Average of 7-10 plants, 9 repeat experiments. Error bars = SE. * = p<0.05 relative to Control² (Randomized block design ANOVA). C. Variation in aerial fresh weight at 250 µM Zn across the 9 replicate experiments.
Figure 30. **Effect of two successive parental treatments on zinc stress tolerance in the F2 progeny.** Parental treatment colour coded as indicated in legend, where C=control and Z=zinc stress (500 µM Zn^{2+} from stratification for 10 days), i.e. ZC = zinc stress treatment in F0 and control growth conditions in F1. Seeds sown onto ATS-agar plates supplemented with 0-500 µM Zn^{2+} and plants weighed 10 days after the end of stratification. 50 plants, n= 5. Error bars = SE. * = p<0.05 (ANOVA, Tukey post hoc).

Figure 31. **Distribution of average plant weights for F2 progeny of zinc stressed plants.** CC and ZC denote the parental treatments as indicated in Figure 30. 34 seeds each from 17 individual CC and ZC lines sown onto media supplemented with 250 µM Zn and seedlings weighed 14 days after the end of stratification. Box = middle quartiles, whiskers = range. •=outlier.
Figure 32. Effect of parental zinc treatment on tolerance to heavy/transitional metals and NaCl in F1 progeny. Parental treatments indicated by bar colour, where Zn(500) = 500 µM Zn$^{2+}$ from stratification for 10 days. The progeny of the repeat parental treatment are denoted with a superscript 2. A. Seeds sown onto ATS-agar supplemented with metal as described and weight measured 10 days after end of stratification. Average of 37-50 plants, 3 repeat experiments, Error bars = SE. B. Seeds sown onto ATS-agar and transferred to ATS media in hydroponics 14 days after stratification and subjected to metal stress 7 days later. Plant weight measured 14 days later. Average of 8-12 plants, 6 repeat experiments. Error bars = SE.
4.1.3 DNA methylation status in stress and in progeny following stress

DNA methylation changes are frequently observed following stress treatment (Karan et al., 2012; Steward et al., 2002; Tan, 2010; Tricker et al., 2012; L. Zhong et al., 2009). Stress-dependent DNA methylation changes may be inherited (Ou et al., 2012; Verhoeven et al., 2010), and these may be associated with an increase in stress tolerance in the progeny (Bilichak et al., 2012; Boyko et al., 2010; H. P. Kou et al., 2011; Ou et al., 2012). To identify if the observed stress memory in response to zinc stress was associated with changes in DNA methylation, the MSAP assay outlined in the previous Chapter was utilised. Aerial and root DNA were extracted at the end of the zinc stress treatment (day 14), after recovery (day 21) and in the following generation. Both HpaII/MspI and MboI MSAP assays were utilized with multiple primer pairs. As NaCl is frequently observed to modify DNA methylation (Karan et al., 2012; Tan, 2010; Verhoeven et al., 2010; Zhong et al., 2009), identical primer pairs were also used to analyse DNA methylation in NaCl treated Col-0 and C24 A.thaliana plants. In total, 534 bands were scored across the two MSAP assays and multiple primer pairs. No consistent band changes were observed compared to control for either zinc or NaCl stress in either aerial or root tissue. An example AseI & MboI MSAP gel is shown in Figure 34.

To further examine a possible role for DNA methylation in the NaCl and zinc stress response, the promoter::GUS lines described in Chapter 3 were utilised to establish if either
stress was correlated with changes in the activity of the MET1, POL IV, RDR2, DCL3 or ROS1 promoters. Plants were sown onto ATS-agar plates supplemented with 100 mM NaCl or 500 µM Zn and stained for GUS expression on day 14. In all instances the expression and localisation of GUS was unchanged in the stressed plants, indicating that neither zinc nor NaCl stress affects the activity of these 5 promoters. Expression of pRDR2:GUS and pPOLIV:GUS are shown in Figure 35.

As mentioned above, an attempt to reproduce a previously observed stress memory (Boyko et al., 2010) using NaCl treatment was unsuccessful. In a follow up publication by the same group, Bilichak, A et al. (2012) identified changes in DNA methylation, histone modifications and genes expression in the progeny of NaCl-treated A. thaliana. Their assessment of DNA methylation involved Methyl-DNA immunoprecipitation (MeDIP) using antibodies against methylated cytosine and hybridisation to a genome array consisting of 90 bp reporters covering the whole of chromosome 2 and partially covering chromosomes 3 and 4. Two of the most extreme examples of DNA methylation change were the hypermethylation of the promoters of miRNA 843A (MIR843A) and MSH6. In the two treatments (25 & 75 mM NaCl) the number of methylated reporters in the progeny reportedly increased from 0 to 6/7 (60/70 %) and 0 to 11 (92%) for MIR843A and MSH6 respectively relative to control progeny. In the case of MSH6, this hypermethylation was also negatively correlated with a decrease in mRNA. In order to independently verify this NaCl-stress dependent hypermethylation, bisulphite sequencing was carried out for the two promoters, both after 21 days of stress and in the following generation. Bisulphite sequencing may be considered superior to MeDIP as it enables single base pair resolution analysis of DNA methylation and can be used to estimate the degree of methylation. As a control to identify if full conversion had taken place, bisulphite sequencing of a 157 bp section of the chloroplast PSAA promoter was performed. Full conversion of all 21 cytosines indicated conversion was complete (data not shown). In contrast to Bilichak, A et al. (2012) who reported 0% methylation for the MIR843A promoter in the absence of stress, of the 148 cytosines examined here, 14 (9%) were fully methylated, and a further 40 (27%) partially methylated. Methylation was unaffected by stress, being unchanged with regards to both the number of methylated cytosines and the degree of methylation (Figure 36). There were a small number of changes in the degree of partial methylation, however these were never observed both during NaCl stress and in progeny of NaCl stress. Similarly, the methylation status of the MSH6 promoter was unaffected by stress, remaining almost completely unmethylated (2.2 % methylated). As bisulphite sequencing was conducted on a mixed sample of PCR products rather than cloned fragments, it is possible that a bias was
introduced for the sequences that amplified during sequencing. No attempts were made to verify the lack of bias in sequence amplification by sequencing individual clones.

Figure 34. **MSAP analysis of methylation in zinc and NaCl stress and in the progeny of zinc stressed plants.**
Band profile shown from 2 representative primer pairs. WGA=Whole genome amplified samples (Col-0). Methylation analysed at the end of the stress treatment (14 or 21 days post stratification depending on the stressor). Methylation analysis repeated in the F1 progeny at 21 days under control conditions. (labelled timepoint = $G_1$)
Figure 35. **GUS staining of the pPOLIV:GUS and pRDR2:GUS lines in NaCl and zinc stress.** Plants sown onto ATS-agar supplemented with NaCl or zinc as described. Staining performed 14 days after stratification. 500µM Zn^{2+} caused retardation in development compared to control conditions. An examination of GUS expression did not identify changes in GUS expression in either line across the early developmental stages (not shown), hence all plants shown here are 14 days old.

Figure 36. **Bisulphite sequencing peaks for the MIR843A promoter.** Sequencing performed directly on the PCR product, hence observed intermediate levels of methylation as methylation varies at some sites across the sequenced fragments within a single sample. Genomic sequence show at top. Methylation status of cytosines is inferred from a comparison of genomic sequence and the sequence peaks in the bisulphite treated DNA samples. The degree of methylation was visually assessed by comparing the peak heights resulting from cytosine and thymine incorporation at each cytosine in the genomic sequence. M=methylated (>75% cytosine), P=partially methylated (25-75%), U=unmethylated (<25%).
4.2 Discussion

4.2.1 Most stressful conditions do not produce a stress memory in *Arabidopsis thaliana*

Although there are now numerous reports of stress memories in *A. thaliana* and other plant species, it is becoming clear that these represent an exceptional response to stress rather than the rule (Pecinka et al., 2009). Nitrogen deficiency, elevated temperature, drought and NaCl, all conditions that have previously been observed to generate a stress memory in plants (Boyko et al., 2010; Kou et al., 2011; Whittle et al., 2009), failed to increase tolerance in the progeny here. Taking each stress condition individually, it is possible to provide plausible explanations why all but one of the conditions tested here were apparently not successful in generating a stress memory. One may conjecture that the stress treatments were not appropriate, being too short, too mild or else introduced at the wrong developmental stage. As very little is known about the sensitivity of the mechanism(s) responsible for the generation of a stress memory, it is difficult to assess how appropriate the stress conditions were. However, as zinc stress did generate a transgenerational stress memory, it is clear that treating plants for a short period early in their lifecycle is a suitable approach. Elevated and reduced temperatures were the only stress conditions tested that were applied here solely at a later developmental stage, however, the application of a milder temperature stress from day 21 was previously observed to be sufficient to generate an increased tolerance in the progeny, albeit after two successive treated generations (Whittle et al., 2009). The increased tolerance detected by Whittle, C. *et al* (2009) was specific to the number of seeds produced, with no increase in plant height, rosette diameter or dry mass observed. This observation hints at another possible explanation that no other stress memories were detected here: the experiments carried out to detect an increased tolerance were too narrow. Indeed, reported increases in stress tolerance in the progeny of stressed plants is often limited to a single measurement, such as plant height, and not to other measurements that one would also expect to be affected such as plant weight (Kou *et al.*, 2011; Ou *et al.*, 2012). It is possible therefore that wider stress tolerance testing here could have established the existence of an increased tolerance where none was detected. What the biological benefit of such a transgenerational stress memory would be is questionable; no increase in plant biomass or seed production was observed for the progeny of nitrogen-deficiency or elevated temperature treated plants, suggesting any unobserved stress tolerance would likely have little impact on fitness traits. The examination of stress tolerance in the progeny of PEG-treated plants was limited to early developmental stages and only included germination and
plant biomass measurements. Here then, it is possible to imagine the existence of an undetected stress memory with plant fitness benefits.

The above considerations are clearly insufficient by themselves, however, as a previously reported stress memory could not be reproduced even when the stress treatment was performed using an identical experimental set up in terms of accession, growth media, NaCl concentration, period of treatment and stress tolerance testing in the following generation (Boyko et al., 2010). An intriguing possibility is that the transgenic C24 line (15d8) used by Boyko et al (2010) may be more liable to generating a stress memory, having been generated by Agrobacterium transformation in plant cell culture. Indeed, plant cell culture has been shown capable of altering the DNA methylome (Tanurdzic et al., 2008) and regeneration from plant cell culture can cause further heritable changes in DNA methylation (Stroud et al., 2013). This could also explain the discrepancy in promoter methylation in non-stress conditions between the bisulphite sequencing reported here and the published MeDIP data (Bilichak et al., 2012). However, given that the original transformation was performed 20 years ago it seems unlikely that line 15d8 has been stably altered in such a way as to encourage a transgenerational response to NaCl stress. A more likely hypothesis would be that differences in the experimental parameters not reported by Boyko et al (2010), such as light intensity or humidity explain the absence of stress memory observed here. A recent report highlights the considerable variability in plant growth even across laboratories using apparently very similar growth conditions (Massonnet et al., 2010), and considerable variation in plant weight was observed here across experiments with apparently identical growth conditions (Figure 29C). It follows that having failed to reproduce the stress memory, the previously published concurrent DNA methylation changes were also not reproduced here. The differences in DNA methylation in non-stress conditions between the bisulphite sequencing reported here and the previous MeDIP data may be due to the aforementioned effects of regenerating from a plant cell culture but more likely reflect the higher resolution of bisulphite sequencing compared to MeDIP. If growth conditions do explain the lack of stress memory observed here, this would suggest that stress memories are dependent upon the plant’s experience of stress conditions in combination with other external factors, and not simply the stress treatment by itself. Recent observations indicate that the circadian clock gates responses to low water potential stress (Seung et al., 2012). Such regulation of stress response pathways in response to a multitude of external factors could also exist for the regulation of plant stress memories. The generation of a stress memory in response to a combination of stress treatment and particular growth conditions may also explain the observation here that, while zinc stress reproducibly results in an increased zinc stress tolerance in the progeny, the developmental stage at which this may be
observed is variable, suggesting a somewhat inconsistent nature to the stress memory. The difficulties in reproducing transgenerational stress memories are most strikingly highlighted by a recent attempt to reproduce the observed increase in HRF in the progeny of *A. thaliana* subjected to a number of stressors (Molinier et al., 2006; Pecinka and Mittelsten Scheid, 2012). Using an identical seed line, Pecinka & Mittelsten-Scheid (2012) were unable to reproduce the observed increase in HR in the progeny of stressed plants in all but a few of the conditions tested, and observed that the transgenerational effects appeared stochastically, being independent of degree of stimulation. Their hypothesis that transgenerational stress memories may be subjected to a gating function that combines defined treatments with other extraneous triggers or internal latches fits with the hypothesis put forward here that they are dependent upon a combination of treatment and other external factors. Unfortunately, the results presented here do not increase our understanding of the specific environmental conditions required to produce a stress memory. However, as the transgenerational response to zinc stress conditions has been shown here to be reproducible, it is hoped other groups may be able to independently reproduce this transgenerational stress memory using the conditions detailed here, enabling a more rigorous study of the molecular mechanism.

If we choose to discount the previously observed stress memories that could not be reproduced here, we must consider whether the observation that only zinc stress was found to produce a transgenerational stress memory reflects a fundamental difference in the response of *A. thaliana* to the other stressors. Although no decrease in fitness was observed in the progeny of zinc stressed plants, it must be assumed that, outside of laboratory growth conditions, a transgenerational stress memory will involve a fitness cost in the absence of the stress. Therefore, the balance between fitness costs and benefit could encourage the evolution of a transgenerational stress memory response for stress conditions which would likely continue into the following generation(s), but not for more transient stressors. In the extreme, a stress condition that provided a strong consistent selective pressure would drive genetic change within the population. Theoretically then, a transgenerational response to a particular stress is most likely to evolve only where the stress is intransient enough for the benefit to outweigh the cost, and where the stress lasts only a few generations. Indeed, transgenerational effects are usually observed to last for only a small number of generations in the absence of stress (Boyko and Kovalchuk, 2010; Lang-Mladek et al., 2010; Rahavi et al., 2011), and as observed here can become “reset” during seed aging (Figure 33) (Lang-Mladek et al., 2010), perhaps reflecting the decreasing benefit to the progeny over time. The selective pressure for mechanisms specific to each possible parental stress would be very weak. A more convincing argument can be made for a general transgenerational stress memory response for a broader range of stressful conditions. In this model, when production
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of a transgenerational stress memory is triggered, epigenetic modifications could feasibly carry a stable mark of the transcriptional element of the stress response into the next generation, modifying either the basal transcriptome and/or the stress response transcriptome. In this way, a general transgenerational mechanism could produce a relatively specific transgenerational stress memory in the following generations. That zinc was the only stress observed here to generate a transgenerational stress memory could indicate that the other stressors did not activate this general mechanism. For transient/seasonal stressors, such as elevated or reduced temperature or drought, it is conceivable that the mechanism is not activated, to avoid generating a stress memory unnecessarily. That nitrogen deficiency and elevated NaCl should fail to activate the same transgenerational mechanism requires another explanation as these stress conditions would be similarly intransient in the field as elevated zinc.

Could there be a fitness benefit to the plant to respond transgenerationally to a single generation of zinc stress but not to other stress conditions? One would expect the relationship between the environmental conditions of the parent and the progeny in the field to depend on three major factors: seed dispersal, seed dormancy and germination, and the nature of the individual environmental conditions. Unlike other Brassicaceae, Arabidopsis has not evolved a mechanism to disperse seeds (Vaughn et al., 2011), which although light enough to be further dispersed in the wind, will usually settle close to the parent plant. Arabidopsis does not require particularly specific conditions to germinate and will break dormancy with either a mild stratification or dry storage (Bentsink and Koornneef, 2008). However, A.thaliana avoids germinating in unfavourable conditions with regards to NaCl concentration, water potential and nitrogen availability. Germination was observed to be inhibited by NaCl concentrations exceeding 100 mM and by low water potentials. A.thaliana is also known to regulate germination in response to nitrogen levels by responding to nitrate and nitric oxide (Alboresi et al., 2005; Arc et al., 2013), although no decrease in germination was observed here when NO\textsubscript{3} was reduced 5-fold. Interestingly, increasing the zinc concentration in the media to 1 mM had no effect on germination, a concentration observed here to lead to a severe stress response a few days later. As A.thaliana regulates germination in response to high NaCl, low water potential and NO\textsubscript{3} deficiency, but not in response to zinc, this may reduce the benefit of generating a transgenerational response to NaCl, drought or NO\textsubscript{3} deficiency as the progeny are capable of avoiding these stressors by regulating germination, a response that is not possible for high concentrations of heavy metals.

