Australian National University

Training Memory: Exploring the Intersection of Plant Stress Signalling and DNA Methylation

Diep Ray Ganguly

A thesis submitted for the degree of
Doctor of Philosophy of
The Australian National University

July 16, 2018

This thesis was typeset using the LaTeX typesetting system originally developed by Leslie Lamport, based on TeX created by Donald Knuth.

Except where otherwise indicated, this thesis is my own original work.

Diep Ganguly
diepganguly@gmail.com
July 16, 2018

Dedicated to my mother, Gargi Ganguly, who gave me everything.

"Do not pray for an easy life, pray for the strength to endure a difficult one."
-Bruce Lee (Lee Jun-fan)

"Basic research is what I am doing when I don't know what I am doing."
-Wernher von Braun

"I bomb atomically, Socrates' philosophies / And hypotheses..."
–Inspectah Deck

Acknowledgements

There are surely a countless number of people that have helped me along the way through this PhD adventure, whether it be providing personal and professional advice, moral support, or stimulating discussions. This section will, no doubt, do far too little to express my gratitude to you all. So for all whom I've missed, I sincerely apologize, but know that your contributions are not forgotten.

The biggest thank you goes to my Mum who continues to work tirelessly to provide me with all the opportunities that I could have asked for throughout my life. You are always there to support me in any way possible, and you laid all the foundations and gave me the freedom when I was young to strive to be who I wanted. You continue to be my biggest source of inspiration. Thanks also to my grandparents who also made sacrifices in support of both my Mum and I.

A tremendous thank you must go to my supervisors and mentors, past and present, Barry, Verónica, Pete, and Steve. I truly have the utmost respect and gratitude for all your patience, guidance, and wisdom over the years, each of you have helped shape my growth and development as a researcher, and I will always reflect on your respective impacts. I truly feel most fortunate and privileged to have had the opportunity to work and learn alongside you. Special thanks go to Barry for providing me the initial opportunity in the beginning and continuing to support my professional development and giving me the freedom to research what I wanted.

A huge thank you to all my fellow lab members over the years. Your companionship, support, snacks, and discussions have been vital throughout this journey. I am most grateful to have been involved with such a smart, motived, and energetic cohort of researchers that feel like a professional family.

Finally a thank you to my patient and loving partner, Rani, for your constant companionship and support throughout this journey. You changed this from a daunting challenge into a vibrant adventure.

This research was supported by an Australian Research Training Program (RTP) Scholarship. The project was also possible due to additional funding sources including the Australian National University, the Australian Research Council Centre of Excellence in Plant Energy Biology, and the Grains Research and Development Corporation. I am grateful for all of the financial support these sources have provided to allow me to undertake this PhD. Furthermore, the added funds from the Grains Research and

Development Corporation were vital in allowing me to perform the ambitious next-generation experiments that I had planned, and for having that opportunity I will always be deeply grateful.

Abstract

Plants are sessile organisms living in a dynamic environment to which they must continually acclimatize in order to maximise their reproductive potential. This plasticity is achieved through many complex and intricate signalling pathways that allow for the continuous perception, response, and adjustments to new environmental stimuli. A growing body of evidence suggests that such pathways are not merely static but dynamic and can be primed following repeated activation, thus affecting enhanced responses to recurring stresses. Such examples of *priming* have led to a notion that plants have some capacity to form stress memories of past environmental perturbations. However, the full extent and nature of such memory, and the machinery involved to store and transmit these, remain enigmatic. One prospective mechanism is the involvement of heritable, yet rapid and reversible, chromatin marks that, theoretically, could be shaped by the environment to convey a regulatory effect on the expression of the underlying genotype, thus acting as an *epi*genetic layer of regulation.

This thesis explores the potential intersection of stress signalling pathways and chromatin variation, specifically DNA methylation, to co-ordinate plant stress responses. First, mechanistic insights into the operation of a SAL1-PAP-XRN retrograde signalling pathway to fine-tune plant physiology under drought are presented. A key finding was that this pathway complements canonical ABA signalling to induce stomatal closure, thus minimising water-loss under water limited conditions. Furthermore, the SAL1-PAP-XRN pathway was found to effect chromatin patterns, specifically DNA methylation at short transposable elements. These observations implicate cross-talk with the RNA directed DNA methylation pathway, however, the exact mechanism for this interaction remains to be identified.

Multiple investigations were performed to test for stress-induced changes in DNA methylation that could potentially regulate responses to recurring stress, thus conveying a memory. A transgenerational recurring drought stress experiment tested whether descendants of drought-exposed lineages displayed greater drought tolerance (transgenerational memory). For the majority of traits tested, including plant growth rate and drought survival, offspring from plant lineages exposed to successive generations of repeated drought stress performed comparably to those from control lineages. However, memory was demonstrated in the form of enhanced seed dormancy, in drought stressed lineages, that persisted at least one generation removed from stress. Whether this ca-

pacity for memory could be related to the type or severity of stress applied, or species examined, remains to be investigated further.

The transgenerational drought experiment was paired with a recurring excess-light stress experiment to investigate memory within a generation. Not only did this treatment lead to priming of plant photosynthetic behaviour, indicative of a greater capacity to withstand abrupt increases in light intensity, but new leaves from stressed plants, developed in the absence of stress, also showed altered photosynthetic characteristics compared to unstressed counterparts. Such observations are consistent with the mitotic transmission of stress-induced traits.

Given multiple demonstrations of memory, comparisons were made to unstressed controls to test for any correlating changes in DNA methylation that might explain the phenomena observed. However, in both experiments, observations of memory were found to be independent of large-scale conserved changes in DNA methylation discounting it as a conveyor of plant stress memories, under these conditions, raising questions regarding the mechanism(s) responsible for the examples of memory observed herein.

Ultimately, this thesis systematically evaluates the notion that plants are able to form genuine memories, potentially underpinned by reversible chromatin marks, that may facilitate acclimation to local environments on a relatively rapid scale compared to the fixation of adaptive genetic polymorphisms. Any capacity for plant stress memories may provide avenues for further epigenomic based agronomic tools to improve crop stress tolerance. However, the nature of such memories observed here appear subtle and nuanced, and are forgotten beyond a generation. Further characterisation and mechanistic understanding of mitotic memory mechanisms, however, may still hold potential. It was also observed that stress signalling pathways can interact with those involved in chromatin modification, giving novel insight into their mechanistic functioning and the how the onset of stress may induce chromatin changes. Despite this potential, the DNA methylome was found to be relatively impervious to stress-induced changes and, thus, is an unlikely memory mechanism.

Guide

This thesis is structured as six chapters: the introduction, materials and methods, three results chapters, and a final summary chapter. The introduction frames the aims of this thesis and the required background information to contextualise their relevance. Each of the core results chapters can be read semi-autonomously, alongside the appropriate sections in the materials and methods, as each presents data on unique experimental systems that all relate to the overall aims of this thesis, as well as a focussed discussion on those chapters' results. The final summary chapter will attempt to provide an integrated discussion regarding the major findings in this thesis. Below is an outline:

- **Chapter 1:** Introduction - background information on the topics investigated in this thesis.
- **Chapter 2:** Materials and methods - information on the experimental procedures performed.
- **Chapter 3:** Mechanistic insight into SAL1-PAP-XRN signalling and potential cross-talk with DNA methylation.
- **Chapter 4:** The Arabidopsis DNA methylome is stable under transgenerational drought stress.
- **Chapter 5:** Maintenance of pre-existing DNA methylation states through recurring excess-light stress.
- **Chapter 6:** Thesis conclusions - An integrated discussion of the major findings.

Additional content:

- **Hyperlinks:** This thesis was compiled in LaTeX as an enhanced PDF with in text hyperlinks (using the *hyperref* package) for all citations, cross-references including appendices, figures, and supplementary data, for example clicking on a heading in the table of contents will navigate to that section. All external hyperlinks, such as a URL, are coloured in blue.
- **Code:** All code used for processing and analyses of next-generation sequencing data can be accessed on GitHub.

- **Supplemental dataset:** Tables and datasets, referred to herein, too large to fit into a printed copy of this thesis are available electronically by following any of the links in the appendices. In the eventuality that these links are broken, these datasets are also available at the corresponding listed open access publications (see below) or can be made available upon request.

Associated publications

The experimental data and materials presented in this work contributed to the following publications, with the associated chapter in brackets:

- [Chapter 1] Crisp PA, **Ganguly D**, Eichten SR, Borevitz JO, Pogson BJ (2016) Reconsidering plant memory: Intersections between stress recovery, RNA turnover, and epigenetics. *Sci Adv*, **2**(2), e1501340–e1501340. doi: 10.1126/sciadv.1501340.

- [Chapter 3] Pornsiriwong W, Estavillo GM, Chan KX, Tee EE, **Ganguly D**, Crisp PA, Phua SY, Zhao C, Qiu J, Park J, et al (2017) A chloroplast retrograde signal, 3'-phosphoadenosine 5'-phosphate, acts as a secondary messenger in abscisic acid signaling in stomatal closure and germination. *Elife*, **6**, e23361. doi: 10.7554/eLife.23361.

- [Chapter 4] **Ganguly DR**, Crisp PA, Eichten SR, Pogson BJ (2017). The Arabidopsis DNA methylome is stable under transgenerational drought stress. *Plant Physiology*, pp.00744.2017. doi: 10.1104/pp.17.00744.

- [Chapter 5] **Ganguly DR**, Crisp PA, Eichten SR, Pogson BJ (2018) Maintenance of pre-existing DNA methylation states through recurring excess-light stress. Plant, Cell & Environment. doi: 10.1111/pce.13324.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Thesis synopsis

Plants show an amazing capacity to perceive and respond to a plethora of environmental stimuli. This is crucial for survival, given that plants are sessile organisms that are unable to avoid fluctuating conditions in a dynamic environment. Indeed, plants have developed complex molecular systems that convey an impressive ability for adaptive plasticity (Mickelbart et al., 2015; van Loon, 2016). A crucial component of these systems are signalling pathways that originate from the perception of a stress-derived signal and culminate in a coordinated response involving a multitude of factors (Fleta-Soriano & Munné-Bosch, 2016). This includes, but is not limited to, protein-protein interactions, for example PYRABACTIN RESISTENCE1-LIKE (PYL) and PROTEIN PHOSPHATASE TYPE-2C (PP2C) interaction upon perception of abscisic acid (ABA) (Komatsu et al., 2013), the induction of intracellular signalling pathways, such as from the chloroplast to the nucleus (Chan, Phua, et al., 2016), biochemical responses, such as the biosynthesis of key hormones to regulate developmental processes, and influencing ribonucleic acid (RNA) dynamics to fine-tune gene expression (Crisp et al., 2016). Ultimately these coordinated factors allow the continual adjustment of plants' developmental and physiological processes to suit the surrounding environment.

Given the dynamic nature of the environment this process of perception and adjustment is not a single static event, rather it is a continual process where new stimuli induce signals that continuously lead to the fine-tuning of plant responses. Furthermore, an emerging concept is that repeated exposures to biotic and abiotic stresses can lead to 'priming', whereby a 'primed' individual displays a more rapid or fine-tuned response enabling acclimation to their local environment (Conrath et al., 2006; Bruce et al., 2007; Crisp et al., 2016). Research into stress priming has uncovered numerous such examples where prior stress exposure leads to altered plant behaviour, suggesting that plants have a capacity for plant stress memory. However, the full extent to which plants can remember their environment, and the mechanisms to store and transmit this memory, are yet

to be fully uncovered. One possibility is the involvement of rapidly reversible, yet reportedly stable and heritable chromatin marks, such as DNA methylation, the variations in which could alter the expression of underlying genetic elements (Lister et al., 2008; Becker et al., 2011; Schmitz et al., 2013). As a result, there has been much speculation for the contribution of DNA methylation to local acclimation and adaptation (Gutzat & Mittelsten Scheid, 2012). However, so too is there ambiguity and uncertainty reflected by conflicting reports (Seymour et al., 2014; Eichten & Springer, 2015; Hagmann et al., 2015; Secco et al., 2015), necessitating a systematic investigation to confirm whether environmentally-induced methylation variation conveys any biological functionality, such as the transmission of plant stress memory.

1.2 Plant abiotic stress

Plant growth can be compromised by a variety of environmental stresses that ultimately impair optimal plant reproductive potential, which is of significant agricultural importance as this diminishes crop productivity and yield (Boyer, 1982; Takahashi et al., 2004; Verslues et al., 2006). Many environmental factors may be considered as biotic or abiotic stresses that cause penalties to plant growth including incoming light energy, ultraviolet irradiance, temperature, soil composition (for example, salinity, nutrient availability, acidity, moisture) or parasitism by various possible pathogens or insects (Jakab et al., 2005; Atkin et al., 2006; Conrath et al., 2006; Gordon et al., 2013; Müller-Xing et al., 2014; Mickelbart et al., 2015). In response to stress, plants must make physiological adjustments, predominantly through the action of molecular and biochemical pathways, to minimize the damage caused by a particular stress without severely impairing growth processes (Takahashi et al., 2004; Skirycz & Inzé, 2010). This often culminates as altered metabolism and gene expression to establish a new equilibrium between growth and resistance (Mazzucotelli et al., 2008). The fundamental impact of abiotic stress is the impairment of plant growth and yield as resources are diverted towards response and repair; nutrient acquisition and utilization is disrupted; photosynthesis and respiration are impaired: ultimately culminating in an altered physiological state slowing growth but maximising survival (Skirycz & Inzé, 2010; Cramer et al., 2011; Mickelbart et al., 2015).

In order to minimise stress-induced damage, there are three predominant models of stress response (or resistance): escape, avoidance, and tolerance (Verslues et al., 2006; Basu et al., 2016). Escape mechanisms entail the completion of a plants' life cycle before the onset of stress (Basu et al., 2016). Avoidance mechanisms, on the other hand, involves short-term physiological adjustments to minimize the impact of stress on plant performance, for example, stomatal closure and solute accumulation in response to water limitation to avoid dehydration (Verslues et al., 2006; Skirycz & Inzé, 2010). Alternatively, additional mechanisms can be engaged to maintain plant function in the presence of stress that are regarded as "tolerance" mechanisms, for example,

the production of osmoprotectants, antioxidants, and reactive oxygen species (ROS) detoxifying enzymes to avoid damage caused by water limitation (Verslues et al., 2006).

Before considering the physiological importance of stress-derived signals or the possibility of plant stress memory, we must first consider the primary impacts of abiotic stresses and the subsequent avoidance and tolerance mechanisms employed by plants, which help identify where plant stress memory could play a functional role to promote success during future events. Both excess-light (EL) and drought induce cellular oxidative stress either by saturating the photosystems with light energy or by limiting the capacity for carbon fixation through water limitation (**Figure 1.1**). Enhancing our understanding of plant abiotic stress signalling pathways and short-term acclimation to dynamic conditions, including the mechanisms involved, is of significant value in developing techniques to maintain and improve agricultural productivity in the face of modern challenges, such as reductions in arable land, and increasingly dynamic and variable climates. This thesis focusses on two agriculturally relevant abiotic factors that induce severe cellular oxidative stress: EL and drought.

1.2.1 Excess-light stress

Sunlight is essential for plant growth, providing the energy to drive photosynthesis that subsequently impacts numerous plant developmental processes. This relies on the absorption of light energy by chlorophyll molecules located in a Photosystem II (PSII)-light-harvesting complex (LHC)II super complex, and the subsequent transfer of electrons [photosynthetic electron transport (PET)] through the reaction centres of two electronically connected photosystems, PSII then Photosystem I (PSI), located on the thylakoid membranes of chloroplasts. This electrochemical energy is ultimately used to generate nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP), both of which are critical for the fixation of carbon dioxide (CO_2) into usable sugars via the Calvin-Benson cycle (Minagawa, 2011; Caffarri et al., 2014). There is, however, a fine balance between the optimum light intensity for carbon fixation (light saturation point) and light intensities exceeding this, termed EL, which can lead to severe oxidative stress, photodamage, and photoinhibition (sustained decrease in photosynthetic efficiency) (Demmig-Adams et al., 1989; Demmig-Adams, 1990; Barber & Andersson, 1992; Niyogi, 1999; Z. Li et al., 2009; Jung et al., 2013).

EL causes photoinhibition by damaging PSII, specifically via light-induced degradation of D1 protein of the PSII reaction centre (Tyystjärvi, 2008). Intriguingly, this is not wholly associated with excessive energy absorption by photosynthetic pigments, highlighted by the finding that the quantum yield and rate of photoinhibition is independent of light intensity and the size of light-harvesting antenna, respectively (Tyystjärvi, 2008; Takahashi & Badger, 2011). Instead, EL causes the release of manganese ions from oxygen evolving clusters in PSII leading to impaired PET (Tyystjärvi, 2008). This leaves PSII reaction centres vulnerable to damage upon absorption of light energy (Takahashi

& Badger, 2011). Chlorophyll molecules are the key photosynthetic pigments that absorb and transfer light energy to PSII reaction centres (Niyogi, 1999). Upon absorption, chlorophyll molecules enter the singlet excited state, which is highly reactive and, if it cannot transfer this energy, can form triplet chlorophyll through intersystem crossing (Niyogi, 1999). Triplet chlorophyll is capable of reacting with molecular oxygen to form ROS, primarily singlet oxygen ($^1O_2$) that can subsequently be converted into hydrogen peroxide (H_2O_2) or hydroxyl radicals, which can readily oxidize many biologically significant molecules causing widespread cellular damage and further photoinhibition (Niyogi, 1999; Triantaphylidès & Havaux, 2009). H_2O_2 is not only produced via $^1O_2$, but also through the dismutation of superoxide radicals generated at PSI (Slesak et al., 2007). Absorption of EL also leads to an acidified thylakoid lumen, reduced components of PET, and perturbation of chlorophyll biosynthesis (Z. Li et al., 2009).

Together, these effects can impair plant productivity and, in severe cases, lead to plant death (Müller et al., 2001; Dietz, 2015). Furthermore, adverse environmental conditions can decrease the light saturation point of afflicted plants, for example, suboptimal temperatures can impair enzyme stability and kinetics, and PET, resulting in exacerbated photoinhibition (Ruelland et al., 2009). Thus, plants must optimize their performance according to their exposed light levels, which can fluctuate as a result of changes in the angling of the sun throughout the day, transient canopy openings, and cloud movements (Gordon et al., 2013; Hirth et al., 2013; Schumann et al., 2017). Indeed, it is well-documented that sun-exposed leaves show distinct photosynthetic profiles compared to those developed in the shade, a process termed photoacclimation, to optimise photosynthesis and limit photoinhibition (Murchie et al., 2009; Zivcak et al., 2014). Additionally, fluctuations in light quantity are often accompanied by temperature fluctuation, which is reflected in the light treatments utilized in this study and causes greater oxidative stress (Jung et al., 2013). It is also important to acknowledge that changes in light quality (or the wavelengths of energy a plant is exposed to) constitute another form of light stress, such as increases in UV-B radiation due to depletion of the ozone layer, that can also have important biological consequences and involve distinct pathways (Müller-Xing et al., 2014; Bornman et al., 2015), however, the focus of this thesis will be in regards to changes in light quantity.

Plants possess a suite of photoprotective mechanisms that guard against EL stress that can result in PSI acceptor-side limitation, including state-transitions, cyclic electron flow and stoichiometric adjustments in photosynthetic complexes (Niyogi, 1999; Munekage et al., 2002; Nilkens et al., 2010; Minagawa, 2011; Schöttler & Tóth, 2014; Allahverdiyeva et al., 2015). It is important to note that there are both short-term and long-term responses for light acclimation, where the former involves reversible modifications to the light harvesting machinery compared to more permanent structural and physiological adjustments (Schöttler & Tóth, 2014; Dietz, 2015). An important example of an immediate short-term response to EL stress are state transitions. This occurs due

to imbalanced excitation of PSII, relative to PSI, and results in phosphorylation-induced re-allocation of PSII antenna to PSI (Niyogi, 1999; Minagawa, 2011). State transitions are part of a key photoprotective mechanism that results in the dissipation of over 75% of EL as thermal energy is measured and referred to as non-photochemical quenching (NPQ) (Demmig-Adams & Adams, 1996; Niyogi, 1999; Müller et al., 2001).

NPQ can be divided into multiple underlying components with characteristic kinetics, including energy-dependent, state-transition (described above), and photoinhibitory quenching (Niyogi, 1999; Müller et al., 2001; Jung & Niyogi, 2009). The major contributor of these is the energy-dependent quench (qE) that occurs rapidly, within seconds to minutes, and is tied to a low thylakoid lumen pH that induces conformational changes in PSII antenna (Demmig-Adams, 1990; Maxwell & Johnson, 2000; Müller et al., 2001). These are administered by the PSII subunit protein PHOTOSYSTEM II SUBUNIT S (PsbS) that acts as a pH sensor in the thylakoid lumen (X.-P. Li et al., 2000, 2002, 2004), and, whose activity also contributes towards Systemic acquired acclimation (SAA) (Ciszak et al., 2015). These rapid, and reversible, adjustments to the PSII antenna promote a quenched state, whereby tight packing of the light harvesting complexes promotes thermal dissipation of excess photochemical energy (Ruban et al., 2007; Horton et al., 2008). This is accompanied by the production of accessory photoprotective pigments, for example the conversion of violaxanthin to zeaxanthin via the xanthophyll cycle, which, upon binding to the light harvesting complexes, allows energy transfer away from chlorophyll pigments, preventing the production of both triplet chlorophyll and subsequent ROS (Demmig-Adams, 1990; Demmig-Adams et al., 1995; Demmig-Adams & Adams, 1996; Demmig-Adams et al., 1996; Horton et al., 2008). Prolonged exposure to EL can result in the accumulation of ROS, which can be averted through the production of ROS detoxifying enzymes and pigments (Telfer, 2002; Rossel et al., 2007; Jahns & Holzwarth, 2012; Dietz, 2015). Additional components involved in the optimization of photosynthetic capacity include DEGP PROTEASE 7 (DEG7), PROTON GRADIENT REGULATION 5 (PGR5), and PROTEIN KINASE STN7 (STN7); which mediate re-arrangement and repair of the photosynthetic machinery (Pesaresi et al., 2009; Munekage et al., 2002; Sun et al., 2010).

1.2.2 Drought stress

Fluctuations in light intensity can be rapid and intense, providing a versatile system with which to examine stress responses. On the other hand, drought (soil moisture deficit as a result of below-average rainfall) is a slow-onset, yet, severe stress that significantly impacts crop yields worldwide and is now occurring with greater frequency and severity, thus having important agronomic, economic, and social consequences (Kramer & Boyer, 1995; Cramer et al., 2011; Walter et al., 2011; Spinoni et al., 2014). Water deficiency also holds numerous physiological implications for plant development and growth either directly or indirectly affecting almost all plant processes, in particular photosynthesis

(Kramer & Boyer, 1995; Osakabe et al., 2014). Physiological effects include loss of turgor, wilting, cessation of cell enlargement, stomatal closure, and impaired metabolism and photosynthesis; ultimately preventing optimal plant productivity and in severe cases leads to death (Kramer & Boyer, 1995). Thus, drought can be a major limiting factor of plant productivity. The magnitude of the impact of drought depends on its duration, severity, and timing; for example, the effects of drought are most severe during plant reproduction (Westgate et al., 1996; Pradhan et al., 2012; Fleta-Soriano & Munné-Bosch, 2016).

A key route for plant water efflux is due to the requirement of atmospheric CO_2 for photosynthesis, which is obtained by gas exchange through stomatal pores on the leaf surface (Murata et al., 2015; Z. Yang et al., 2016). Opening of stomata promotes plant growth and photosynthesis due to increased CO_2 uptake allowing for maximal carbon fixation as described previously (Murata et al., 2015). However, accompanying this is a large efflux of water vapour that must be maintained through plant transpiration (Z. Yang et al., 2016). In order to absorb water, plant roots must generate water potentials low enough relative to the soil water potential (Kramer & Boyer, 1995). Thus, water uptake by plant root systems can efficiently replace the lost water when soil moisture is high, and plant growth can continue unimpeded (Kramer & Boyer, 1995; Z. Yang et al., 2016). Alternatively, when water is limited, such as during periods of drought, there can be drastic reduction in soil water potential that makes it more difficult for plants to absorb water from the soil (Kramer & Boyer, 1995; Verslues et al., 2006).

This forces an adjustment of plant processes to more efficiently utilize the water available for essential metabolic processes, while slowing plant growth to minimize water loss and compromising plant productivity for survival (Boyer, 1982; Kramer & Boyer, 1995; Verslues et al., 2006; Z. Yang et al., 2016). A key short-term drought avoidance mechanism is to minimize water loss by closing stomata, which also slows transpiration necessitating other cellular adjustments (Verslues et al., 2006). Key signalling cascades control the development and sensitivity of guard cells, which surround stomatal pores on the leaf epidermis and control its aperture, enabling fine-tuned regulation of this avoidance response (Dong & Bergmann, 2010; Chen, Hills, et al., 2012; Osakabe et al., 2014). Thus guard cells are a key cell type controlling plant physiology during changes in water availability, among other environmental fluctuations such as ambient CO_2 levels (Zhao et al., 2008; Osakabe et al., 2014). The signalling events involved in guard-cell mediated stomatal control will be explored in greater detail in this thesis, in particular, the identification of a new secondary messenger that acts as an agonist of the canonical ABA signalling pathway.

In the case of prolonged drought, longer term avoidance mechanisms, including maximising root growth, increasing tissue water storage capacity and cuticle thickness (to prevent water evaporation), are engaged to preserve plant water status (Verslues et al., 2006). However, maintained avoidance of water limitation and optimal metabolic

activity is not feasible during a prolonged drought and low soil water potential will eventually result in reduced plant water content and, consequently, impaired photosynthetic performance. Drought tolerance mechanisms primarily involve the protection of cellular structures, such as cell membranes, through the production of osmoprotectants and protective proteins, like dehydrins that can act as chaperones, solutes and detoxifying enzymes (Verslues et al., 2006).

Understanding the mechanisms employed by plants to withstand such abiotic stresses helps us appreciate the dynamic and flexible nature of plant physiology, which is required for survival in dynamic and flexible environments (**Figure 1.1**). Many of the responses are carried out through a variety of integrated transcriptional and post-transcriptional changes that underlie, for example, the production of antioxidants for detoxification, osmoprotectants for maintaining cellular water potential, or manipulation of the light harvesting complexes to prevent excess photodamage (Verslues et al., 2006; Ruban et al., 2007; Cramer et al., 2011). It is also important to note that many of the responses described are part of an integrated response, rather than occurring independently, and, depending on plant species, can be engaged to different extents (Verslues et al., 2006). The ability for these responses to be carried out in an intricate and integrated manner relies on the operation of signalling pathways that relay information between important cellular components, such as the nucleus and chloroplast.

1.3 Plant stress signalling

Plants must respond to abiotic stresses to minimise disruption of plant growth and reproduction. Plant stress signalling pathways are paramount in activating the molecular and biochemical mechanisms required for stress avoidance or tolerance (**Figure 1.2**) (Takahashi et al., 2004; Cramer et al., 2011; Chan, Phua, et al., 2016). These pathways are initiated by the perception of stress, or environmental fluctuations, which relies on the production of signals that are typically a direct biochemical consequence from the stress itself, for example, the over-production of ROS from photoinhibition during EL stress (Tyystjärvi, 2008). Additionally, a wide range of plant-derived chemicals and metabolites can also act as stress signals including, but not limited to, ROS, carotenoid oxidation products, intermediates of sulfur metabolism, calcium, and ABA (Ramakrishna & Ravishankar, 2011; Ramel et al., 2012; Carmody et al., 2016; Chan, Phua, et al., 2016; Hou et al., 2016). These signals are mobile and they themselves can be recognised by secondary transducers or receptors that can propagate the signals. Ultimately, this induces biochemical adjustments culminating into a physiological response, such as the production of ROS paired with intracellular calcium transport and the subsequent activation of ion channels to induce stomatal closure in response to ABA (Osakabe et al., 2014; Murata et al., 2015). An important destination for many of these signalling pathways is the nucleus, where changes in gene expression can be made to affect cellular

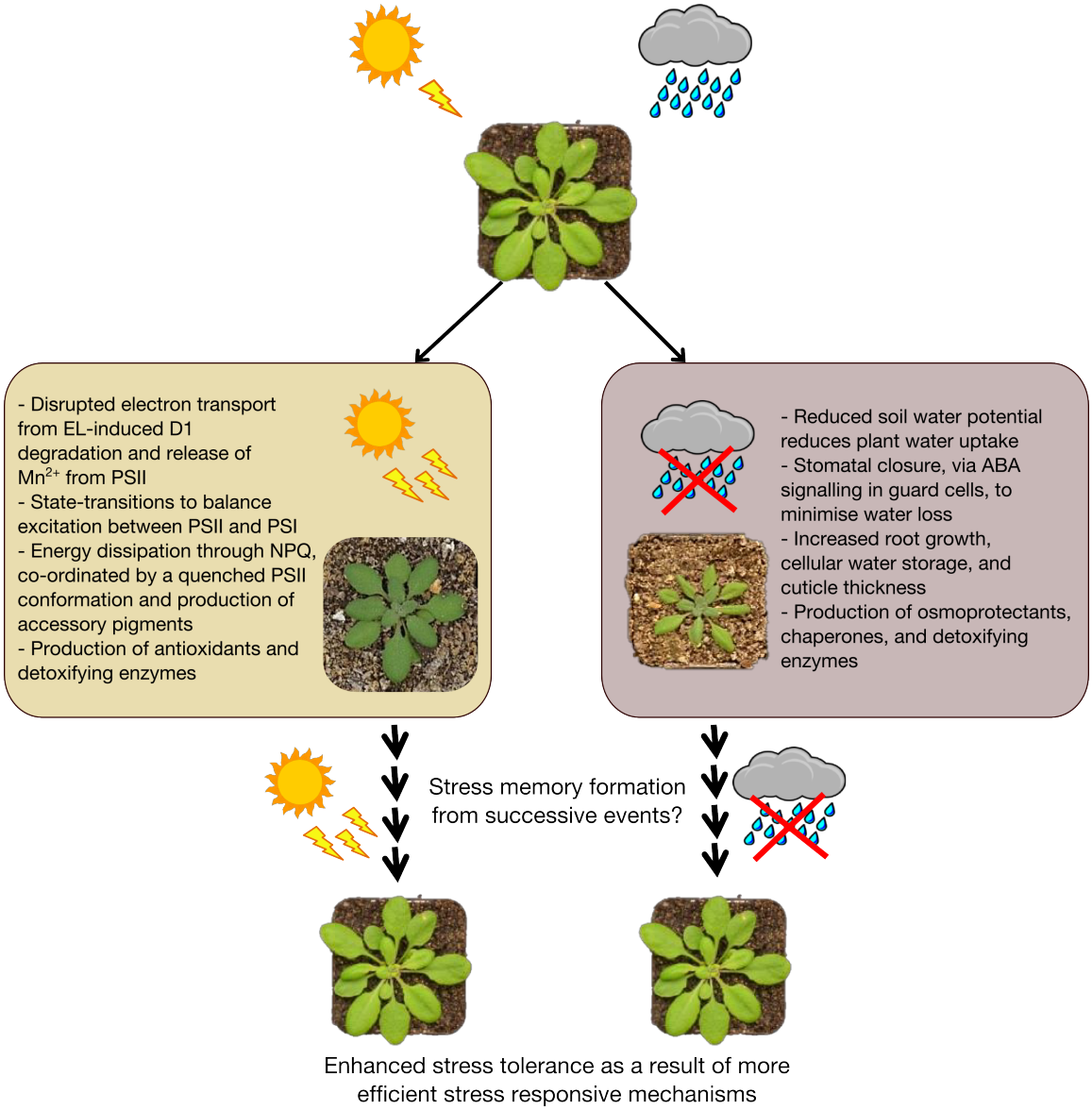

Figure 1.1: Responses to excess-light and drought stress
Comparison of the physiological adjustments plants must make in response to EL or drought stress. Memory formation against such stresses could facilitate stronger or more rapid responses to promote tolerance and survival.

adjustments, for example, many transcripts that encode ROS detoxifying enzymes, or their regulators, are responsive to the accumulation of ROS including H_2O_2 and $^1O_2$ (Gordon et al., 2013; Carmody et al., 2016). A subset of these nucleus-bound signals can be derived from organelles, such as the chloroplast or mitochondrion, allowing for intracellular communication. These are referred to as retrograde signals and allow for the fine-tuning of molecular processes that adjust organelle function to promote adaptation to new environmental conditions (Chan, Phua, et al., 2016). Further dissection of the pathways, including chloroplast-to-nucleus retrograde signalling, investigated in this thesis follows below.

1.3.1 Systemic acquired acclimation signals for whole plant photoacclimation

SAA has been elucidated as a key signalling mechanism for relaying photoacclimation to a whole plant level to guard against fluctuating light intensity. Leaves experiencing EL stress actively signal, through a variety of metabolic and oxidative molecules (Ramel et al., 2012; Gordon et al., 2013; Suzuki et al., 2013; Carmody et al., 2016), to unexposed leaves to convey a "primed" state (Conrath et al., 2006) in anticipation for incoming EL stress (Karpinski et al., 1999; Rossel et al., 2007). Indeed, PSII has also been shown to be primed in response to repeated EL stress (Karpinski et al., 1999), as well as improved NPQ activation in both exposed and newly developed tissue, suggestive of the potential for memory (Szechyńska-Hebda et al., 2010; Gordon et al., 2013). Similar experiments utilizing continuous cold treatments limited to pre-existing leaves provided evidence for systemic signalling such that naive newly developed leaves exhibited the induction of cold-responsive genes (Gorsuch et al., 2010). A key untested hypothesis (Gordon et al., 2013) is whether there could be an epigenetic component (Eichten et al., 2014) contributing to the memory and transmission of this acclimation (**Figure 1.3**). Indeed, there is now evidence for stress memory against a variety of biotic and abiotic stresses including insect herbivory and oxidative stress (Cayuela et al., 1996; Agrawal, 2002; Jakab et al., 2005; Gorsuch et al., 2010; Rasmann et al., 2012; Ding et al., 2013; X. Wang et al., 2014). Pertinently, there is growing evidence that plants are able to form light stress memory also (Szechyńska-Hebda et al., 2010; Gordon et al., 2013; Crisp et al., 2017). This occurs regardless of the area of tissue exposed as targeted, or partial rosette, EL treatments still resulted in acclimation across a whole plant demonstrable through enhanced induction of light-response transcripts, and improved photoprotective capacity and oxidative tolerance (Karpinski et al., 1999; Rossel et al., 2007; Gordon et al., 2013; Carmody et al., 2016). Indeed, using a partial rosette treatment revealed an 87% overlap in transcriptional changes between exposed and distal tissues (Rossel et al., 2007). Accompanying this reprogramming were alterations in auxin homeostasis across the rosette, despite EL treatment of a single leaf (Gordon et al., 2013) and many SAA induced transcripts being unresponsive to hormone treatments (Rossel et al.,

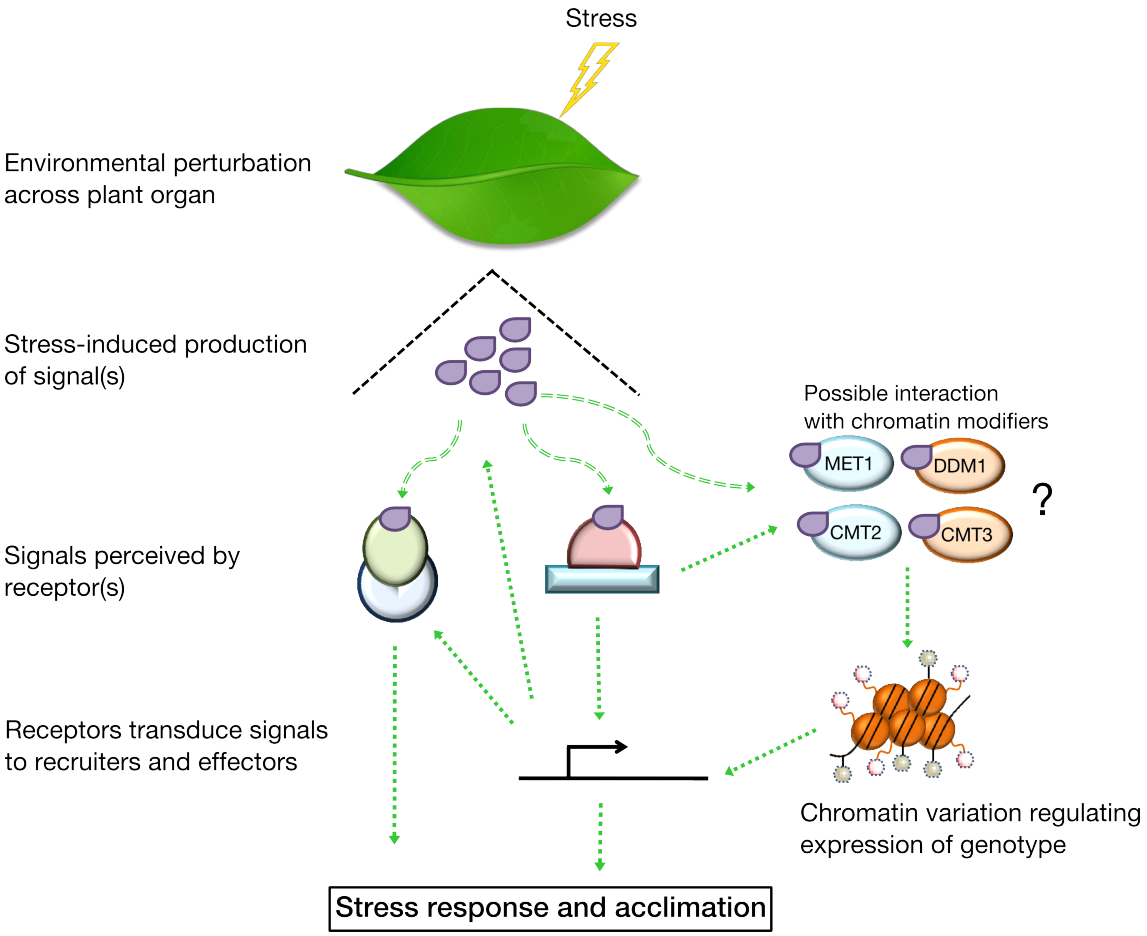

Figure 1.2: Signalling pathways perceive environmental fluctuations
Schematic depiction of a signalling cascade responding to environmental stimuli including abiotic stress. This is perceived by millions of cells across a plant organ leading to the biosynthesis of a signal, often as a by-product of the stress itself. This signal binds to receptors that act as transducers to propagate the signal to recruiters or effectors that ultimately elicit a response. This can involve physical (e.g. protein-protein interactions), biochemical (e.g. ion gradients), or molecular changes (e.g. gene expression) all of which can feedback on the pathway. There is an emerging notion that chromatin variation can contribute to stress response. This thesis investigates the possibility that components involved in maintaining chromatin state (e.g. *METHYLTRANSFERASE 1 (MET1)*, *DECREASE IN DNA METHYLATION 1 (DDM1)*, *CHROMOMETHYLASE (CMT)2*, *CMT3*) are responsive to stress signals, or to intermediate signalling components, to result in stress-induced chromatin variation that contribute to stress tolerance by regulating expression of underlying genetic elements. Adapted from Pastori & Foyer (2002).

2007). Such observations suggest a capacity for the storage and transmission of these acclimatory events, into new tissue post-stress, to form a memory.

1.3.2 ABA signalling in guard cells

Stomata are pores on the leaf epidermis that regulate gas exchange with the surrounding environment, which has important consequences for plant growth (Mickelbart et al., 2015; Murata et al., 2015). Thus, stomatal closure is one of the most important responses to calibrate plant growth through environmental fluctuations, including changes in light intensity, atmospheric CO_2, and water availability (Hetherington & Woodward, 2003). This regulation is achieved through physical manipulations in aperture size of the stoma controlled by hydraulic changes in a pair of specialized gatekeeper cells bordering stomata (T.-H. Kim et al., 2010). These cells, termed guard cells, respond to a variety of stress-induced signals, such as ROS, calcium (Ca^{2+}), hormones, and metabolites; to regulate stomatal aperture through controlling membrane ion transport causing solute influx or efflux that ultimately changes guard cell turgor pressure (Farquhar & Sharkey, 1982; Murata et al., 2015). One of the best characterised mechanisms operating in guard cells to regulate stomatal closure is the ABA signalling pathway (**Figure 1.4**).

In addition to stomatal closure, ABA signalling plays important physiological roles in many aspects of plant development including embryogenesis, germination, reproduction, and developmental transitions (Xiong et al., 2001; Finkelstein et al., 2002; Shinozaki et al., 2003; Xiong & Zhu, 2003; Jakab et al., 2005; Kurahashi et al., 2009). Its mode of action, particularly in guard cells, has been the target of much active research. The accumulation of ABA occurs by *de novo* biosynthesis, primarily in shoot vascular tissue cells under conditions of osmotic stress (Zeevaart & Boyer, 1984; Xiong & Zhu, 2003; Endo et al., 2008). The first step of this reaction occurs in the plastids where 9-*cis*-neoxanthin or 9-*cis*-violaxanthin are cleaved by 9-*cis*-epoxycarotenoid dioxygenases (NCED), to produce the C_{15} intermediate xanthoxin that is subsequently converted to ABA in the cytosol by ABA DEFICIENT 2 (ABA2) (Schwartz et al., 1997; Xiong & Zhu, 2003; Nambara & Marion-Poll, 2005; González-Guzmán et al., 2002). The synthesized ABA can then be transported to target sites of action, via the xylem and phloem, potentially in an inactive conjugated form that subsequently triggers further ABA production (Zeevaart & Boyer, 1984; Sauter et al., 2001; Nambara & Marion-Poll, 2005; Boursiac et al., 2013). Additional sites of ABA biosynthesis beyond vascular tissues have also been postulated based on the expression of key biosynthetic proteins, including directly in guard cells (Tan et al., 2003; Koiwai et al., 2004; Nambara & Marion-Poll, 2005). This has brought suggestions that root-derived mobile compounds could be transported through the xylem to target sites to induce ABA biosynthesis (Goodger & Schachtman, 2010). Recent investigations on sulfate content and transport in xylem sap under drought suggest that this could be a genuine mechanism for triggering ABA accumulation directly at functionally relevant sites, such as at guard cells (Malcheska et

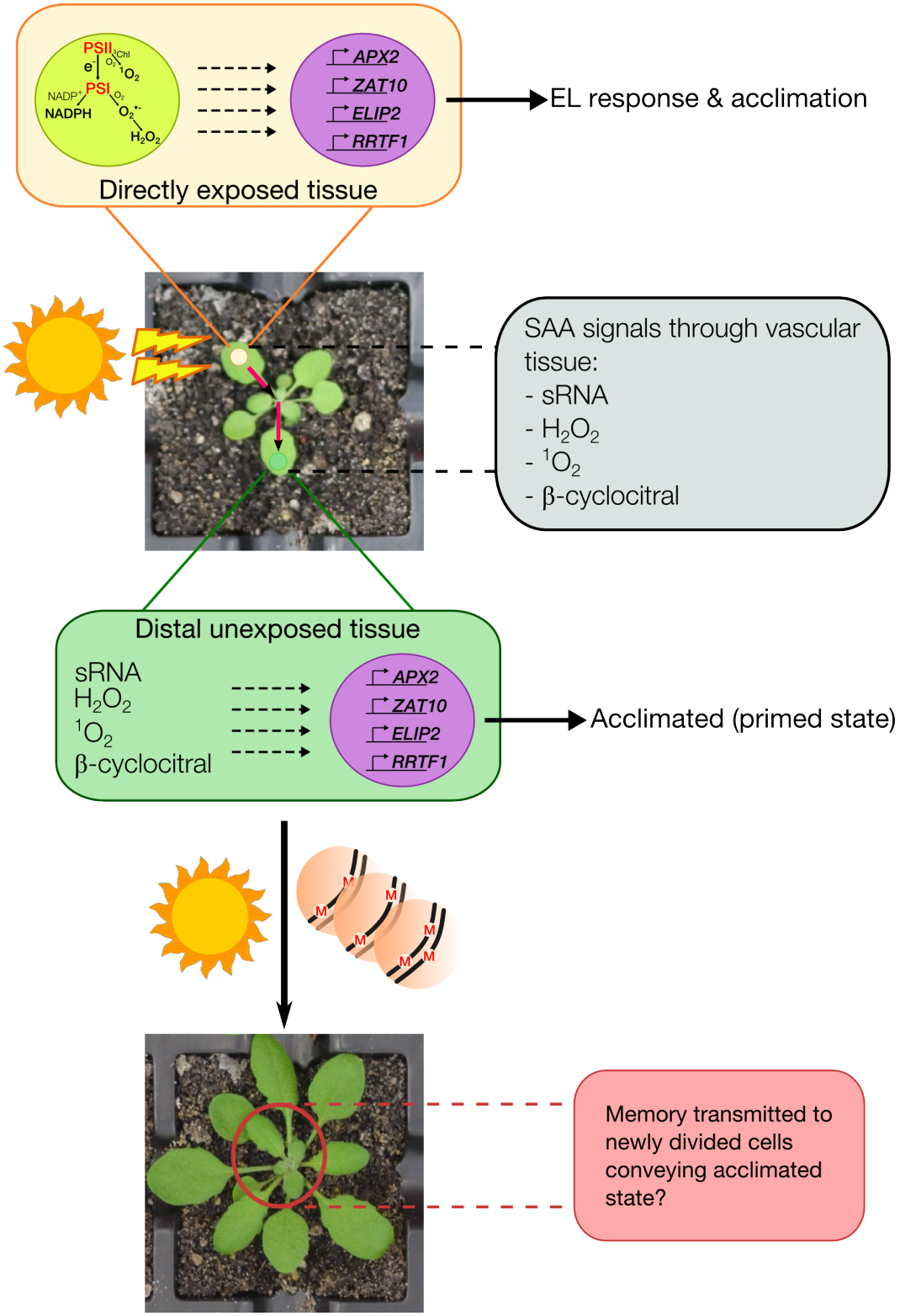

Figure 1.3: Systemic acquired acclimation and memory
Outline of SAA signalling to facilitate whole plant acclimation for incoming EL. Furthermore, an untested hypothesis is whether there could be an epigenetic component to convey mitotic transmission, or memory, of this acclimation. Adapted from Gordon (2012).

al., 2017).

ABA signalling in guard cells involves various levels of redundancy allowing for compensatory feedback regulation while also adding a layer of complexity (Finkelstein et al., 2002; Hetherington & Woodward, 2003). Once inside guard cells, ABA binds to ABA receptors from the PYL protein family, which subsequently forms a complex with, thus inactivating, PP2C phosphatases (Park et al., 2009). Active PP2Cs inhibit SNF1-RELATED PROTEIN KINASE 2 (SnRK2) kinase activity through dephosphorylation. Inactivation of PP2Cs releases this inhibition allowing activation of SnRK2 via autophosphorylation (Boudsocq et al., 2007). Subsequently, active SnRK2 kinases phosphorylate various target proteins including a range of ion channels, such as SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1), POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA (KAT)1, and KAT2; and transcription factors triggering an ABA response (Johnson et al., 2002; Mustilli et al., 2002; Furihata et al., 2006; Geiger et al., 2009; S. C. Lee et al., 2009) (**Figure 1.4**).

The two key outputs of ABA signalling are: *(I)* in the case of stomatal closure, SnRK2-mediated regulation of ion channel activity causing depolarisation of the guard cell plasma membrane and the efflux of cellular solutes that results in reduced guard cell turgor that shrinks the stomatal pore (Chen & Blatt, 2010); and *(II)* gene expression changes in the nucleus via ABA-RESPONSIVE ELEMENT BINDING FACTOR (ABF) activity modulated through SnRK2- or CALCIUM-DEPENDENT PROTEIN KINASE (CDPK)-mediated phosphorylation (Furihata et al., 2006; S.-Y. Zhu et al., 2007; Lynch et al., 2012; Yoshida et al., 2015). ABFs comprise a family of basic-domain leucine zipper (bZIP) transcription factors that bind to ABA-RESPONSIVE ELEMENT (ABRE) *cis*-acting elements, inducing ABA-dependent gene expression to promote stress tolerance (Johnson et al., 2002; Cutler et al., 2010; Murata et al., 2015). Examples of proteins encoded by ABA-activated genes include enzymes involved in ROS detoxification, transporters, dehydrins, and other signalling transducers such as protein kinases (Choi et al., 2000; Shinozaki et al., 2003; T.-H. Kim et al., 2010). Many of the ABA-activated genes are co-regulated by other stimuli, including temperature and salinity, suggesting interaction with other signalling pathways (Seki et al., 2002). Although many of these signalling pathways have been intimately mapped out (Chan, Phua, et al., 2016), how multiple signals are integrated into a synergistic response is not well understood. Thus, this thesis will explore the mechanism of eliciting plant acclimatory responses via stress-responsive signalling pathways, and how multiple signals can synergistically promote stress tolerance. In addition to the regulation of stomatal closure, it is worthwhile noting that the development of stomata itself is an environmentally responsive and tightly regulated long-term process that has critical biological consequences for plant growth and development. This is because the efficiency of gas exchange can also be affected by alterations in stomatal number and density (Dong & Bergmann, 2010). Interestingly, this developmental program has also been reported to be under epigenetic control (Tricker et al.,

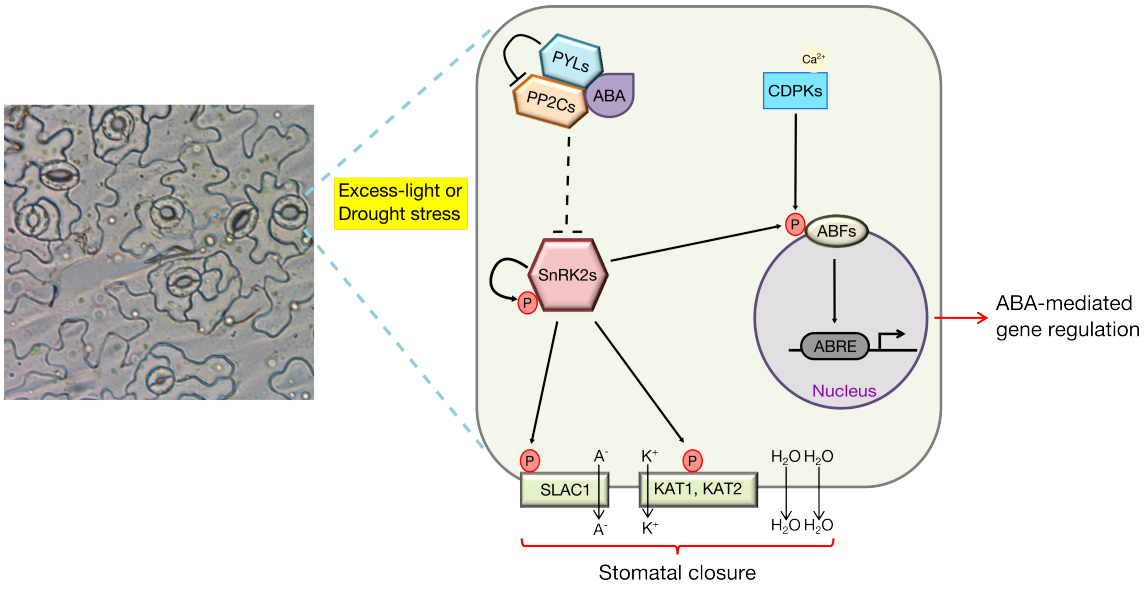

Figure 1.4: ABA signalling in guard cells
Guard cells are critical for regulating leaf gas exchange. A key mechanism involved is the ABA signalling pathway that, upon perception of stress-induced ABA, initiates a signalling transduction cascade that promotes the mechanical closure of stomata and ABA-induced gene expression to, collectively, promote stress tolerance. Adapted from Chen & Blatt (2010); Cutler et al. (2010); T.-H. Kim et al. (2010); F. Hauser et al. (2011).

2012, 2013), which will be relevant for considerations of stress signalling and memory.

1.3.3 Retrograde signalling and the SAL1-PAP Pathway

Retrograde signalling refers to communication from cell organelles, including mitochondria, chloroplasts, to the nucleus, allowing for co-ordination between organellar and nuclear-encoded gene expression according to organelle developmental and physiological state (Fernández & Strand, 2008; Pogson et al., 2008; Chan, Phua, et al., 2016; Martín et al., 2016). There are multiple examples of changes to nuclear gene expression upon genetic or chemical perturbation of plastid function (Bradbeer et al., 1979; Oelmüller et al., 1986; Oelmüller & Mohr, 1986; Susek et al., 1993; Hess et al., 1994; Xiao et al., 2012). Thus, chloroplasts can act as stress sensors for the cell, particularly in the case of EL-induced oxidative stress, by relaying this information to influence nuclear gene expression. Intermediates of various biochemical or metabolic pathways have been identified as having secondary roles as retrograde signals, such as intermediates of the tetrapyrrole biosynthetic pathway Fe-protoporphyrin (heme) and Mg-protoporphyrin IX (Strand et al., 2003; Woodson et al., 2011), the carotenoid oxidation product β-cyclocitral, the isoprenoid precursor methylerythritol cyclodiphosphate (Ramel et al., 2012; Xiao et al., 2012), and a by-product of sulfur metabolism 3′-phosphoadenosine 5′-phosphate (PAP) (Klein & Papenbrock, 2004; Estavillo et al., 2011). Despite the identification of numerous retrograde pathways and signals, there is still only limited mechanistic insight into how these signals are translated into improved stress tolerance from initial perception.

Nonetheless, the extent to which these signals are integrated with canonical stress signalling pathways, such as the ABA pathway, remain an interesting field for exploration. Interestingly, Exposito-Rodriguez et al. (2017) demonstrated that light-induced H_2O_2 accumulation in chloroplasts could move directly to the nucleus to impact nuclear gene expression in tobacco epidermal cells, bypassing the cytosol.

Using a screen for constitutive *ASCORBATE PEROXIDASE 2 (APX2)* up-regulation, as an indicator of oxidative stress, a point mutation in the nuclear-encoded *SAL1 PHOSPHATASE-LIKE PROTEIN (SAL1)* gene was observed to convey enhanced drought and EL stress tolerance (herein referred to as *sal1*-8; Rossel et al. 2006; Wilson et al. 2009). The *SAL1* gene encodes a chloroplast and mitochondria localized phosphatase that degrades the phosphonucleotide PAP, a by-product of secondary sulfur metabolism, that would otherwise inhibit sulfotransferase activity, consequently having feedback regulation on overall sulfur metabolism (B.-R. Lee et al., 2012; Chan et al., 2013). If allowed to accumulate, either due to lesions in *SAL1* (Estavillo et al., 2011) or inactivation through altered chloroplast redox status (Chan, Mabbitt, et al., 2016), PAP is considered to inhibit the action of the 5′-3′ exoribonuclease (XRN) family of RNA processing enzymes, including nuclear-localized XRN2 and XRN3, and cytosol-localized XRN4; thereby altering post-transcriptional RNA metabolism (Dichtl et al., 1997; Gy et al., 2007; Kurihara et al., 2012). This is further evidenced by *xrn2xrn3* double and *xrn2xrn3xrn4* triple mutants phenocopying *sal1* mutants (Estavillo et al., 2011). However, action through the XRN enzymes does not fully explain *sal1* phenotypes. For example, while there is a substantial overlap (>50%) between the *sal1* and *xrn2xrn3* trancriptomes, there are still over 2000 genes uniquely differentially regulated in *sal1* (Estavillo et al., 2011). This suggests alternative, or combinatorial, modes of action for PAP. Pertinently, a subset of ABA-responsive genes are differentially regulated in *sal1* mutants, alongside decreased stomatal conductance, raising the possibility of PAP participating in ABA-mediated processes (**Figure 1.5**; Rossel et al. 2007; Wilson et al. 2009; Estavillo et al. 2011).

1.4 Plant stress priming and memory

In addition to plant phenotypic plasticity, actioned by the aforementioned signalling pathways, plants show an ability to be 'primed' by stresses. That is, prior exposure to a stress or priming stimulus conveys an enhanced ability to respond to future events (Conrath et al., 2006; Bruce et al., 2007; Crisp et al., 2016). This notion has been extended to numerous considerations of the formation of plant 'stress memory', in which a state of altered stress responsivity is mitotically or meiotically transmissible (Bruce et al., 2007; M.-T. Hauser et al., 2011; Probst & Mittelsten Scheid, 2015; Crisp et al., 2016; van Loon, 2016). There is much interest in plant stress memory, including the underlying molecular mechanism(s) and its potential to impact crop yields, particularly in harsh and

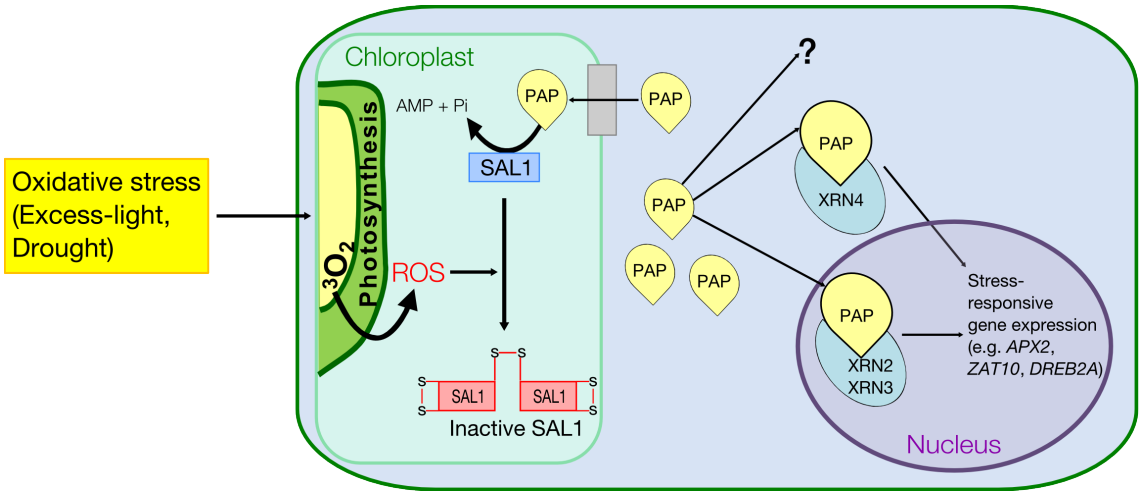

Figure 1.5: The canonical SAL1-PAP retrograde signalling pathway
The secondary messenger PAP has been identified as a chloroplast to nucleus retrograde signal that promotes stress tolerance, particularly during drought. Changes in chloroplastic redox state, induced by oxidative stress, inactivates SAL1 resulting in PAP accumulation, which can subsequently be transported into the cytosol and nucleus where it has the potential for multiple molecular interactions. A canonical mode of action is inhibition of XRN enzymes, altering RNA metabolism and promoting stress-responsive gene expression, such as through the transcription factors *ZINC FINGER PROTEIN ZAT10 (ZAT10)* and *DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2A (DREB2A)*. Adapted from Estavillo et al. (2011); Gigolashvili et al. (2012); Chan, Mabbitt, et al. (2016).

variable environments (Springer, 2013; Ji et al., 2015; Mickelbart et al., 2015). Such a priming or memory response could be evolutionarily beneficial, facilitating acclimation to local environmental conditions (**Figure 1.6**), albeit these must be balanced with the costs of maladaptive memories (Crisp et al., 2016).