In summary, most of the stress conditions tested did not result in any transgenerational increase in stress tolerance. The hypothesis put forward to explain the failure to reproduce
previously observed stress memories is that the stress memory mechanism responds to a combination of the defined treatment and other environmental conditions. A second hypothesis has been put forward to explain the results here without making reference to the published stress memories that could not be reproduced: Stress memories are triggered only by intransient stress conditions that do not significantly affect germination and are therefore likely to be experienced by the progeny. This hypothesis is not supported by experimental data but rather has been formulated to explain the observed results here in isolation within an evolutionary framework. It is suggested here that A. thaliana has evolved a transgenerational response to zinc because it has not evolved a seed dispersal mechanism and hence progeny will likely be exposed to similar zinc concentrations as the parent plant and it does not regulate germination in response to zinc. Zinc stress generates a transgenerational stress memory. Experiments to test these hypotheses are put forward in Chapter 6.

Whilst most stressors examined here did not result in an increased stress tolerance in the progeny, zinc stress was found to generate a transgenerational increase in stress tolerance. Although heavy metals have previously been observed to increase tolerance in the progeny (Ou et al., 2012; Rahavi et al., 2011), the observed transgenerational stress memory in response to elevated zinc is, to the author’s knowledge, novel. Further experiments indicate that it is reproducible, stable over one untreated generation and is not stochastic with regards to the individual treated plants. The increased metal tolerance also appears to be specific to zinc.

The original observation of an increased tolerance in the progeny of zinc stressed plants was not definitively heritable through multiple generations, as it was possible the hypertolerance was related to zinc stress-dependent changes in seed composition. However, the stability of the stress tolerance over an untreated generation suggests an epigenetic mechanism and a truly transgenerational stress memory. It is clear that the hypertolerance within the F2 progeny of treated plants is not a consequence of a subset of highly tolerant lines, indicating that the transgenerational stress memory mechanism has been activated at the level of the treated population rather than stochastically with respect to the individual plants treated.

The observation that the transgenerational response, whilst reproducible, is inconsistent with regards to the developmental stage at which an increased tolerance is detected, is difficult to explain. Although the two treatments were identical with respect to the defined chemical treatment, there were some differences in environmental conditions after they had been transferred to soil. In the first experiment, the plants remained in the same growth room as used during stress treatment, with seeds collected by enclosing the aerial portion of the
plants in glassine bags, in the second experiment, the plants were placed in a temperature and light controlled greenhouse and seed collected by enclosing the aerial portions of the plants in micro perforated cellulose. Thus, during the period post-stress, the plants would have been exposed to slightly different light intensities and temperatures, and seeds would likely have developed under different humidities. If these differences in growth conditions post-stress were found to consistently produce the observed differences in the stress tolerance in the progeny, this would fit with the hypothesis that stress memories are generated in response to a combination of defined treatment and other environmental factors, and extend the period during which the plant is sensitive beyond the end of the stress treatment. This could also suggest that particular conditions post-stress may reduce or even erase the stress memory in the treated generation.

The examination of cytosine DNA methylation at CCGG and GATC loci failed to detect any changes in DNA methylation either at the end of the stress treatment, after a recovery period, or in the following generation. As in excess of 500 loci were examined it is reasonable to assume wide-scale changes in DNA methylation levels did not occur. However, DNA methylation remains the most likely mechanism, as it is the only known epigenetic modification which could transmit a transgenerational stress memory in A.thaliana. As described in section 1.7, C.elegans have been shown to utilise a RNAi-related pathway whereby the heritable element is a sRNA rather than an epigenetic mark (Alcazar et al., 2008; Buckley et al., 2012; Luteijn and Ketting, 2013; Shirayama et al., 2012) and a similar mechanism operates in Drosophila melanogaster (Grentzinger et al., 2012). A.thaliana has the prerequisite RNA-dependent RNA polymerase to potentially inherit sRNAs through a similar mechanism although this has not been observed to occur. Thus, whilst DNA methylation changes were not observed, this is still considered to be the most likely mechanism underlying the transgenerational stress memory in response to zinc stress. Such a proposed change in the epigenome would necessarily function to increase stress tolerance through modifying the transcriptome. Given the considerable overlap between stress responses to excesses of different heavy metals (Maestri et al., 2010), it is surprising to note how specific the increased tolerance appears to be. Along with the lack of global DNA methylation changes, this suggests the tolerance is a manifestation of very specific changes in the transcriptome of the progeny. Directly analysing the transcriptome has the advantage of detecting transcriptome changes regardless of the mechanism involved and more directly examines the functional differences in the progeny of zinc stressed plants. Another approach would be to perform whole genome bisulphite sequencing to detect changes in DNA methylation and to follow this up with gene expression analysis. Were DNA methylation differences observed, this would provide direct evidence for an epigenetic change in the
Chapter 4. Identification and characterisation of a novel transgenerational stress memory

...progeny. Furthermore, it is possible that discrete regions of the genome observed to be differentially epigenetically modified may be correlated with stress response genes. However, it is not possible to accurately predict the effect of changes in the DNA methylome on the transcriptome, as DNA methylation status is not directly correlated with expression levels even in promoter regions (Vining et al., 2012; Xiaoyu Zhang et al., 2006). Thus, progressing from DNA methylome changes to changes in the transcriptome to functional biochemical changes in the plant is not straightforward. Furthermore, it is possible that the transgenerational stress memory mechanism is novel. Transcriptome changes would still be a possible way in which a novel mechanism could increase the stress tolerance in the progeny. As such, transcriptomics may identify functional differences where a DNA methylome approach would fail to do so. Although the developmental stage at which the increased stress tolerance is observed differs between the two independent experiments, Chapter identifies similarities in the transcriptome and biochemical changes in the two independent zinc stress progenies.
Chapter 5. Transcriptome and biochemical changes in the progeny of zinc stressed plants
5.1 RNA-sequencing analysis of the progeny of zinc stressed plants

In order to detect transcriptome differences between the progenies of zinc stressed and unstressed *A. thaliana* plants, RNA-sequencing was carried out on RNA extracted from whole CC and ZC 14 day old seedlings grown on either full strength ATS media, or ATS supplemented with 500 µM Zn. This experimental design enables identification of genes differentially expressed in ZC relative to CC in both zinc-stress and non-stress conditions, and allows correlation of these genes with the transcriptional changes that occur during zinc stress.

RNA sequencing samples were prepared as explained in Figure 37. For each RNA-Seq sample, RNA was extracted from 3 batches of seedlings (Control=37-40 plants per batch, +Zn = 100-113 per batch) and pooled in equal measure. Plant weight was observed to be significantly higher in ZC than CC at 500 µM Zn (*p*<0.001, Student’s t-test), although the increase in biomass was less than previously observed with the same seeds (11.2 % compared to 21.0 %; Figure 37). A significant difference in plant weight under zinc stress was still observed between the parental treatments when comparing just those seedling batches used for RNA extractions (*p*<0.05, Student’s t-test). RNA-Seq samples will be referred to as Mock(C), Mock(Z), Zinc(C) and Zinc(Z) to denote whether they received a zinc stress treatment in their parental treatment (Mock = CC, Zinc = ZC) and the conditions the F2 progeny were grown under for transcriptome analysis (C = control, Z = zinc stress), as described in Figure 37.

RNA-Seq samples were analysed by Illumina HiSeq 2500 on a single lane by the Exeter University sequencing service. In total 327 M 100 bp paired end sequence reads were obtained by Exeter University (Mock(C) =120 M, Mock(Z)=68 M, Zinc(C)=68 M, Zinc(Z)=71 M). Exeter University filtered and trimmed reads to remove low quality reads and to remove low scoring base calls, leaving 267 million reads (82%). All further bioinformatics was conducted by the author. 199 million (75%) of the filtered and trimmed reads provided by Exeter University mapped onto the *Arabidopsis thaliana* reference genome for quantification of transcript abundance.
Figure 37. RNA-seq sample preparation, seedling weight and RNA sample nomenclature. Top panel: RNA-seq sample preparation. CC and ZC seeds sown onto ATS and ATS + 500 µM Zn²⁺ agar plates. RNA extracted and pooled as indicated 14 days after stratification. Bottom: Weight of seedlings used for RNA extraction and back-up tissue samples= 37-46 plants per ATS weight measurement, 100-113 plants per ATS + 500 µM Zn²⁺ weight measurement. N=6. *p<0.001 (Student’s t-test). RNA-Seq sample nomenclature based on parental treatment and growth conditions.
5.1.1 Differentially expressed genes

RNA-Seq was performed without repeats, preventing estimation of expression variance and statistical testing of differential transcript abundance between two samples. To select genes differentially expressed in a pairwise comparison of samples, a cut off was set to the calculated fold changes. It was noted that negative correlations existed between gene expression or gene length and the absolute fold-difference in expression between the two control samples, Mock(C) and Zinc(C), such that short and/or lowly expressed transcripts were more likely to be differentially expressed (Figure 38). In part, these correlations could reflect an increased biological variability in expression for genes with a low expression level; however, these relationships are most likely due to the reduced accuracy in transcript abundance quantification for short and lowly expressed genes. Applying a simple fold-change cut-off would therefore preferentially yield short and/or lowly expressed genes. Instead, after removing genes with a low expression and short genes (<256 bp), parameters from the above correlation curves were used to adjust the fold-change values for the remaining 18 697 genes, thereby removing the aforementioned relationships between gene expression or gene length and fold change (Figure 38). After adjustment, genes with a log₂ fold change greater than 5 standard deviations (SD) from the mean were selected as differentially regulated. This cut off is equivalent to a p value of <5.8 x 10⁻⁷. For comparison, a conservative Bonferroni correction (Abdi, 2007) of α=0.05 with 18 697 comparisons (the number of genes analysed) gives a corrected α of 2.7 x 10⁻⁶.

For other pairwise comparisons, fold changes were adjusted using the correlation curves fitted to the control samples data and a cut off applied of pairwise mean +/- 5 control samples SD. In this way, 1935 and 2030 genes were selected as being upregulated and downregulated respectively in Mock(Z) relative to Mock(C). In a pairwise comparison of the progeny of the two parental treatments under control conditions, Zinc(C) vs. Mock(C), 90 genes were differentially regulated (78 downregulated, 12 upregulated). A comparison of the two parental treatments under zinc stress condition yielded 520 differentially expressed genes, of which 259 were upregulated in Zinc(Z) relative to Mock(Z), and 261 were downregulated. All reported log₂ fold changes are from the raw data without adjustment. Only genes passing the cut off are discussed below.
Figure 38. Adjustment of data to remove relationships between gene expression or gene length and fold-difference between the two control samples. Expression values given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Red = Transcripts with Log₂ Zinc(C)/Mock(C) values +/- 5 SD from the mean after adjustment. A. Raw data presented as gene expression vs. fold-difference. B. Correlation curve from plotting average fold-difference (sliding window of 500 genes) vs. expression. C. Data adjusted using parameters from correlation curve in C. and plotted as per A. D. Once adjusted data presented as gene length vs. fold-difference. E. Correlation curve from plotting average fold-difference (sliding window of 100 genes) vs. gene length in once adjusted data. F. Once adjusted data adjusted a second time using parameters from correlation curve in C and plotted as per D. Red lines = Cut off of mean Log₂ Zinc(C)/Mock(C) +/- 5 SD after double adjustment.
Chapter 5. Transcriptome and biochemical changes in the progeny of zinc stressed plants

The 50 most highly zinc-stress upregulated and downregulated genes (derived from the Mock(Z) vs. Mock(C) comparison) are listed in Table 5 & Table 6. The highly upregulated genes include 3 basic helix-loop-helix transcription factors which control the expression of ion homeostasis genes \((\text{BHLH038}, \text{BHLH039} & \text{BHLH100})\), one of their downstream targets which functions in iron uptake in the roots \((\text{IRT1})\), and \text{PLANT CADMIUM RESISTANCE 1} \((\text{PCR1})\). The highly down regulated genes include \text{FE SUPEROXIDE DISMUTASE 1} \((\text{FSD1})\), \text{FERRITIN 4} \((\text{FER4})\) and genes responsive to the hormone jasmonic acid (JA), including \text{VEGETATIVE STORAGE PROTEIN 2} \((\text{VSP2})\) and the peroxidase superfamily protein \text{NATA1}. All genes showing differential expression in the progeny of the zinc stressed plants under control conditions are listed in Table 7 and Table 8. Genes with a reduced expression in Zinc(C) compared to Mock(C) include \text{FERRIC REDUCTION OXIDASE 2} \((\text{FRO2})\), \text{NRAMP METAL ION TRANSPORTER 6} \((\text{NRAMP6})\), JA biosynthesis genes \text{ALLENE OXIDE CYCLASE 1} \((\text{AOC1})\), \text{AOC2} and \text{LIPOXYGENASE 2} \((\text{LOX2})\), and JA-responsive genes including \text{VSP1} & \text{VSP2}, \text{N-ACETYLTRANSFERASE ACTIVITY 1} \((\text{NATA1})\) and \text{TYROSINE AMINOTRANSFERASE 3} \((\text{TAT3})\).

Genes with differential expression in the progeny of zinc stressed plants under zinc stress conditions (Zinc(Z) vs. Mock(Z)) are not listed here as these genes do not show enrichment for particular functions and the observed differences between Zinc(Z) and Mock(Z) are best considered at the level of the expression distribution for stress-responsive genes rather than individual genes in isolation, as explained in section 5.1.1.3.

5.1.1.1 Enriched GO terms within the differentially regulated genes

Gene ontology terms describe the known and predicted function and localisation of gene products. Determining which gene ontology terms are significantly overrepresented within a gene set provides an indication of what pathways are most severely affected. A straightforward analysis of each term in isolation (term-by-term), with a confidence correction for multiple comparisons will usually yield a large number of significantly enriched terms from which one must establish trends in the GO terms. A more sophisticated approach taking into account the relationships between the GO terms will yield a smaller, more useful set of GO terms. For instance, if a parent GO term and of all its children are enriched, a term-by-term analysis will report all the child terms along with the parent term, whereas an analysis taking account of GO term relationships will report only the parent term. The model-based gene set analysis (MGSA), as outlined by Bauer, S. et al, was used here to identify enriched GO terms (Bauer et al., 2010). This approach will yield only a small number of terms, however, each reported term will be more directly relevant to the
whole get set. MGSA associates a marginal posterior probability to each term that reflects the likelihood of its involvement in the process. A cut off of 0.5 is suggested by the authors.

Table 5. Genes upregulated in zinc stress as derived from the Mock(Z) vs. Mock(C) comparison. Top 50 most differentially expressed are listed in order of fold change. Expression values are given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Where the full gene name is not available, a gene family description is given in lower case.
Chapter 5. Transcriptome and biochemical changes in the progeny of zinc stressed plants

Table 6. Genes downregulated in zinc stress as derived from the Mock(Z) vs. Mock(C) comparison. Top 50 most differentially expressed are listed in order of fold change. Expression values given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Where the full gene name is not available, a gene family description is given in lower case.

<table>
<thead>
<tr>
<th>Adi code</th>
<th>Gene full name (Where available)</th>
<th>Gene short name</th>
<th>Mock(Z) FPKM</th>
<th>Mock(C) FPKM</th>
<th>Log_{2} fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATIG05940</td>
<td>POLY-1</td>
<td>PEL3</td>
<td>0.4</td>
<td>3.8</td>
<td>3.4</td>
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<tr>
<td>AT1G12180</td>
<td>MYB DOMAIN PROTEIN 14</td>
<td>MBR24</td>
<td>0.6</td>
<td>3.3</td>
<td>2.5</td>
</tr>
<tr>
<td>AT1G24090</td>
<td>Protein of unknown function</td>
<td>ATAP4</td>
<td>2.3</td>
<td>3.8</td>
<td>1.3</td>
</tr>
<tr>
<td>AT1G25990</td>
<td>LOW EXPRESSION OF COSMODIVISORY GENES 1</td>
<td>LCE1</td>
<td>5.1</td>
<td>12.6</td>
<td>1.3</td>
</tr>
<tr>
<td>AT2G02790</td>
<td>SHOOT GRAVITROPISM 3</td>
<td>SIG3</td>
<td>9.9</td>
<td>20.7</td>
<td>1.1</td>
</tr>
<tr>
<td>AT2G16600</td>
<td>TYPE ONE SERINE-THREONINE PROTEIN PHOSPHATASE 4</td>
<td>TSP4</td>
<td>0.4</td>
<td>7.7</td>
<td>0.9</td>
</tr>
<tr>
<td>AT2G18600</td>
<td>PRODUCTION OF ANTIMYCOTIC PHENOL 1</td>
<td>PAP1</td>
<td>10.1</td>
<td>19.4</td>
<td>0.9</td>
</tr>
<tr>
<td>AT2G24420</td>
<td>PROTEIN PHOSPHATASE 2CA</td>
<td>PPA2</td>
<td>10.2</td>
<td>18.5</td>
<td>0.9</td>
</tr>
<tr>
<td>AT3G47340</td>
<td>GLUTATHIONE-DEPENDENT ASPARAGINE SYNTHETASE 1</td>
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<td>101.0</td>
<td>179.0</td>
<td>0.8</td>
</tr>
<tr>
<td>AT3G67870</td>
<td>FRAGRANT HOMOLOG 6</td>
<td>FHS</td>
<td>5.4</td>
<td>9.5</td>
<td>0.8</td>
</tr>
<tr>
<td>AT3G39210</td>
<td>CYPRESS-LIKE PROTEIN KINASE DZETA</td>
<td>SKDZETA</td>
<td>7.8</td>
<td>13.0</td>
<td>0.7</td>
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<tr>
<td>AT1G75310</td>
<td>AUXIN-LIKE 1</td>
<td>AUX1</td>
<td>12.5</td>
<td>18.9</td>
<td>0.6</td>
</tr>
</tbody>
</table>

Table 7. Genes with higher expression in Zinc(C) relative to Mock(C) listed in order of fold change. Expression values given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Where the full gene name is not available, a gene family description is given in lower case.

<table>
<thead>
<tr>
<th>Adi code</th>
<th>Gene full name (Where available)</th>
<th>Gene short name</th>
<th>Zinc(C) FPKM</th>
<th>Log_{2} fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1G24090</td>
<td>Protein of unknown function</td>
<td>ATAP4</td>
<td>2.3</td>
<td>3.8</td>
</tr>
<tr>
<td>AT1G25990</td>
<td>LOW EXPRESSION OF COSMODIVISORY GENES 1</td>
<td>LCE1</td>
<td>5.1</td>
<td>12.6</td>
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<tr>
<td>AT2G02790</td>
<td>SHOOT GRAVITROPISM 3</td>
<td>SIG3</td>
<td>9.9</td>
<td>20.7</td>
</tr>
<tr>
<td>AT2G16600</td>
<td>TYPE ONE SERINE-THREONINE PROTEIN PHOSPHATASE 4</td>
<td>TSP4</td>
<td>0.4</td>
<td>7.7</td>
</tr>
<tr>
<td>AT2G18600</td>
<td>PRODUCTION OF ANTIMYCOTIC PHENOL 1</td>
<td>PAP1</td>
<td>10.1</td>
<td>19.4</td>
</tr>
<tr>
<td>AT2G24420</td>
<td>PROTEIN PHOSPHATASE 2CA</td>
<td>PPA2</td>
<td>10.2</td>
<td>18.5</td>
</tr>
<tr>
<td>AT3G47340</td>
<td>GLUTATHIONE-DEPENDENT ASPARAGINE SYNTHETASE 1</td>
<td>ASN1</td>
<td>101.0</td>
<td>179.0</td>
</tr>
<tr>
<td>AT3G67870</td>
<td>FRAGRANT HOMOLOG 6</td>
<td>FHS</td>
<td>5.4</td>
<td>9.5</td>
</tr>
<tr>
<td>AT3G39210</td>
<td>CYPRESS-LIKE PROTEIN KINASE DZETA</td>
<td>SKDZETA</td>
<td>7.8</td>
<td>13.0</td>
</tr>
<tr>
<td>AT1G75310</td>
<td>AUXIN-LIKE 1</td>
<td>AUX1</td>
<td>12.5</td>
<td>18.9</td>
</tr>
</tbody>
</table>
Table 8. Genes with lower expression in Zinc(C) relative to Mock(C) listed in order of fold change. Expression values given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Where the full gene name is not available, a gene family description is given in lowercase.
Enriched gene ontology terms for the genes differentially expressed in zinc stress (Mock(Z) relative to Mock(C)) are shown in Table 9. GO terms enriched within the upregulated genes included terms expected in response to zinc stress: “iron ion homeostasis”, “hydrogen peroxide biosynthetic process” and 5 response terms: cyclopentenone, heat, karrakin, fungus and UV-B.

As expected, enriched GO terms within the downregulated genes included terms relating to regulating plant growth: “root epidermal cell differentiation”, “regulation of meristem growth” and “cell cycle cytokinesis”, as well a stress response term, “Response to desiccation”, and a term relating more specifically to zinc stress, “transition metal ion transport”.

### Table 9. Gene ontology terms enriched for genes upregulated and downregulated in zinc stress (Mock(Z) relative to Mock(C)). Terms listed in order of marginal posterior probability. Biological function, molecular function and cellular localisation terms listed separately.

<table>
<thead>
<tr>
<th>Upregulated</th>
<th>GO term</th>
<th>Fold enrichment</th>
<th>Marginal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response to cyclopentenone</td>
<td>4.4</td>
<td>0.97</td>
<td></td>
</tr>
<tr>
<td>Response to heat</td>
<td>3.7</td>
<td>0.99</td>
<td></td>
</tr>
<tr>
<td>Amino acid transport</td>
<td>3.2</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Response to karrakin</td>
<td>2.7</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Iron ion homeostasis</td>
<td>4.4</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Hydrogen peroxide biosynthetic process</td>
<td>4.1</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>Response to fungus</td>
<td>3.4</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Nucleotide biosynthetic process</td>
<td>3.6</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Response to UV-B</td>
<td>3.0</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>Rhythmic process</td>
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<td></td>
</tr>
<tr>
<td>Female gamete generation</td>
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<td>0.52</td>
<td></td>
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<tr>
<td>ATPase activity</td>
<td>3.2</td>
<td>0.51</td>
<td></td>
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<td>UDP glycosyltransferase activity</td>
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<td></td>
</tr>
<tr>
<td>ADP binding</td>
<td>2.9</td>
<td>0.83</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Downregulated</th>
<th>GO term</th>
<th>Fold enrichment</th>
<th>Marginal</th>
</tr>
</thead>
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<tr>
<td>Transition metal ion transport</td>
<td>3.9</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td>Acid phosphatase activity</td>
<td>5.7</td>
<td>0.86</td>
<td></td>
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<tr>
<td>Microtubule-based movement</td>
<td>4.7</td>
<td>0.79</td>
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</tr>
<tr>
<td>Root epidermal cell differentiation</td>
<td>3.2</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>Cell wall biogenesis</td>
<td>3.8</td>
<td>0.74</td>
<td></td>
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<tr>
<td>Lignin metabolic process</td>
<td>3.4</td>
<td>0.67</td>
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<tr>
<td>Regulation of meristem growth</td>
<td>3.6</td>
<td>0.61</td>
<td></td>
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<tr>
<td>Cell cycle cytokinesis</td>
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<td>0.60</td>
<td></td>
</tr>
<tr>
<td>Response to fructose stimulus</td>
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<td>0.57</td>
<td></td>
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<tr>
<td>Symptom formation</td>
<td>5.7</td>
<td>0.54</td>
<td></td>
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<tr>
<td>Cyclin-dependent protein kinase regulator activity</td>
<td>4.1</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td>Response to desiccation</td>
<td>5.0</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td>Alkylation/alkylglycerol transferase activity</td>
<td>5.5</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Plant-type cell wall</td>
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<tr>
<td>Chloroplast stroma</td>
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<td>0.78</td>
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</tbody>
</table>

Enriched GO terms for genes with a reduced expression in Zinc(C) relative to Mock(C) included the related terms “iron ion binding” and “iron ion homeostasis”, as well as “jasmonic acid biosynthetic process” and the related “response to cyclopentenone”, and an additional response term, “response to light intensity (Table 10).

Analyses for genes with an increased or decreased expression in Zinc(Z) vs. Mock(Z) did not identify any enriched GO terms. However, approximately 40% of the genes differentially expressed in Zinc(Z) vs. Mock(Z) were identified as zinc stress-responsive (Mock(Z) vs. Mock(C)) and overall trends were observed in the expression of zinc stress-responsive genes in Zinc(Z) vs. Mock(Z), as discussed in section 5.1.1.3. GO enrichment analysis was therefore performed on genes differentially expressed in Zinc(Z) vs. Mock(Z)
against a background population of all zinc stress response genes. This analysis identified
the single GO term “response to hydrogen peroxide” as being enriched.

<table>
<thead>
<tr>
<th>GO term</th>
<th>Enrichment</th>
<th>Marginal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase activity</td>
<td>24.8</td>
<td>1.00</td>
</tr>
<tr>
<td>Carbohydrate binding</td>
<td>1.8</td>
<td>0.99</td>
</tr>
<tr>
<td>ADP binding</td>
<td>4.4</td>
<td>0.97</td>
</tr>
<tr>
<td>Iron ion binding</td>
<td>4.8</td>
<td>0.75</td>
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<tr>
<td>Heme binding</td>
<td>3.0</td>
<td>0.70</td>
</tr>
<tr>
<td>Response to light intensity</td>
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<tr>
<td>Response to cyclopentenone</td>
<td>8.9</td>
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</tr>
<tr>
<td>Ornithine metabolic process</td>
<td>124.0</td>
<td>0.94</td>
</tr>
<tr>
<td>Iron ion homeostasis</td>
<td>11.6</td>
<td>0.90</td>
</tr>
<tr>
<td>Lipid transport</td>
<td>5.4</td>
<td>0.86</td>
</tr>
<tr>
<td>Jasmonic acid biosynthetic process</td>
<td>19.4</td>
<td>0.64</td>
</tr>
<tr>
<td>Vacuole</td>
<td>6.1</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Table 10. Gene ontology terms enriched for genes with reduced expression in Zinc(C) relative to Mock(C).
Terms listed in order of marginal posterior probability. Biological function, molecular function and cellular
localisation terms listed separately.

5.1.1.2 Stress response genes differentially expressed in the progeny of zinc stressed plants.

The zinc treatments employed here to examine the transcriptome of Mock and Zinc progeny under zinc stress is identical to the zinc stress treatment two generations previously. This allows comparison and correlation between zinc stress-responsive genes with genes whose expression is altered in the F2 progeny of zinc stressed plants.

Venn diagrams are shown in Figure 39 depicting the overlap between the genes
differentially regulated by zinc stress (Mock(Z) vs. Mock(C)) and the genes showing
differential expression between the parental treatments in non-stress conditions (Zinc(C) vs. Mock(C)). Many genes with a lower expression in Zinc(C) relative to Mock(C) are
differentially expressed in response to zinc stress (45/79 = 57 %, of which 25 are zinc stress upregulated and 20 are zinc stress downregulated). These genes are re-listed in Table 11.
Genes upregulated in zinc stress with a reduced expression in Zinc(C) compared to Mock(C) include a class II heat shock protein (HSP17.6II), a cytochrome P450 (CYP71A12), the ion homeostasis gene BHLH038 and the ferric reductase FRO2.

Genes downregulated in zinc stress with a reduced expression in Zinc(C) relative to Mock(C) include the JA-responsive genes NATA1, VSP1 & VSP2 and the ferric reductase FRO6.
Chapter 5. Transcriptome and biochemical changes in the progeny of zinc stressed plants

Genes observed to be differentially expressed in the progeny of zinc stressed plants compared to the progeny of mock treated plants when grown under non-stress conditions will be referred to as SMTs (transgenerational Stress Memory Transcripts). Little overlap was observed between genes differentially expressed in Zinc(C) relative to Mock(C) and those differentially expressed in Zinc(Z) relative to Mock(Z). A few genes, AVRRPT2-INDUCED GENE 1 (AIG1), GRX480, EIF4A-2 and TSK-ASSOCIATING PROTEIN 1 (TSA1), were observed to have a reduced expression in Zinc relative to Mock in both control and stress growth conditions (Table 11).

Figure 39. Venn diagrams of overlap between zinc stress responsive genes and genes with an increased or decreased expression in the progeny of zinc stressed plants relative to control progeny under non-stress conditions. Top and bottom right circles = stress responsive genes (Mock(Z) vs. Mock(C)). Bottom left circle = Differential expression in the progeny of zinc stressed plants in non-stress conditions (Zinc(C) vs. Mock(C)), Red = Zinc stress-downregulated, Green=Zinc stress-upregulated, Blue =Reduced expression in Zinc(C) vs. Mock(C), Yellow = Increased expression in Zinc(C) vs. Mock(C). Arrows highlight direction of change relative to Mock(C).

5.1.1.3 The progeny of zinc stressed plants display a reduced zinc stress response.

As mentioned above, there is very little enrichment of functional groups within the genes passing the cut-off for differential expression in the Zinc(Z) vs. Mock(Z) comparison. Instead, when we observe the overall distribution of fold difference in expression for zinc stress-responsive genes it is apparent that zinc-stress response genes are less activated/repressed in Zinc(Z) compared to Mock(Z) (Figure 40). On average, genes upregulated in response to zinc stress show a 16.8 % reduced expression in Zinc(Z) relative to Mock(Z), whilst downregulated genes show a 16.2 % increased expression in Zinc(Z) relative to Mock(Z).
Table 11 Zinc stress responsive genes with a reduced expression in Zinc(C) relative to Mock(C). Expression values given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Fold changes (Log2) given where they passed the cut off of +/- 5 SD from the mean after adjustment. Fold changes colour coded, red = downregulated, green = upregulated, blue = reduced expression, yellow = increased expression. Genes ordered by fold difference in Zinc (C) vs. Mock (C). Where the full gene name is not available, a gene family description is given in lower case.
5.1.2 Analysis of functional gene groups

5.1.2.1 DNA methylation and demethylation proteins in zinc stress

As suggested in section 4.1.3, DNA methylation remains the most likely mechanism by which a transgenerational stress memory could be passed onto the next generation. Assuming that changes in DNA methylation occur during stress, one might expect changes in the expression of DNA methylation and/or demethylation genes. To establish if this was the case, the expression of 17 genes involved in DNA methylation or DNA demethylation (Furner and Matzke, 2010) was analysed under zinc stress conditions. These genes included the DNA methyltransferases, MET1, CMT3, DRM2 & DRM1, the DNA demethylases, DME1, 2 & 3 and ROS1, the histone H3 K9 lysine methyltransferase and demethylase, KYP and IBM1, and the methylation DNA binding proteins VIM1, 2 & 3.

Table 12 gives results for all genes investigated. The DNA methyltransferases CMT3 and MET1 were observed to be downregulated in zinc stress, as was VIM1 which is required for maintenance of CG methylation (Woo et al., 2007). Conversely, IBM1 and DME1, and DML2 were upregulated in zinc stress.
None of the 17 DNA methylation/demethylation genes were differentially expressed between the two progenies. Many components of the *A. thaliana* de novo DNA methylation pathway have been identified (Greenberg *et al*., 2011). None of these were found to be zinc stress-responsive (data not shown).

Table 12. Expression of genes involved in DNA methylation and DNA demethylation in zinc stress (Mock(Z) vs. Mock(C)). Expression values given as fragments per kilobase exon gene model per million mapped fragments (FPKM). Fold changes (Log2) given where they passed the cut off of +/- 5 SD from the mean after adjustment. Fold changes colour coded, red=downregulated, green = upregulated. Genes ordered by fold difference in Mock (Z) vs. Mock (C).