As discussed above, the primary modes of plant stress response can be categorised into measures of avoidance or tolerance. It is possible that the prior stress exposure can prime plants, altering its responsivity in engaging avoidance and/or tolerance mechanisms. For example, prior stress exposure can lead to enhanced transcriptional responses allowing for more rapid activation of stress responsive transcripts, such as observed from β-aminobutyric acid (BABA)-induced priming (Jakab et al., 2001, 2005; Ton et al., 2005) or through stress training experiments (Ding et al., 2012; Sani et al., 2013; Crisp et al., 2017), which could contribute to a more rapid engagement of the related avoidance or tolerance mechanisms. On the other hand, priming could have variable effects rather than just the hyper-induction of stress responses (Ding et al., 2013; Sani et al., 2013). More subtle changes that do not involve a stronger response, thus minimising the growth penalties associated with stress tolerance, could lead to maximising reproductive potential.

This investigation also seeks to delineate between stress priming (Conrath et al., 2006) and heritable (mitotically or meiotically) memory (Eichten et al., 2014; Crisp et al., 2016). A key concern is that the term *'memory'* is often used synonymously with *'priming'*, which confounds observations, making it difficult to properly compare between responses. Priming is typically measured as the enhanced accumulation of tolerance-

associated transcripts, proteins, and/or by demonstrating enhanced stress tolerance in treated tissues after a period of recovery or in naive systemic tissues with regards to SAA-mediated priming (Rossel et al., 2007; Gorsuch et al., 2010; Ding et al., 2013; Gordon et al., 2013; Suzuki et al., 2013; Carmody et al., 2016; Crisp et al., 2017). With respect to mitotic memory, stress-induced molecular or physiological traits should be evident in new tissue that were absent prior to, or during, the initial stress to demonstrate transmission across cell divisions. Transgenerational memory will rely on observations of altered traits in not only in the direct offspring of stressed parents, but in descendants at least one generation removed from the directly stressed maternal plant to delineate between heritable memory and maternal effects (Gutzat & Mittelsten Scheid, 2012; Piskurewicz et al., 2016). Regardless, such mechanisms, whether systemic signalling and priming, or genuine forms of memory, are considered to convey an evolutionary advantage (Gutzat & Mittelsten Scheid, 2012; Mittler & Blumwald, 2015). Thus, this thesis aims to validate a capacity for plant stress priming and memory as genuinely observable plant responses, both within a generation and across generations.

1.4.1 Mechanisms for priming and memory

Plant stress priming and memory is often associated with an enhanced response to recurring stress, for example, the hyper-induction of stress-responses transcripts underpinned by maintained stress-induced structural, biochemical, or molecules changes (**Figure 1.7A**). There are now extensive examples of stress priming across a variety of plant species, in response to a multitude of stresses across multiple times-scales, including both somatic and transgenerational stress priming (reviewed extensively Tricker 2015; Crisp et al. 2016; Secco et al. 2017; Lämke & Bäurle 2017). Despite the numerous documented examples of plant stress priming and memory (Cayuela et al., 1996; Jakab et al., 2001; Agrawal, 2002; Jakab et al., 2005; Iqbal & Ashraf, 2007; Slaughter et al., 2012; Rasmann et al., 2012; X. Wang et al., 2014), the underlying molecular mechanisms are still being uncovered, however, various candidates have been posited (**Figure 1.7B-F**). One possibility is the persistence of stress-induced changes in chromatin to convey altered regulation of underlying elements until resetting of the memory occurs (**Figure 1.7B**). This is a pre-eminent hypothesis as a molecular memory mechanism based on the canonical example of cold-induced silencing of *FLOWERING LOCUS C (FLC)*, allowing the transition to reproductive growth, that persists for the remainder of the plant's life through persistent histone methylation across the locus (**Figure 1.7C**; Sheldon et al. 2000; Bastow et al. 2004; Sung & Amasino 2004). The recovery period is a critical window for this memory as return to warmth induces spreading of repressive histone methylation across *FLC* (De Lucia et al., 2008). Alterations in RNA metabolism also provide potential memory pathways. For example, inhibiting RNA decay, or stabilising stress-induced transcripts, provides a direct mechanism of stress memory through persistent accumulation of transcripts until resetting occurs to pre-stress lev-

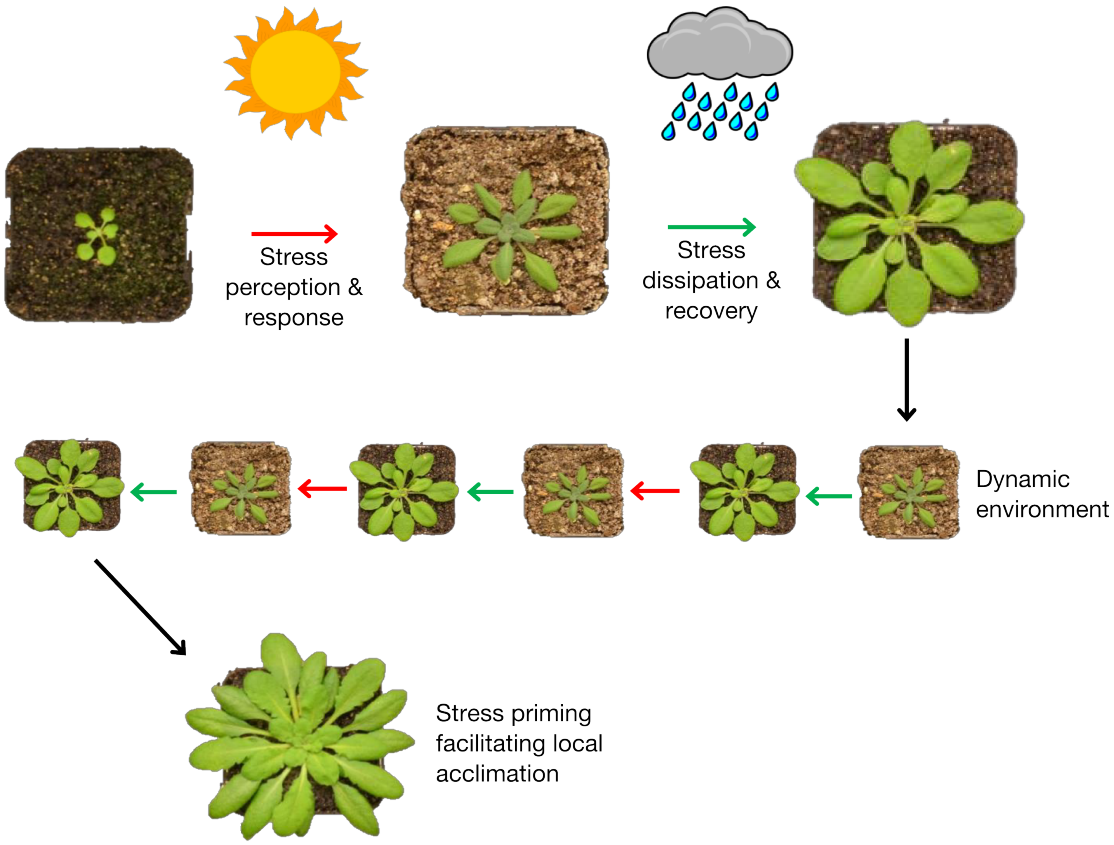

Figure 1.6: Stress priming could facilitate acclimation to local environmental conditions
Plants are constantly sensing and responding to stresses arising from the surrounding dynamic environment. Often these are transient events that eventually dissipate allowing for recovery to optimal growth conditions. Over a plants' lifetime, however, these transient fluctuations may persist or occur repeatedly, necessitating repeated physiological adjustments. Such conditions may lead to the formation of stress priming or memory that allows for more efficient responses to local fluctuations. While underlying genetic variation can contribute to local adaptation, these changes occur over longer geological timescales whereas environments have the ability to change much more rapidly. Thus the involvement of faster acting mechanisms, including variable chromatin modifications, have been proposed to facilitate short term stress priming allowing for local acclimation.

els (**Figure 1.7D**) as was observed upon genetic impairment of XRN-mediated decay (Nguyen et al., 2015). Another priming mechanism may be the sustained accumulation of key signalling metabolites, proteins, or transcription factors that can prime activity of those pathways (**Figure 1.7E**), akin to components required for BABA-induced priming that are involved in the salicylate and ABA pathways (Ton et al., 2005). Alterations of such metabolic or biochemical activity might also be coupled to chromatin maintenance, for example, through the unavailability of essential metabolites ATP and nicotinamide adenine dinucleotide (NAD), or the cofactors *S*-adenosylmethionine (SAM) and acetyl co-enzyme A required for the reactions involved (Vriet et al., 2015; Groth et al., 2016). Structural and biochemical memory factors may also pertain to sustained changes in photosynthetic performance, such as those mediated by PsbS and VIOLAXANTHIN DE-EPOXIDASE (VDE) to engage NPQ under EL. These can be reset by the activity of proteins such as ZEAXANTHIN EPOXIDASE (ZEP) or K^+ EFFLUX ANTIPORTER 3 (KEA3), which promote recovery by disengaging NPQ activity (**Figure 1.7F**; Z. Li et al. 2009; Armbruster et al. 2014, 2016). While various mechanisms have been posited here it is unlikely that any act alone as a single master mechanism. Stress priming and memory will inevitably rely on an integrated response involving chromatin, structural, and biochemical changes (Fleta-Soriano & Munné-Bosch, 2016). This investigation focusses on the potential contribution of sustained stress-induced changes in DNA methylation towards stress priming and memory.

1.4.2 Distinguishing chromatin modifications and epigenetics

Notions of plant stress memory are commonly associated with *epigenetics*, a broad term that continues to evolve and encapsulate multiple non-Mendelian phenomena. Its origin is often attributed to Conrad Waddington's use of the term "epigenotype" to encapsulate the processes, relating to both differentiation and development, that result in the expression of a given genotype (Goldberg et al., 2007; Waddington, 2012). The continual broad use of this term, however, interferes with the ability to properly analyse and compare specific phenomena, such as paramutation, transposon activity, non-Mendelian inheritance, and gene regulation by chromatin state/modifications (Eichten et al., 2014). *This thesis regards the definition of epigenetics to pertain to heritable changes in phenotype that are not solely attributable to genetic sources* (Eichten et al., 2014). A further distinction can be made between *transgenerational epigenetic effects*, pertaining to phenotypes present in successive generations that show no genetic basis, and *transgenerational epigenetic inheritance*, where phenotypes are the traceable result of heritable chromatin modifications (Youngson & Whitelaw, 2008). Furthermore, an important but often overlooked component of epigenetic phenomena is the requirement for heritability over mitotic and/or meiotic cell divisions (Eichten et al., 2014). This thesis is focussed on whether abiotic stress can prime plants for subsequent exposure, and to investigate whether any such priming traits might be transmitted across cell divisions,

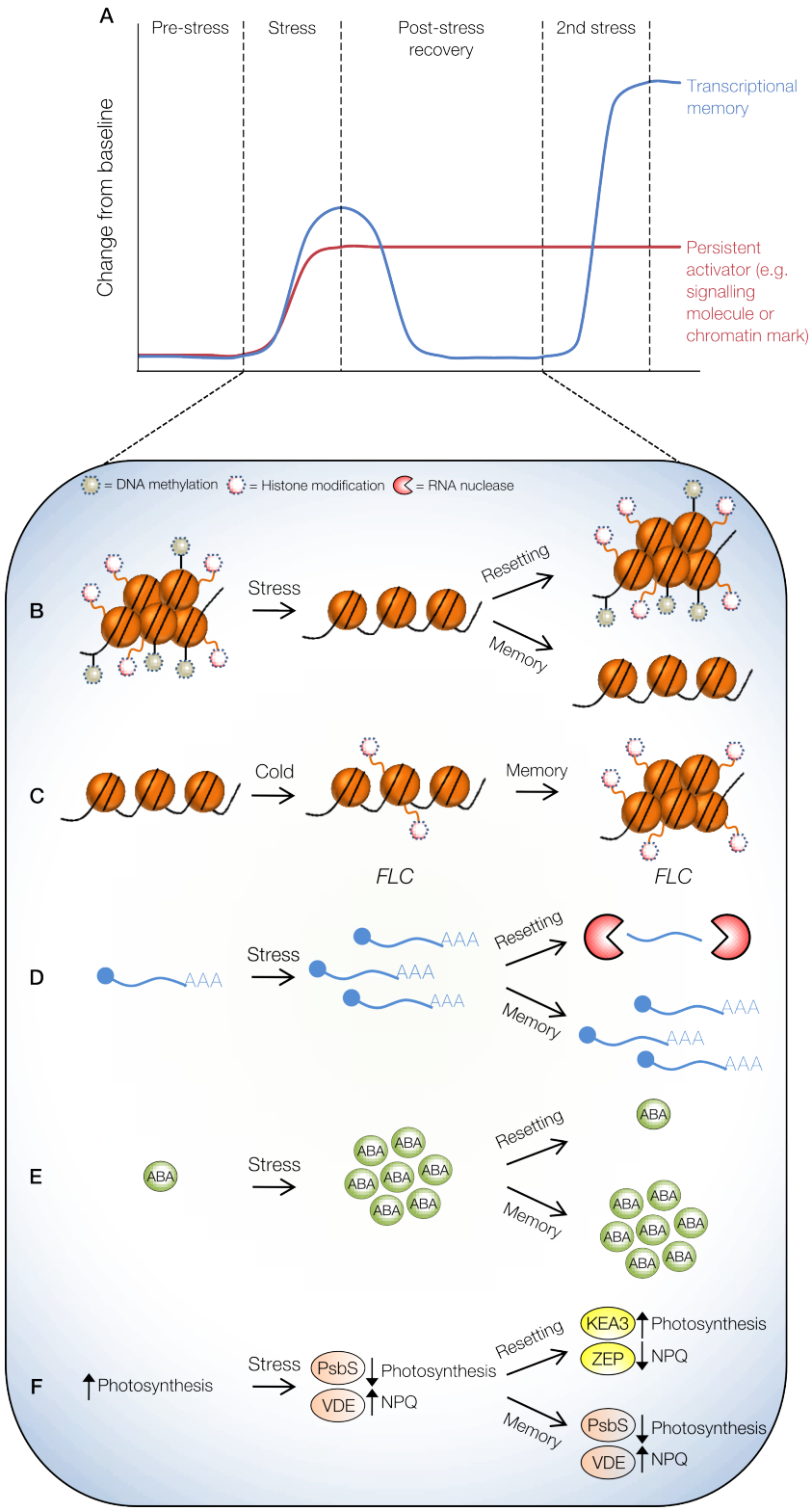

Figure 1.7: Molecular pathways for stress memory
A Theoretical example of memory formation where a strong transcriptional response (blue line) occurs upon an initial stress, with a concurrent accumulation of signalling molecules and the release of repressive chromatin (red line) facilitating future enhanced responses. Depending on the nature of stress, memory formation and consolidation might occur or resetting can occur to avoid the growth costs of maintained tolerance.
B-F Possible structural, biochemical, and molecular mechanisms to convey stress memory.
Adapted from Crisp et al. (2016).

both mitotic and meiotically, as evidence for plant stress memory. Correlating a change in chromatin state may suggest functional relevance and potentially fulfil the criteria for truly epigenetic phenomena.

Chromatin broadly refers to the structure and packaging of DNA within cells, which governs the accessibility of enzymes and interacting proteins to certain portions of the genome thus allowing chromatin state to govern the expression of the underlying elements and, consequently, a range of biological processes (Kouzarides, 2007; S. Feng et al., 2014; Rodriguez-Granados et al., 2016). The nucleosome is the basic unit of chromatin and is composed of approximately 147 base pairs of DNA wrapped around a histone octamer core, which consists of two copies of each of the core histones (H2A, H2B, H3 and H4) (Kouzarides, 2007; Taudt et al., 2016). Across this nucleosome structure are the potential addition of a plethora of chemical marks and modifications that contribute to overall chromatin state including histone tail modifications, replacement of histone core variants, altered nucleosome positioning, and direct DNA chemical modification (Kouzarides, 2007; Eichten et al., 2014). Of pertinence to this thesis is the malleability and regulatory potential of DNA methylation through environmentally-induced variations (Lister et al., 2008; Gutzat & Mittelsten Scheid, 2012). These may originate from differential regulation of the components or pathways involved, either directly or indirectly, since DNA methylation is ultimately a biochemical reaction that requires an enzyme (for example MET1), substrate (cytosine DNA base), and co-factors (for example SAM); all of which are influenced by other metabolic processes that can be sensitive to environmental conditions. This is illustrated by studies reporting altered DNA methylation profiles underpinned by abnormalities in sulfur assimilation pathways or processes (Neuhierl et al., 1999; Rocha et al., 2005; Duan et al., 2015; Groth et al., 2016).

1.4.3 The molecular pathways for establishing and maintaining DNA methylation

DNA methylation is a largely evolutionarily conserved process, across many eukaryotic organisms, that constitutes the addition of a methyl group (-CH_3) to a cytosine base and, in plants, occurs in three sequence contexts: mCG, mCHG, and mCHH (H is any base except G) (Law & Jacobsen, 2010; Varriale, 2014). Establishment and maintenance, in each sequence context, is performed by a suite of DNA methyltransferases. In plants, methylation in the mCG context is catalysed by MET1, mCHG by CMT2 and CMT3 guided by DDM1, and asymmetric mCHH by CMT2-DDM1 or DOMAINS REARRANGED METHYLTRANSFERASE (DRM)1 and DRM2 targeted by the RdDM pathway (**Figure 1.8**; Law & Jacobsen 2010; Zemach et al. 2013; Stroud et al. 2014; M. A. Matzke et al. 2015). Although such distinctions between functions are suggested, there is also overlap between the targets of different methyltransferases, such as the capacity for DRM1 and DRM2 to also influence mCHG, and to a lesser extent mCG,

patterns (Cao & Jacobsen, 2002; Stroud et al., 2013). This multi-targeting capacity of the DRM enzymes are also critical as these are the predominant *de novo* acting methyltransferases that establish DNA methylation (He et al., 2011). A catalytically inactive homologue of DRM2, DRM3, has also been identified to be required for proper methylation at a subset of RdDM targets, putatively through stabilisation of small RNA (sRNA) transcripts (X. Zhong et al., 2015). The nucleosome remodeler DDM1 is also critical for facilitating accessibility of methyltransferases to their target cytosine base, including the otherwise inaccessible DNA wrapped into nucleosomes (Zemach et al., 2013; Lyons & Zilberman, 2017). Furthermore, the CMT non-CG methyltransferases appear to be directed by deposition of histone 3 lysine 9 mono- and di-methylation (H3K9me1/2), thus acting as a feedback loop to reinforce silenced heterochromatic regions (Du et al., 2012; Stroud et al., 2014).

RNA-mediated gene and chromatin silencing stems from the discovery of RNA-mediated viral defence, whereby recognition of viral RNA by plant machinery leads to the silencing of virus-encoded proteins by a variety of actions including enzymatic cleavage and targeting by DNA methyltransferases (Lindbo et al., 1993; Wassenegger et al., 1994; X.-B. Wang et al., 2010). Building on these initial observations, genome-wide analyses revealed a capacity for non-coding RNA to direct DNA methylation based on sequence homology, although a lack of perfect correlation suggests multiple contributing factors (Cokus et al., 2008; Lister et al., 2008; M. Matzke et al., 2009). This so-termed RdDM pathway has since been elucidated as a key mechanism for the *de novo* DNA methylation, directed by a subclass of non-coding sRNA molecules (small interfering RNA (siRNA)), that is particularly important for targeting non-CG methylation, although all sequence contexts can be methylated, to establish heterochromatic regions and silence otherwise active transposable element (TE)s (Pélissier et al., 1999; Stroud et al., 2013, 2014; M. A. Matzke & Mosher, 2014; M. A. Matzke et al., 2015; Panda et al., 2016). The RdDM pathway predominantly targets younger and shorter TEs, the edges of larger TEs, and repetitive elements in euchromatic regions; whereas the action of DDM1 facilitates methylation in an RdDM-independent manner, involving MET1, CMT2, and CMT3, at already established heterochromatin (Tran et al., 2005; X. Zhong et al., 2012; Zemach et al., 2013; Ito & Kakutani, 2014). Thus DDM1 and RdDM collaboratively mediate DNA methylation of transposons where RdDM acts as genome surveillance to establish silencing at relatively recent TE insertions or mobilizations whereas DDM1 acts to maintain heterochromatic areas (X. Zhong et al., 2012; Zemach et al., 2013; Panda et al., 2016).

The mechanism for RdDM has been divided into the canonical (RNA Polymerase (RNA Pol) IV-RDR2) and non-canonical (RNA Pol II-RDR6) pathways (**Figure 1.8**; M. A. Matzke et al. 2015). Both of these rely on the generation of siRNA molecules that direct the activity of DRM methyltransferases towards targets through sequence homology (Cokus et al., 2008; Lister et al., 2008; M. Matzke et al., 2009). However,

differences lie in the machinery involved in the generation, and nature, of the siRNA molecules, which also influences the regions targeted for methylation.

The canonical RdDM pathway relies on the activity of two plant-specific RNA Pol II paralogs, RNA Pol IV and RNA Pol V (Haag & Pikaard, 2011). Canonical RdDM initiates with RNA Pol IV transcription, preferentially recruited towards heterochromatic regions by SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) (Law et al., 2013; Blevins et al., 2014), followed by RNA-DIREECTED RNA POLYMERASE (RDR)2 action on this primary transcript to produce double-stranded RNA (Haag et al., 2012; Blevins et al., 2015). The putative chromatin remodeler CLASSY 1 (CLSY1) is also required for correct RNA Pol IV and RDR2 recruitment and generation of corresponding transcripts (Smith et al., 2007; S. Li et al., 2015). These double-stranded molecules are subsequently converted to 24nt-siRNA through the activity of DICER-LIKE (DCL)3, which are stabilised by the RNA methyltransferase HUA ENHANCER 1 (HEN1) (Xie et al., 2004; J. Li et al., 2005; Haag et al., 2012; Blevins et al., 2015). The guide strand from the stabilised siRNA duplex is incorporated into ARGONAUTE (AGO)4 allowing target recognition via sequence homology (Qi et al., 2006; Havecker et al., 2010). In the nucleus, the siRNA-loaded AGO4 is recruited to target regions through binding with nascent RNA Pol V transcribed scaffold RNAs (Wierzbicki et al., 2009; Q. Zheng et al., 2013). AGO4 also associates with, thus recruiting the activity of, DRM1 and DRM2 to its site of binding (Zilberman et al., 2003; X. Zhong et al., 2014). Through this action, the canonical RdDM machinery acts to maintain methylation levels, predominantly mCHH, through *de novo* methylation at heterochromatic regions for continued silencing (S. Li et al., 2015; Q. Li, Gent, et al., 2015).

Conversely, the non-canonical pathway could be considered to act as a surveillance mechanism identifying and targeting actively transcribed regions for silencing, such as at active TE elements potentially due to loss of silencing factors or recent transposition (Nuthikattu et al., 2013; McCue et al., 2015; Panda et al., 2016). This mechanism utilizes transcripts produced by RNA Pol II to *de novo* initiate new regions for silencing independently of RNA Pol IV (Stroud et al., 2013). These RNA Pol II-mediated transcripts are converted into double-stranded RNA molecules by RDR6 that are subsequently processed by DCL2 and DCL4 to produce 21-22nt siRNA (McCue et al., 2012; Nuthikattu et al., 2013). These 21-22nt siRNA can then be loaded into either AGO4 or AGO6 to direct mCHH in a RNA Pol V- and DRM-dependent manner as described above (Havecker et al., 2010; Stroud et al., 2013; McCue et al., 2015).

RdDM not only represses transposon activity but can also affect gene expression levels, due to the presence of RdDM targeted transposons in regions adjacent to genes including at upstream of transcription start sites (X. Zhong et al., 2012; Zemach et al., 2013; Q. Zheng et al., 2013; Groth et al., 2014; R. Yang et al., 2017). It has also been reported that only a small portion of RdDM-mediated regulation of gene expression acts in *cis*, instead RdDM can affect the interaction of promoter regions with distal regulatory

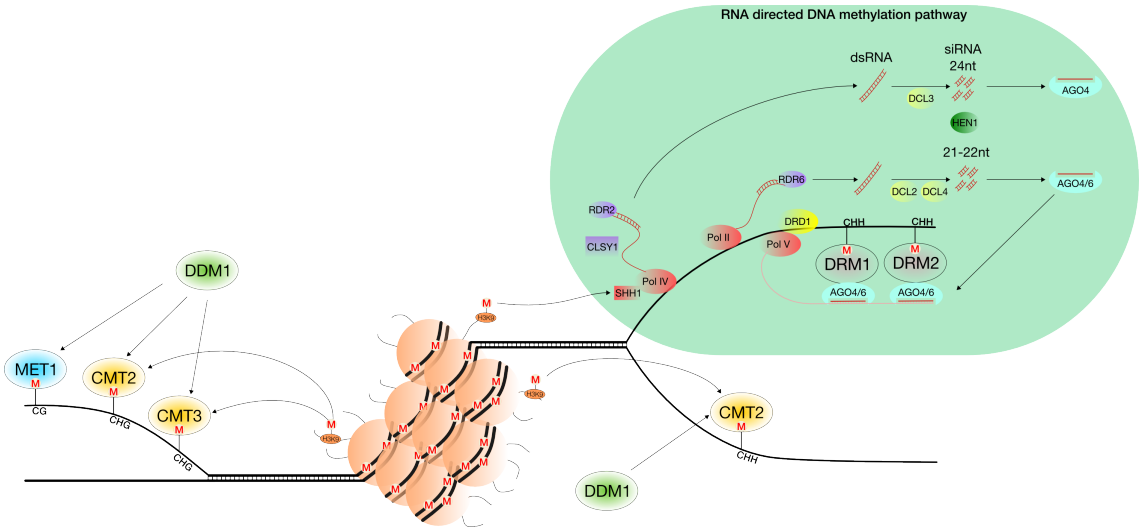

Figure 1.8: DNA methylation pathways
Diagram of the predominant pathways responsible for the establishment and maintenance of genome wide DNA methylation patterns, and the action of siRNA-based targeting of DRM activity via the RdDM pathway for *de novo* DNA methylation. Adapted from M. A. Matzke & Mosher (2014); M. A. Matzke et al. (2015).

elements by preventing chromosomal interactions (Rowley et al., 2017).

Because there is positive feedback between methylation and further RdDM activity, there must be a regulatory mechanism to prevent aberrant hyper-methylation. Indeed, the activity of RdDM is antagonised, and thus moderated, by that of REPRESSOR OF SILENCING 1 (ROS1) (Lei et al., 2015; Williams et al., 2015). ROS1 is a DNA glycosylase, involved in the base-excision repair pathway, which preferentially excises methylated cytosines and, thus, is crucial for the removal of methyl groups that prevents hyper-methylation of, and the spreading of methylation from methylated TEs into, genes (Gong et al., 2002; Tang et al., 2016). Regulation of proper *ROS1* expression and activity has been linked to the methylation status of a 5′ proximal helitron TE (*AtREP5*, *AT2TE68230*), controlled by an antagonistic relationship between the activity of *ROS1* and the RdDM pathway, which negatively controls *ROS1* expression (Lei et al., 2015; Williams et al., 2015). Thus, ROS1 is considered to serve as a 'Rheostat' for RdDM-mediated methylation.

1.4.4 DNA methylation as a mechanism for stress memory

DNA methylation is largely considered a repressive mark that contributes to TE silencing, maintaining genome stability and integrity, and potentially regulating gene expression (Reinders et al., 2009; Law & Jacobsen, 2010; Jones, 2012; Mirouze et al., 2012; Yelina et al., 2012; Eichten et al., 2014; Niederhuth & Schmitz, 2017). Regardless of its precise role, appropriate maintenance of genome-wide patterns in DNA methylation (methylome) is critical for proper plant development and long-term viability (Finnegan et al., 1996; Saze et al., 2003; Stroud et al., 2014; Yamamuro et al., 2014).

Stable propagation of DNA methylation states has been suggested as a possible mechanism for the formation of plant stress memory (Boyko et al., 2010; Gutzat & Mittelsten Scheid, 2012; Probst & Mittelsten Scheid, 2015). The potential for regulating gene expression has raised the proposition that DNA methylation could complement genetic variation, as a mode for transferring heritable information, to contribute to phenotypic variation (Molinier et al., 2006; Heard & Martienssen, 2014; Quadrana & Colot, 2016). Indeed, DNA methylation states can be faithfully maintained over both mitotic and meiotic cell divisions by a suite of pathways and enzymes as described above (Probst et al., 2009; Law & Jacobsen, 2010; Stroud et al., 2013). It is unclear the extent to which the methylome, in plants, are reset. Rather, it appears that the parental methylome is re-established and propagated during gametogenesis and spermatogenesis (Slotkin et al., 2009; Calarco et al., 2012). Since these processes occur within post-embryonic growth in plants (Boavida et al., 2005), any variations in the methylome (epi-allele), either environmentally-induced or spontaneous, have the potential to be carried over generations. DNA methylation state has shown stable heritability (Dubin et al., 2015; Hagmann et al., 2015; Q. Li et al., 2014) with the documented appearance of epi-alleles (in the form of differentially methylated region (DMR)s) to occur at a frequency comparable to genetic polymorphisms (Becker et al., 2011; Schmitz et al., 2011, 2013), potentially at an elevated rate under abiotic stress (Jiang et al., 2014). Furthermore, the development and use of epigenetic recombinant inbred lines (epiRILs), near isogenic lines that segregate for DNA methylation patterns, have also provided empirical evidence for the contribution of DNA methylation variance towards plant traits (Johannes et al., 2009; Reinders et al., 2009; Zhang et al., 2013).

Increased responsiveness, or a constitutive alteration, of stress-responsive transcripts is a common memory trait (Ding et al., 2012; Gordon et al., 2013; Ding et al., 2013; Virlouvet et al., 2014; Firtzlaff et al., 2016; Lämke et al., 2016; Crisp et al., 2017) and the persistent activity of transcription factors are likely important contributors (Rossel et al., 2007; Lämke et al., 2016). A pre-eminent hypothesis is that these changes can be underpinned by relatively rapid, yet reversible, chromatin variations that have the potential to be maintained stably through cell divisions thus acting as a mechanism for plant stress regulation as well as memory (**Figure 1.7**; Probst et al. 2009; Gutzat & Mittelsten Scheid 2012; Crisp et al. 2016; Lämke & Bäurle 2017). Indeed, numerous priming responses, including transcriptional memory, have been associated with altered chromatin marks, however, these largely pertain to histone modifications and RNA stability (Ding et al., 2012; Sani et al., 2013; Crisp et al., 2017; J.-M. Kim et al., 2017; Lämke & Bäurle, 2017).

Pertinent to this thesis is the popular, yet enigmatic, association between DNA methylation and gene expression that has been fuelled by conflicting reports on stress-induced variation in DNA methylation and its contribution towards stress responses and memory (S. Zhong et al., 2013; Dubin et al., 2015; Le et al., 2014; Seymour et al.,

2014; Eichten & Springer, 2015; Yong-Villalobos et al., 2015; Al-Lawati et al., 2016; Wibowo et al., 2016). Furthermore, there is limited empirical evidence of the regulatory effects of DNA methylation on gene expression (Secco et al., 2015; Meng et al., 2016). This is complicated by the context-dependent (location and sequence context) nature of methylation-mediated gene regulation, such as the potential expression promoting role of gene body methylation compared to the expected repressive effects of promoter methylation (Bewick et al., 2016, 2017).

There have been many investigations for both stress priming and memory that are mediated by environmentally induced epi-alleles, which could open exciting possibilities for crop (epi)genomics (M.-T. Hauser et al., 2011; Springer, 2013; Ji et al., 2015). However, bona fide examples of transgenerational methylation changes leading to altered plant behaviour remain a rare observation (Pecinka et al., 2009; Schmitz et al., 2011; Becker et al., 2011; Jiang et al., 2014; Seymour et al., 2014) with the majority of methylome variation attributable to underlying genetic differences rather than being truly *epigenetic* (Eichten et al., 2013; Schmitz et al., 2013; Q. Li et al., 2014; Seymour et al., 2014; Dubin et al., 2015; Hagmann et al., 2015; Q. Li, Song, et al., 2015). Additional studies, across multiple species and stresses, have also documented a lack of stress-induced variation in the methylome (Pecinka et al., 2010; Seymour et al., 2014; Eichten & Springer, 2015; Hagmann et al., 2015; Secco et al., 2015). Despite this, there are numerous reports demonstrating the ability for plants to be primed against short-term abiotic stress, including EL and drought, in a DNA methylation-independent manner (Cayuela et al., 1996; Jakab et al., 2005; Rossel et al., 2007; Dyer et al., 2010; Ding et al., 2012; Gordon et al., 2013; Sani et al., 2013; J.-M. Kim et al., 2017).

On the other hand, support for DNA methylation-mediated stress priming is growing in both mitotic and meiotic time-scales (Tricker et al., 2013; Le et al., 2014; Yong-Villalobos et al., 2015; Al-Lawati et al., 2016; Herman & Sultan, 2016; Nosalewicz et al., 2016; Wibowo et al., 2016; X. Zheng et al., 2017). Furthermore, there are numerous reports of the importance of DNA methylation changes towards developmental processes, including fruit development (Luo et al., 1996; Manning et al., 2006; S. Zhong et al., 2013; Liu et al., 2015; Ong-Abdullah et al., 2015; Daccord et al., 2017). However, empirical evidence for "proactive" DNA methylation changes to support its role in stress response, rather than as a passive by-product of transcriptional changes, are lacking (Secco et al., 2015; Meng et al., 2016).

This thesis seeks to clarify these conflicts in the literature, which represent the complex nature of plant stress responses. Indeed, an important underlying consideration is that observations are being made of an integrated response across millions of non-uniform cells comprising multiple cell types, even within a single leaf, that can be further confounded by the heterogeneous effects of abiotic stress across a whole plant (Mustroph et al., 2009; Cramer et al., 2011). Therefore, it is critical to use rigorous methods for quantifying responses to make appropriate biological conclusions, while also being

mindful that observations on a model species may not be broadly applicable.

1.5 Thesis aims

The overall goal of this thesis is to: *(I)* improve our understanding of the molecular basis of light and drought responses, particularly in regards to retrograde signalling, and, *(II)* investigate the contribution of DNA methylation towards stress responses, in the context of priming and memory, in the model plant *Arabidopsis thaliana* (Arabidopsis). This investigation can be divided into sections with corresponding aims:

1. Investigate the potential for cross-talk between stress signalling pathways and the machinery involved in maintaining the methylome to lead to signalling-induced changes in DNA methylation by:
   (a) Exploring the mechanism of the SAL1-PAP-XRN chloroplast-to-nucleus retrograde signalling pathway to complement ABA signalling to promote stress tolerance and,
   (b) Explore the down-stream nuclear effects of PAP accumulation to observe how a signalling pathway could impact on the methylome.
2. Systematically investigate the potential for stress-induced DNA methylation variation and its contribution towards stress responses, in the context of priming and memory:
   (a) Test the potential for rapid priming by observing whether a recurring EL stress, within a generation, can influence future responses and how such transient stresses might impact DNA methylation to form memory (mitotic stress memory).
   (b) Test whether parental experience can influence offspring performance by comparing descendants of plants propagated under recurring drought to unstressed counterparts, and whether this correlates with altered DNA methylation patterns (transgenerational stress memory).
   (c) Systematically test for stress-induces changes in the Arabidopsis methylome against recurring EL and drought stress.
   (d) Quantify any contribution of stress-induced epi-alleles towards any observed stress priming or memory.

Chapter 2

Materials and methods

This section provides information on the materials and methods used to make the conclusions in this thesis.

2.1 Plant germplasm, growth conditions, and stress treatments

2.1.1 Plant germplasm

For most experiments, Arabidopsis plants used were in the Columbia (Col-0) background and were derived from a common inbred parent to minimise genetic variation and stochastic DNA methylation variation (Schmitz et al., 2013; Crisp et al., 2017). The exceptions were the use of *abi1*-1 (*abi1*; Koornneef et al. 1984) and *ost1*-2 (Mustilli et al., 2002) that were generated in the Landsberg erecta (Ler) background. The *sal1*-8 mutant was crossed to both of the aforementioned lines to generate double homozygous mutants, and were validated and maintained using derived cleaved amplified polymorphic sequence (dCAPS) markers (Wilson et al., 2009; Estavillo et al., 2011). Where germplasm were derived from multiple backgrounds, comparisons were made to a Col-0 x Ler F_1 hybrid (ColLer) . As the original *sal1*-8 mutant was derived from a mutagenesis screen, an independent SALK T-DNA mutant in the Col-0 background was also used (SALK_020882; *sal1*-6). The *xrn2*-1 x *xrn3*-3 double mutant (*xrn2xrn3*) was maintained and provided by P.A. Crisp (formerly, The Australian National University)[1]. The *ost1*-2 line was crossed with *xrn2xrn3* to generate a triple mutant (*ost1xrn2xrn3*), generated and maintained by P.A. Crisp. An *ost1* SALK T-DNA mutant, in the Col-0 background, was also obtained from TAIR (SALK_008068 and maintained by K.X. Chan (The Australian National University). This was crossed to the *sal1*-6 mutant to create another *ost1sal1* line derived from the Col-0 background, which was generated and maintained by N. Nisar (formerly,

[1]Current: University of Minnesota

The Australian University[2]).

2.1.2 Control growth conditions

Prior to light, seed were sown onto moist soil and kept at 4°C for three nights to allow for seed stratification. Plants were cultivated on soil (seedling raising mix, Debco, Australia) supplemented with Osmocote Exact Mini slow release fertilizer (Scotts Australia) at 3g/L dry soil using 1mg/L. Plants were grown under a 12-hour photoperiod (8:00am – 8:00pm) of 100 – 150 μmol photons m^{-2} s^{-1}, 20°($\pm$0.5°) C, and 55 ($\pm$5)% relative humidity. The desired light intensity was achieved using 250W metal halide lamps (Venture Lighting, MH 250W/U). For epidermal peels, plants were grown under higher ($\approx$ 80%) relative humidity (Eisenach et al., 2012; Chen, Eisenach, et al., 2012).

2.1.3 Excess-light stress treatment

For EL treatments, exposure to approximately 10X growth irradiance (1000 μmol photons m^{-2} s^{-1}) was applied, across the adaxial side of whole rosettes, using a mixture of 250W metal halide lamps (Venture Lighting, MH 250W/U) and high pressure sodium lamps (Phillips, SON-T 250W E E40 SL/12) providing a source of 'warm' light (simulating sunlight) that effectively induces oxidative stress (Jung et al., 2013). For Week Long Recurring Stress (WLRS) this was applied for one hour and repeated thrice daily at 9:30am, 1:30pm, and 5:30pm. Plant PSII performance under EL was monitored using chlorophyll fluorescence measurements (see below). Whole rosettes were harvested and flash-frozen in liquid nitrogen at the appropriate time-points (**Figure 5.1**).

2.1.4 Within generation drought stress

A slow onset water deprivation treatment ('drought stress') was imposed, after saturating soil moisture, by withholding watering for the desired length of time optimized using non-destructive means by observing the extent of leaf wilting paired with chlorophyll fluorescence measurements, in particular F_v/F_m and R_{fd}. For a within generation drought stress, watering was withheld for nine days causing a drop in relative water content (RWC) to approximately 60%.

2.1.5 Propagating transgenerational drought lineages

Growth conditions for propagation of lineages by single seed descent, used in the trans-generational drought experiment, were identical to control growth conditions described above, with the exception of a 16-hour photoperiod (8:00am – 12:00am) to promote rapid cycling. All lineages were initiated from a common inbred G_0 progenitor to min-imise genetic difference and stochastic DNA methylome variations. An extended version

[2]Current: Australian Government Department of Agriculture and Water Resources

of the water deprivation treatment, described above, was applied twice every generation to lineages propagated under drought stress (**Figure 4.4**). The first treatment was applied at one week of age, which involved saturating soil moisture and subsequently withholding water for two weeks. Plants were then watered and allowed to recover for five days. The second treatment was repeated following recovery, however, this time for only 12 days to minimise plant death. Plants were then watered until rosette leaf senescence and the appearance of dried, mature siliques for seed harvesting following the guidelines set by the Arabidopsis Biological Resource Centre.

2.2 High-throughput phenotyping

PlantScreen^{TM} (Photon Systems Instruments; Brno, Czech Republic), a platform for high throughput phenotyping, was used to measure plants traits (plant area and compactness) and monitor plant photosynthetic performance (chlorophyll fluorescence, see below) (Humplík et al., 2015; Rungrat et al., 2016). An analysis of variance (ANOVA) with subsequent Tukey's honest significant difference test (Tukey's HSD) *post-hoc* analysis was utilized to test for statistically significant ($p < 0.05$) differences at single timepoints. Additionally, a second order mixed effect polynomial model was constructed to statistically compare differences in growth rate.

2.3 Whole rosette ABA treatments

For *in vivo* ABA treatments on whole intact rosettes, 20 μM ABA (Sigma-Aldrich) was dissolved in a modified infiltration buffer (1 mM PIPES KOH pH 6, 1 mM Sodium citrate, 1 mM KCl, 15 mM Sucrose; Seeley et al. 1992). Either mock buffer or 20μM ABA was sprayed directly onto leaves using a Studio Series IS-875 airbrush with a 0.5mm nozzle opening (Iwata). Leaf temperature was subsequently monitored over time with an infra-red camera FLIR A600-Series, IR lens f=13.1 mm (FLIR Systems AB, Sweden). Single leaves were harvested for total RNA extractions to quantify gene expression.

2.4 Stomatal bio-assays

Stomatal apertures in response to individual and combinatorial chemical treatments [ABA, 3′-ethylsulfanyl-ABA (AS2), PAP, ATP, 3′-deoxyadenosine (cordycepin), AS2] were measured in epidermal peels of newly expanded leaves of three to four-week old plants (Chen, Hills, et al., 2012). Stomatal images were taken using a bright field microscope capable of 400X magnification for 10 min in opening buffer (OB: 50 mM KCl, 5 mM MES titrated to pH 6.1 with NaOH) as a pre-treatment to ensure the stomata stay open and responsive before subsequent assays on the signal of interest dissolved in a physiological measuring buffer (MB: 10 mM KCl, 5 mM MES titrated to

pH 6.1 with $Ca(OH)_2$; $[Ca^{2+}]_{final} \approx 1$ mM) (Blatt et al., 1990; Armstrong et al., 1995; Chen, Eisenach, et al., 2012). The epidermal peels were under the same light intensity (150 mmol m^{-2} s^{-1}) as in the growth chamber to avoid dark-induced stomatal closure. Stomata aperture width and length were measured using *ImageJ* (NIH, USA). The stomatal pore area was calculated using these values under the assumption that the area of a stomatal pore was that of an ellipse. Data are expressed as percentage compared to time = 0 min. A linear mixed-effect model was produced on log-transformed data between 10-30 minutes (predominant period of closure), taking into account random variation within and between leaf peels, to statistically compare rates of closure between treatments. Steady state stomatal closure was statistically compared ($p<0.05$) using an ANOVA with Tukey's HSD *post-hoc* analysis across the final 20 minutes of each time-course. This method was also employed for single time-points measures when comparing multiple genotypes.

2.5 RNA isolation using TRIzol and quality assessment

For gene expression analysis by quantitative RT-PCR (qRT-PCR), RNA was extracted using the TRIzol reagent (Life Technologies Australia Pty Ltd) using a procedure adapted from Allen et al. (2010). Briefly, up to 100 mg of snap frozen tissue was ground then lysed in 1 ml of TRIzol with gentle agitation. Following 5 min incubation at room temperature, the organic phase was extracted twice with 200 μl of chloroform. The RNA was precipitated by addition of an equal volume of 100% isopropanol and incubated overnight at -20°C. RNA was recovered by centrifugation and washed with 70% ethanol, air dried at room temperature, re-suspended in H_2O and stored at -80°C. RNA quality was assessed by separation of semi-denatured RNA (approximately 500 ng; incubate 5 min at 65°C), subsequently mixed with a loading buffer [approximately 98% (v/v) deionized formamide, 2% (v/v) EDTA (0.5M, pH 8), 0.01% (w/v) xylene cyanol, 0.01% (w/v) bromophenol blue], on an agarose gel via electrophoresis. RNA quantification was performed by spectrophotometric analysis using either the ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, USA) or the DropletQuant/LabChip DS (Perkin Elmer, MA, USA), including determination of sample concentration, A_{260} to A_{280} ratio, and spectral discrimination between single-stranded nucleic acids, double-stranded DNA and RNA, and protein (performed with DropletQuant).

2.6 Gene expression analysis by semi-quantitative RT-PCR

Total RNA was extracted from leaf tissue as described above, and reverse-transcribed into cDNA using the Invitrogen Superscript III cDNA Synthesis Kit (LifeTechnologies, USA) according to manufacturer's instructions. Exactly 1 mg total RNA was incubated with 50 pmol oligoDT (dT18VN) primer (65°C, 10 min). Complementary DNA was then synthesised in a 20 mL reaction containing 1 mM dNTPs, 1X first strand reaction buffer [250 mM Tris-HCl pH 3.8, 375 mM KCl, 15 mM $MgCl_2$], RNase inhibitor, and 100 units of Superscript III Reverse Transcriptase. The reaction was incubated at 50°C for 60 min, then stopped by heating (70°C, 15 min) and placing on ice. cDNA samples were stored at -20°C. Gene expression was monitored on the Roche LightCycler480 (Roche Diagnostics, Germany) based on fluorescence obtained from a PCR reaction incorporating SybrGreen fluorescent intercalating dye (Sybr Green I; Roche Diagnostics, Germany), performed in 384-well plates. Raw fluorescence data was exported and analysed using LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009) to perform background subtraction, determine PCR efficiency, and calculate starting concentration (N0; in arbitrary fluorescence units). Samples were then normalized against *PROTEIN PHOSPHATASE 2A SUBUNIT A3 (PP2AA3)* (*AT1G13320*); and expressed as fold changes against the appropriate WT control. Melt curve analyses were also utilized to test for single products using the *Melt Curve* and T_m *Calling* analysis modules from the *LightCycler480* software (v1.5). At least three biological replicates (individual plants) per treatment per genotype per experiment were sampled, and each reaction was performed in technical triplicate. Gene-specific primer sequences and cycling conditions are provided in **appendix B**.

2.7 Gene expression localisation

Visualising tissue localized gene expression was performed using *in situ* RT-PCR on epidermal cells (Athman et al., 2014). Epidermal peels were performed as described above, however, they were not fixed to a glass bottom chamber with the silicon adhesive. Instead, they were incubated in a fixation solution (2% formaldehyde, 63% ethanol, 5% acetic acid) on a microscope slide (Sigma Aldrich, USA). After fixation, the formaldehyde was removed by rinsing with two wash solutions (A: 63% (v/v) ethanol, 5% (v/v) acetic acid; B: 0.01 M Na_2HPO_4, 0.13 M NaCl). Subsequently, the epidermal peels were DNAse treated using Ambion TURBO DNAse (LifeTechnologies, USA) as per the manufacturer's instructions. For first strand synthesis, SuperScript III (ThermoFisher Scientific, USA) was used with polyT/oligodT primers, as per the manufacturer's instructions. On these products a PCR reaction, incorporating DIG-11-dUTP, was performed using gene-specific primers and cycling conditions as outlined in **appendix B**. To detect PCR products, peels were incubated with an anti-DIG antibody with a conju-

gated alkaline phosphatase (Roche, Switzerland), which binds to the DIG-labelled PCR products. Staining was achieved through incubation with a substrate of alkaline phosphatase, BM purple (Roche, Switzerland), for 1 hr. Epidermal peels were then washed and mounted in 40% glycerol and viewed under a Leica DM5500B Bright Field Microscope, with attached camera, at 40X magnification. For *in situ* qRT-PCR, expression of nuclear-encoded 18S rRNA was used as a positive control.

2.8 Monitoring PSII performance using chlorophyll fluorescence measurements

Measures of chlorophyll fluorescence were used to monitor PSII performance using a PSI FluorCam (Photon System Instruments; Brno, Czech Republic). Images were analysed using the accompanying *FluorCam7* (Photon System Instruments; Brno, Czech Republic) imaging software, which also allowed analysing fluorescence traces from specific regions of the intact rosette. Measurements were taken from across the adaxial side of dark-adapted (30 minutes) plants for seven minutes under actinic light (approx. 800 μmol photons m^{-2} s^{-1}) followed by three minutes in the dark (select measurements were adjusted to ten minutes under actinic light and four minutes dark for measurements after WLRS treatment), with regular measures of chlorophyll fluorescence induced by a saturating pulse (approx. 3000 μmol photons m^{-2} s^{-1}), as well as minimal fluorescence in the presence of measuring light only (Humplík et al., 2015; Rungrat et al., 2016). Measurements on 30 minute dark adapted plants allowed measures of base fluorescence (F_0), the fluorescence immediately prior to a saturating pulse (F_t), maximal fluorescence after a saturating pulse (F_m), and the variable fluorescence (F_v). Subsequently, the Kautsky effect is induced with the initial signal giving peak fluorescence (F_p) as PSII activity engages. Regular saturating pulses occur under actinic light allowing measurement of the light-adapted counterparts: F_0', F_m', F_v', and F_t'. These values were used to calculate the parameters shown in **Table 2.1** with the corresponding equations (Haitz & Lichtenthaler, 1988; Lichtenthaler & Miehé, 1997; Maxwell & Johnson, 2000; Lichtenthaler et al., 2005; Baker, 2008; Brestic & Zivcak, 2013; Murchie & Lawson, 2013). An ANOVA with Tukey's HSD *post hoc* analysis was used to determine significant differences ($p<0.05$) for single time-point measures. A linear mixed-effects model was fitted, to account for variance from random effects (e.g. blocking design), across time-course data (treating time as a factor) for each PSII parameter measured. Statistical significance between plants exposed to differing conditions was determined using pairwise comparisons at each time-point with a Bonferroni *post hoc* correction for multiple hypothesis testing.

Table 2.1: PSII parameters, and corresponding equations, used to non-destructively assay impacts of stress

Parameter	Equation	Interpretation
F_v	F_m-F_0	Variable fluorescence, the ability for PSII to perform photochemistry, in dark adapted plants
F_v'	F_m'-F_0'	Variable fluorescence, the ability for PSII to perform photochemistry, under actinic light
F_q'	F_m'-F_t'	Photochemical quenching of fluorescence by open PSII centres
F_v/F_m	$(F_m\text{-}F_0)/F_m$	Maximum quantum efficiency of PSII
F_v'/F_m'	$(F_m'\text{-}F_0')/F_m'$	Estimate of maximum quantum efficiency of PSII under actinic light
ϕ_{PSII}	F_q'/F_m'	PSII quantum efficiency: proportion of light absorbed by chlorophyll used for photochemistry
qP	F_q'/F_v'	Coefficient of photochemical quenching: relates PSII maximum efficiency to PSII operating efficiency
qL	$(F_q'/F_v')/(F_0'/F_t')$	Estimates fraction of open PSII centres
NPQ	(F_m/F_m')-1	Estimates rate constant for heat loss from PSII
R_{fd}	(F_p/F_t')-1	Fluorescence decline ratio calculated using steady state fluorescence: correlates with CO_2 fixation rate with values > 3 indicative of highly efficient PSII and < 1 reflecting negligible net CO_2 gain

2.9 Plant biomass and rosette dehydration assay

Rosette dehydration assay was performed as previously reported (Wilson et al., 2009). Briefly, rosettes of approximately four week old plants, grown under control growth conditions as described above, were excised at the base and weighed on a five-digit fine balance (Mettler Toledo; Melbourne, Australia). This was used as the measurement of fresh biomass. An ANOVA with Tukey's HSD *post hoc* test was used to determine significant differences ($p < 0.05$) in fresh rosette biomass. The mass of excised rosettes was then monitored at regular intervals for one hour. A mixed effect second order polynomial model was constructed to test for significant differences in rate of water loss ($p < 0.05$).

2.10 Seed dormancy assay

Seed dormancy was tested on fresh seed from mature, dried siliques from senescing plants using the recommended methods (McNair et al., 2012). Each silique was taken from an individual plant and considered as a single biological replicate. At least 20 seeds per individual silique was released onto a 0.8% agar plate and kept immediately under control growth conditions. For the first five days, photos of the plates were taken twice daily, thereafter only once daily. At each time-point, all seeds per plate were scored as either germinated or ungerminated. To statistically compare seed dormancy, a Cox-proportional hazards model was produced. From this model, calculation of the hazard ratio (HR) provides a comparative value between treatment groups. For the transgenerational drought experiment, the hazards ratio was calculated for drought lineages relative to unstressed lineages (HR_D).

2.11 Survival under terminal drought

Length of survival under drought was tested by performing a terminal drought experiment. Plants were grown under control growth conditions to approximately three weeks of age. Subsequently, soil was watered to saturation and excess water was drained. Watering was thereafter withheld and plant vitality was monitored non-destructively with chlorophyll fluorescence measurements (Woo et al., 2008). The parameter R_{fd} was utilized as a vitality index where plants demonstrating values < 1 were considered dead (**Table 2.1**, Haitz & Lichtenthaler 1988).

2.12 Quantification of chlorophylls and xanthophylls

Quantification of chlorophylls and xanthophyll was performed based on an established method utilising high performance liquid chromatography (HPLC) with some modifications (Pogson et al., 1998). Harvested, flash-frozen whole rosettes were ground into a powder using a $1/8''$ steel ball bearing in a 1.5mL Eppendorf tube, with shaking at 25Hz for one minute in the Tissue Lyser II (Qiagen, Germany). Pigments were extracted by mixing ground tissue with 400 μl acetone-ethyl acetate (3:2 [v/v]). Water (320 μl) was added, the mixture was centrifuged (5 minutes; 20,000 rcf), and the upper phase was recovered (repeated twice, approximately 150 μl final supernatant recovered) into a glass HPLC vial for analysis on the Agilent 1200 Series HPLC System (Agilent Technologies, Germany). A total 20 μl of extract was injected and separated by reverse phase HPLC on a GraceSmart RP18, 4 micron, 4.6x250mm column (W.R. Grace & Company, USA) using an ethyl acetate gradient in acetonitrile: water: triethylamine (9:1:0.01 [v/v]) at 1 mL min^{-1} using the following timetable (optimised for separating xanthophylls): 0–3 min, 0% ethyl acetate; 3–31 min 0–66.7%, 31–31.2 min 66.7–100%,

31.2–34 min 100%. Carotenoids were identified based on their retention time and UV absorption spectra compared to known standards, with detection at λ_{max} 440 nm using an inline photodiode-array detector. Quantifications are based on integrated peak areas (via Agilent Chemstation software). β-carotene and xanthophyll levels are presented as a fraction of the total carotenoid pool. Chlorophyll *a* and *b* levels are presented as percent of total chlorophylls (chl *a* + chl *b*). An ANOVA with Tukey's HSD *post-hoc* analysis was used to determine significant differences ($p < 0.05$).

2.13 Re-analysis of mRNA sequencing datasets

Next-generation sequencing datasets were obtained from either the NCBI or EBI data repositories (*SRA Toolkit*; see **Appendix A**).

Quality control was performed with *FastQC* (v0.11.2). Adapters were removed using *scythe* (v.0.991) and reads were quality trimmed with *sickle* (v.1.33). The resulting trimmed reads were aligned to the Arabidopsis genome (TAIR10) using either *(I) subjunc* aligner with the flags -u -H for mRNA sequencing reads or the *subread* aligner with flags -t 1 -u -H for ChIP sequencing reads (v.1.5.1; Liao et al. 2013). Aligned reads were sorted, indexed, and compressed using *samtools* (v1.5; H. Li et al. 2009). Raw expression levels were assigned to annotated gene and transposable element features of the TAIR10 assembly using *Bedtools* (v2.25.0; Quinlan & Hall 2010) and the Araport11 genome re-annotation (Cheng et al., 2017), which could subsequently be converted into reads per kilobase per million reads (RPKM) or fragments per kilobase per million reads (FPKM) for single- or paired-end sequencing, respectively. *BigWig* files were generated using *bedGraphToBigWig* for visualizing mRNA expression in the *Integrated Genomics Viewer* (*IGV*; Robinson et al. 2011).

Re-analysis and comparisons to differentially expressed gene (DEG)s were based on previous analyses and published differentially expressed gene lists (Crisp, 2015; Pornsiriwong et al., 2017; Crisp et al., 2017). In short, read counts were assigned to "gene" and "transposable_element" features in the TAIR10 GFF3 or Araport11 GFF references using *featureCounts* with flags -p -c for uniquely mapping reads only (v.1.4.6; Liao et al. 2014). Statistical testing for relative gene expression was performed in using *edgeR* (v.3.4.2; McCarthy et al. 2012. Reads mapping to rRNA were removed (contamination rate $< 1\%$ for all samples); organelle transcripts were removed, and only loci with an abundance of at least 1 counts per million (CPM) in at least three samples ($\approx$ 10–20 reads for each replicate in one sample group) were retained.

2.14 Whole genome bisulfite sequencing

2.14.1 Library preparation

Whole genome bisulfite sequencing was performed from snap-frozen leaf tissue of harvested whole rosettes. Genomic DNA was extracted using the Qiagen DNeasy Plant Mini Kit (Limburg, Netherlands), as per the manufacturer's instructions, and quantified using the ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, USA). 100-200 ng of fragmented (Covaris) and purified gDNA was bisulfite converted using the Zymo DNA-Gold bisulfite conversion kit (Zymo Research; CA, USA). Whole genome bisulfite sequencing libraries were constructing using the Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences; MI, USA) as per the manufacturer's instructions. All purification steps were performed using Sera-mag SpeedBeads (GE Healthcare; Buckinghamshire, UK). The concentration and size distribution of bead-purified libraries were quantified on the Perkin Elmer GXII using a DNA High Sensitivity kit (MA, USA). Libraries were subsequently pooled equal-molar, in six-sample pools, and sequenced across a HiSeq2500 (100bp single end; Illumina; CA, USA), with a 5-10% spike in of PhiX DNA, depending on sample complexity, at the ACRF Biomolecular Research Facility (Australian National University, Canberra, Australia).