<table>
<thead>
<tr>
<th>AGI code</th>
<th>Gene full name</th>
<th>Gene short name</th>
<th>locus</th>
<th>Mock(Z) FPKM</th>
<th>Mock(C) FPKM</th>
<th>Log fold change</th>
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</thead>
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<tr>
<td>AT1G07000</td>
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<td>CH3M1</td>
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<td>DEM</td>
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</tr>
</tbody>
</table>

5.1.2.2 The JA precursor 12-oxo-phytodienoic acid (OPDA) partly functions independently to JA in response to zinc stress

As many genes with a reduced expression in Zinc(C) relative to Mock(C) were JA biosynthesis genes, and JA-responsive genes were downregulated in response to zinc stress, the potential function of JA in zinc stress was explored. As the JA biosynthesis genes with a reduced expression in Zinc(C) all encoded proteins that function in the production of the JA-precursor OPDA (Dave and Graham, 2012), the RNA-Seq data was interrogated to investigate if OPDA or JA-regulated genes are a component of the zinc stress response. OPDA-regulated and JA-regulated genes have previously been identified based on gene expression in response to OPDA, JA or Methyl-JA stimulus (Taki *et al*., 2005). Using these identifications, the 135 genes upregulated by OPDA were on average 2.47-fold upregulated in zinc stress and the 316 JA-upregulated genes were unaffected (Figure 41A).
5.1.2.3 Specific JA and OPDA-regulated genes have a reduced expression in the progeny of zinc stressed plants in non-stress conditions

The expression of both OPDA and JA-upregulated genes is generally unaffected in Zinc(C) relative to Mock(C), suggesting that the reduced expression of OPDA synthesis genes under non-stress conditions does not have an overall effect on the expression of either OPDA or JA regulated genes in non-stress conditions (Figure 41B). However, there are a number of OPDA and JA-regulated genes whose expression is affected, as observed by the left hand tails in the distributions. Looking back at the GO term analysis the term “response to cyclopentenone” is enriched in the genes with a lower expression in Zinc(C) vs. Mock(C) (Table 10). Although these genes are apparently not regulated by the cyclopentenone OPDA (Taki et al., 2005), it is possible that they are regulated by a OPDA-derived compound or OPDA precursor.

A subset of the JA-upregulated genes are affected in non-stress conditions; 21 out of 78 genes with reduced expression under non-stress conditions are identified as JA-upregulated (Taki et al., 2005), a 30-fold enrichment over the background population. The GO term “response to jasmonic acid” was not reported by Gene Ontology enrichment analysis with MGSA as it is designed to report as few terms as possible and some of these genes are annotated as being involved in JA biosynthesis, therefore the term "jasmonic acid biosynthetic process" was reported instead (Table 10). Analysing the GO terms on a term-by-term basis with Benjamini-Hochberg correction (Benjamini, Y, Hochberg, 1995), the enrichment of “response to jasmonic acid” in the Zinc(C) vs. Mock(C) comparison is very significant (4.73e-06). In summary, the expression of the vast majority of OPDA and JA-regulated genes were unaffected by the parental treatment, although a subset of cyclopentenone and JA-regulated genes were expressed at a lower level in Zinc(C) relative to Mock(C).
5.1.2.4 Spliceosome differences could not be established

One of the advantages of RNA-Seq over microarrays for transcriptomics is that RNA-Seq can provide additional information on which splice variants are expressed. Unfortunately,
analysis of differential expression of splice variants yielded a great many results that were not confirmed by visual assessment of the mapped reads. The accuracy of the parsimonious set of transcripts constructed to explain the mapped reads is greatest when combining all the possible gene models into a single gene expression estimate. It appears that due to the lower accuracy of the splice variant expression estimates compared to gene expression estimates, the analysis of splice variants is much more negatively affected by the lack of repeats.

5.1.3 Validation of RNA-Seq results

5.1.3.1 Elevated zinc and iron deficiency cause similar transcriptome changes

Similarities in the transcriptional responses to elevated zinc and iron deficiency have been demonstrated previously (Shanmugam et al., 2011; T. J. W. Yang et al., 2010). To verify that zinc stress produced a transcriptome change in line with expectations, the presence of a Fe-deficiency transcriptome signature was examined. The GO terms “iron ion homeostasis” and “transition metal ion transport” are enriched in the zinc stress responsive genes (Table 9). Additionally, a significant positive correlation was observed between the expression changes identified by Yang et al (2010) in Fe-deficiency and the expression changes identified in zinc stress, even across different transcriptomics analysis platforms (Figure 42, \( p < 0.05 \), Pearson's r test of significance).

![Figure 42. A comparison of the Fe-deficiency and Zn stress transcriptome responses.](image)

Expression relative to control (Log2) in zinc stress and Fe-deficiency for genes with 2-fold up/down regulation in response to Fe-deficiency by microarray analysis.
5.1.3.2 Quantitative reverse transcriptase-PCR

The RNA-Seq samples were derived from equal pooling of 3 RNA samples from at least 37 plants each, which should ensure the RNA-Seq quantification is a reasonable estimate of mean expression. However, without repeats, it is not possible to establish the biological variation which make statistical testing of expression differences impossible. In order to independently validate some of the RNA-Seq results reported in 5.1.1, (SYBR-green) quantitative reverse transcriptase PCR (qRT-PCR) was performed in triplicate using the same 12 RNA samples that were used for RNA-Seq, with 3 technical replicates of each. Genes were selected based on their assumed biological relevance in the observed transgenerational stress memory; hence most of the selected genes were SMTs. Expression quantification was achieved by normalising expression to 3 reference genes, AT2G28390, AT5G15710 and AT5G14030. The first two reference genes were selected for their stable expression in metal stress (Remans et al., 2008) and stable expression across the RNA-Seq results obtained. The third reference gene was selected based upon its stable expression in the RNA-Seq data and across the perturbations available in Genevestigator (Hruz et al., 2008).

A visualisation of gene expression relative to Mock(C) shows that the qRT-PCR data closely fits the RNA-Seq data (Figure 43). A linear line of best fit between RNA-Seq and qRT-PCR data gives a correlation coefficient of 0.81. Whilst the qRT-PCR data for most genes closely fits the RNA-Seq data, there were a few instances of disagreement. For example, whereas in the RNA-Seq data, ATGPX1 appeared to be downregulated in response to zinc stress in Mock(Z) but not Zinc(Z) (Figure 43A), the qRT-PCR data indicated ATGPX1 was downregulated in both samples (Figure 43B).

In total, 5 out of 8 SMTs were confirmed to have significantly lower expression in Zinc(C) relative to Mock(C) by qRT-PCR (AOC1, EIF4A-2, VSP1, and BHLH038 & BHLH039). In addition, JAL23 was found to have a lower expression by qRT-PCR, however, the difference was not significant (p=0.067), largely due to considerable variation in the Mock(C) samples.
Figure 43. Independent validation of RNA-Seq by qRT-PCR. A & B: RNA-Seq and PCR data expressed at log₂ fold change relative to Mock(C). B. Error bars = SE, 3 repeat experiments with 3 technical repeats each. * p<0.05, **p<0.01, ***p<0.001. ND=No data. JAL23, BHLH038 & BHLH039 transcript abundances not measured in Mock(Z) and Zinc(Z) samples. C. Correlation of log₂ fold change (relative to Mock(C)) from RNA-Seq and qRT-PCR. Error bars = qRT-PCR SE.
5.1.4 SMT expression in the progeny of the independent zinc stress experiment

As observed in section 4.1.2.1, zinc stress reproducibly results in a transgenerational stress memory in the following generation, however, the developmental stage at which this increased tolerance is observed is inconsistent. To examine whether the same SMTs were consistently differentially expressed in the progeny of zinc stressed plants, qRT-PCR was carried out on RNA samples extracted from 35 day old Zn(500)\(^2\) and Control\(^2\) plants which were previously observed to be hypertolerant to zinc stress at this developmental stage (Figure 29A & B). Expression of SMTs validated by qRT-PCR, VSP1 and AOC1 was examined in the root and aerial portions of plants grown under control and zinc stress conditions. In addition, two further SMTs, JAL23 and NATA1, which showed 13-fold and 47-fold reduced expression in Zinc(C) by RNA-Seq, respectively, were examined. Figure 44 shows transcript abundances as measured by qRT-PCR in relative expression units. AOC1, VSP1 and NATA1 were all observed to be differentially expressed in aerial tissue in response to zinc stress when assessed at day 35. However, all three genes showed increased expression in Zn(500)\(^2\) relative to Control\(^2\) when assessed at day 35, although they show decreased expression in Mock(Z) relative to Mock(C) when assessed at day 14 (section 5.1.3.2). For example, VSP1 was observed to be downregulated \(>8\)-fold when plants were subjected to zinc stress from day 0-14, but was 2.4-fold upregulated in aerial tissue when plants were subjected to zinc stress from day 21-35 (Figure 41 & Figure 44). Looking at the three SMTs at day 35 in control conditions, AOC1, VSP1 and NATA1 expression is 1.6, 1.5 and 2.7-fold higher, respectively, in Zn(500)\(^2\) relative to Control\(^2\). For AOC1, the increase in expression in Zn(500)\(^2\) relative to Control\(^2\) in non-stress conditions is similar to the increase which occurs during zinc stress. For VSP1 and NATA1 the increase in expression in Zn(500)\(^2\) relative to Control\(^2\) is less than the increase which occurs in zinc stress. It appears that, at least looking across these three SMTs, the expression of SMTs in the progeny of zinc stressed plants reflects a mild zinc stress-like response, similar to the expression profiles of these genes in the RNA-Seq data.
Figure 44. qRT-PCR quantification of AOC1, VSP1, NATA1 and JAL23 transcripts in root and aerial portions of the progeny of zinc stressed (Zn(500)2) and control plants (Control2) in zinc stress (500 µM Zn²⁺; +Zn) and control (Zn) growth conditions. Expression values are expression relative to the mean expression of the 3 reference genes. Error bars = SE. Three repeat experiments were performed with 3 technical replicates each. *p<0.05, Zn(500)2 compared to Control2 (Student’s t-test with Benjamin & Hochberg False Discovery Rate correction).
5.2 Ferric reductase oxidase activity in the roots of zinc stress memory plants

One of the most obvious functional groups within the SMTs relates to iron uptake and distribution and includes the basic helix loop helix (bHLH) transcription factor BHLH038, the root ferric reductase gene it regulates, FRO2 (Colangelo and M. Lou Guerinot, 2004; Y. Yuan et al., 2008), and two further ferric reductases, FRO6 and FRO7. Additionally, BHLH039, BHLH100 and IRT1 which function in the same iron uptake pathway as BHLH038 and FRO2 (Y. Yuan et al., 2008) were just below the z-score cut off of +/- 5, at 4.24, 4.97 and 4.26 respectively. FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR 1 (FIT1) - the bHLH binding partner of BHLH038 and BHLH039, required for their transcription factor activity - was not differentially expressed in Zinc(C) relative to Mock(C).

Since reduced expression of FRO2 in the progeny of zinc stressed plants would be expected to reduce ferric reductase activity in the roots and zinc stress is known to increase root ferric reductase activity (Shanmugam et al., 2011), a root FRO activity assay was performed across a range of Zn\(^{2+}\) concentrations to quantify the reduction of Fe(III) to Fe(II). The Zn(500)\(^{2+}\) progeny were utilized for the experiment as the transgenerational stress memory had reset in the ZC line (Figure 33).

Seedlings were transferred on day 7 onto media supplemented with 250-500 µM Zn. Root weight was higher in Zn(500)\(^{2+}\) relative to Control\(^{2+}\) at 250 and 500 µM Zn, although parental treatment did not have a significant effect on root weight either by itself or in interaction with zinc concentration \((p>0.05)\). Root ferric reductase enzyme activity was measured on day 14 (Figure 45). The parental zinc treatment had a significant effect on Fe(III) chelate reductase activity \((p<0.01, 2\text{-way ANOVA})\).

Reduced ferric reductase enzyme activity in the roots of zinc-stressed plants would be expected to impact on chlorophyll production in the aerial portion of the plant as reduced iron is known to encourage chlorosis (García-Mina et al., 2013; Sivitz et al., 2012). To test if parental treatment influenced the ability of the progeny to produce chlorophyll under iron limited conditions and how they responded to zinc stress under iron limited or excess iron conditions, plants were grown under 3 concentrations of iron for 14 days (5, 50 & 500 µM Fe), with zinc stress (500 µM) introduced for half the plants at day 7. At 14 days, root
weight, aerial chlorophyll content, and root Fe(III) chelate reductase activity were all measured.

As expected, increased Fe alleviated the zinc stress associated chlorosis (Figure 46, bottom left) and lead to a slight increase in root weight under zinc stress conditions (Figure 46, top left and right). Root weight was significantly influenced by Zn and Fe concentrations (p=0.011, p=0.003, 2-way ANOVA), being highest under 50 µM Fe with no added Zn. Root weight was also influenced by Zn and Fe (p<0.000), with 500 µM Fe reducing root weight but alleviating the negative effect of Zn on root weight. Root FRO activity was significantly affected by Zn and the Fe*Zn interaction (p=0.015, p=0.003, respectively), with root FRO activity higher upon addition of Zn. Chlorophyll content was significantly affected by Zn, Fe and Zn*Fe (all p<0.000) with chlorophyll content reduced to less than 50% with addition of 500 µM Zn in 5 or 50 µM Fe but only slightly reduced with addition of 500 µM Zn in 500 µM.

Whilst root weight and root ferric reductase activity were both affected by parental treatment, chlorophyll content was not. Root weight was affected by parental treatment (p<0.000), being higher in Zn(500)² under all conditions. Root FRO activity was affected by parental treatment (p<0.000) and the parental treatment*Zn interaction (p< 0.000), being lower in Zn(500)² with the difference between the parental treatments most prominent in 500 µM Zn and 5 µM Fe. Chlorophyll content was not affected by parental treatment.
In summary, parental zinc stress treatment reduces the root ferric reductase activity under zinc stress and increases root weight, whilst having no impact on chlorophyll content.

Figure 46. Root weight, root Fe(III) chelate reductase activity and aerial chlorophyll content in F1 progeny of zinc stressed plants under varying concentrations of zinc and iron. Parental treatment denoted by bar colour, where Zn(500) = 500 µM Zn for 10 days from stratification. The superscript 2 indicates that these are the F1 progeny of the repeat parental zinc stress experiment. Seedling were transferred 7dps from control conditions (1 µM Zn$^{2+}$) onto solidified media containing the indicated concentrations of Zn. Fe$^{3+}$ was maintained at the indicated concentration throughout the experiment. Root weight, root FRO activity and aerial chlorophyll concentrations were measured 7 days later. Error bars = SE, 28-30 plants, 3 repeat experiments.
5.3 Discussion

In section 4.1.2, an increased zinc stress tolerance in the progeny of zinc stressed plants was discussed. The hypothesis put forward was that epigenetic modifications would be the most likely mechanism for such a transgenerational memory of stress. Such a proposed change in the epigenome would necessarily function to increase stress tolerance through modifying the transcriptome. Therefore, to detect the hypothesised transcriptome changes, RNA-Seq was performed on RNA samples from the F2 progeny of zinc stressed plants, both in zinc stress and non-stress conditions. This approach has the advantage of detecting transcriptome changes regardless of the mechanism involved and more directly examines the functional differences in the progeny of zinc stressed plants. Before considering the possible biological significance of the transcriptome changes observed in this Chapter, it is important to note that no attempt has been made to correlate changes in the transcriptome changes with the proteome. Whilst changes in DNA transcription would be expected to partially correlate with changes in protein level, buffering can act between the transcriptome and proteome (Fu et al., 2009). For example, a recent report of transcriptome and proteome changes in A. thaliana upon phosphate starvation in roots found that while the correlation between gene and protein expression changes was significant across differentially expressed transcripts, the correlation was most pronounced where transcriptome changes were large (>4 fold) (Lan et al., 2012). Of the transcriptome changes observed in this Chapter, only the reduced expression of BHLH038, BHLH039 and FRO2 was followed up with an analysis of the biochemical differences between the progenies. For all other transcriptome changes observed, the impact of the transcriptome on the proteome, metabolome, and ultimately the phenome remain unknown. For the purposes of all further discussion the transcriptome differences observed are assumed to generate changes in the proteome in the same direction. However, bearing in mind the absence of proteomic data, the hypotheses put forward here to explain the RNA-Seq data are largely concerned with functional groups rather than individual genes.