2.14.2 Sequencing analysis

Raw sequencing reads were quality controlled and trimmed using *Trim Galore!* (v0.3.7), *Cutadapt* (v1.9), and *FastQC* (v0.11.2). Trimmed reads were aligned to the TAIR10 reference genome using *Bismark* (v0.14.5; Krueger & Andrews 2011) and *Bowtie2* (v2.3.3; Langmead & Salzberg 2012). Methylated cytosines were extracted from aligned reads using *Bismark methylation extractor* with default parameters. Bisulfite conversion efficiency was calculated from the proportion of unconverted cytosines in the mCHH context from the chloroplast genome. The proportion of mCG, mCHG, and mCHH was determined as weighted methylation (Schultz et al., 2012) across reads at single cytosine resolution and across 100bp tiles for genome-wide comparisons. Pearson's correlation coefficient (r) of methylation levels, between samples, was performed on mean methylation levels across 100bp tiles in all sequence contexts. Methylation levels were assigned to annotated gene and transposable element features of the TAIR10 assembly using *Bedtools* (v2.25.0; Quinlan & Hall 2010) and the Araport11 genome re-annotation (Cheng et al., 2017). Details for all samples generated in this thesis, including summary sequencing statistics, are provided in the online supplemental datasets.

2.14.3 Identifying differentially methylated regions

Two unbiased methods were utilized in a combinatorial approach, based on established work (Eichten et al., 2016), to explore differential methylation between samples.

First, to look at stochastic variation in the DNA methylome, regardless of treatment, DMRs were identified using pairwise comparisons employing a method based on average methylation binned to 100bp tiles across the genome (Eichten & Springer, 2015). In brief, pairwise comparisons were performed between corresponding 100bp tiles in all samples. For each pairwise sample comparison, all 100bp tiles were called differentially methylated if the absolute difference in methylation levels met a given threshold (mCG: 70%; mCHG 50%; mCHH 40%) alongside a minimum coverage and number of cytosines (10X coverage, 3 cytosines). Adjacent tiles identified DMRs were collapsed into a single tile. All results were compared and the largest region was kept for any overlapping DMRs between pairwise comparisons. A Kruskal-Wallis rank sum test was performed to identify significant differences between sample groups with a Bonferroni *post-hoc* p-value adjustment for multiple comparisons.

Second, a more conservative approach was used to identify statistically significant treatment or genotype associated DMRs utilizing Bayesian hierarchical modelling, incorporating technical and biological variation at the individual cytosine level, with the *R* package *DSS* (v2.10.0; H. Feng et al. 2014). This was performed using the recommended default settings (with smoothing to allow for imputation of missing data) except for a reduced smoothing tile size (smoothing.span = 100). The threshold methylation difference for DMRs in each sequence context (delta) was defined as 40% for mCG, 20% for mCHG and 20% for mCHH based on published thresholds with the exception that mCHH DMRs were called with greater stringency (Stroud et al., 2013). *DSS* calculates an adjusted p-value (q-value) based on the posterior probability that the differential methylation is greater than the specified thresholds (delta); DMRs were considered significant at q-value < 0.05.

2.15 Data visualisation and statistical analyses

Data visualisation and statistical analyses were conducted in *R* (v3.3.2) using the appropriate packages (Wickham, 2007, 2009, 2011; Bache & Wickham, 2014; R Core Team, 2016; Warnes et al., 2016). To test for statistically significant fixed-effects, ANOVAs were fitted using the *aov* function with *post-hoc* computation of Tukey's HSD, between factors, performed using the function *TukeyHSD*. The *lme4* package (v1.1 Bates et al. 2015) was used for producing linear mixed effects models measuring both fixed (e.g. condition) and random effects (e.g. blocking design). Model fit was assessed using the conditional R^2 value (R^2_C), calculated using the *piecewiseSEM* package (Lefcheck, 2016). Relative model fit was also assessed, for model selection, using Akaike's information criterion, Bayesian information criterion, and the log-likelihoods from the computed analysis of variance tables using the *anova* function. The *anova* function was also used to compute analysis of variance tables from fitted models to test whether the fixed effects were significant. The *lsmeans* package (v2.26 Lenth 2016) was used to compute

least squares means, derived from the aforementioned mixed effects models, with 95% confidence intervals and conduct *post-hoc* contrasts between factors with appropriate p-value correction (Tukey or Bonferroni methods). Hypergeometric tests were computed using the *phyper* function. Expression-based clustering of drought-responsive transcripts was achieved using the *kmeans* function (centers = 10). For non-parametric ANOVAs, Kruskal-Wallis rank sum tests were performed to determine statistically significant fixed-effects using the *kruskal.test* function. The *p.adjust* function (method="bonferroni") allowed for *post-hoc* Bonferroni p-value adjustment to account for multiple pairwise comparisons. Alternatively, a Dunn's test of multiple comparisons with Benjamini–Hochberg p-value adjustment was performed using the *dunnTest* function (method='bh') from the *FSA* package (Ogle, 2017). Survival analyses to compare seed dormancy between lineages, using a Cox proportional hazards model, were performed using the *survival* package (v2.41 Therneau 2015). Pearson's *r* was calculated using the *cor* function (method="pearson"). All statistical analyses, including modelling, were produced on single raw data points. Biological replication, unless otherwise stated, was considered to be independent whole plants. The *DiGGer* package (v0.2.31 Coombes 2011) was used to produce spatially optimised complete randomised experimental designs. Next-generation sequencing data, such as DMRs, were viewed using (*IGV* Robinson et al. 2011).

2.16 Dataset repositories

The next-generation sequencing datasets utilized herein are available at the following NCBI data repositories: PRJNA368978 and PRJNA391262. All bioinformatic pipelines are freely available on GitHub. All information, including summary alignment metrics and repository ID, regarding utilized publicly accessible next-generation sequencing datasets are available in **Appendix A**. For all publicly accessed datasets, raw data was downloaded and re-analysed using the same pipelines as for the samples generated in this study. Unless specifically stated, WT methylation patterns are based on the Col-0 samples generated herein. Independent Col-0 methylome data were used for DMR calling with corresponding mutant samples, or for producing normalized methylation levels to allow for appropriate comparisons.

Chapter 3

Mechanistic insight into SAL1-PAP-XRN signalling and cross-talk with RNA directed DNA methylation

---

This chapter, in part, comprises my component of the complete results, which are published and presented in full in Pornsiriwong et al. (2017) available online at eLife.

3.1 Synopsis

Intracellular communication between different organelles and compartments is considered vital, regulating appropriate plant growth and development. Indeed, a variety of signals and pathways, collectively referred to as retrograde signalling, have now been identified that relay information between the energy producing organelles, such as the chloroplast and mitochondria, and the overarching control hub, the nucleus (Pogson et al., 2008; Chan, Mabbitt, et al., 2016). The SAL1-PAP-XRN pathway has recently been identified as a chloroplast-to-nucleus retrograde signalling pathway that promotes drought tolerance (Wilson et al., 2009; Estavillo et al., 2011). However, the exact downstream mechanism and effects of PAP, including its co-operation with other bona fide signalling pathways, remain enigmatic. The pre-eminent hypothesis is that accumulated PAP can be transported between the chloroplast and cytosol (Gigolashvili et al., 2012) and diffuse into the nucleus where it induces stress-responsive gene expression, through inhibition of XRN function (Dichtl et al., 1997; Gy et al., 2007; Kurihara et al., 2012), to promote abiotic stress tolerance (Rossel et al., 2006; Wilson et al., 2009; Estavillo et al., 2011). However, multiple clues suggest that PAP may also be interacting with other pathways. Firstly, whilst *sal1*-8 displays a striking transcriptional overlap with *xrn2xrn3* double mutants, there are still a large number of genes uniquely differentially regulated in *sal1*-8 (Estavillo et al., 2011), a distinct subset of which are ABA-responsive (Wilson et al., 2009). Additionally, introducing the *sal1*-8 lesion into ABA insensitive mutants,

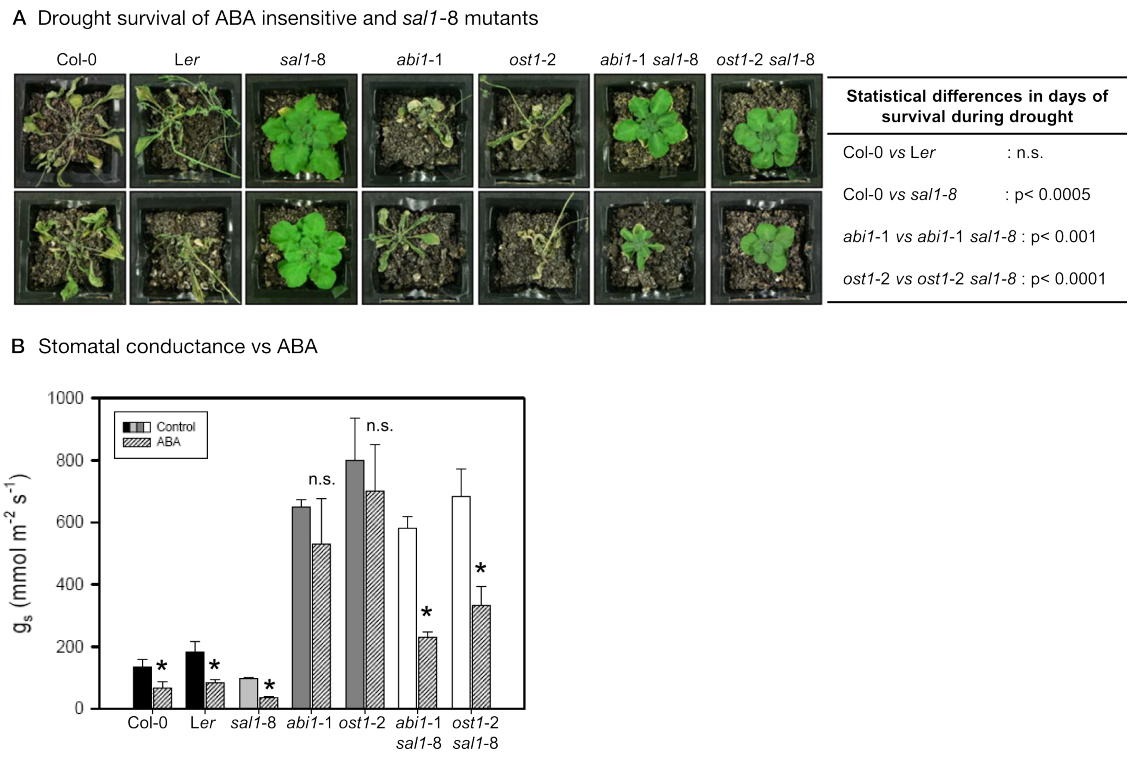

Figure 3.1: Genetic restoration of ABA sensitivity
A Representative photos of two plants per genotype exposed to 10 days of drought. Statistically significant differences in survival between genotypes are indicated (n = 4).
B Effect of 20μM ABA, root fed to hydroponically grown plants, on stomatal conductance (g_s) after 2 h feeding through the roots of hydroponically-grown plants. Bars denote means from two independent experiments (n = 3); errors bars denote standard error of the mean.
Experiments performed by Wannarat Pornsiriwong and Gonzalo Estavillo, and full results are presented in Pornsiriwong et al. (2017).

such as *abi1*-1 and *ost1*-2 (two key regulators of ABA signalling Figure 1.4), rescues ABA sensitivity and restores drought tolerance (**Figure 3.1**[1]). Lastly, using an affinity chromatography approach involving PAP-agarose beads, a number of diverse cellular components, including those involved in ABA signalling, ROS production, and RNA regulation, were found to be bound by, and potentially interact with, PAP (collectively referred to as the PAP-interactome, Crisp 2015). Thus, it was clear that PAP might have diverse cellular interactions that could be contributing to the phenotypes observed in *sal1*-8. Thus, this chapter extends on previous work to provide mechanistic insight on SAL1-PAP-XRN signalling, in particular, how accumulated PAP improves drought tolerance. Subsequently, evidence for cross-talk of the SAL1-PAP-XRN and RdDM pathways are examined, extending the direct or indirect effects of PAP within the nucleus.

[1]Experiments performed by Wannarat Pornsiriwong and Gonzalo Estavillo, and is presented in Pornsiriwong et al. (2017)

3.2 Results

3.2.1 Exogenous PAP treatment induces stomatal closure

A key short-term response to drought is stomatal closure to minimize water loss (Verslues et al., 2006). One hypothesis for PAP-mediated improvement of drought tolerance was through altered stomatal control, however, genetic evidence showed no constitutive effect as *sal1* mutants displayed stomatal morphologies that were comparable to wild-type under well watered conditions (Wilson et al., 2009). On the other hand, *sal1* mutants were found to have reduced stomatal conductance (Rossel et al., 2006) and genetic evidence suggested that PAP may be involved in ABA-mediated processes (**Figure 3.1**), such as stomatal closure (Cutler et al., 2010). Thus, there was potential for PAP to act as a signalling molecule that, in and of itself, could induce stomatal closure.

If PAP is a bona fide signalling molecule that can induce stomatal closure, then exogenous PAP application should elicit responses akin to known guard cell regulators, such as ABA. Thus, a significant undertaking was to establish and validate protocols for observing the effects of direct PAP application to leaves either via petioles or directly onto epidermal leaf peels. An initial method to evaluate the physiological effects of PAP on stomata was via petiole feeding. Here, detached leaves are placed, petiole first, into a solution allowing uptake into the leaf. This allows physiological responses to be observed in response to chemical treatments. Pertinent to ABA and PAP treatment was to monitor leaf temperature, as an increase is indicative of stomatal closure (Rossel et al., 2006). However, to effectively induce PAP accumulation using this method requires the co-feeding of ATP and/or LiCl, which may confound the exact effects of PAP itself (**Figure 3.2**[2]).

An alternative method was the direct application of PAP onto guard cells, by performing epidermal leaf peels and pairing it with light microscopy. This circumvents the necessity of chemical co-treatments to facilitate PAP uptake and allows for direct observations of stomatal dynamics (Chen, Eisenach, et al., 2012). Indeed, this technique is routinely utilized to measure stomatal responsiveness to bona fide signalling molecules, including ABA, Ca^{2+}, and ROS (Kinoshita et al., 1995; Hosy et al., 2003; Sierla et al., 2016). First, different concentrations of PAP were tested (10, 50, and 100μM exogenous PAP) and stomatal closure was monitored. All tested concentrations of PAP led comparable extents of closure (**Figure 3.3A**). This demonstrated that the stomata were responsive from the shift from the opening buffer (OB) pre-treatment to the physiological measuring buffer (MB) containing PAP.

Next, PAP- and ABA-mediated stomatal closure were compared. In order to make direct comparisons between ABA and PAP, both chemicals were applied at a standardized concentration of 100μM in all subsequent experiments utilizing epidermal peels. A time-

[2]Experiments performed by Kai Xun Chan and presented in Pornsiriwong et al. (2017)

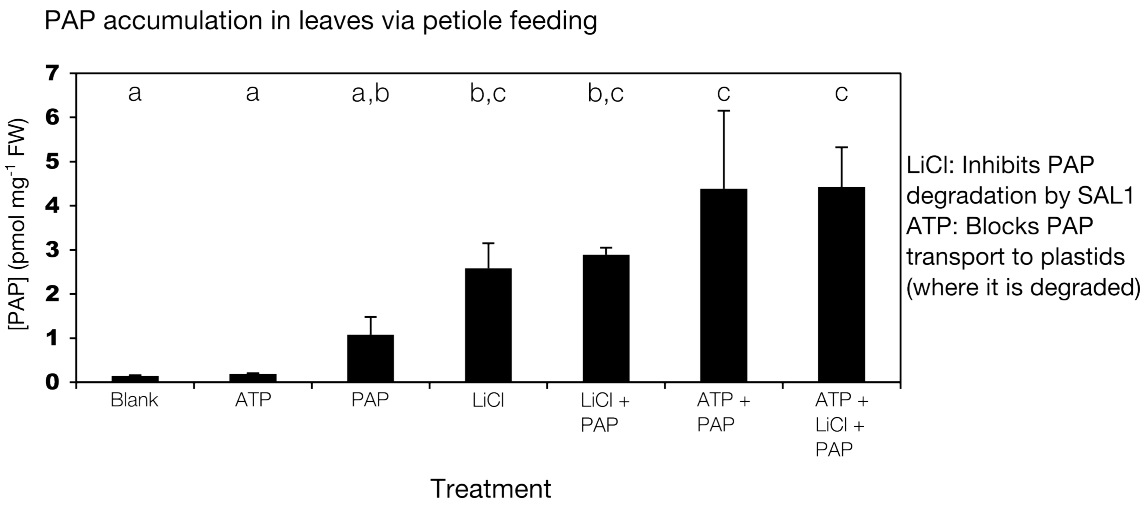

Figure 3.2: Accumulation of PAP in leaves via petiole feeding
Petiole feeding of PAP for 1h results in some accumulation of PAP in leaves. Levels can be enhanced by co-application with LiCl, an inhibitor of SAL1, or with ATP, which outcompetes PAP for transport into plastids where PAP is degraded. Bars denote means (n=3); error bars denote standard deviation. Letters denote statistically significant groups ($p < 0.05$).
Experiment performed by Kai Xun Chan.

course of ABA, PAP, and MB (buffer only control) measurements showed that both ABA and PAP stimulated stomatal closure to an extent greater than MB only, which contains some Ca^{2+} (Ca^{2+} itself induces some stomatal closure; Blatt et al. 1990). Under this system, both ABA and PAP treatments induced stomatal closure within 10 minutes of application. The rate of closure during this period post-treatment was statistically similar for both treatments, however, PAP showed a greater final extent of closure as compared to ABA (**Figure 3.3B**). Next, it was investigated if the kinetics of stomatal closure could be improved through biochemical manipulation of PAP transport, using co-treatments on epidermal peels, based on previous petiole-feeding experiments (**Figure 3.2**). A known co-substrate for the PAP transporter is ATP that is predicted to out compete PAP for import into chloroplasts, thus preventing its degradation by SAL1 (Estavillo et al., 2011; Gigolashvili et al., 2012). To see if this lead to enhanced stomatal closure ATP co-feeding was repeated on epidermal peels and, as expected, co-treatment with PAP and ATP showed enhanced closure, with respect to a faster rate and a greater final extent of closure, compared to PAP alone (**Figure 3.3C**). Taken together, these results demonstrate that PAP is a signal that, in and of itself, induces stomatal closure to a similar extent as ABA.

3.2.2 PAP complements ABA signalling and restores ABA sensitivity in ABA signalling mutants

Since PAP was established to induce stomatal closure and its effects could be directly monitored, the mechanism for this PAP-induced closure was investigated. Genetic analyses show that lesions in *SAL1* lead to elevated levels of PAP (Estavillo et al., 2011), and

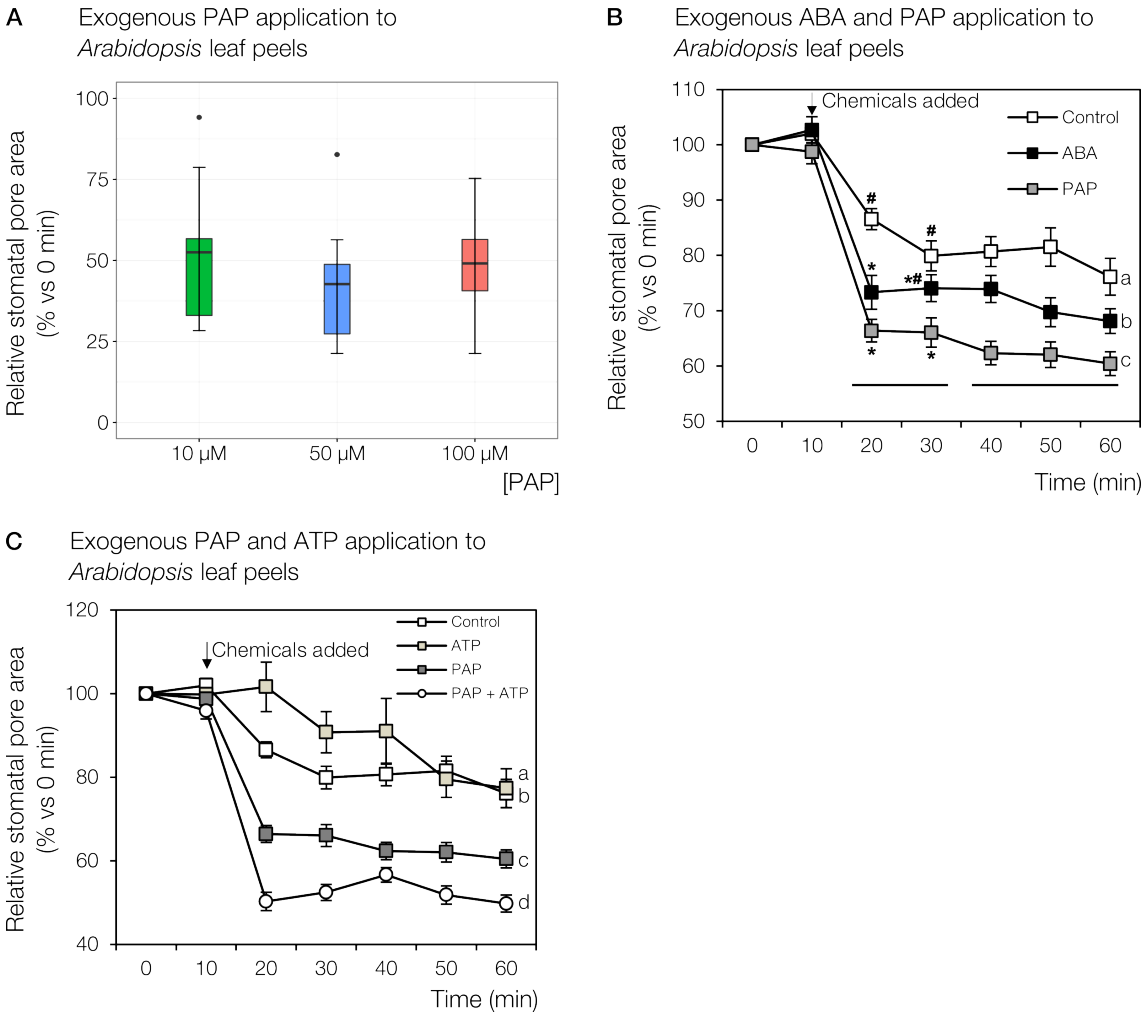

Figure 3.3: Exogenous PAP induces stomatal closure
A Stomatal aperture after 60 mins incubation in 10, 50, or 100 μM PAP, dissolved in MB, relative to 0 min. Standard box plots are presented (n = 9-13 stomata): upper and lower hinges denote the first and third quartiles, mid-band denotes the median, upper and lower whiskers denotes 1.5x interquartile range, respectively. No statistical significance was determined.
B Stomatal aperture, relative to 0 min, in epidermal peels of wild type (ColLer) plants treated with either MB only (control), 100μM PAP, or 100μM ABA over a period of 1 h. Points denote means (n $>$ 20 stomata), error bars denote standard error of the mean. Rates of closure were compared by linear mixed-effect modelling of closure between 10 – 30 min (log-transformed data), significant difference groups ($p < 0.05$) are denoted by #, *. Final level of closure was also considered by ANOVA across the final 20 min; significant difference ($p < 0.05$) denoted a, b, c.
C Stomatal aperture as in **A** but treated with either MB only (control), 100 μM PAP, or 1 mM ATP alone or in combination. Points denote means (n $>$ 8 stomata), error bars denote standard error of the mean. Letters denote significance groups ($p < 0.05$) based on an ANOVA and Tukey's HSD *post-hoc* analysis across the final 30 min.

restore drought tolerance and stomatal conductance in the ABA insensitive, and drought intolerant, mutants *abi1* and *ost1* (**Figure 3.1**). To investigate an interaction between the SAL1-PAP-XRN pathway and ABA signalling, the epidermal treatment system was used to confirm that genetically elevated PAP levels restored ABA-mediated stomatal closure in *ost1* (**Figure 3.4**). Whereas the stomates on WT epidermal peels were responsive to ABA, counterparts from *ost1* mutants, of both Col-0 and Ler backgrounds, showed ABA insensitivity (**Figure 3.4A**). The *ost1*-2 (Ler) appeared to have increased closure to MB only compared to WT or *ost1* (Col-0), possibly due to enhanced sensitivity to the low concentration of Ca^{2+} used in the physiological buffer, which was also observed in the *ost1*-2*sal1*-8 double mutant. Importantly, *ost1*-2*sal1*-8 demonstrated ABA responsiveness with greater stomatal closure after ABA incubation confirming that ABA responsivity has been restored.

To dissect whether PAP mediated restoration of ABA sensitivity required the PYL cascade of ABA receptors, the functionality of specific PYLs in *ost1sal1* double mutants was tested using ABA analogues. The ABA analogue AS2 is a limited-spectrum ABA agonist that largely activates dimeric PYL receptors (PYR1, PYL1, PYL2, PYL3), weakly activates the monomeric receptors PYL4, PYL5, and PYL11; but cannot activate PYL6, PYL9, and PYL10 (Takeuchi et al., 2014). Thus, to test whether the reversion of ABA sensitivity was occurring through specific PYL receptors, 50μM AS2 was applied to each of the genotypes, with the addition of the *ost1sal1* double mutant in the Col-0 background (**Figure 3.4B**). Application of AS2 induced stomatal closure in a comparable manner as ABA in WT, whereas *ost1* mutants maintained open stomata indicative of insensitivity. The key test was whether closure was attenuated in the *ost1sal1* double mutants that, if observed, would implicate PYL6, PYL9, or PYL10 as being important for the reversion in ABA sensitivity. However, both of the double mutants showed typical stomatal closure, comparable to ABA treatments, ruling out this hypothesis but also suggesting that PYL1/2/3 may be important. Whether specific receptors are required for the restoration of ABA signalling in *ost1sal1* double mutants observed requires further systematic testing. Next, we tested if PAP could function independently of ABA, that is, whether PAP induced stomatal closure in the absence of the ABA signalling machinery. Interestingly, exogenous PAP applied to guard cells, of the ABA insensitive *ost1*-2 single mutant, was able to induce stomatal closure. This suggests that PAP can either circumvent, or directly activate downstream components of, the canonical ABA pathway (**Figure 3.4C**).

3.2.3 Rapid PAP-induced stomatal closure does not rely on transcriptional changes

The canonical model of PAP-mediated signalling is through transcriptional reprogramming via XRN inhibition (Dichtl et al., 1997; Estavillo et al., 2011; Crisp, 2015). Whilst this interaction may, in part, contribute towards the constitutive restoration of ABA

signalling in the *ost1*-2*sal1*-8 double mutant, it remains unclear whether this might contribute towards the direct, rapid closure induced by PAP. Indeed, nuclear transcription can be altered within minutes or even seconds of EL stress (Suzuki et al., 2015; Crisp et al., 2017) and eukaryotic translation rates are also sufficiently rapid as only three minutes are required for the *de novo* synthesis of a typical protein (Milo & Phillips, 2015). Therefore, to test whether transcriptional changes contributed to PAP-induced stomatal closure, the transcriptional inhibitor, cordycepin, was co-treated alongside PAP (Gutierrez et al., 2002). Stomatal closure was still induced from this co-treatment and occurred in a very similar manner as the PAP only treatment, which could suggest a non-transcriptional mechanism for PAP-mediated closure (**Figure 3.4D**). However, the cordycepin only treatment also induced closure in a very similar manner, and there was no additive effect in the co-treatment leading to faster closure.

As an alternative approach, changes in ABA-responsive guard cell-localized transcripts were measured within 10 minutes of treatment to test for transcriptional changes that might contribute towards stomatal closure. This was not performed using PAP due to its lack of penetrance in whole plant treatments; and the epidermal peel system lending itself poorly for observing short-term molecular changes. Specifically, the process of sampling epidermal peels can be tedious, time-intensive, particularly to harvest sufficient tissue, and the resulting nucleic material is of low yield and quality. Given such issues with PAP application, ABA treatments were performed on whole intact rosettes. Treatment of 20μM ABA was paired with thermal imaging to ensure appropriate ABA responses were being observed. Rosette temperatures were measured, independently, for both 10 and 60 minutes post ABA treatment. Both time-points showed expected increases in rosette temperature, indicative of ABA-induced stomatal closure in all genotypes except for *ost1*-2 (**Figure 3.5A**). Interestingly, the *ost1*-2 mutant showed a minor response 10 minutes post-ABA treatment, however, this did not reach statistical significance. This suggests that there may be an initial attenuated response that cannot be maintained.

A selection of ABA-induced, guard cell localized, and ABA signalling component encoding transcripts, particularly those with increased expression with elevated PAP (i.e. in the *sal1* background) that may explain the rapid stomatal closure, were selected as markers for a transcriptional response within 10 minutes of 20μM ABA treatment[3]. Largely, there were no strong changes in expression in any of the transcripts tested, although some variability was observed and *KAT2* was determined to be statistically down-regulated post 10 minutes of ABA treatment. Whether or not such changes are biologically meaningful and could contribute towards the stomatal closure observed is unclear, however, is largely unlikely.

[3]see Supplementary File 1 presented in Pornsiriwong et al. 2017

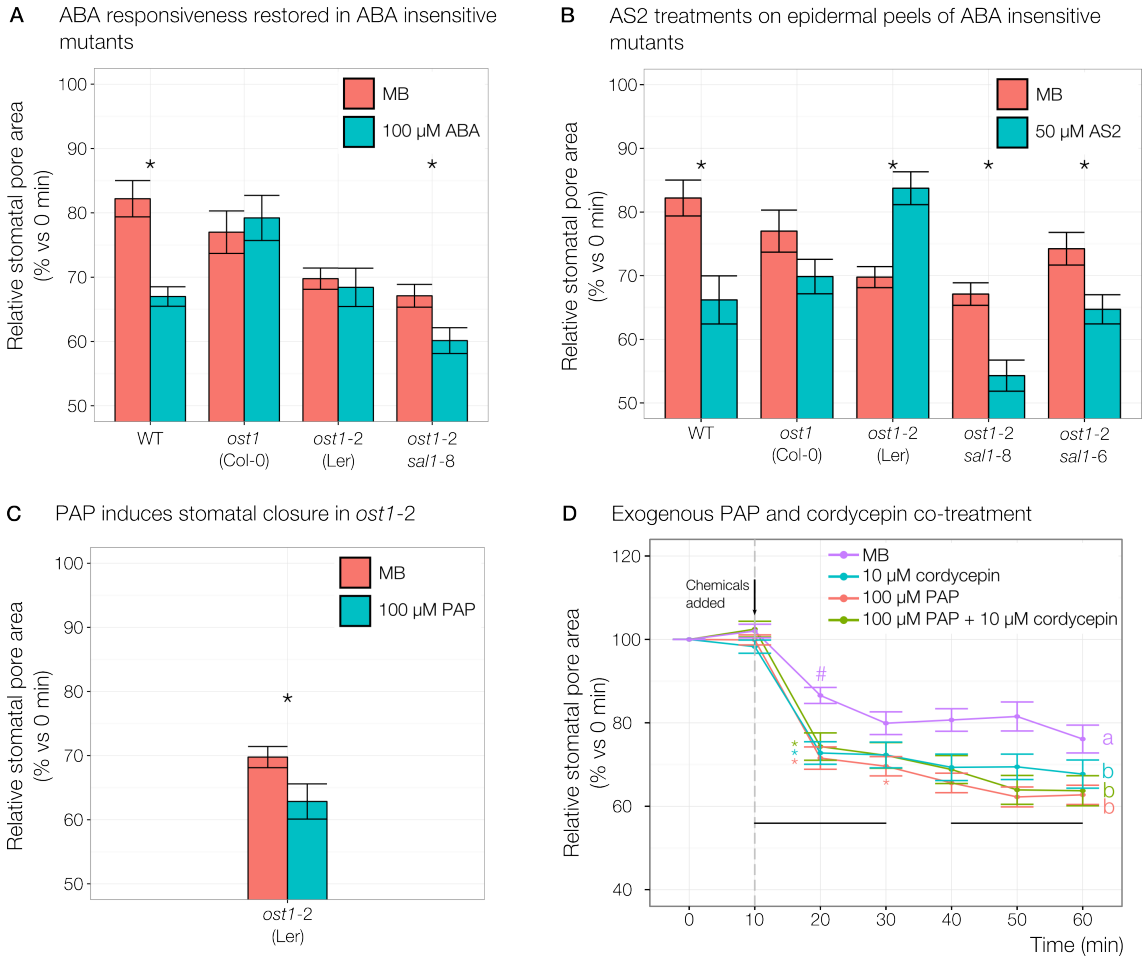

Figure 3.4: Lesions in SAL1 restores response to exogenous ABA
A Stomatal aperture, relative to 0 min, in epidermal peels of wild type (ColLer), *ost1* (Col-0), *ost1*-2 (Ler), and *ost1*-2*sal1*-8 plants after 30 minutes incubation with either MB only (control) or 100μM ABA. Bars denote means (n $>$ 10 stomata), error bars denote standard error of the mean. * denotes statistical significance ($p < 0.05$) between treatments within each genotype.
B Stomatal aperture, relative to 0 min, in epidermal peels of WT (ColLer), *ost1* (Col-0), *ost1*-2 (Ler), and *ost1*-2*sal1*-8 plants after 30 minutes incubation with either MB only (control) or 50μM AS2, a limited spectrum ABA analogue. Bars denote means (n$>$10 stomata), error bars denote standard error of the mean. * denotes statistical significance ($p < 0.05$) between treatments within each genotype.
C Stomatal aperture, relative to 0 min, in epidermal peels of *ost1*-2 (Ler) after 30 minutes incubation with either MB only (control) or 100μM PAP. Bars denote means (n = 41-75 stomata), error bars denote standard error of the mean. * denotes statistical significance ($p < 0.05$).
D Stomatal aperture, relative to 0 min, in epidermal peels of wild type (ColLer) plants treated with either MB only (control), 10μM cordycepin, 100μM PAP, or 100μM PAP + 10μM cordycepin over a period of 1 h. Points denote means (n=34-43 stomata), error bars denote standard error of the mean. Rates of closure were compared by linear mixed-effect modelling of closure between 10–30 min (log-transformed data), significant difference groups ($p < 0.05$) are denoted by #, *. Final level of closure was also considered by ANOVA and Tukey's HSD *post-hoc* analysis across the final 20 minutes; letters denote significance groups ($p < 0.05$).
Experiments performed and analysed with assistance from Estee Tee, Chenchen Zhao, and Kai Xun Chan.

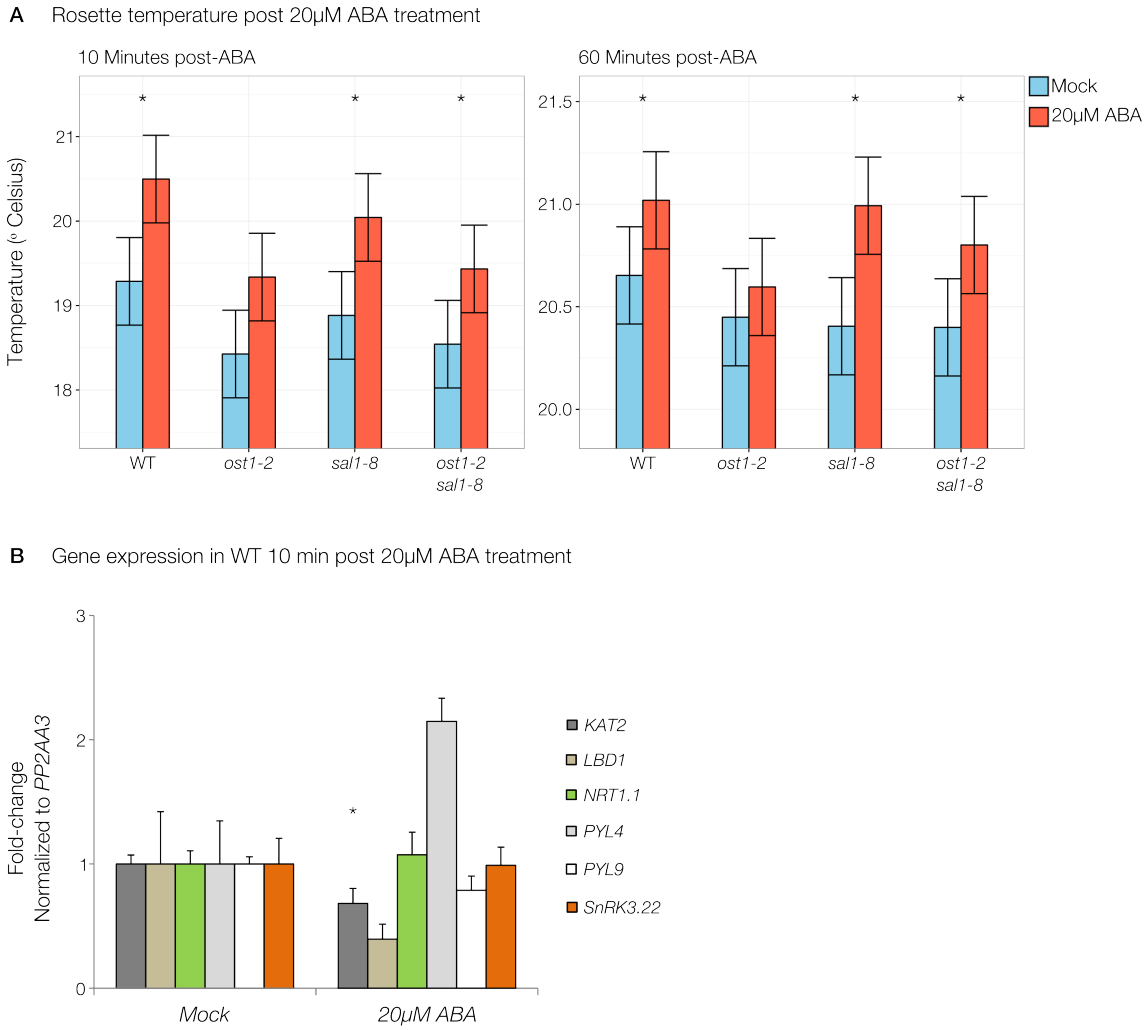

Figure 3.5: Transcriptional changes mediated by sal1-8 contribute towards constitutive restoration in ABA sensitivity

A Rosette temperature post-treatment of 20μM ABA. Independent experiments were performed to measure rosette temperature 10 and 60 minutes post ABA treatment. Bars denote computed least-squares means based off linear mixed-effects modelling ($R^2_C = 0.78$); error bars denote 95% confidence intervals; * denotes statistically significant differences (Tukey adjusted $p < 0.01$, n = 5-10) between treatments within each genotype.

B Normalized fold-changes in ABA-responsive guard cell localized transcripts that are highly up-regulated in *ost1*-2*sal1*-8 post 10 minutes of 20μM ABA treatment. Bars denote mean; error bars denote standard error of the mean (n=3); * denotes statistical significance ($p < 0.05$) as determined by a Student's t-test per transcript. WT = ColLer.

Experiments were performed with assistance from Kai Xun Chan.

3.2.4 Transcriptome analysis reveals constitutive PAP-mediated up-regulation of select CDPKs

Alongside the restoration of ABA sensitivity, global transcriptome reprogramming was also documented whereby *ost1*-2*sal1*-8 double mutants showed WT-like transcriptional response to ABA in comparison to the insensitive mutant *ost1*-2 (Pornsiriwong et al., 2017). This restoration of ABA-dependent gene expression is likely a consequence of the complementation by *sal1*-8, rather than the cause. If there is a transcriptional contribution towards PAP complementation of ABA sensitivity, then candidate gene(s) would need to collectively restore ABA responsiveness, and be either ABA-inducible in WT but constitutively up-regulated in *ost1*-2*sal1*-8 or be transcriptionally ABA-inducible in *ost1*-2*sal1*-8 but not in WT.

Mining of the *sal1*-8 and *ost1*-2*sal1*-8 transcriptomes identified a set of up-regulated loci encoding both characterized and putative ABA signalling components that match either of the previously described criteria[4]. Among the candidates were transcription factors and multiple kinases, including numerous CDPKs, which have the potential to restore ABA sensitivity as many are documented as key ABA signalling components (Boudsocq & Sheen, 2013). Significantly, many of the CDPKs in this list are related to group II CDPKs known to regulate the SLAC1 ion channel, a key target of SnRK2.6 (OST1), but their function remains unverified (Boudsocq & Sheen, 2013).

Thus, four largely uncharacterised CDPKs [CDPK32, CDPK34, CDPK-RELATED KINASE (CRK)2 and CRK8], which were also constitutively up-regulated in *ost1*-2*sal1*-8, were investigated to verify their ability to activate SLAC1. Towards this, qRT-PCR was performed to validate the original transcriptome profiling and confirm *CDPK* up-regulation in *ost1*-2*sal1*-8 and *ost1*-2*xrn2xrn3*. Indeed, all investigated *CDPKs* were constitutively up-regulated, irrespective of ABA treatment, in both *ost1*-2*sal1*-8 and *ost1*-2*xrn2xrn3* but did not show ABA induction in WT (**Table 3.1**). Consistent up-regulation in *ost1*-2*xrn2xrn3* aligns with the notion of transcriptional control via PAP-XRN signalling (Estavillo et al., 2011). Of the four CDPKs, CDPK34 showed the strongest up-regulation.

While guard cell expression of *CDPK32*, *CRK2*, and *CRK8* has been documented (Y. Yang et al., 2008; R.-S. Wang et al., 2011), it was unknown whether *CDPK34* was also expressed in guard cells. To confirm this, *in situ* RT-PCR (Athman et al., 2014) was performed in leaf peels of *ost1*-2*sal1*-8 (**Figure 3.6A**). Staining of *CDPK34* and *18S rRNA* transcripts showed a diffuse blue pattern across both pavement and guard cells, whereas the negative control (-RT) showed only large precipitates or no staining as expected. Importantly, staining of *CDPK34* transcript was evenly blue or punctate in guard cells consistent with guard cell localized *CDPK34* expression. When coupled to the observation that CDPK34 can activate SLAC1, to a greater extent that OST1,

[4]see Supplementary file 2 in Pornsiriwong et al. 2017

Table 3.1: Transcriptional up-regulation of select *CDPK* encoding genes in *ost1*-2*sal1*-8 and *ost1xrn2xrn3*

Gene	Fold-change vs WT				
	ost1-2*sal1*-8	*ost1xrn2xrn3*	*ost1*-2*sal1*-8 + ABA	*ost1xrn2xrn3* + ABA	WT + ABA
CDPK34	5.6±0.30	19.2±0.02	4.4±1.20	11.4±0.10	1.2±0.08
CDPK32	1.8±0.10	1.5±0.02	1.7±0.10	1.4±0.03	1.1±0.20
CRK2	2.0±0.40	1.8±0.10	3.1±0.70	3.7±0.08	1.0±0.10
CRK8	1.5±0.30	1.8±0.30	1.3±0.10	1.6±0.40	0.9±0.20

in *Xenopus laevis* oocytes (Pornsiriwong et al., 2017); constitutive up-regulation of CDPK34 in guard cells provides a mechanism for PAP-mediated restoration of stomatal closure.

3.2.5 mCHH hypo-methylation at short TEs in the sal1-8 methylome

The canonical mechanism for PAP-mediated gene expression changes is via the inhibition XRN enzymes based on *in vitro* evidence in yeast and *in planta* genetic evidence (Dichtl et al., 1997; Estavillo et al., 2011). The XRNs are a family of 5′-3′ exoribonucleases that are responsible for suppressing post-transcriptional gene silencing through 5′ processing of aberrant RNA species, including those arising from messenger RNA (mRNA) precursors (Gy et al., 2007; Kurihara et al., 2012; Nagarajan et al., 2013). Inhibition of these enzymes by PAP leads to the proliferation of sRNAs that, through a currently unidentified mechanism, regulates gene expression based on genetic evidence (Estavillo et al., 2011; Nguyen et al., 2015; Basbouss-Serhal et al., 2017). One potential is that the proliferation of such RNA molecules could also provide potential substrates for RDR enzymes, such as RDR2 and RDR6, which could lead to DCL-mediated siRNA molecules (Gazzani, 2004; Martínez de Alba et al., 2015; Tsuzuki et al., 2017). These could regulate gene expression either by inducing post-transcriptional gene silencing or act in the RdDM pathway to affect the methylome.

Whole genome bisulfite sequencing (WGBS) was performed on Col-0, *sal*-8, and *xrn2xrn3*[5] (**Appendix A**-Dataset 1 Table 1). Summary plots of mean methylation across the genome revealed prolific non-CG hypo-methylation in *sal1*-8, particularly across the body of TEs (**Figure 3.7A**). Surprisingly, this was not observed in *xrn2xrn3* that had a methylome more consistent to that of WT suggesting a mechanism independent of XRN2 and XRN3. Subsequently, *sal1*-8 mRNA-sequencing based transcriptome data (Crisp, 2015) was utilized to search for potential candidates causing the mCHH hypo-

[5]Data generated in conjunction with Peter Crisp

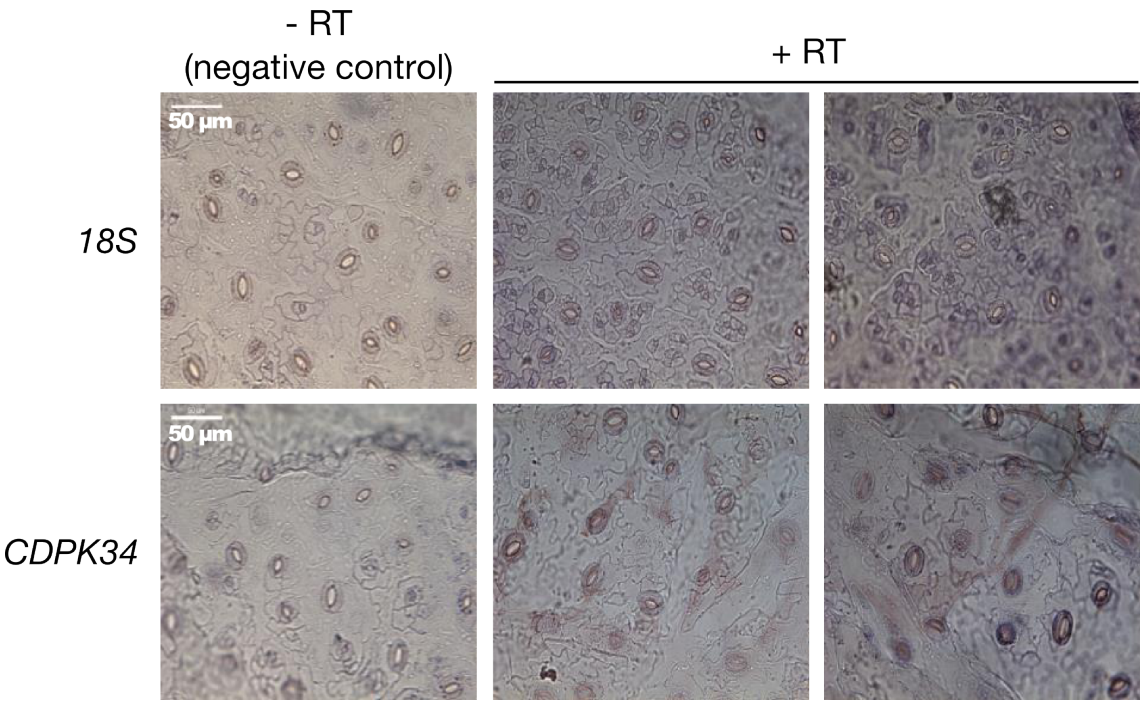

Figure 3.6: CDPK34 expression in guard cells
Representative images for guard cell expression of *CDPK34* and 18S rRNA (housekeeper) in *ost1-2sal1-8*, as detected by *in situ* RT-PCR on leaf peels. -RT denotes negative controls in which the reverse transcriptase was omitted, thus any staining in these slides occur from non-specific binding or precipitation of the stain. Similar results were observed in at least two biological replicates per gene.

methylation observed. A range of epigenomic factors including those involved in DNA (de)methylation, histone (de)methylation, chromatin remodelling, and sRNA biogenesis were collated to investigate if any such components were differentially expressed in *sal1-8* and *xrn2xrn3* (M. Matzke et al., 2009; Law & Jacobsen, 2010; Kurihara et al., 2012; Stroud et al., 2013, 2014; M. A. Matzke & Mosher, 2014; Ye et al., 2016). Indeed, several epigenomic machinery were differentially expressed including *ROS1*, which was strongly down-regulated, and a range of factors involved in RdDM, including the largest RNA Pol IV subunit *NUCLEAR RNA POLYMERASE D2B (NRPD2B)*, which were predominantly up-regulated (**Figure 3.7B**). Contrastingly, only a handful of components were differentially expressed in *xrn2xrn3* including both *XRN2* and *XRN3* themselves (**Figure 3.7C**). In both germplasm, expression of *SAL1* remained unperturbed. Interestingly, some factors were commonly differentially regulated between *sal1*-8 and *xrn2xrn3*, such as *ROS1*, *AGO9*, or *NRPD2B*, albeit the extent of altered expression was not comparable. For example, although *ROS1* was strongly down-regulated in *sal1*-8 it exhibited relatively attenuated down-regulation in *xrn2xrn3*; whereas stronger up-regulation of *AGO9* is observed in *xrn2xrn3* relative to *sal*-8. Regardless, the greater number of loci differentially expressed in *sal1*-8 may reflect the altered methylome evident in this mutant. Furthermore, the differential regulation of *ROS1* alongside numerous RdDM factors indicates potential molecular abnormalities potentially causing the mCHH hypomethylation.

The regulatory antagonism between *ROS1* and RdDM was investigated, in *sal1*-8 and *xrn2xrn3*, to test whether a decoupling of this relationship could explain the hypo-methylation observed. The methylomes of *ros1*-4 (Qian et al., 2012), and RNA Pol IV and V mutants (*nrpd1* and *nrpe1* respectively; R. Yang et al. 2017), important for the maintenance of methylation at *AT2TE68230*, were compared to that of *sal1*-8 and *xrn2xrn3*. Transcriptomes of *nrpd1* and *nrpe1* were also generated in the same study and thus was available for comparison (R. Yang et al., 2017). Both mRNA-sequencing and WGBS reads, from both datasets, were aligned to the TAIR10 genome and their profiles across *ROS1* were visualized in *IGV* (**Figure 3.7D**). *ROS1* down-regulation in *sal1*-8 is comparable to what is observed in *nrpd1* and *nrpe1*, where expression is almost negligible. Interestingly, *xrn2xrn3* shows attenuated *ROS1* down-regulation compared to what is observed in *sal1*-8. Comparable to both *nrpd1* and *nrpe1*, *sal1*-8 also shows mCHH hypo-methylation in the 5′ upstream region of *ROS1*, although the former mutants show almost complete depletion of mCHH, whereas *xrn2xrn3* methylation patterns remain intact. Although no mRNA-sequencing data was available for *ros1*-4, WGBS data revealed hyper-methylation, in all three methylation contexts, in the region 5′ of *ROS1*. Such observations are consistent with a relationship between *ROS1* expression and methylation levels in the adjacent TE, whereby reduced *ROS1* correlates with *AT2TE68230* hypo-methylation (Williams et al., 2015).

3.2.6 The sal1-8 methylome exhibits aberrant RdDM potentially involving factors beyond XRN2 and XRN3

The RdDM pathway has been described to target shorter euchromatic TEs as opposed to longer heterochromatic TEs that are methylated by a DDM1-CMT2 dependant pathway (Zemach et al., 2013; Stroud et al., 2014). Therefore, to test for a broader impairment in RdDM, methylation levels were measured across TE defined as short (length $<$ 1kb) or long (length $>$ 1kb) (**Figure 3.8A**). Indeed, mCHH levels were almost eliminated across short TEs in *sal1*-8 consistent with abnormal RdDM activity. Interestingly, this was not observed in *xrn2xrn3*, which displayed only minute differences suggesting that this observation is independent from altered XRN2 or XRN3 activity. A broad range of methylome datasets were analysed to further investigate a mechanism for hypo-methylation in *sal1*-8 (see **Appendix A**-Dataset 1 Table 1). From these datasets, genome-wide non-CG methylation patterns were compared across a range of mutants with lesions in key methylation machinery, such as *MET1*, *CMT2*, *CMT3*, and an array of RdDM factors with varying severity on mCHH levels (**Figure 3.8B**; Groth et al. 2014; M. A. Matzke & Mosher 2014; M. A. Matzke et al. 2015). From this broad exploratory analysis, the methylome of *sal1*-8 was found to correlate closely with that of moderate RdDM mutants, in particular the *dcl2dcl3dcl4* triple mutant. This is most evident in the mCHH context, where *sal1*-8, *dcl2dcl3dcl4*, *dcl1dcl2dcl3dcl4*, *idn2*, and *ago6* form a tight subcluster, which was part of a broader cluster with more severe RdDM impairments, such

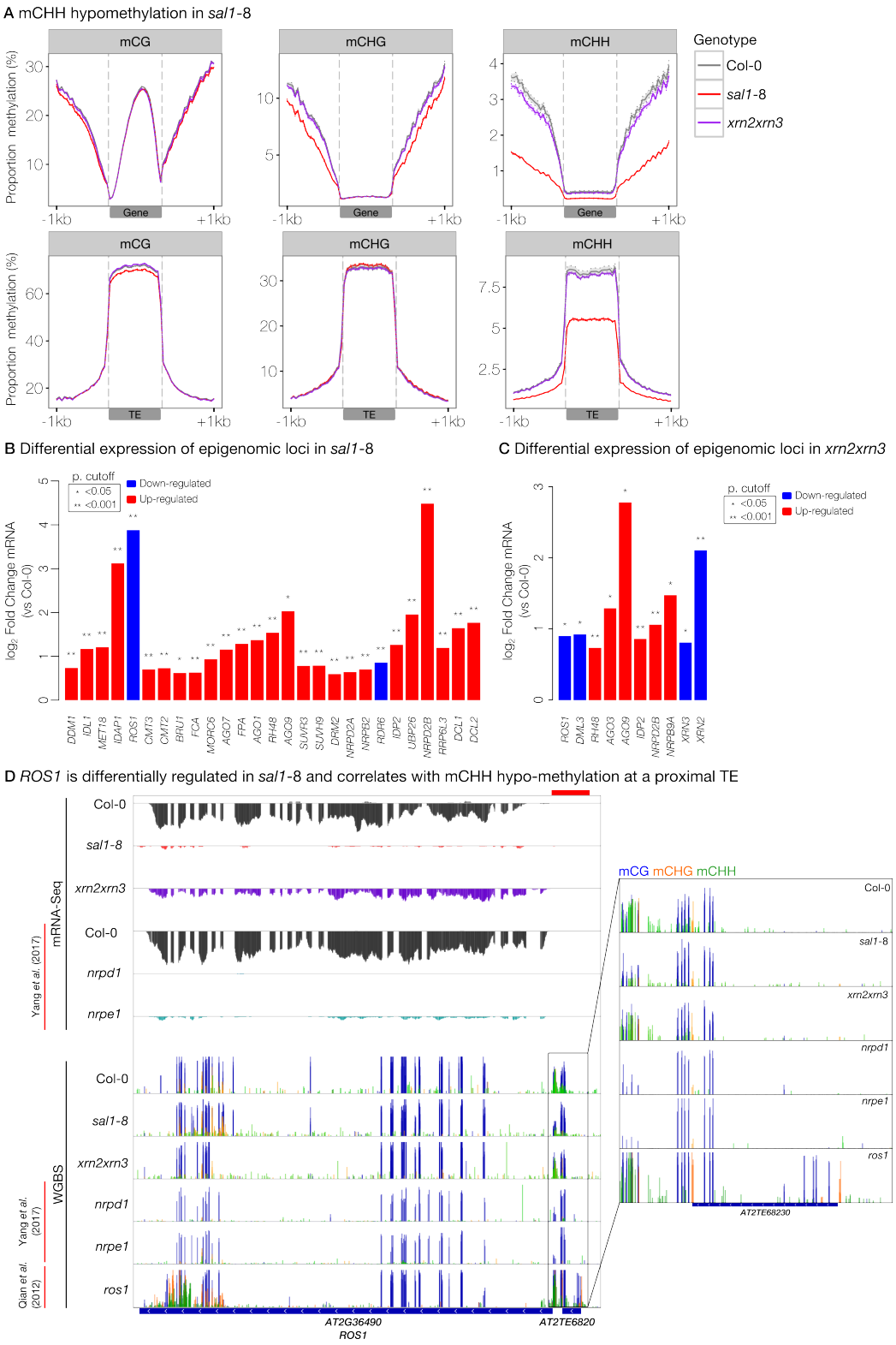

Figure 3.7: Lesions in SAL1 cause hypo-methylation in the methylome
A Meta plots of average methylation levels, in each sequence context, across all genes (top) and TEs (bottom) in the genome for Col-0, *sal1*-8, and *xrn2xrn3*. Lines denote mean proportion methylation, shaded region denotes standard error of the mean (n=3).
B-C fold-change (FC) in expression of epigenomic loci in *sal1*-8 (**B**) and *xrn2xrn3* (**C**) (vs Col-0) based on a previous mRNA sequencing dataset (Crisp, 2015). Bars denote mean $\log_2$FC, * denotes statistical significance, colour denotes direction of expression change (blue=down, red=up).
D Strand specific mRNA abundance (read depth) and DNA methylation levels (blue: mCG, orange: mCHG, green: mCHH; proportion methylation at single cytosines) across *ROS1* and a 5′ proximal TE (*AT2TE68230*), the methylation status of which has been linked to regulation of *ROS1* expression (denoted by horizontal red bar). Supplementary sequencing data was obtained from the corresponding references.

as *nrpd1* or *drm1drm2*. Conversely, the *xrn2xrn3* methylome correlated closer to that Col-0, particularly in mCHG. However, when observing mCHH levels, *xrn2xrn3* appeared to cluster most closely with the subtle *drm3* and *frg1frg2* RdDM mutants, which is consistent with an attenuated mCHH hypo-methylation compared to *sal1*-8. Genes encoding components of the RdDM are often co-expressed (Groth et al., 2014). Thus, the *sal1*-8 methylome correlates well with mutants containing lesions in known RdDM factors consistent with aberrant RdDM function, and potentially linked to the observed *ROS1* down-regulation.