Before the RNA-Seq data was analysed the expectation was that the zinc hypertolerance observed in the second generation progeny of zinc stress plants would be correlated with either an altered transcriptome response to zinc stress or an altered transcriptome in the absence of stress (Referred to as mechanisms 1 and 2 in Figure 2). Interestingly, in the hypertolerant progeny of zinc stressed plants, zinc stress response genes are on average 16.5 % less up- or downregulated (Figure 40). This reduced stress response is observed across the whole range of zinc stress response genes, although the GO term “response to
hydrogen peroxide” is over-represented amongst the genes passing the cut off for differential expression in Zinc(Z) relative to Mock(Z). Given that the reduced stress response is observed across all aspects of the stress response, the most likely explanation is that the plants are suffering a lower degree of stress. This could account for the observed increase in biomass relative to the control progeny (Figure 26). Within this lower severity of stress experienced, the enrichment for “response to hydrogen peroxide” indicates that reactive oxygen species may be particularly reduced. Since the progeny of zinc stressed plants do not appear to display an altered response to zinc stress, the hypertolerance is likely to be dependent on transcriptome differences in the absence of stress.

Differences were indeed observed by RNA-Seq between the non-stress transcriptomes of the two progenies, even though the stress occurred two generations previously for only 14 days, and with the transcriptome of whole seedlings being studied. The vast majority of the changes are of genes with a lower expression in the progeny of zinc stressed plants. As the majority of these genes are zinc-stress responsive, the biochemical pathways affected by the transgenerational stress memory appear to be a subset of the pathways usually activated or repressed in zinc stress. Three major groups of genes exist within the SMTs: genes involved in the biosynthesis of the JA-precursor OPDA, genes whose expression is regulated by JA, and genes involved in the uptake and homeostasis of iron. It appears that zinc stress produces repressive changes in the transcriptome of the progeny in the early developmental stages, such that it is “stress adjusted” and more capable of coping with zinc stress.

The observation that many of the SMTs are defence genes (e.g. NATA1, GPX1) is surprising, especially given the apparent specificity of the increased tolerance to exposure to stressful concentrations of zinc or iron, but not to other heavy metals. However, defence genes have previously been shown to be capable of conferring a zinc specific stress tolerance, indicating that some defence genes may possess additional functions in conveying specific metal tolerance (Mirouze et al., 2006). This stress adjustment is in contrast to transgenerational biotic stress priming which appears to involve alterations in the stress response transcriptome but not the basal transcriptome (Luna et al., 2012; Slaughter et al., 2012).

Three main hypotheses for the mechanism underlying the increased stress tolerance will be discussed here; changes in the expression of JA-regulated genes that cause a mild zinc stress like response under normal growth conditions, changes in OPDA biosynthesis modify the OPDA response to elevated zinc, or changes in iron homeostasis improve the iron deficiency tolerance. The three hypotheses are considered in turn, starting with the role of the JA-regulated SMTs in zinc stress and the implications this could have for their altered
expression in the progeny of zinc stressed plants. Following this, the other possible indirect effects of reduced OPDA synthesis gene expression are considered, and finally, the possible benefit of altering iron homeostasis to improve tolerance to the iron deficiency component of zinc stress is discussed. These three hypotheses are not mutually exclusive.

Genes responsible for two of the three steps involved in the synthesis of OPDA from membrane derived octadecatrienoic acid were affected in the progeny of zinc stressed plants. LOX2, one of six LOX genes in A.thaliana, and AOC1 & AOC2, two of four AOC genes (Stenzel et al., 2012) have reduced expression (Table 8). Additionally, AOC3 was just outside the cut off for differential regulation. Although OPDA responsive genes are upregulated in zinc stress (Figure 41A), LOX2 is the only SMT gene involved in OPDA biosynthesis that is zinc stress-responsive. This is similar to a previous report that cadmium stress induces expression of LOX1, LOX3, LOX4 and LOX6, although changes in LOX2 transcript abundance were not detected (Keunen et al., 2013). The reduced expression of these genes under non-stress conditions appears to affect the expression of a discrete subset of JA-upregulated genes, including VSP1 & VSP1, NATA1 and JAL23, but does not have a general effect on JA- or OPDA- responsive genes (Figure 41B).

Unlike AOCs1-3, AOC4 was not affected in the second generation progeny of zinc stressed plants. It is possible that this specificity limits the observed JA-regulated gene expression changes to particular tissues as the AOCs have been observed to display tissue and organ specific expression based on promoter activities (Stenzel et al., 2012). Additionally, in vivo dimerization of AOCs has been observed, with AOC4 showing the strongest homo- and hetedimerisation capability (Stenzel et al., 2012). As AOC4 is expressed specifically in the vascular-bundle in the leaves and is expressed in the roots along with AOC3 (Stenzel et al., 2012), it is possible that AOC4’s capacity for homodimerisation maintains OPDA/JA production in these tissues, limiting gene expression changes observed to other plant tissues. Indeed, NATA1 and VSP1 were observed to have an altered expression in the aerial portion of zinc stressed progeny under non-stress conditions, with no difference observed in the root tissues (Figure 44). However, as an aerial-specific altered AOC1 expression change was also observed (Figure 44), it is also possible that the tissue specific difference in JA-upregulated genes is due to a tissue specific difference in AOC1 expression, and perhaps AOC2, AOC3 and LOX2 also. As whole seedlings transcriptomes were analysed here, localised transcriptome changes as a result of localised reductions in OPDA biosynthesis may not be detected.
VSP1 and VSP2 are members of the family of vegetative storage proteins that serve to sequester amino acids during development, buffering nutrient availability (Staswick et al., 1991). VSP proteins are induced in response to a wide range of stimuli and stressors including JA, wounding, phosphate deficiency, herbivory and osmotic stress (Berger et al., 2002; Z Gong et al., 2001; Estrella Luna et al., 2012; Mason and John E Mullet, 1990). The downregulation of VSP1 & VSP2 during zinc stress at an early developmental stage (Table 6) may function to release stored amino acids to compensate for reduced nutrient uptake, enabling rapid production of stress-responsive proteins. Furthermore, in keeping with its induction by JA and herbivory, VSP2 has also been shown to possess an insect defence functionality dependent upon its acid phosphatase capability (Liu et al., 2005). Given the 82% sequence identity between the VSP2 and VSP1 genes, it is likely VSP1 possesses the same defence functionality. The low specificity of acid phosphatases (Van Etten and Waymack, 1991) may enable the acid phosphatase capacity of VSP1 and VSP2 to be of biological significance during a wide range of stressors, perhaps including heavy metal stress. Why VSP1 and VSP2 expression should be downregulated in response to zinc stress at an early developmental stage but upregulated at a later developmental stage (Figure 44) is unclear. However, the increased expression of VSP1 and VSP2 when the stress is experienced at a later developmental stage could be beneficial in sequestering unused amino acids as the plant enters a period of senescence.

Similar to VSP2, NATA1 has been shown to have a defence function; N-acetylation of orthinine by NATA1 yields N⁵-acetylorthinin, a compound which inhibits herbivory (Adio et al., 2011). While a function for N⁵-acetylorthinin in zinc stress is difficult to imagine, one possible function of NATA1 in zinc stress could be to regulate the pool of ornithine, a compound which functions in abiotic stress responses (Kalamaki et al., 2009; Haitao Shi et al., 2013). Again, interpretation of the role this gene may play in zinc stress from the observations in this Chapter is complicated by the observation that the expression of NATA1 is repressed in zinc stress in an early developmental stage and elevated in zinc stress in a later developmental stage.

As stated above, the role of the JA-upregulated defence genes in zinc stress is unclear given that the three genes (VSP1, JAL23 & NATA1) examined at two developmental stages all show reduced expression when zinc stress is introduced at an early developmental stage and increased expression when it is introduced at a later developmental stage. However, the progeny of zinc stressed plants show a mild zinc-stress-like expression level of two of these genes, VSP1 and NATA1, in non-stress conditions, at both developmental stages. This suggests that regardless of the function of these genes, the progeny of zinc stressed plants
display a mild zinc-stress-like transcriptome in the absence of stress. Presumably, this zinc-stress adjusted basal transcriptome reduces the initial shock of zinc stress and also reduces the time between stress signalling pathway activation and achieving sufficient activation/repression of stress-responsive genes. Although the increased tolerance was observed in plants sown directly onto high zinc, it is apparent that the high zinc concentration is not immediately stressful to the plant; germination is unaffected by high zinc, and symptoms of zinc stress such as chlorosis and retarded root growth are not observed within the first few days, presumably because excess zinc has not yet accumulated in the tissues. In this short window after germination and before zinc stress signalling pathways are activated, adjustments to the basal transcriptome could prepare the plants for zinc stress, thus reducing the overall extent of stress experienced, as observed when comparing the stress responses of the progeny of zinc stressed and control plants (Figure 40). Under laboratory growth conditions the altered basal transcriptome does not appear to affect growth under non-stress conditions, which could suggest there is no fitness cost to the plants in activating the transgenerational response to zinc. As discussed in Chapter 3, a more plausible explanation is that the fitness cost is not observed because of the growth conditions. It is very possible that the adjusted basal transcriptome confers an increased zinc tolerance but reduces tolerance to other stressors given the enrichment in GO response terms in the downregulated genes (Table 8).

Alterations to the OPDA/JA biosynthesis pathway will also affect other chemical signalling pathways beyond those directly regulated by OPDA or JA. The green leaf volatiles (GLV) pathway cross-talks with the OPDA synthesis pathway (Hirao et al., 2012) and could have implications in the OPDA-regulated response to zinc stress given the ability of GLVs to prime the OPDA response (Vicedo et al., 2009). The product of LOX-mediated oxidation of octadecatrienoic acid is a precursor to GLVs, chemical signals that function to repel insects, attract foraging predators (Mumm et al., 2008) or signal between different parts of the plant or even between plants (Heil and Ton, 2008). In Nicotiana attenuate, the specificity and temporal responses of the LOX enzymes has been clearly demonstrated, with NaLOX2 expression affecting GLV but not JA biosynthesis, and NaLOX3 expression having the opposite effect (Allmann et al., 2010).

In A.thaliana, LOX2 is required for wound-induced JA biosynthesis(Bell et al., 1995); whether LOX2 also functions in GLV synthesis is unclear. Interestingly, cross talk between the GLV and OPDA/JA synthesis pathways has been observed, with some GLVs capable of priming A.thaliana (Col-0) to be more responsive to future Me-JA treatments (Hirao et al., 2012). However, Col-0 cannot produce C_6 GLVs, which are most effective in priming, as it
possesses a 10-nucleotide deletion in exon 1 of the prerequisite \textit{CYP74B2} gene, which renders it non-functional (Duan \textit{et al.}, 2005). Thus, it is unclear how the reduced expression of \textit{LOX2} and AOC genes will affect GLV synthesis. Given that reduced expression of a particular LOX may influence the synthesis of JA without affecting GLV, it is possible that the reduced expression of \textit{LOX2}, \textit{AOC1} and \textit{AOC2} could reduce JA biosynthesis whilst maintaining or perhaps increasing C\textsubscript{9} GLV production through decreasing the competition of the AOC genes for LOX products. Interestingly, hexanoic acid, a GLV-related compound, has been shown to prime tomato plants to produce OPDA more rapidly in response to pathogen attack (Vicedo \textit{et al.}, 2009). It is plausible then that decreased OPDA biosynthesis could increase GLV production in non-stress conditions, priming \textit{A.thaliana} to respond more rapidly to zinc stress by modifying the OPDA response. If this were the case, the specificity of AOC gene expression may play a role in limiting the downstream effects to particular tissues, as mentioned previously.

The genes discussed so far display a mild zinc-stress-like expression in the progeny of zinc stressed plants. The opposite expression is observed for the iron ion homeostasis genes, \textit{BHLH038}, \textit{BHLH039} and \textit{FRO2}, which are all upregulated in zinc stress but show a reduced expression in the progeny of zinc stress plants in non-stress conditions relative to control progeny. Additionally, further members of this functional group, \textit{IRT1} and \textit{bHLH100} show a similar expression pattern but do not pass the cut off for reduced expression in the progeny of zinc stress plants in non-stress conditions.

The upregulation of iron homeostasis genes in zinc stress was to be expected. A component of the transcriptomic response to zinc stress in \textit{A.thaliana} has been observed to correlate with the transcriptomic response to Fe-deficiency ((Yang \textit{et al.}, 2010) & Figure 42). Furthermore, elevating iron in growth media has previously been shown to reduce Zn tissue content and increase the biomass of plants subjected to zinc stress (Shanmugam \textit{et al.}, 2011), the latter of which was confirmed here (Figure 46).

Given zinc stress increases ferric reductase activity in \textit{A.thaliana} roots (Becher \textit{et al.}, 2004; Shanmugam \textit{et al.}, 2011), the reduced expression of \textit{FRO2} in the progeny of zinc stressed plants was intriguing. The investigation of FRO enzyme capacity in the roots indicated that the progeny of zinc stressed plants display a reduced root ferric reductase activity, in agreement with the reduced expression of \textit{FRO2}. Furthermore, root FRO activity was less responsive to elevated zinc or reduced iron without impacting chlorophyll content, whilst root weight was greater. Reduced \textit{FRO2} would not be expected to correlate with increased zinc tolerance as zinc stress can be alleviated by increasing Fe supply, as shown in Figure
46, and co-overexpressing FIT1 and BHLH038 or BHLH039 has previously been shown to increase cadmium stress tolerance by increasing Fe uptake (Wu et al., 2012). However, Arabidopsis halleri, a zinc hypertolerant species, shows a 5-fold reduction in root ferric reductase activity compared to A.thaliana, and ferric reductase activity is not increased in Fe deficiency or zinc stress conditions (Shanmugam et al., 2011), which indicates that metal stress tolerance may be achieved through many routes including both increasing and limiting the activity of ferric reductases in the roots. Taken together, these results suggest the progeny of zinc stressed plant may be more efficiently distributing iron either subcellularly or between the plant organs. One advantage of responding less to elevated zinc would be to limit the uptake of zinc. The FRO2 and IRT1 proteins are transcriptionally and post-transcriptionally regulated in parallel (Connolly et al., 2003), and although FRO2 is not known to function in zinc uptake, IRT1 has been implicated (Vert et al., 2002). Thus, the reduced expression of IRT1 in non-stress conditions observed in this Chapter would be expected to correlate with reduced zinc uptake into the roots.

Whilst the function of FRO2 as the prerequisite ferric reductase for uptake of iron from the soil has been firmly established (Connolly et al., 2003; N. J. Robinson et al., 1999), the functions of the other FRO genes with reduced expression in the progeny of zinc stressed plants, FRO6 and FRO7, are less clear (Jeong and Connolly, 2009). A recent report indicates that iron homeostasis in plant species including A.thaliana may involve a reduction step for each membrane crossed (Guelke and Von Blanckenburg, 2007), highlighting the importance of FRO enzymes in iron homeostasis in A.thaliana. On current evidence, FRO6 is postulated to be the major leaf plasma membrane ferric reductase, while FRO7 appears to deliver iron into the chloroplasts (Jeong and Connolly, 2009; Jeong et al., 2008; Li et al., 2011). FRO6 and FRO7 are both Fe-deficiency sensitive although FRO6 is downregulated and FRO7 upregulated (Mukherjee et al., 2006). Overexpression of AtFRO6 in Nicotiana tabacum confers Fe-deficiency tolerance (Li et al., 2011), and FRO6 was is downregulated approximately 8-fold in zinc stress (Table 6).

The observed reduction in expression of a NRAMP gene in the progeny of zinc stressed plants would also be expected to affect iron homeostasis. NRAMP3 & NRAMP4 have previously been shown to function in regulating iron storage release from the vacuole (Lanquar et al., 2005), whilst NRAMP6, the gene with reduced expression in this Chapter, has been proposed to provide a pathway for iron transport into the chloroplast (Duy et al., 2007). Concurrent reduced expression of FRO7 and NRAMP6 in the progeny of zinc stressed plants would therefore be expected to reduce the transport of iron into the chloroplast. Unlike in mammals, ferritins are not considered the major iron storage proteins
in *A. thaliana* (Briat *et al*., 2010; Ravet *et al*., 2009), however, reduced expression of FRO7 and NRAMP6 may significantly reduce ferritin-dependent storage of iron in the chloroplast. Given that *fro7* chloroplasts contain 33% less iron than wild type (Jeong *et al*., 2008), it is surprising that the reduced expression of FRO7 observed here in the progeny of zinc stressed plants did not result in a lower chlorophyll concentration. This may reflect an improved regulation of iron distribution throughout the plant via localised changes in FRO enzyme expression that could not be detected as the whole plant transcriptome was examined.