To further investigate whether the correlation of methylome patterns may reflect aberrant functioning of the corresponding factors (e.g. *DCL2/3/4*), a co-expression analysis was performed between components of the RdDM and SAL1-PAP-XRN pathways; available in a collated mRNA-sequencing dataset through the ATTED-II database (Obayashi et al., 2017). Interestingly, *SAL1* expression (down-regulation leads to increased PAP) was strongly anti-correlated with numerous key RdDM components, including *DCL2/3/4* and *FRG2*; whereas *XRN2/3/4* showed only a modest positive correlation with these factors (measured by mutual rank index), suggesting that XRN function may contribute towards *sal1*-8 mCHH hypo-methylation. This observation also corresponds with the methylome correlations of *sal1*-8 with *dcl2dcl3dcl4* and *xrn2xrn3* with *frg1frg2*, however, whether this is indicative of a functional interaction requires further elucidation.

Methylation levels in sub-contexts, of canonical sequence contexts, have been reported to be informative about the mechanisms involved (Gouil & Baulcombe, 2016). Pertinently, contextual biases in mCHH methylation was found between the various CMTs and components of the RdDM pathway. To explore whether particular sub-contexts of mCHH methylation are depleted across TEs in *sal1*-8, thus potentially providing information about components impaired in *sal1*-8, re-analysis of tri-nucleotide sub-contexts of mCHH was performed. As a form of quality control, methylation bias in CAA and CTA tri-nucleotide sequences was re-established across TE subsets in the Col-0 methylomes generated here (**Figure 3.8C**). Subsequently, a focussed subset of the analysed methylomes were re-analysed based on the two-dimensional clustering of methylome patterns in Figure 3.8B, including *frg1frg2*, *dcl2dcl3dcl4*, *nrpd1*, *drm1drm2*, and *cmt2* (**Figure 3.9**). A comparison of the RdDM mutants revealed a depletion of all sub-contexts of mCHH that is particularly pronounced across the short TE subset, as expected, with varying severity from weaker (e.g. *frg1frg2*) to more severe RdDM lesions (e.g. *drm1drm2*). These patterns were mimicked in *sal1*-8 albeit to an attenuated extent compared to *drm1drm2*. The patterns between each of the RdDM components is difficult to distinguish with the exception of the severity of methylation loss, of which *sal1*-8 most closely resembles that of *dcl2dcl3dcl4* across short TEs corroborating the similarity observed in Figure 3.8B. However, there is also an attenuated, yet variable, depletion in CAA and CTA methylation that is comparable to levels in *frg1frg2* across

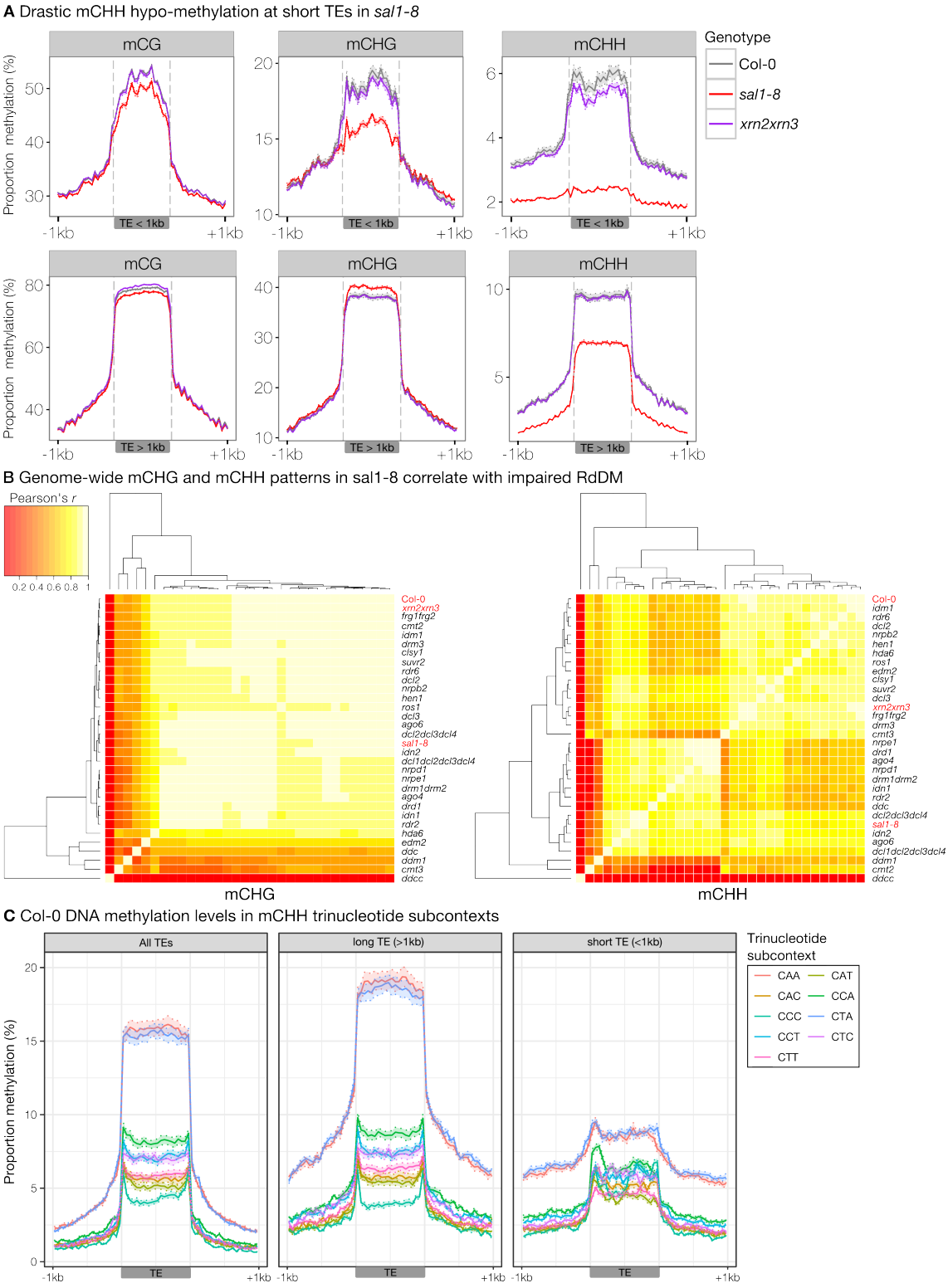

Figure 3.8: mCHH hypo-methylation in sal1-8 correlates with impaired RdDM
A Meta plots of average methylation levels, in each sequence context, across TEs shorter than 1 kb (top) and longer than 1kb (bottom) in the genome for Col-0, *sal1*-8, and *xrn2xrn3*. Lines denote mean proportion methylation, shaded region denotes standard error of the mean (n=3).
B Heat maps representing two-dimensional hierarchical clustering of correlations (Pearson's *r*) in genome-wide mCHG and mCHH levels, averaged across 100 bp bins, of a range of mutants with lesions effecting the methylome machinery alongside Col-0, *sal1*-8, and *xrn2xrn3*.
C Meta plots of average mCHH sub-context methylation levels, in Col-0, across TE subsets: all, short, (length $<$ 1kb), and long (length $>$ 1kb). Lines denote mean proportion methylation, shaded region denotes standard error of the mean (n=3).

short TEs. Despite a clustering of *xrn2xrn3* alongside *frg1frg2* previously, here *xrn2xrn3* shows WT levels of all sub-contexts of mCHH. Collectively, *sal1*-8 clearly demonstrates an impairment in RdDM that cannot be linked, as yet, with any single component. Instead, there may be an impairment in upstream processes, independent of XRN2 and XRN3, that is more broadly affecting downstream components. This would be consistent with the similarities observed between *sal1*-8 and the other RdDM mutants investigated here.

To determine the nature of TE mCHH hypo-methylation in *sal1*-8 and *xrn2xrn3*, heatmaps of average mCHH methylation per TE were produced. This allowed visualisation of whether the mCHH hypo-methylation observed was affecting all methylated TEs or a specific subset, as well as further exploring for any attenuated hypo-methylation in *xrn2xrn3*. A total of 3,599 TEs were identified as methylated, defined as exhibiting at least 5% mCHH (averaged across TE body) in all Col-0 samples. It is likely that many more TEs are methylated to this extent but were not captured due to a relative lack of sequencing depth, particularly crucial for accurately assaying mCHH (Eichten et al., 2016), which could be improved through re-sequencing. The assayable TEs were clustered into groups based on their length using the *k-means* method, allowing for discrimination of short and long TEs. The vast majority of the TEs captured were shorter in length, likely reflecting a lack of mapping to larger elements as opposed to fewer longer TEs meeting the mCHH cut-off. From this, the distinct targeting of the *drm1drm2* and *cmt2* pathways is reinforced (**Figure 3.10**). The *sal1*-8 methylome shows striking similarity to that of *dcl2dcl3dcl4*, where hypo-methylation can be observed to be broadly affecting TEs, not just at a specific subset. However, the severity of hypo-methylation appears to be intermediate between that of *dcl2dcl3dcl4* and *drm1drm2*, where mCHH is completely lost at short TEs. The methylome of *xrn2xrn3* and *frg1frg2* also show a striking similarity, reflective of a WT methylome thus conflicting with previous observations. This disparity likely the reflects differences between datasets as raw methylation levels are presented here as opposed to normalized values relative to the corresponding WT methylome, which has much higher levels of methylation (**Appendix A**-Dataset 1 Table 1). Regardless, the notion of impaired RdDM in *sal1*-8 is further reinforced here, effecting all methylated short TEs in a comparable manner as *dcl2dcl3dcl4*.

To further characterise the sites of mCHH hypo-methylation, DMR calling was performed using the *R* package *DSS* (see **Methods**, H. Feng et al. 2014). As expected, the *drm1drm2* and *cmt2* mutants showed an extensive number of mCHH DMRs, almost exclusively hypo-DMRs, despite the use of relatively stringent DMR criteria reflected in the reduced number of DMRs compared to previous studies (**Figure 3.11A**, **Appendix A** - Dataset 1 Table 4; Stroud et al. 2013, 2014). The *sal1*-8 mutant also exhibited prolific mCHH hypo-DMRs that were not evident in *xrn2xrn3*, which had far fewer DMRs (*sal1*-8: 4,038; *xrn2xrn3*: 104). Of the DMRs in *xrn2xrn3*, 67/104 (64%) sites were in common with *sal1*-8. Given a lack of detection of hypo-DMR in *xrn2xrn3*, further

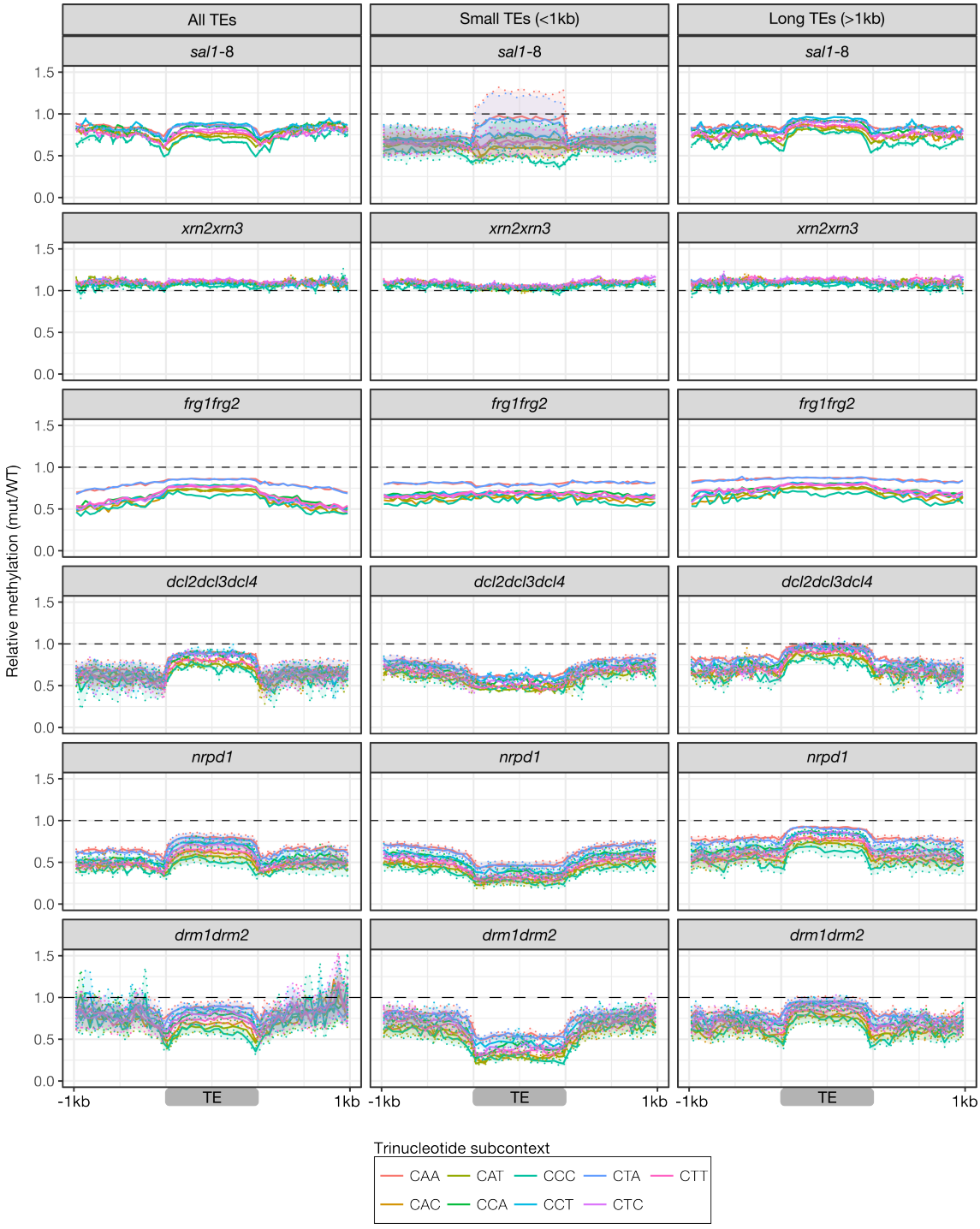

Figure 3.9: TE methylation levels in tri-nucleotide sub-contexts of mCHH in sal1-8
Meta plots of mean relative methylation (mut/WT), in tri-nucleotide sub-contexts of mCHH, in *sal1*-8 (n=3), *xrn2xrn3* (n=3), and comparable RdDM mutants (*frg1frg2*, n=1; *dcl2dcl3dcl4*, n=4; *nrpd1*, n=3; *drm1drm2*, n=3) based on two-dimensional clustering in **Figure 3.8**. Lines denote mean relative methylation (normalized to corresponding WT samples per experiment), shaded regions denote standard error of the mean.

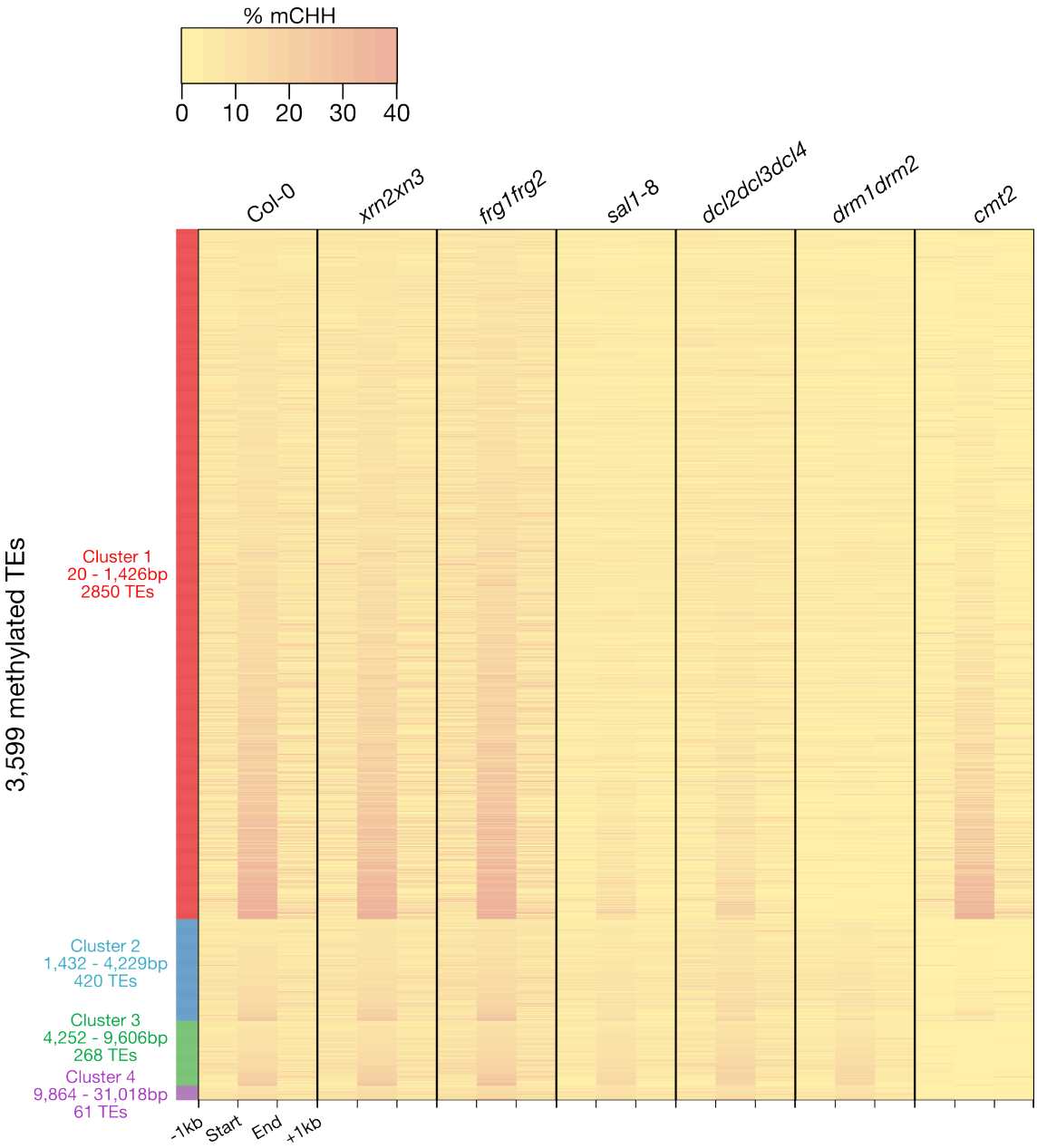

Figure 3.10: mCHH levels across methylated TEs
Mean mCHH levels at methylated TEs (minimum mCHH $>$ 5% in all Col-0 samples), grouped by length using *k-means* clustering (centers=4, method="MacQueen"); in Col-0 (n=4), *xrn2xrn3* (n=3), *frg1frg2* (n=1), *sal1*-8 (n=3), *dcl2dcl3dcl4* (n=4), *drm1drm2* (n=3), *cmt2* (n=2).

analyses focussed on hypo-methylated mCHH sites in *sal1*-8.

When mapped to all genomic elements, *sal1*-8 mCHH hypo-DMR were located predominantly within the body of short TEs (TE: 3,587; short TE: 2,428; long TE: 1,159; **Figure 3.11B**). However, there were still a substantial number (1,163) of DMRs within, or adjacent to, annotated protein coding genes potentiating the possibility of influencing gene expression. Furthermore, many of the DMRs mapping to TEs are also likely surrounding protein coding genes (explored further below).

Hypo-methylation mCHH regions in *cmt2* and *drm1drm2* were overlapped with *sal1*-8, and methylation levels across regions in each category of DMR (*sal1*-8 only, overlapping, or *drm1drm2*/*cmt2 only*) were visualized. There is a strong overlap in regions hypo-methylated in *sal1*-8 and *drm1drm2*, which are distinct from the regions hypo-methylated in *cmt2*. Furthermore, mCHH hypo-methylation in *sal1*-8 is most evident at sites where *drm1drm2* is also hypo-methylated, albeit to a differing magnitude, though it is not restricted to only these regions. Indeed, *sal1*-8 mCHH hypo-methylation is also evident at *cmt2* hypo-methylated regions. Interestingly, *xrn2xrn3* also exhibits reduced mCHH at *sal1*-8 hypo-methylated sites, consistent with having an attenuated mCHH hypo-methylation observed in *sal1*-8. Collectively, these results support the notion that *sal1*-8 is a moderate RdDM mutant.

3.2.7 Effects of aberrant RdDM on gene and TE expression

Given the nature of RdDM targeting to short TEs adjacent to protein-coding genes (X. Zhong et al., 2012; Zemach et al., 2013; Q. Zheng et al., 2013), aberrant functioning has the potential to effect gene expression. To test if loss of mCHH in *sal1*-8 was associated with gene expression changes, mRNA-sequencing data was re-analyzed to measure mRNA abundance at sites in *sal1*-8 and *drm1drm2* exhibiting mCHH hypo-methylation (**Figure 3.12A**). When a random set of 5,000 genes were observed, there was only minor deviation in mRNA abundance (measured as FPKM) that did not meet statistical significance. However, when mRNA abundance at *sal1*-8 and *drm1drm2* mCHH hypo-DMRs were observed, *sal1*-8 and *xrn2xrn3* both showed a statistically significant increase in mRNA abundance, across these sites, as compared to Col-0, but not in comparison with each other. These results suggest that mRNA abundance is effected at mCHH hypo-methylated sites in *sal1*-8. However, that *xrn2xrn3* also showed altered mRNA abundance, despite having attenuated mCHH hypo-methylation, may not fit this hypothesis.

Changes in DNA methylation are considered to convey regulatory effects on adjacent genes, such as by defining local chromatin state that could either facilitate or inhibit transcription (euchromatin vs heterochromatin) or by stabilizing transcription factor binding sites (Maurano et al., 2015; O'Malley et al., 2016; Niederhuth & Schmitz, 2017). To further investigate whether the mCHH hypo-methylation could contribute to altered gene expression, *sal1*-8 hypo-DMRs were overlapped with DEGs identified from previously

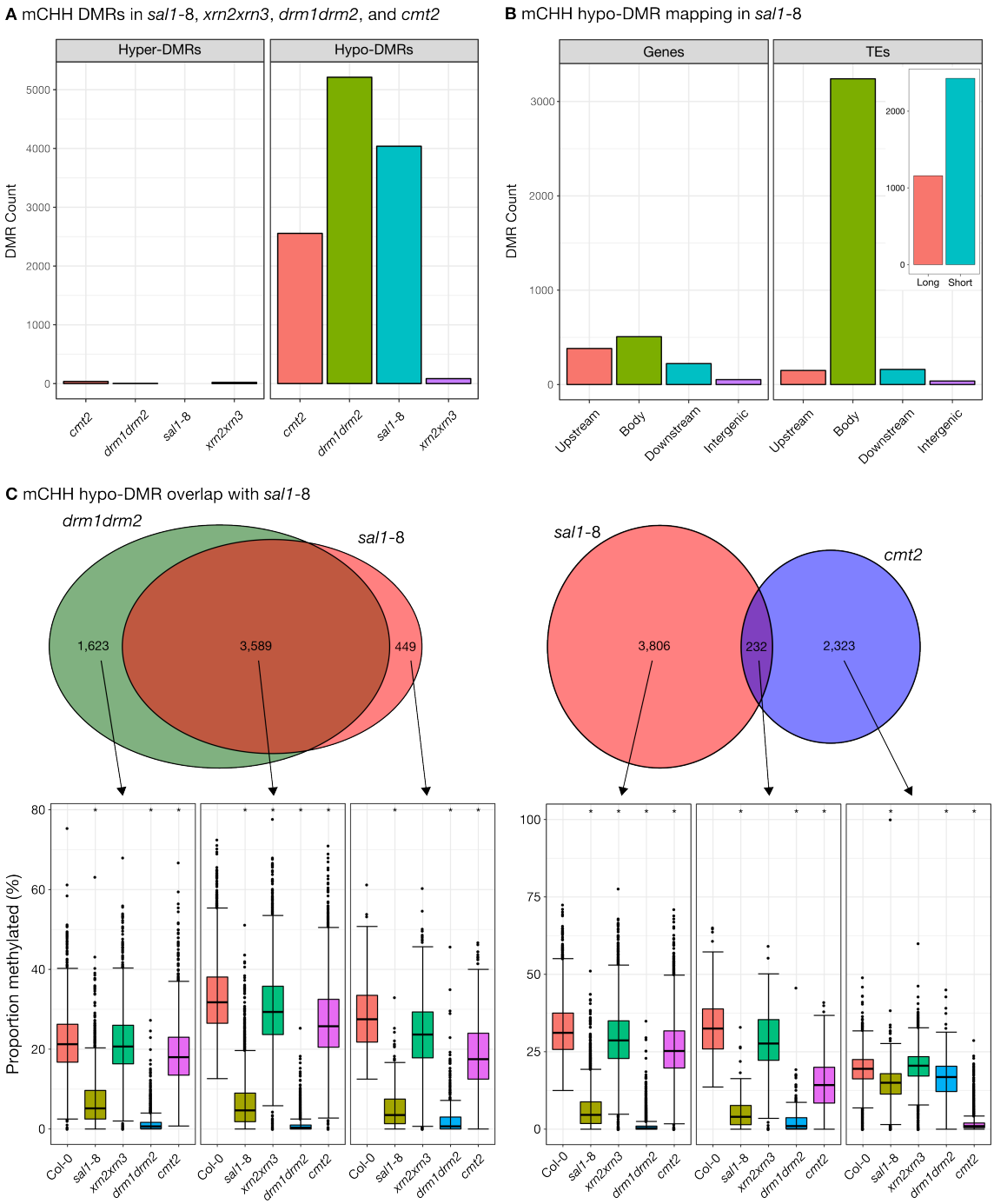

Figure 3.11: sal1-8 mCHH hypo-DMRs overlap with drm1drm2
A Number of hyper- and hypo-mCHH DMRs in *sal1*-8, *xrn2xrn3*, *drm1drm2*, *cmt2*.
B Detailed mapping of *sal1*-8 mCHH hypo-DMRs to the nearest genomic element, including annotated protein coding genes and TE. Body refers to DMRs occurring within an element. Upstream and downstream refers to DMRs within 1kb, at either 5′ or 3′ end respectively, of a genomic element. Intergenic DMRs are those further than 1kb away from the nearest element. Inset shows the counts of each size class of TEs (short, $<$ 1kb; long, $>$ 1kb) to which mCHH hypo-DMRs mapped.
C Overlap between *sal1*-8 and *drm1drm2* or *cmt2* mCHH hypo-DMRs. Box plots of mean mCHH methylation levels across regions in each DMR category for all mutants. * denotes a significant difference in distribution of mean methylation levels compared to Col-0 (Benjamini-Hochberg adjusted P $< 10^{-10}$) as determined by Dunn's test of multiple comparisons.

analysed mRNA-sequencing data (**Appendix A** - Dataset 1 Table 5[6], Crisp 2015). The 4,038 mCHH hypo-DMRs in *sal1*-8 were mapped to 3,148 annotated protein coding genes (where multiple DMRs might map to the same nearest gene), of which 2,010 were measurable in the mRNA-sequencing dataset (**Appendix A** - Dataset 1 Table 6). From the 2,010 measurable genes, to which a mCHH hypo-DMR was mapped, 819 ($\approx$ 41%) were significantly differentially expressed in *sal1*-8. To test whether there was an association between the presence of a hypo-DMR and gene expression, the position of DMRs relative to all genes and significant DEGs, in *sal1*-8, were compared. One hypothesis could be that hypo-methylation in the promoter regions could lead to up-regulated gene expression, which might be reflected in an enrichment of hypo-DMRs in the promoter regions of DEG in *sal1*-8. However, a comparison of DMR positions revealed a similar distribution for all genes and DEGs (**Figure 3.12B**).

While no overall enrichment of DMRs in promoter regions of DEGs was observed, this does not rule out the potential for individual associations with gene expression changes. Indeed, there are multiple characteristics of DMRs that might effect such an association, including their position, distance to transcriptional start site, and the magnitude of change observed. Thus, the difference in mRNA abundance ($\log_2$ absolute change in FPKM; *sal1*-8 - Col-0), for all significant DEGs in *sal1*-8 to which a DMR was mapped within 1kb distance, was plotted by DMR position relative to the transcriptional start site and magnitude of methylation difference (**Figure 3.12C**). The majority of DMRs were found to map to non-DEG with a slight bias towards the promoter region (as defined here), which is unsurprising given the targeting of RdDM towards such regions (X. Zhong et al., 2012; Q. Zheng et al., 2013). As the differential methylation observed is exclusively hypo-methylation, it was expected that it should correlate with up-regulated gene expression. However, there were equal numbers of up- and down-DEGs observed. Indeed, the strongest DEGs were found to be down-regulated, opposite to what might be expected. This does not preclude changes in transcription factor binding, as a consequence of differential methylation, leading to altered gene expression (Maurano et al., 2015). Indeed, an analysis to see if there are *cis*-elements, upstream of DEGs, that are targeted by methylation sensitive transcription factors, using the epicistrome dataset (O'Malley et al., 2016), may reveal a novel form of gene regulation in *sal1*-8. In the case of up-regulated DEGs, an obvious hypothesis is that there is a release of repressive DNA methylation leading to increased transcription. In both cases, further investigation is required validate any true interactions, as well as delineate other regulatory mechanisms. Regardless, of the prolific hypo-methylation observed in *sal1*-8, there is clearly the potential for some of these changes to be effecting gene expression.