In summary, it appears the progeny of zinc stressed plants are less responsive to elevated zinc/Fe-deficiency. In these plants, genes involved in transporting iron into the chloroplast show a reduced expression without negatively impacting on chlorophyll content, whilst increasing plant biomass, indicating that the reduced expression may lead to more efficient iron homeostasis under Fe-limited conditions including zinc stress.

The changes in the transcriptome of the progeny of zinc stressed plants are assumed to reflect changes in the DNA methylome that occurred during the initial zinc stress and were inherited in the following generations. Both of the DNA methyltransferases responsible for symmetrical DNA methylation, *CMT3* and *MET1*, were slightly downregulated in response to zinc stress, as was *VIM1* which operates with *MET1*. Furthermore, two DNA demethylases, *DME* and *DME2* were both upregulated.

Demethylation by the DME family of genes was originally suggested to function solely in reproductive development, imprinting genes including *FWA* and *MEA* (Choi *et al*., 2002; T. Kinoshita *et al*., 2004), however, there are now indications that it may also function in regulating stress responses (Kim *et al*., 2010).

The observations in this Chapter are insufficient by themselves to conclude that the original zinc stress resulted in DNA methylation changes, although they do offer an initial indication that DNA methylation changes may occur in response to zinc stress. Given that two methyltransferases are repressed during zinc stress, whilst two demethylases are activated, it seems likely that, if DNA methylation changes do occur, hypomethylation would be most prevalent, although it is possible that the analysis of whole seedling transcriptomes missed localised increases in the activity of these and other genes involved in DNA methylation. The most interesting hypothesis in relation to the possible implications is that the expression changes in *MET1*, *CMT3*, *VIM1*, *DME* and *DME2* drives DNA hypomethylation which is inherited by the progeny, in turn increasing zinc stress tolerance. Given that wide-scale DNA hypomethylation was not observed (Figure 34) and the stress tolerance appears to be specific...
to elevated zinc/iron deficiency, it is clear that functional DNA methylation changes would have to be directed towards specific loci. Interestingly, the majority of genes affected in the progeny of the zinc stressed plants show reduced expression which would require the proposed DNA hypomethylation to increase the expression of repressive factors or expose repressive promoter elements. Whilst the de novo DNA methylation pathway is known to be directed towards specific loci by sRNA sequence (Matzke et al., 2009), a similar mechanism to direct DNA methylation changes via MET1, CMT3, DME or DME2 has not been identified. The maintenance of CMT3 CHG methylation has been shown to be siRNA-dependent in some specific cases (Enke et al., 2011), which could indicate a possible mechanism by which directed changes could occur. However, it is difficult on current evidence to hypothesise how an overall changes in the expression of these DNA methyltransferases/demethylases could lead to DNA methylation changes at specific loci.

A simpler hypothesis which does not involve DNA methylation changes could be put forward. For example, expression of symmetrical DNA methyltransferases would be expected to be coupled with the cell cycle, as DNA methylation must be re-established on the nascent daughter strand. Therefore, under stress, when plant growth is reduced, expression of MET1, VIM1 and CMT3 may be decreased in coordination with a decreased expression of cell cycle factors. Likewise, if DME and DME2 display higher expression in later developmental stages, then retarding development by introducing stress may decrease their expression relative to unstressed plants of the same age.

The ferric reductase genes are suggested as candidates to identify correlated methylation changes as the reduced expression of this gene has been confirmed to have a detectable effect on the root biochemistry. However, the reduced FRO2 expression is unlikely to be a direct effect of DNA methylation changes as two of its regulatory transcription factors BHLH038 and BHLH039 also show a reduced expression. Little is known about the regulation of FRO6 and FRO7 expression beyond their respective expression localisation in aerial portions of the plant and their expression changed in response to heavy metals (Mukherjee et al., 2006). The promoters of the two bHLH genes show large regions of significant sequence identity (by BLAST analysis), indicating their expression may be regulated in unison via shared regions in their promoters. This would likely suggest their reduced expression in the progeny of zinc stressed plants is due reduced binding of a transcription factor(s), rather than both promoters being hypermethylated in unison. Furthermore, B.oleracea bHLH genes can be identified based on the alignment of their upstream sequence to the A.thaliana BHLH038 promoter alone (by BLAST), strongly indicating this sequence is under purifying selection. Interestingly, the central motif appears
to be a non-palindromic 24-mer. Given its assumed role in regulating gene expression in $cis$, this is somewhat unexpected as transcription factor and repressor binding sites are usually palindromic, although there are examples of transcription factors binding to non-palindromic or pseudopalindromic sequences (Assunção et al., 2010; Jakoby et al., 2002; Ringli and Keller, 1998; Viola et al., 2011).

In summary, the reduced ferric reductase activity in the roots of the progeny of zinc stressed plants is believed to be the result of reduced $FRO2$ expression, in turn resulting from reduced $BHLH038$ and $BHLH039$ expression. The promoter regions of these genes share a considerable sequence identity with each other and with bHLH genes in other Brassicae, and a motif which is highly conserved has been identified. If the expression of $BHLH038$ and $BHLH039$ is controlled by the binding of a single factor to this motif, a suitable route to start looking for changes in DNA methylation which could explain the transgenerational stress memory would be to identify this factor and analyse the DNA methylation of its genomic loci in the progeny of zinc stressed plants.
Chapter 6. General discussion and future directions
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6.1 DNA methylation is relatively unresponsive to stress

The original aim of analysing DNA methylation in response to stress was to identify conditions which could lead to changes in DNA methylation that could be inherited by the progeny, in the hope that these would correlate with an enhanced stress tolerance. In section 1.5.7, examples were given in which DNA methylation was observed to be dynamic in response to stress in *A.thaliana* and other plant species. In many cases these DNA methylation changes are observed at the genome wide level. It was therefore expected that the methylation sensitive amplified polymorphism assay developed here would enable identification of DNA methylation change in response to stress with relative ease. Instead, the DNA methylation profile of *A.thaliana* was observed to be unchanged by most stressors. Treatments which have frequently been reported to generate changes in DNA methylation in plants including NaCl stress (Baek *et al*., 2010; Cao *et al*., 2011; Choi and Hiroshi Sano, 2007; Dyachenko *et al*., 2006; Karan *et al*., 2012; Tan, 2010) and heat and cold stress (Boyko *et al*., 2010; Hashida *et al*., 2006; Steward *et al*., 2002) failed to generate DNA methylation changes that were detectable by MSAP. Although it’s possible that DNA methylation changes went undetected, previous reports have suggested abiotic stressors induce wide scale changes in DNA methylation profiles of aerial tissue (Boyko *et al*., 2010; Kou *et al*., 2011; Steward *et al*., 2002; Tan, 2010). In experiments with maize, Tan *et al* (2010) observed that over 10% of MSAP bands were affected by osmotic and/or NaCl stress, whilst Steward *et al* (2002) observed an overall decrease in methylation following eight days of cold treatment (4 ºC. The results presented here suggest the DNA methylation profile of *A.thaliana* is relatively unresponsive to stress.

The only condition which was observed to generate wide scale changes in DNA methylation was treatment with 5-15% PEG. Whilst it’s clear that regulation of DNA methylation plays an important role in biotic stressors in *A.thaliana* (Dowen *et al*., 2012), there have been few observations of dynamic DNA methylation in response to abiotic stressors in *A.thaliana*. Indeed, two such observations involve PEG stress and low humidity (Dowen *et al*., 2012; Tricker *et al*., 2012), a stress condition which produces a similar physiological response to PEG/drought stress (Casson and Hetherington, 2010; Levin *et al*., 2009; Macková *et al*., 2013). It seems possible then that *A.thaliana*, unlike many other plant species, does not commonly utilise DNA methylation as an abiotic stress response, except in water-limiting stressors. The hypermethylation in response to PEG was detected solely in the roots and suggested to be partly dependent upon DRM2 and other RdDM components. The activity of the *DRM2*, *POL IV* and *RDR2* promoters was relatively unchanged in the roots.
Additionally, the activity of the ROS1 promoter was unaffected by PEG stress. Therefore, it is suggested that the PEG-induced root DNA hypermethylation is predominantly the result of changes in the siRNA pool from which RdDM targets genomic loci or gene expression changes for other RdDM components not examined, both of which could lead to DRM2-dependent methylation at previously unmethylated loci. The restriction of the detected DNA methylation changes to the roots fits with previous observations of DNA methylation changes in response to alkali and NaCl stress in *Gossypium hirsutum* (Cao et al., 2011) NaCl and heavy metal stress in *Oryza sativa* (Karan et al., 2012; Ou et al., 2012) and cold and NaCl stress in *Zea mays* (Steward et al., 2002; Tan, 2010) in which the changes were wholly or largely restricted to the root tissues. Thus, the observed ease at which root DNA methylation may be perturbed (with as little as 5% PEG) relative to the aerial tissues may be common across the plant kingdom. As the root DNA methylation profile will not be inherited in the following generation, it is possible that the methylation status is more easily perturbed as there is no risk to the plant that the considerably altered DNA methylation profile will be inherited. Therefore, wide scale DNA methylation changes could be utilised to regulate some aspects of the stress response without risking inheritance of the alterations into the following generations where they may prove to be deleterious. Future experiments should help to establish whether stress-induced changes in DNA-methylation predominantly occur in non-meristemic tissue and the regulatory pathways which restrict changes in DNA to particular tissue types.
6.2 Is DNA methylation involved in the transgenerational stress memory?

Chapter 1 introduced various mechanisms by which a stress event could lead to changes in DNA methylation which enhance stress tolerance in the progeny. These include the generation of stress-induced siRNAs which could target genomic loci for *de novo* RdDM, the expression of stress-induced RdDM pathway components which could modifying the activity of RdDM at specific loci, and the coupling of DNA methylation to gene expression via the proposed dependency of MET1 CG methylation at gene bodies on POL II activity and the passive methylation of vacant transcription factor binding sites. Although no wide scale changes in DNA methylation were detected in the results presented in Chapter 5 in response to zinc stress, this is still considered the most likely mechanism for the transmission of the transgenerational stress memory that was observed, as no other epigenetic modification has been observed to be inherited. As such, the changes in the transcriptome of the G2 progeny of zinc stressed plants are assumed to reflect changes in the DNA methylome that occurred during the initial zinc stress and were inherited in the following generations. Unfortunately, without whole genome DNA methylation data, it is not possible to assess the mechanism of inheritance in detail. The observation that greater than 50% of the SMTs are zinc stress responsive clearly indicates that the transcriptome changes are biased towards genomic regions that are differentially regulated during stress. This could suggest that the mechanisms coupling DNA methylation and gene expression in zinc stress drive heritable changes in the DNA methylation of stress-responsive loci which in turn modifies their expression levels. However, it is also possible that stress-induced siRNAs or RdDM genes target stress-responsive genes for differential DNA methylation as part of the plant’s stress response, and that these changes in DNA methylation are inherited, again leading to heritable modification of stress-responsive gene expression levels.

The RNA-Seq data identifies loci that could be affected by differential methylation due to their differing expression. On this basis, the transcription factors *BHLH038* and *BHLH039* are candidates since their expression is reduced under non-stress conditions and the *FRO2* gene which they control shows reduced expression in the G2 progeny of zinc stress plants. As the promoters of these genes share significant homology and do not appear to contain repeat elements, it is possible they are jointly regulated by the expression of an upstream transcription factor rather than co-differential methylation of their promoters. If, as expected, their promoter methylation was unaltered, a suitable approach to identify any common transcription factors for *BHLH038* and *BHLH039* would be a yeast one-hybrid assay using
their promoter sequences and a cDNA library of *A.thaliana* TFs (Gaudinier *et al*., 2011; Mitsuda *et al*., 2010).

The other main candidates for DNA methylation analysis are those STMs which are stress-responsive, as their differential expression in the progeny may be the result of an epigenetic memory fixed onto the gene following the change in gene expression during zinc stress. For all candidates, the hypothesis would be that their expression is responsive to zinc stress and that they retain an altered DNA methylation state once the zinc stress has ceased. To test this, promoter and gene body methylation status and mRNA expression of these TFs could be examined during zinc stress and in the progeny of zinc stress plants.

Clearly, a whole genome DNA methylation analysis of the DNA methylation status of the G₂ progeny of zinc stressed plants would be ideal as 45 zinc stress-responsive SMTs have been identified (Figure 39). An additional advantage of a whole DNA methylome analysis is that it would allow DNA methylation and gene expression data to be overlaid across the entire genome which may identify further regions of interest. It is expected that regions of the genome would show differential DNA methylation between control and zinc stress progeny with correlated changes in gene expression. The DNA methylation could then be examined at selected regions during and after zinc stress by bisulphite sequencing and correlated with qRT-PCR transcript abundance data over a time course to establish whether the DNA methylation change or gene expression change occurred first at each individual loci, and whether the DNA methylation change was maintained in its entirety once the change in gene expression has been reversed. These experiments would begin to identify regions of the genome which can carry a memory of zinc stress through to the following generation via alterations in DNA methylation.

### 6.3 Heritable effects of stress are rare in *A.thaliana*

Given the diversity of stress conditions which have been reported to produce heritable effects in plants (Boyko *et al*., 2010; Lang-Mladek *et al*., 2010; Estrella Luna *et al*., 2012; Molinier *et al*., 2006; Ou *et al*., 2012; Rahavi *et al*., 2011; Rasmann *et al*., 2012a; Slaughter *et al*., 2012; Whittle *et al*., 2009), one could easily come to the conclusion that plants frequently pass on information about past stress events to their offspring. Having failed to repeat some of these observations, in one case with a near identical experimental set-up, it is the author’s opinion that heritable effects of stress in fact represent an exceptional long-term response to stress. This is also the position reached by Pecinka *et al* after failing to reproduce the reported heritable effects of stress on HRF (Molinier *et al*., 2006; Pecinka *et al*., 2009).
Given the publication bias against negative results, it is quite possible that other researchers have also failed to reproduce published heritable effects of stress in plants. In the light of the apparent irreproducibility of many transgenerational stress memories, it is tempting to believe that they represent false positives resulting from the testing of multiple parental treatments followed by significance testing of multiple trait measurements in the offspring, with only the positive results being published. However, there are reasons to believe this is not the case. Importantly, transgenerational stress memories are frequently associated with changes in DNA methylation in the progeny and observed to be dependent upon RdDM components (Boyko et al., 2010; Kou et al., 2011; Rasmann et al., 2012a). This suggests hypertolerant progeny may possess an epigenetic memory of the parental stress treatment, and provides a plausible mechanism for transgenerational stress memory inheritance. In Chapter 4, a transgenerational stress memory is reported in the second generation offspring of zinc stress treated plants. Crucially, transcriptome differences are observed in the second generation progeny of zinc stressed plants which suggests an epigenetic mechanism may be operating.

Two hypothesis are put forward in Chapter 4 to explain why a memory of zinc stress was observed, whilst no memory of a NaCl, PEG, heat, cold or nitrogen deficiency stress was observed. The first hypothesis was that transgenerational stress memories are dependent upon an interaction between the defined stress treatment and other environmental conditions such as light intensity, humidity, etc. In this hypothesis, the failure to reproduce stress memories such as that observed by Boyko et al in response to NaCl stress (Boyko et al., 2010), is postulated to be due to differences in the growth conditions. The second hypothesis is that stress memories must involve a cost to the plant and therefore evolutionary pressures will select for a transgenerational stress memory mechanism that is triggered by stress conditions that are intransient enough to be experienced by the following generations, but not permanent enough to represent a consistent selective pressure. As such, the longevity of the stress conditions in the environment, the capacity of the seed to regulate germination in response to the stress, and the seed dispersal mechanism of the plant are all hypothesised to affect the likelihood of a stress generating a transgenerational stress memory. In this hypothesis, the previously published stress memories in A.thaliana are not included as the results could not be reproduced. Zinc stress is proposed to generate a stress memory in A.thaliana as it is relatively stable within the immediate environment of the parent plant and the seeds cannot regulate germination in response to excess zinc. In contrast, the other stressors tested are transient and/or A.thaliana seeds regulate their germination in response to the stress condition.
When faced with competing hypotheses, the “Occam’s razor” principle is usually applied, by which it is stated that the simplest hypothesis should be selected. What constitutes the simplest hypothesis is debated (Riesch, 2010) but a sensible approach is to follow Karl Popper’s position and select the hypothesis which can be most easily tested (Popper, 2002). Hence, the hypothesis that transgenerational stress memories are dependent upon the defined treatment in interaction with other environmental conditions should be tested first as it is can be tested relatively simply. This would involve attempting to prevent or facilitate the production of a transgenerational stress memory by altering growth conditions only, with the defined treatment remaining the same. The most straightforward approach would be to alter the growth conditions during the zinc treatment outlined here. For example, does elevating Fe content in the growth media inhibit the transgenerational effects of zinc stress? Or can the transgenerational effect be inhibited by supplementing the growth media with sucrose, thereby negating some of the impact of the zinc-stress induced chlorosis? Or can more subtle changes in light intensity, day length or humidity affect the generation of the stress memory? These experiments would start to test the hypothesis put forward and start to better define the conditions required to generate a transgenerational stress memory in Arabidopsis thaliana. As discussed previously, our poor understanding of the conditions required to generate a transgenerational stress memory makes interrogation of the mechanism(s) involved very difficult. Currently, we have a collection of largely one-off observations from which to attempt to draw conclusions. If the conditions required to generate a transgenerational stress memory could be determined, model stress treatments could then be established for future examinations of the mechanism(s) involved.

If the transgenerational zinc stress memory was observed regardless of variations in other environmental conditions, the second hypothesis should then be tested. Experiments should first establish if other heavy metals which do not affect germination also generate a transgenerational stress memory. As mentioned in Chapter 1, a previous observations of stress memories in response to heavy metals in A. thaliana is complicated by the apparent use of lethal heavy metal concentrations (Rahavi et al., 2011). Assuming Rahavi, M. et al’s observations are reproducible at non-lethal concentrations, it appears many heavy metals can produce a stress memory in plants, including zinc, mercury (Ou et al., 2012), cadmium, copper and nickel (Rahavi et al., 2011). If transgenerational responses to excess heavy metals or transitional metals were observed to be a general response, it would be interesting to investigate whether other stress conditions which do not affect germination, such as phosphate starvation (Sánchez-Calderón et al., 2005), also lead to a transgenerational response. Testing another assumption of the hypothesis, a comparison of Brassicaceae with ballistic seed dispersal, such as Cardamine hirsute (Vaughn et al., 2011), Cardamine...
parviflora (Hayashi et al., 2010) and Lepidium campestre (Thiede et al., 2013), with those without a seed dispersal mechanism, such as A.thaliana, could indicate if there is a relationship between seed dispersal and the transgenerational response to zinc stress as suggested. In the extreme, it would interesting to investigate if costal Brassicaceae capable of dispersing seeds over much larger distance after immersion in sea water, such as Cakile edentula - the first plant species to colonise the island of Surtsey over a distance of least 20 km (Edentula and Fridriksson, 1966) - generate transgenerational stress memories, given the potentially much greater distances between parent and progeny. These experiments would test the hypothesised correlation between the similarity in parental:progeny environmental conditions and the evolutionary benefit of responding to stress with a transgenerational response.

6.4 A novel transgenerational zinc stress memory

One of the aims of this project was to identify how changes in the transcriptome could enhance the stress tolerance of the progeny of stressed plants. Two mechanisms were proposed that were described as “stress adjustment” and “stress priming” with the difference being whether the improved stress tolerance related to changes in the transcriptome in the absence of stress or upon triggering of the stress response. RNA-Seq data strongly suggests that the transgenerational zinc stress memory is due to a stress adjusted transcriptome in the progeny. This adjusted basal transcriptome appears to confer a lower zinc stress sensitivity on the plants, as observed by a reduction in the severity of gene expression changes in response to zinc stress. This is in contrast with observations of biotic stress memories in which increased expression of defence genes is observed in response to stress, with no change under non-stress condition (Pastor et al., 2013; Slaughter et al., 2012). It is possible then that abiotic and biotic stressors generate transgenerational stress memories in which the stress tolerance is increased through fundamentally different means. However, there are some striking similarities in the transcriptome changes.

As discussed in section 1.2, biotic stress memories involve alterations in the synthesis of jasmonates and the expression of JA-responsive genes (Estrella Luna et al., 2012; Rasmann et al., 2012a). Increased P.rapae-induced expression of LOX2 is observed in the progeny of A.thaliana plants previously challenged with P.rapae herbivory (Rasmann et al., 2012a), whilst the progeny of A.thaliana plants challenged with Pst. exhibit lower expression of the JA-responsive defence gene VSP2 in response to JA (Estrella Luna et al., 2012). As there was a lower expression of both of these genes in the G2 progeny of zinc stressed plants in the
results presented in Chapter 5, this suggests that modifications to the jasmonate synthesis and signalling pathways may be a common mechanism across transgenerational stress memories. It has been suggested that jasmonate compounds such as OPDA could act as a memory buffer of previous stress events by enhancing the speed of the JA-dependent response in subsequent stress events (Gáliš et al., 2009). The results presented here indicate that if such a mechanism does exist, it could extend over multiple generations and allow the plant to fine tune its stress signalling pathways based upon the experience of its progenitors.

Three non-mutually exclusive hypotheses are proposed by which the altered transcriptome could increase the zinc stress tolerance of the progeny. Firstly, changes in the expression of jasmonate-regulated genes could cause a mild zinc stress-like response under normal growth conditions. Secondly, alterations to the OPDA biosynthesis pathway could modify the OPDA response to elevated zinc. Thirdly, enhanced iron homeostasis could improve the iron deficiency tolerance. Within these hypotheses, the possible effects of the differential expressions of many genes were explored. It is unlikely that all the explanations consider in the discussion in Chapter 5 will be proved true as the transcriptome data presented here contains no temporal element, and has not been correlated with metabolome or proteome data. Indeed, it is distinctly possible that some of the observed transcriptome changes confer no increased tolerance but are instead indirect consequences of functional transcriptome changes, especially given the cross-talk between the stress response pathways. For instance, it is possible that the reduced expression of a variety of defence responsive genes has no role in increasing zinc stress tolerance but instead reflects a fine-tuning of the stress response pathways which raises tolerance to zinc but reduces tolerance to herbivores in response to a perceived shift in relative likelihood of zinc stress. Likewise, the reduced root FRO activity may have no direct benefit but rather reflect altered iron homeostasis through which the plants are more prepared for zinc stress conditions, therefore resulting in a reduced root FRO response.

The RNA-Seq data presented here clearly implicates OPDA in the zinc stress response, which could suggest the reduced expression of OPDA synthesis genes is responsible for the zinc tolerance either through reduced synthesis of OPDA or by priming the OPDA response through the production of C9 GLV, as discussed in section 5.3. However, OPDA appears to regulate a range of metal stress response genes, thus, priming of the OPDA response would not be expected to confer a zinc-stress-specific tolerance (Taki et al., 2005). Additionally, the JA-regulated genes with reduced expression in the progeny, VSP1 and NATAl, are not known to confer zinc-specific stress tolerance. The observed zinc-specificity combined with differential expression of genes with roles in biotic stress has some similarities with a recent
observation that defensins confer zinc-specific tolerance in *A.halleri* (Mirouze et al., 2006). Attempting to explain the specificity, Mirouze, M. et al (2006) proposed that the defensins may confer zinc tolerance by interfering with divalent metal cation trafficking. Although the JA-regulated defence genes with reduced expression in the progeny of zinc stressed plants cannot be proposed to function in the same manner, it is clear that there is still much to learn about the function of supposed biotic stress genes in metal stress. The author’s view is that JA and OPDA-regulated genes will be shown to possess zinc stress specific functions alongside their biotic stress functions.

There are a multitude of straightforward future experiments that could be carried out to test the hypotheses put forward here. Examination of the iron homeostasis pathways should be prioritised, since the transcriptional changes have already been correlated with biochemical changes in the progeny. At a minimum, the tissue ion content should be examined. The root and aerial tissue accumulation of Fe and Zn in the progeny of zinc stressed plants under elevated Zn and reduced Fe should be determined. This would establish whether reduced root ferric reductase activity is correlated with reduced Fe and Zn uptake as expected. If so, the next hypothesis to test would be whether there is a change in subcellular iron homeostasis. The reduced *FRO6, FRO7* and *NRAMP6* expression is hypothesised to reduce chloroplast Fe content without affecting chlorophyll levels. Chloroplast Fe content has been quantified previously in the *fro7* mutant and found to be 33% lower (Jeong et al., 2008). If a similar reduction was observed in the progeny of zinc stressed *A.thaliana*, without impacting on chlorophyll production under elevated Zn/Fe-deficiency, it would strongly suggest improved iron homeostasis in the progeny.

Quantification of OPDA, JA and GLVs within the progeny of the zinc stressed plants would be the obvious first experiment in establishing whether the observed reduced expression of *AOC1, AOC2* and *LOX2* has biochemical consequences to the plant. If, as hypothesised, the G2 progeny of the zinc stressed plants produce greater quantities of GLVs, the next step would be to examine whether GLVs can “zinc-prime” plants in the absence of a prior zinc stress, as has been observed for chemical signals such as BABA, which can prime pathogen responses (Slaughter et al., 2012). Analysis of OPDA response genes observed here to be upregulated in response to stress could confirm the hypothesised GLV-dependent priming of the zinc stress response. The other hypothesis surrounding the reduced expression of OPDA synthesis genes and JA-responsive genes is that this may have a negative impact on herbivore/wounding tolerance. A simple experiment to test the herbivore tolerance and JA-regulated gene expression in the progeny of zinc stressed plants grazed on by species known
to activate JA-responsive genes, such as *Plutella xylostella* or *Spodoptera littoralis*, would test this hypothesis (Berger *et al.*, 2002).

Ideally, temporal data to follow the progression of transcriptome changes during the progress of the zinc stress response, correlated with proteome and ionome data, would allow construction of networks to establish which of the observed changes are functional and which are indirect effects of the increased tolerance. Beyond hopefully confirming some of the trends observed here, this more extensive analysis could potentially identify a small number of hub genes whose expression is crucially altered in the progeny of zinc stressed plants, enabling a more directed examination of the mechanism behind the increased zinc stress tolerance.

To summarise, the results presented indicate that the transcriptome of *A.thaliana* can be modified by stress two generations previously. The tolerance appears to involve an altered transcriptome in the absence of stress which reduces stress sensitivity. The are some similarities with biotic stress memories in that modifications to the jasmonate signalling pathways appear to be involved, although further experiments are required to determine which transcriptome changes are critical for the increased stress tolerance and whether inherited differences in the DNA methylome are involved.
6.5 Concluding remarks

As discussed previously, the lack of a well-established model for abiotic stress memories has hindered investigations into the mechanisms involved. It is the author’s opinion that this irreducibility is best explained by underlying stochastic processes which may hindered a satisfactory explanation of transgenerational stress memories for many years to come. However, the novel transgenerational stress memory presented here has the potential to become a general model for abiotic stress memories in plants as the treatment is simple and the effects transmissible through to the F2 progeny. Furthermore, the transcriptome analysis of this stress memory in the F2 progeny yielded many interesting observations and suggests a number of changes due to grandparental stress. Based on observed changes in the expression of iron deficiency response genes in the F2 progeny, the stress memory is postulated to increase zinc stress tolerance partially through modifying iron homeostasis. Additionally, the transcriptomics analysis suggests a role for jasmonate signalling and possible crosstalk with defence response pathways. The presence of a transcriptional mark of parental stress under non-stress conditions is indicative of an epigenetic change in the progeny, with DNA methylation the most plausible candidate mechanism. The analyses performed did not establish if DNA methylation was involved, although it is clear the stress event does not cause wide-scale changes in DNA methylation. The author expects that loci-specific changes in DNA methylation are responsible for the observed changes in the transcriptome of the zinc stress progeny and hopes that future experiments will identify the crucial genomics regions. The transcriptomic analysis has identified candidate regions to which the follow-up analyses should be directed in the first instance.

Wide-scale heritable changes in DNA methylation were not detected in response to any of the stressors tested, suggesting DNA methylation is less responsive to stress than the literature may suggest. In order to study DNA methylation in response to stress and its inheritance, the commonly utilised MSAP assay was modified. The introduction of the whole genome amplification step in order to allow conclusive assignment of methylation change from changes in band profile represents a valuable improvement on current practice. It would benefit other researchers currently employing MSAP analyses to adopt this modification to increase the value of their DNA methylation data analyses.

Transgenerational stress memories have attracted much attention recently, with some even suggesting they re-open the debate about the possibility of acquired characteristics. Whilst published plant stress memories are now commonplace, it is still unclear how frequently
plants inherit a mark of parental stress. During the course of this research, multiple attempts were made to identify transgenerational effects of stress in *A. thaliana*. The results presented here indicate that parental stress rarely leads to an improved stress tolerance in the progeny. It is the author’s belief that stress memories in plants will ultimately be shown to be rare events which persist for only one or two generations outside of the laboratory.

For the presence of transgenerational stress memories in plants to become widely accepted, there are a number of key areas to be addressed. Firstly, a suitable model of transgenerational stress needs to be established. It is vital that this model is sufficiently robust to be reproducible between independent laboratories. As suggested previously, the zinc stress model may be suitable, however, it has not yet been replicated in an independent laboratory. Once model(s) have been established, concerted efforts will be required to understand the underlying molecular mechanism by which transgenerational stress memories are transmitted through the germline and the mechanisms by which they enhance stress tolerance in the progeny. Given the heritability of DNA methylation changes in response to stress, this remains the most likely molecular mechanism underlying the transmission of transgenerational stress memories. However, for most transgenerational stress memories, there is only weak evidence that DNA methylation is involved. Researchers should focus efforts on attempting to identify causative changes in DNA methylation that are required for transmission of transgenerational stress memories. This may be best achieved by identifying the physiological basis of the increased stress tolerance in the progeny and then directing analysis towards genomic loci that are implicated by the physiological changes. For the zinc stress memory presented here, an altered transcriptome was observed in the absence of stress, with iron homeostasis the clearest physiological change. If the zinc stress memory described here could be conclusively shown to depend on stress-induced changes in DNA methylation at one of the candidate genomic regions identified, this would provide the first firm evidence that DNA methylation represents the crucial transgenerational mark of stress.

It is also currently unclear whether the collection of published transgenerational stress memories are a manifestation of similar responses to diverse stressors, or whether there are fundamental differences between them. The transcriptome results presented here suggest an altered transcriptome in the absence in stress, in contrast with the progeny of biotic stress treated plants which show an altered transcriptomic response to subsequent stress treatment. Further experiments are required to establish whether biotic and abiotic transgenerational stress memories involve the activation of the same response mechanism or if multiple mechanisms exist by which stress may leave a heritable mark on the plant.
Cleary the elucidation of the mechanisms by which transgenerational stress memories are transmitted, and how they enhance stress tolerance, is only just beginning. The findings of this research further our understanding of this elusive and intriguing stress response and will hopefully provide many fruitful avenues of exploration for future research.
VII. Appendices
### Oligonucleotides

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asel adapter 1</td>
<td>CTCTGACTGGACCTGTTG</td>
<td>N/A</td>
</tr>
<tr>
<td>Asel adapter 2</td>
<td>TACACACGCTGTGGGAC</td>
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</tr>
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</tr>
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</tr>
<tr>
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### Bisulphite Sequencing

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<tr>
<td>MH66 R1</td>
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<td>MH66 R2</td>
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<td>Psa R</td>
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Table 13 continued on following page
The table provides a list of oligonucleotides used in PCR reactions. All oligonucleotides except the MSAP adapters were used in PCR reactions, and the annealing temperatures used are specified. +2 and +3 MSAP oligonucleotides were used in a touchdown PCR, and the annealing temperature specified is the final annealing temperature at the end of the touchdown cycles. H/M = HpaII/MspI. M/S/D = MboI/Sau3AI/DpnII.
## Chemicals, enzymes, kits, consumables and equipment

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<tr>
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<td>Commercial bleach (Sodium hypochlorite; NaOCl)</td>
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<td>Ethanol (≥99.9%)</td>
<td>Fisher Scientific</td>
<td>E9050/0/PL17</td>
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<tr>
<td>Ethylenediaminetetraacetic acid ferric sodium salt (FeIII·NaEDTA)</td>
<td>Duchefa</td>
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<tr>
<td>Ferric Zine (3-[(2-Pyridyl)-5-[(2-phosphonic acid)-1,2,4-triazine-5-p]dipropionic acid monosodium salt hydrate</td>
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<td>D06001</td>
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<td>Ficoll</td>
<td>Sigma</td>
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<tr>
<td>Formaldehyde (CH₂O; MB)</td>
<td>Sigma</td>
<td>F775,500-1f</td>
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<td>Glycolipid (CAUS, MBG)</td>
<td>Sigma</td>
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<td>Hydrochloric acid (HCl); 36.5-38%</td>
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<td>G9060/17</td>
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<td>Hygromycin B</td>
<td>Invitrogen</td>
<td>21667-037</td>
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<td>Interferon-α (NIPG570-1)</td>
<td>Novo Nordisk</td>
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<td>Isopropanol (C₃H₇OH; 99.95%)</td>
<td>Acros Organics</td>
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<tr>
<td>Kambic</td>
<td>Life Technologies</td>
<td>18131-024</td>
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<tr>
<td>Magnesium sulphate (dihydrate; USP; 99.99%)</td>
<td>Sigma</td>
<td>M7505</td>
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<tr>
<td>Manganese chloride (MnCl₂; 99%)</td>
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<td>MS salts</td>
<td>MYD</td>
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<td>N2, N2-DIMF (≥99%)</td>
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<td>NaNH₂PO₄·2H₂O</td>
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<td>2164579811</td>
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<tr>
<td>NaN₃PO₄·5H₂O</td>
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<td>Polyethylene glycol (MW 8000; MBG)</td>
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<td>Potassium dihydrogen phosphate (KH₂PO₄; 99%)</td>
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<td>Potassium chloride (KCl; MB)</td>
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<td>Potassium nitrate (KNO₃; MBG)</td>
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<td>SequaQWAX complete buffer</td>
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<td>M18073</td>
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<td>SequaQWAX JR Monomer Solution</td>
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<td>Sheath L-37</td>
<td>Life Technologies</td>
<td>V5-20</td>
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<tr>
<td>Sodium chloride (NaCl; MBG)</td>
<td>VWR International</td>
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<tr>
<td>Sodium molybdate dihydrate (Na₂MoO₄·2H₂O; 99%)</td>
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<td>Spectrin</td>
<td>AppliChem</td>
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<td>Triton X-100 Reagent</td>
<td>Invitrogen</td>
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<td>Water [sterile, nuclease free]</td>
<td>AMRESCO</td>
<td>7732-18-1</td>
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<td>X-Gal (X-Gal)</td>
<td>Novafect</td>
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<tr>
<td>Zinc sulphate heptahydrate (ZnSO₄·7H₂O; 98%)</td>
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<td>New England Biolabs</td>
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<td>MnCl₂</td>
<td>New England Biolabs</td>
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<td>PfuUltra™ II Fusion</td>
<td>Stratagene</td>
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<td>Hbsf</td>
<td>New England Biolabs</td>
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<td>Invitrogen</td>
<td>18064-014</td>
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<td>T4 DNA ligase</td>
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<td>Taq DNA polymerase</td>
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<table>
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<tr>
<td>Gel Extraction Kit</td>
<td>GeneCruz™</td>
<td>SC-49048</td>
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<tr>
<td>Genesy Plant Mini Kit</td>
<td>QIAGEN</td>
<td>21004</td>
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<tr>
<td>H/DNA Methylation Light™ Kit</td>
<td>Zyga Research</td>
<td>20092</td>
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<tr>
<td>Gateway® LR Clonase™ II Enzyme Mix</td>
<td>Invitrogen</td>
<td>11751-020</td>
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<tr>
<td>pET™/D/D-TOP10 Cloning Kit with One Shot® TOP10 Chemically Competent E. coli</td>
<td>Invitrogen</td>
<td>K391-20</td>
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<tr>
<td>Gateway Spin Miniprep Kit</td>
<td>QIAGEN</td>
<td>21704</td>
</tr>
<tr>
<td>GSK24QUik system extraction kit</td>
<td>QIAGEN</td>
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<tr>
<td>Ramsey Plant Mini Kit</td>
<td>QIAGEN</td>
<td>24953</td>
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Table 14 continued on following page
Table 14. Details of chemicals, enzymes, kits, consumables, equipment and other items used. Chemical purity or grade given where specified by supplier. MBG = Molecular Biology grade.

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<thead>
<tr>
<th>Consumable</th>
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<tr>
<td>1.5 ml microcentrifuge tubes</td>
<td>Starstedt</td>
<td>71,690,001</td>
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<tr>
<td>1.75 ml Glass Vials</td>
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<td>71,500,060</td>
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<tr>
<td>16/20 pl, Graduated Tip</td>
<td>StarLab</td>
<td>51110-3700</td>
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<tr>
<td>10 pl Graduated Tip</td>
<td>StarLab</td>
<td>51111-3700</td>
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<tr>
<td>1250 pl, Graduated Tip</td>
<td>StarLab</td>
<td>51112-3700</td>
</tr>
<tr>
<td>1L Plastic Box</td>
<td>The Plastic Box Company</td>
<td>WF-LALL</td>
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<tr>
<td>5 ml Microcentrifuge tubes</td>
<td>Starstedt</td>
<td>71,691</td>
</tr>
<tr>
<td>30 pl Tip</td>
<td>StarLab</td>
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<tr>
<td>8-Strip PCR Cassettes, Dotted</td>
<td>StarLab</td>
<td>51400-0800</td>
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<tr>
<td>8-Strip PCR Tubes</td>
<td>StarLab</td>
<td>51402-3500</td>
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<tr>
<td>96-well Ice Loading Combs</td>
<td>NAB Scientific</td>
<td>CA99</td>
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<td>96-well plate (semi-skirted with raised rims)</td>
<td>StarLab</td>
<td>E1400-8200</td>
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<tr>
<td>Accessori™ PCR Tubes</td>
<td>Corning Incorporated</td>
<td>PCR-925-C</td>
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<tr>
<td>Covette Cuvette Semi-Micro 50 ul Disposable</td>
<td>NL5</td>
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<td>Covette UV-Cuvette UV-disposable plastic 70 ul</td>
<td>FisherScientific</td>
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<td>Glassine bags (140 x 193 cm)</td>
<td>Kenko</td>
<td>N8007</td>
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<td>Microscope Slide Coverslips No 1.5 (12x50mm)</td>
<td>SLS</td>
<td>M13246</td>
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<td>Microscope tape</td>
<td>IM</td>
<td>13300</td>
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<tr>
<td>Microscope slides</td>
<td>FisherScientific</td>
<td>7001</td>
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<tr>
<td>Plantpaks Sheet Trays and Unit Paks</td>
<td>Neotech/Platypus</td>
<td>PST &amp; PS &amp; PS &amp; P60</td>
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<tr>
<td>Square Petri Dishes</td>
<td>Greiner Bio-one</td>
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<table>
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<td>ATP, CTX, GTP, TIP</td>
<td>Promega</td>
<td>U1221, U1223, U1225</td>
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<td>Bact™ Agar</td>
<td>BD Biosciences</td>
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<td>GeneScan™ 800 Lr Size Standard (Capillary electrophoresis marker)</td>
<td>Life Technologies</td>
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<tr>
<td>Iota Efficiency® DHLa™ Compliant Cells</td>
<td>Invitrogen</td>
<td>12625-012</td>
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<tr>
<td>IRby270 50-70 String Standard</td>
<td>Li-COB Biosciences UK</td>
<td>4200-80</td>
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<td>Origin CT12-14</td>
<td>Invitrogen</td>
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<td>PRIME Green</td>
<td>PCR Master Mix</td>
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<table>
<thead>
<tr>
<th>Equipment</th>
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<tbody>
<tr>
<td>54150 Microcentrifuge</td>
<td>Eppendorf</td>
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<tr>
<td>5415A Temperature Controlled Microcentrifuge</td>
<td>Eppendorf</td>
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<tr>
<td>ABI Prism 3130</td>
<td>Applied Biosystems</td>
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<tr>
<td>ABI Prism 3130 Sequence Detection Systems</td>
<td>Applied Biosystems</td>
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<tr>
<td>Automatic MII2200 Dimerometer</td>
<td>Roche</td>
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<tr>
<td>High-Engine Dyed Dual Thermocycler</td>
<td>Bio-Rad</td>
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<tr>
<td>Fume Hood</td>
<td>Fumevac</td>
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<tr>
<td>Gene Pulser apparatus</td>
<td>Bio-Rad</td>
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<tr>
<td>Microbiological Incubator</td>
<td>SLS</td>
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<tr>
<td>MLR-351 Growth Cabinet</td>
<td>Sanyo</td>
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<tr>
<td>NanoDrop 2000/800</td>
<td>Thermo Scientific</td>
</tr>
<tr>
<td>Pipetten (0.5 - 1000 µl)</td>
<td>Gilson</td>
</tr>
<tr>
<td>Pipettor</td>
<td>Aquilabo</td>
</tr>
<tr>
<td>Pocket PEA Portable Chlorophyll Fluorometer</td>
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<td>Temperature Controlled Orbital Shaking Incubator</td>
<td>Environ</td>
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<tr>
<td>Ultraviolet 2000 Spectrophotometer</td>
<td>Pharma BioTech</td>
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<tr>
<td>Vacuum</td>
<td>KNF</td>
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<td>Microbiological Incubator</td>
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<td>MLR-351 Growth Cabinet</td>
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<td>Pharma BioTech</td>
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<tr>
<td>Vacuum</td>
<td>KNF</td>
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Abbreviations

HSP17.6II  17.6 KDA CLASS II HEAT SHOCK PROTEIN
5-azaC   5-azacytidine
Ac     Activator
AOC1   ALLENE OXIDE CYCLASE 1
AOC2   ALLENE OXIDE CYCLASE 2
AOC3   ALLENE OXIDE CYCLASE 3
AOC4   ALLENE OXIDE CYCLASE 4
AFLP   Amplified fragment length polymorphsim
MSH6   MUTS HOMOLOG 6
AG10   ARGONAUTE 10
AGO4   ARGONAUTE 4
AGO6   ARGONAUTE 6
AGO9   ARGONAUTE 9
AIG1   AVRRPT2-INDUCED GENE 1
bHLH   Basic helix loop helix
BLAST  Basic local alignment search tool
BABA   Beta-aminobutyric acid
PSCS   CARBOXYLATE SYNTHETASE
CMT3   CHROMOMETHYLASE 3
cdd    dmt3 drm2 drm1
CNR    COLOURLESS NON-RIPENING
cDNA   Complimentary DNA
CTD    C-terminal domain
Ct     Cycle threshold
CYP71A12 CYTOCHROME P450, FAMILY 71, SUBFAMILY A,
dps    Days post stratification
DRD1   DEFECTIVE IN RNA-DIRECTED DNA METHYLATION 1
DME    DEMETER
DML1   DEMETER-LIKE 1
DML2   DEMETER-LIKE 2
DCL1   DICER-LIKE 1
DCL2   DICER-LIKE 2
DCL3   DICER-LIKE 3
DCL4   DICER-LIKE 4
DNMT1  DNA Methyltransferase 1
DRM2   DOMAINS REARRANGED METHYLTRANSFERASE 2
dsRNA  Doubles stranded RNA
epiRILs Epigenetic Recombinant Inbred Lines
EVD    Evadé
FSD1   FE SUPEROXIDE DISMUTASE 1
FIT1   FE-DEFICIENCY INDUCED TRANSCRIPTION FACTOR 1
FRO2   FERRIC REDUCTION OXIDASE
FRO6   FERRIC REDUCTION OXIDASE 6
FRO7  FERRIC REDUCTION OXIDASE 7
FER4  FERRITIN 4
FWA  FLOWERING WAGENINGEN
FPKM  Fragments per kilobase exon gene model per million mapped fragments
FW  Fresh weight
GLV  Green leaf volatiles
H3K9me2  Histone 3 lysine 9 dimethylation
H3K4me3  Histone 3 lysine 4 trimethylation
H3K9ac  Histone 3 lysine 9 acetylation
HRF  Homologous recombination frequency
HEN1  HUA ENHANCER 1
HPA  Hypothalamo-pituitary-adrenal
ISR  Induced systemic resistance
IR  Inverted repeats
JA  Jasmonic acid
KYP  KRYPTONITE
LOX2  LIPOXYGENASE 2
LRH  Low relative humidity
LB  Lysogeny Broth
MECP2  MBP METHYL CGP BINDING PROTEIN 2
MEL1  MEIOSIS ARRESTED AT LEPTOTENE1
Tm  Melting temperature
MSAP  Methylation sensitive amplified polymorphism
MBPs  Methyl-binding proteins
MeDIP  Methyl-DNA immunoprecipitation
MET1  METHYLTRANSFERASE 1
MIR843A  miRNA 843A
MGSA  Model-based gene set analysis
MDS  Multidimensional scaling approach
MS  Murashige & Skoog
Mu  Mutator
NATA1  N-ACETYLTRANSFERASE ACTIVITY 1
NRAMP6  NRAMP metal ion transporter 6
NRPD1  NUCLEAR RNA POLYMERASE D 1A
NRPE1  NUCLEAR RNA POLYMERASE D 1B
δ-OAT  ORNITHINE-DELTA-AMINOTRANSFERASE
OPDA  12-oxo-phytodienoic acid
PCR1  PLANT CADMIUM RESISTANCE 1
PDF1.2  PLANT DEFENSIN1.2
PEG  Polyethylene glycol
PTGS  Post-transcriptional genes silencing
psm.  Pseudomonas syringae
Pst.  Pseudomonas syringae
qRT-PCR  Quantitative Reverse-Transcriptase PCR
rfu  Relative florescence units
ROS1  REPRESSOR OF SILENCING 1
RNAi  RNA interference
POLII RNA POLYMERASE II
POL IV RNA POLYMERASE IV
RdRP RNA-dependent RNA polymerase
RDR2 RNA-DEPENDENT RNA POLYMERASE 2
RISC RNA-induced silencing complex
SA Salicylic acid
SD-AFLP Secondary digest AFLP
ssRNA Single stranded RNA
siRNA Small interfering RNA
transgene SMTs
SD Standard deviations
SI Stomatal index
S.O.C Super Optimal Broth
SDC SUPPRESSOR OF DRM2, DRM1, CMT3
SAR Systemic acquired resistance
TGS Transcriptional genes silencing
TEs Transposable elements
TEs Transposable elements
TSA1 TSK-ASSOCIATING PROTEIN 1
TAT3 TYROSINE AMINOTRANSFERASE 3
UHRFI Ubiquitin-like, containing PHD and RING finger domains 1
VIM1 VARIANT IN METHYLATION 1
VIM2 VARIANT IN METHYLATION 2
VIM3 VARIANT IN METHYLATION 3
VSP1 VEGETATIVE STORAGE PROTEIN1
VSP2 VEGETATIVE STORAGE PROTEIN2
VOC Volatile organic compounds
WGA Whole genome amplification
WT Wild type
ZDP ZINC FINGER DNA 3’ PHOSPHOESTERASE
GUS β-glucoronidase
VIII. References


Hayashi, M., Gerry, S.P., Ellerby, D.J., 2010. The seed dispersal catapult of Cardamine parviflora (Brassicaceae) is efficient but unreliable. American journal of botany 97, 1595–601.


Law, J. a, Vashisht, A. a, Wohlschlegel, J. a, Jacobsen, Steven E. 2011. SHH1, a homeodomain protein required for DNA methylation, as well as RDR2, RDM4, and chromatin remodeling factors, associate with RNA polymerase IV. PLoS genetics 7, e1002195.


Teixeira, Felipe Karam, Heredia, F., Sarazin, A., Roudier, F., Bocca, M., Ciaudo, C., Cruaud, C., Poulain, J., Berdasco, M., Fraga, M.F., Voinnet, O., Wincker, P., Esteller,


Viola, I.L., Uberti Manassero, N.G., Ripoll, R., Gonzalez, D.H., 2011. The Arabidopsis class I TCP transcription factor AtTCP11 is a developmental regulator with distinct DNA-
binding properties due to the presence of a threonine residue at position 15 of the TCP domain. The Biochemical journal 435, 143–55.


correlation between multiple histone marks and gene expression. Plant molecular biology 72, 585–95.