A key site of hypo-methylation in *sal1*-8 is at short TEs, reflective of the role of RdDM to survey the genome and silence such elements by establishing repressive non-CG methylation (Stroud et al., 2014). In the case of *sal1*-8, this seemed unlikely given that only one

[6]DEG analysis performed by Peter Crisp

context of methylation had been effected (whereas *ddcc* has almost eliminated nonCG methylation), and, to an attenuated extent compared to many canonical RdDM mutants, that can also show hypo-methylation in multiple contexts of methylation (Stroud et al., 2013, 2014). Nonetheless, to investigate for aberrant TE transcription, mRNA abundance across the three investigated TE subsets was measured (**Figure 3.12C**). The canonical RdDM mutants *nrdp1* and *nrpe1* as well as the *drm1drm2cmt2cmt3* quadruple mutant (*ddcc*), which demonstrates prominent TE de-repression (Stroud et al., 2014), were included as points for comparison. As expected, *xrn2xrn3* showed no expression of TEs. On the other hand, *sal1*-8 showed TE expression to a comparable extent as the *ddcc* quadruple mutant. Not only did this contradict the initial expectation, but the expression seemed to largely originate from longer TEs. Whether or not these are truly active and mobile TEs, however, remains to be validated but are an interesting point for further investigation, such as the identification of those TEs contributing to the signal observed here.

3.3 Discussion

The SAL1-PAP-XRN pathway has long been identified as promoting oxidative stress tolerance in Arabidopsis, particularly in response to drought (Xiong et al., 2001; Rossel et al., 2006; Wilson et al., 2009; Estavillo et al., 2011), however, the exact mechanism for this remained enigmatic. Some evidence suggested that transcriptional changes may be facilitated through the PAP-mediated inhibition of its targets, the XRN enzymes. Indeed, various studies report altered RNA metabolism in *sal1* mutants, where PAP can no longer be catabolized and thus accumulates, leading to altered expression of stress-inducible genes that is phenocopied in the *xrn2xrn3* mutant (Rossel et al., 2006; Gy et al., 2007; Estavillo et al., 2011; Kurihara et al., 2012; Kurihara, 2017). A hitherto unknown was whether the proliferation of aberrant RNA molecules might also trigger altered RdDM, although preliminary evidence suggested otherwise (Kurihara et al., 2012). Here, data is presented confirming PAP as a signalling molecule that is capable of inducing stomatal closure in and of itself. Furthermore, PAP shows the ability to cross-activate downstream components of the ABA pathway transcriptionally, however, the mode of action for this requires further investigation. It was also observed that genetically elevated PAP led to an impairment of RdDM, likely involving factors beyond the XRN2/3, potentiating the existence of novel PAP targets or effects that require elucidation. A new model of the SAL1-PAP-XRN pathway is proposed with the added insight of its contribution towards guard cell closure and potential point of interaction with the RdDM pathway (**Figure 3.13**).

PAP perception induces rapid stomatal closure

The PAP signalling pathway has been characterised over the previous decade (Xiong et

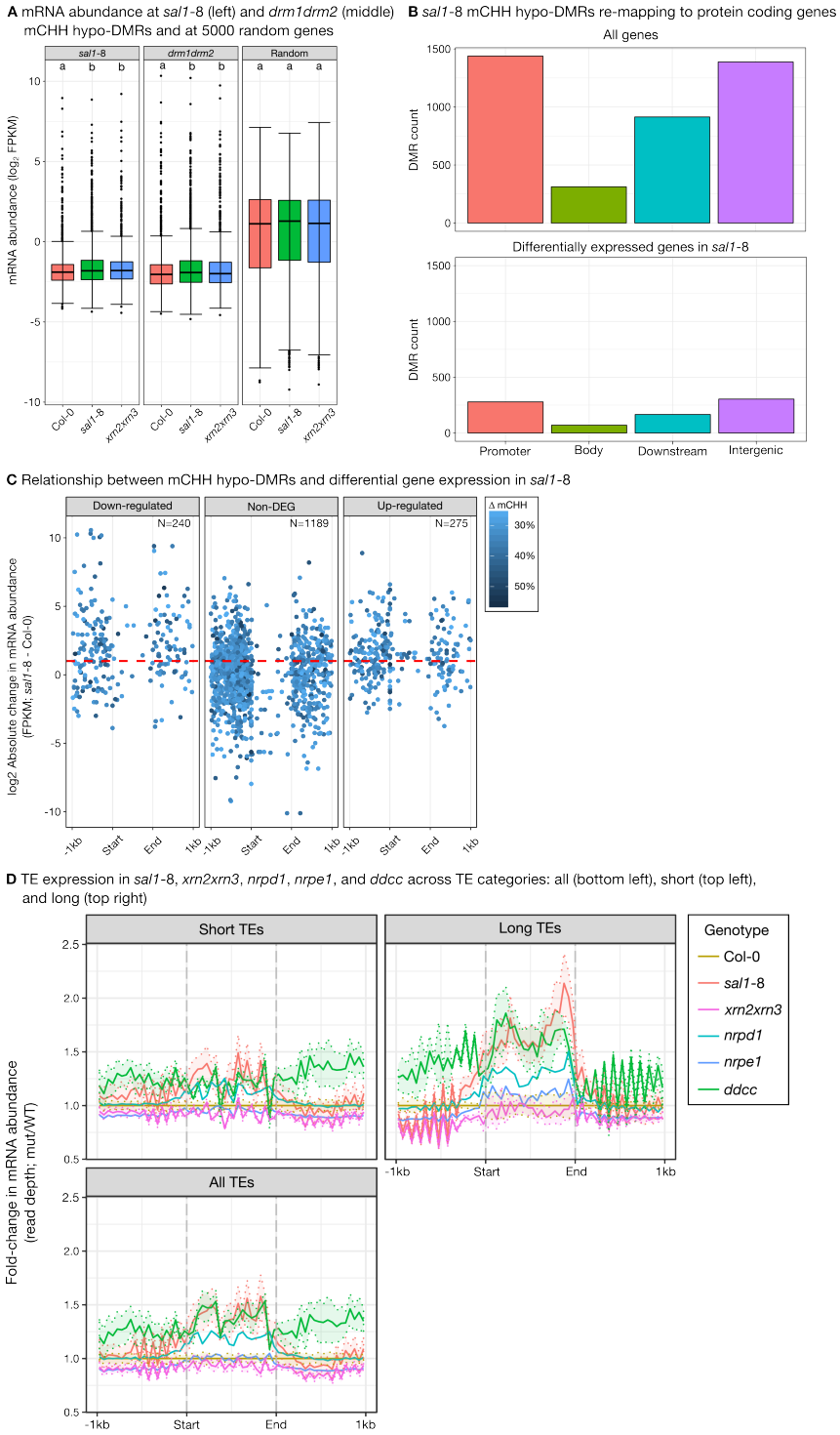

Figure 3.12: Overlapping mCHH hypo-DMRs with gene expression in sal1-8
A mRNA abundance (log_2FPKM) at *sal1*-8 (left) and *drm1drm2* (middle) mCHH hypo-DMRs, and at 5,000 random genes (right). Letters denote significance groups (adjusted $P < 10^{-10}$) as determined by Dunn's test of multiple comparisons.
B Mapping of *sal1*-8 mCHH hypo-DMRs to all annotated protein coding genes and DEGs in *sal1*-8. Body refers to DMRs occurring within a gene. Promoter and downstream refers to DMRs < 1kb away from the 5′ or 3′ end of the gene, respectively. Intergenic DMRs are those further than 1kb away from the nearest gene.
C Scatter plot of change in mRNA abundance (log_2 absolute difference in FPKM; *sal1*-8 - Col-0) by DMR position relative to the transcriptional start site (start). Separate plotting was performed for non-DEG, up-regulated, and down-regulated genes, in *sal1*-8. Colour denotes mCHH change at the DMR. Red dashed line denotes 2x FC in FPKM.
D Meta plots of mean FC in mRNA abundance (read depth) across TE categories (all, short, long TEs) in *sal1*-8 (n=3), *xrn2xrn3* (n=3), *nrpd1* (n=1), *nrpe1* (n=1), and *ddcc* (n=2) normalized to corresponding WT samples (mut/WT). Lines denote means, shaded region denotes standard error of the mean.

al., 2001; Rossel et al., 2006; Wilson et al., 2009; Estavillo et al., 2011; Gigolashvili et al., 2012; Chan, Mabbitt, et al., 2016). However, whilst its accumulation and transport are well elucidated, its exact cellular effects remain enigmatic despite physiological observations on genetic mutants showing developmental abnormalities, enhanced drought stress tolerance, and the differential regulation of >2000 genes that were enriched as stress-responsive or involved in ABA signalling (Xiong et al., 2001; Rossel et al., 2006; Wilson et al., 2009; Estavillo et al., 2011). One hypothesis was that PAP affects guard cell responsivity as the stomatal index in *sal1* mutants was unchanged under well-watered conditions, however, could maintain viable tissue for longer periods of time under drought (Wilson et al., 2009). Indeed, a key finding here was the direct observation that PAP induced guard cell closure to a similar extent as ABA. In both cases, closure was observed to be relatively rapid, occurring within 10 minutes, matching observations of rapid ABA-induced anion currents in intact guard cells (Y. Wang et al., 2013). Whether PAP signalling is functional beyond Arabidopsis, and the nature of PAP-mediated guard-cell closure in other species, warrants further investigation. ABA signalling is an ancient conserved pathway that is prolific across land plant species, including multiple terrestrial ferns (Cai et al., 2017). Emerging evidence suggests that PAP signalling could also be conserved as *SAL1* homologs, with the inclusion of key cysteine residues for SAL1 redox regulation and PAP accumulation, can be found in multiple plant species beyond Arabidopsis, including grasses and rice, and is even present in yeast and humans (Chan, Mabbitt, et al., 2016). However, this might reflect the conservation of the components utilized for sulfur metabolism, thus whether there is a conserved response, including both molecular and physiological responses, to PAP requires further investigation.

PAP-mediated closure was expedited in WT plants when co-treated with ATP, which prevents PAP transport into the chloroplast where it is degraded by SAL1 (Estavillo et al., 2011; Gigolashvili et al., 2012), consistent with the notion that PAP is a signal whose efficacy can be altered through biochemical manipulation of its transport. Additionally, this result is also consistent with the notion that PAP is being sensed outside of, although its catabolism occurs within, the chloroplast (Estavillo et al., 2011). Alongside the observations of altered guard cell dynamics, the enrichment of differentially regulated ABA signalling genes in *sal1* mutants raised the possibility for cross-talk between these pathways (Wilson et al., 2009). Indeed, using the epidermal peel system, it was confirmed that lesions in SAL1 restored ABA sensitivity in the otherwise ABA insensitive *ost1* mutants giving credence to the possibility that PAP might be activating components down-stream of SnRK2.6 to promote stomatal closure. Further evidence of this came from exogenous application of PAP directly onto guard cells of *ost1*-2, which also induced guard cell closure whereas application of ABA did not.

In order to mediate such cellular responses, PAP must first be perceived. Whereas the highly conserved PYL family of ABA receptors have now been extensively researched and elucidated (Cutler et al., 2010; M.-T. Hauser et al., 2011; Murata et al., 2015), there is

less information regarding *bone fide* receptors of PAP. The selective PYL activator AS2 (Takeuchi et al., 2014) was used to test whether PAP was effecting the functionality of the PYL-mediated ABA signaling cascade in *ost1*-2*sal1*-8 and *ost1*-2*sal1*-6. However, exogenous application of AS2 still induced stomatal closure in both double mutants, suggesting that the specific PYL receptors that are not activating by AS2 (PYR1, PYL1, PYL2 and PYL3) are not involved in the restoration of ABA responsiveness. It is also unclear how the activation of select PYL proteins induces stomatal closure in the absence of functional SnRK2.6 but presents an intriguing possibility for SnRK2.6-independent PYL induction of guard cell channels to induce stomatal closure, potentially through the stimulation of ROS or Ca^{2+} channels (Y. Wang et al., 2013).

Hitherto, the exact mechanism for PAP perception has largely been considered to be through interaction with their canonical targets, the XRN enzymes (Dichtl et al., 1997; Gy et al., 2007; Estavillo et al., 2011; Kurihara et al., 2012; Kurihara, 2017). However, primary evidence for this interaction was identified in yeast and analogous *in planta* evidence is lacking. A recent attempt failed to identify such an interaction also, although it did reveal the potential for PAP to be bound by a range of other enzymes (Crisp, 2015). Furthermore, an *xrn2xrn3xrn4* triple mutant is morphologically distinct from *sal1*, for example, showing relatively WT-like growth patterns compared to the stunting observed in *sal1* (Kurihara, 2017), further potentiating the possibility of PAP targets beyond the canonical XRN enzymes. Nonetheless, there are still significant molecular commonalities between *xrn2xrn3* and *sal1* mutants in favour of a SAL1-PAP-XRN pathway, and an intermediate factor(s) acting between PAP and XRNs, in plants, cannot be discounted but requires identification.

While the transcriptome changes induced by lesions in *sal1* may be reflective of constitutive PAP-mediated inhibition of the nuclear XRNs, given the high degree of transcriptional overlap previously observed (Estavillo et al., 2011), it is questionable whether such a mechanism is responsible for the rapid closure observed within 10 minutes of exogenous PAP application. In support of a transcriptionally-independent mechanism, co-treatments with cordycepin did not attenuate PAP-mediated stomatal closure; nor were there appreciable changes in transcript levels for genes that might readily explain the induction of stomatal closure, after 10 minutes of ABA treatment, despite an appropriate physiological response (stomatal closure indirectly assayed through increased leave temperature upon ABA treatment). The transcriptional inhibition via cordycepin co-treatment was not the ideal experiment, however, due to the closure induced by cordycepin alone, thus it would be worthwhile to repeat this and find an optimal concentration of cordycepin that has no effect alone. An alternative transcriptional inhibitor, actinomycin D, might also prove more suitable if it does not induce closure itself (Bensaude, 2011). Regardless, it is possible that PAP is binding a yet to be identified protein(s), from a range of putative candidates (Crisp, 2015), that is responsible for PAP perception resulting in stomatal closure, akin to PYL receptors for ABA allowing for the activation

of SnRK2 kinases (Boudsocq et al., 2007; Park et al., 2009; Cutler et al., 2010). Thus, it is likely that the broad transcriptome reprogramming observed in *sal1*-8*ost1*-2, compared to *ost1*-2, might be a consequence of restored stomatal closure. However, this does not preclude a PAP-XRN-mediated activation of specific components to restore ABA sensitivity.

PAP recovers ABA signalling by transcriptionally priming down-stream components of the pathway

Similar to ABA, perception of PAP could also be considered to have multiple effects. While the primary impact is to induce mechanical closure of stomates, there is also the potential for down-stream PAP-XRN mediated changes in gene expression, akin to those induced ABA-ABF in addition to the transcriptional reprogramming evident in *sal1* mutants. While the majority of this reflects restoration of gene expression to wild type[7], there are a category of genes constitutively up-regulated in *ost1*-2*sal1*-8 by PAP. In particular, *CDPK34* was constitutively up-regulated, to comparable extents, in both the *ost1*-2*sal*-8 and *ost1*-2*xrn2xrn3* double mutants. *CDPK34* was also shown to be expressed in guard cells, to have the capacity to activate the SLAC1 anion channel, and is known to be expressed on the plasma membrane consistent with SLAC1 localization (Boudsocq et al., 2007; Negi et al., 2008). Thus, increased activity of *CDPK34* could circumvent the loss of SnRK2.6 (*in the ost1-2*), the predominant SnRK2 kinase controlling guard cell turgor (Virlouvet & Fromm, 2015), especially as *CDPK34* itself has greater propensity to be activated through Ca^{2+} sensing rather than by SnRK2.6 phosphorylation (Boudsocq & Sheen, 2013; Simeunovic et al., 2016).

While *CDPK34* was the strongest up-regulated CDPK tested, this does not rule out involvement from other CDPKs. Indeed, the restoration of SLAC1 activation could involve combinatorial action, between multiple CDPKs, to compensate for loss of SnRK2.6. It remains unclear if up-regulation of CDPK34 is sufficient to restore SLAC1 activity or whether any of the other up-regulated CDPKs may also be contributing towards SLAC1 activation. Indeed, there is now a growing list of CDPKs that are capable of activating the SLAC1 channel (Geiger et al., 2010). One hypothesis might be a direct interaction with PAP, enabling post-transcriptional control, however, a previous study found no CDPKs being bound by PAP (Crisp, 2015). To test whether CDPK34 is key for the restoration of ABA sensitivity, an*ost1sal1cdpk34* triple mutant could be generated in which ABA sensitivity , or over-express *CDPK34* in *ost1*-2, and characterise ABA sensitivity. Furthermore, CDPKs are also capable of activating additional transporters on the guard cell plasma membrane, including various potassium transporters, as well as numerous ABF transcription factors (Boudsocq & Sheen, 2013; Simeunovic et al., 2016). Whether these are additional functional mechanisms that contribute towards restored ABA sensitivity and stomatal closure in *ost1*-2*sal1*-8 requires further validation.

[7]see Pornsiriwong et al. 2017

SAL1 stress signalling interacts with the RdDM pathway

While *SAL1* was originally identified as a regulator of stress signalling and tolerance (Xiong et al., 2001; Rossel et al., 2006); it has also been identified from screens implicating it as a suppressor of RNA silencing (also referred to as *FIERY1/FRY1*), through its inhibition of the XRNs (Gy et al., 2007). While the proliferation of XRN-dependent non-coding RNAs has been characterised in an independent *sal1* mutant (Gy et al., 2007; Kurihara et al., 2012), it has also been hypothesized that such molecules could be substrates for RDRs resulting in the proliferation of siRNAs that could have further regulatory effects, namely transcriptional gene silencing via RdDM (Gregory et al., 2008; Crisp, 2015). Indeed, methylome profiling of *sal1*-8 revealed prolific hypo-methylation, contrary to the expected hyper-methylation, specifically in the mCHH context at RdDM sites (defined by regions targeted by DRM1/2). The severity of this hypo-methylation was not as severe as that found in canonical RdDM mutants, including *nrpd1* or *drm1drm2*, and shows characteristics similar to other moderate RdDM mutants, such as *frg1frg2* (Groth et al., 2014), *drm3* (X. Zhong et al., 2015), and, in particular, *dcl2dcl3dcl4* (Stroud et al., 2013). Each of these factors contribute towards proper RdDM, however, are not as essential as other core components, thus exhibiting attenuated, but not eliminated, methylation levels at RdDM sites. The observations of the *sal1*-8 methylome here are similar to those documented for PICKLE (PKL), an annotated chromatin remodeler involved in regulating plant development that is also required for proper RdDM through stabilization of nucleosomes to facilitate RNA Pol V function (R. Yang et al., 2017). However, in this case, SAL1 itself does not directly associate with chromatin, but is, instead, an established component of the SAL1-PAP-XRN retrograde stress signalling pathway that is localized to the chloroplast, a completely different sub-cellular compartment (Estavillo et al., 2011). Thus, this is the first report of a stress signalling component that shows cross-talk with the RdDM pathway providing a potential point of interaction between abiotic stress and the methylome.

While the mechanism for the interaction between PKL and RdDM has been elucidated, through its nucleosome positioning activity leading to RNA Pol V stabilization (R. Yang et al., 2017), that between the chloroplast-localized SAL1 and RdDM has proved elusive. Indeed, previous investigations failed to identify an altered methylome in an independent *sal1* allele (*sal1*-6; Kurihara et al. 2012). This raises concerns regarding whether PAP is truly responsible for the impaired RdDM observed here. Re-sequencing of the *sal1*-6 methylome using recent library preparation techniques would establish whether the previous report was affected as a result of early WGBS methods. Additionally, sequencing a Col-0 methylome after long-term PAP treatment may also provide evidence towards confirming an affect of PAP on RdDM. Given that PAP levels are known to be strongly elevated in *sal1*-8 (Estavillo et al., 2011; Pornsiriwong et al., 2017), the results presented here will be discussed in the context of presenting evidence towards the poten-

tial for the SAL1-PAP-XRN pathway to influence the Arabidopsis methylome, without the aim of diminishing the importance for further validation using one of aforementioned strategies.

Based on the canonical SAL1-PAP-XRN retrograde pathway, an initial hypothesis was that PAP-mediated inhibition of XRN activity could lead to the proliferation of aberrant RNA molecules providing substrates for siRNA biogenesis that could then be recruited by AGO1 or 4 for post-transcriptional gene silencing or RdDM, respectively (M. A. Matzke et al., 2015). However, in such a scenario, one might expect to observe mCHH hyper-methylation due to a proliferation of siRNA guide molecules given the expected strong positive correlation between siRNA abundance and methylation (Cokus et al., 2008; Lister et al., 2008). However, in light of the complex nature of siRNA biogenesis and RdDM, there may yet be more complex interactions leading to hypo-methylation despite a hypothetical proliferation of siRNA. Whether or not RNA metabolism, specifically siRNA biogenesis, has been perturbed in a manner consistent with aberrant RdDM remains unclear. Previous attempts to characterise sRNAs in *sal1* mutants did not show an accumulation of 24nt siRNA clusters but, instead, suggested an increase in mRNA cleavage products and non-coding 3′ extensions of transcripts that was phenocopied by *xrn* mutants (Gy et al., 2007; Kurihara et al., 2012; Crisp, 2015). Performing sRNA-sequencing in *sal1*-8, *xrn2xrn3*, and additional RdDM mutants, with closely correlative methylomes, to correlate 24nt siRNA clusters with mCHH hypo-DMRs would verify this hypothesis.

From the analysis performed here, there is still some evidence for the involvement of XRN2/3. Indeed, *xrn2xrn3* still demonstrated attenuated mCHH hypo-methylation at RdDM targets, especially at mCHH hypo-methylated sites in *sal1*-8. However, it was seemingly attenuated to an extent where these sites did not meet the DMR significance thresholds nor was the decrease observable in genome-wide meta-plots, despite the *xrn2xrn3* methylome still correlating closely with attenuated RdDM mutants. One possible explanation for this is that the *xrn3* allele is a knock-down, as opposed to a knock-out, and leaky expression of *XRN3* may be sufficient to restore a WT-like methylome (Gy et al., 2007; Kurihara et al., 2012). Notwithstanding, current evidence suggests that, while there may be some contribution, there is the potential for factors beyond XRN2 and XRN3 to be responsible for the mCHH hypo-methylation observed in *sal1*-8.

SAL1 interaction with RdDM involves factors beyond XRN2 and XRN3

Multiple lines of evidence suggest that the interaction between SAL1-PAP involves additional factors besides XRN2/3. Indeed, the *sal1*-8 and *xrn2xrn3* methylome did not cluster together, with the latter showing correlation with weaker RdDM mutants. A caveat here is that PAP-mediated inhibition of XRNs has largely been characterized with respect to the nuclear XRNs (XRN2/3), which is also true with respect to stress tolerance and guard cell signalling in *sal1*-8. However, the importance of XRN4 cannot

be marginalized and warrants further investigation as a putative PAP target based on both biochemical and genetic evidence (Dichtl et al., 1997; Gy et al., 2007; Estavillo et al., 2011; Hirsch et al., 2011; Kurihara et al., 2012; Kurihara, 2017). Although it is largely considered to contribute towards co-translation mRNA decay in cytosolic P-bodies (Chantarachot & Bailey-Serres, 2017; Tsuzuki et al., 2017), its function has also been reported to contribute to plant stress responses (Merret et al., 2013; Nguyen et al., 2015), seed germination (Basbouss-Serhal et al., 2017), and to suppress transcriptional gene silencing via 5′-3′ exonucleolysis of uncapped RNA (Gazzani, 2004). Thus, inhibition of XRN4 is likely to contribute towards *sal1*-8 phenotypes, including enhanced stress tolerance and aberrant RdDM. Indeed, the sRNA-ome and methylome of *xrn4*, or ideally the *xrn2xrn3xrn4* triple mutant, may uncover the difference between the *xrn2xrn3* and *sal1*-8 methylome, as well as the contribution of XRN inhibition towards siRNA proliferation and mCHH hypo-methylation.

Clues towards an interaction with RdDM can also be searched for in the *sal1*-8 transcriptome, which revealed differential expression of various loci encoding epigenomic factors. The most striking change was the down-regulation of *ROS1*, a DNA glycosylase involved in the active removal of methylated cytosines that interacts, antagonistically, with the RdDM pathway both across the genome and at an upstream region (*AT2TE68230*) that regulates *ROS1* expression (Huettel et al., 2006; Penterman et al., 2007; X. Zheng et al., 2007; Otagaki et al., 2013; Lei et al., 2015; Williams et al., 2015; Tang et al., 2016). Indeed, *sal1*-8 exhibited mCHH hypo-methylation at this region, albeit to a lesser extent than other RdDM mutants. Accompanying this was negligible expression of *ROS1* in *sal1*-8 that was more comparable to RdDM mutants. Thus, one potential mechanism for mCHH hypo-methylation was through an uncoupling of *ROS1* regulation with RdDM. However, given these observations, ROS1 activity might be expected to be decreased in *sal1*-8 leading to a hyper-methylation as observed in the *ros1*-4 mutant, predominantly in the mCG context of methylation (Qian et al., 2012). This does not align with the changes observed in the *sal1*-8 methylome. Thus, whether mCHH hypo-methylation in *sal*-8 is a consequence of reduced ROS1 activity, or whether reduced *ROS1* expression is a consequence of impaired RdDM at *AT2TE68230* remains unclear. The latter hypothesis is favoured here given the contrasting phenotype and methylomes between *sal1*-8 and *ros1*-4 (Gong et al., 2002; Qian et al., 2012). Indeed, *ROS1* down-regulation is likely a secondary effect, potentially a result of feedback regulation in response to hypo-methylation, in *sal1*-8, rather than a primary driver for the methylome differences observed. A potential strategy to confirm this is to restore *ROS1* expression, such as using conventional over-expression of *ROS1* using the CaMV 35S promoter, in the *sal1*-8 mutant. Alternative strategies include using CRISPR-Cas9 promoter mutagenesis to fine-tune *ROS1* expression (Rodríguez-Leal et al., 2017) or to artificially restore methylation levels in the *ROS1* promoter region adjacent to *AT2TE68230* (Ford et al., 2017), in *sal1*-8, and subsequently test for restored *ROS1* expression and mCHH

levels. However, such a result seems counter-intuitive and a similar strategy, used in an *rdr2* mutant, lead to a exacerbated morphological defects and methylation losses (Williams & Gehring, 2017).

Consistent with the role of *SAL1* as a suppressor of RNA silencing, there is an up-regulation of various factors involved in post-transcriptional gene silencing, including AGO1/7/9 and DCL1/2. However, these components are not considered to be involved in the canonical RdDM pathway (M. A. Matzke & Mosher, 2014; M. A. Matzke et al., 2015). Interestingly, *sal1*-8 also shows a minor, yet significant, up-regulation of *DRM2*, the predominant functioning DRM (Cao & Jacobsen, 2002), potentially as a form of feedback regulation to counter the mCHH hypo-methylation in its methylome. Strong up-regulation of the RNA Pol IV subunit NRPD2B might also reflect an attempt to produce the required transcripts to induce RdDM mediated methylation at DRM targets. Both CMT2 and 3 also showed a minor up-regulation, again likely as a counter to the minor hypo-methylation observed at larger repetitive elements. Given the modest differential expression of epigenomic loci, it appears unlikely that the mCHH hypo-methylation is a consequence of altered transcriptional regulation but, instead, likely reflects a response to a release of RNA silencing and aberrant RdDM.

There is some evidence that the inhibition of XRNs can lead to RNA Pol II read-through, in *sal1*-8, leading to 3′ transcript extensions and intergenic transcription (Kurihara et al., 2012; Crisp, 2015). Additionally, a relationship has been identified between RNA Pol II, IV, and V, whereby the function of RNA Pol II effects RNA Pol IV and V targeting and vice versa (B. Zheng et al., 2009; McKinlay et al., 2017). In particular, intergenic RNA Pol II transcription can induce siRNA biogenesis, and the recruitment of AGO4, resulting in transcriptional gene silencing (B. Zheng et al., 2009). An inhibition of this process may lead to aberrant distributions of the three key polymerases, leading to the aberrant RdDM observed. Although this mechanism cannot be completely accounted for by the *xrn2xrn3* double mutant, further analyses of *xrn4* would be required to test this hypothesis.

Correlations of broad mCHH patterns across the methylome, alongside sub mCHH tri-nucleotide biases, reinforce the similarity of *sal1*-8 to RdDM mutants, in particular *dcl2dcl3dcl4*, and *xrn2xrn3* to those with reduced severity, such as *frg1frg2* or *drm3*. Co-expression analyses showed patterns consistent with the notion that these might be of functional consequence. As expected, *SAL1* showed a strong negative correlation with almost all the RdDM loci tested, consistent with its role as a suppressor of RNA silencing. Interestingly, *XRN2*, *XRN3*, and *XRN4* all showed modest co-regulation alongside RdDM factors, corresponding to mutants that correlated with the *sal1*-8 and *xrn2xrn3* methylomes, including *dcl2dcl3dcl4* and *frg1frg2*. In particular, *XRN4* showed the strongest co-regulation, further pointing to an interaction requiring XRN4. Collectively, these further promote the possibility of altered siRNA metabolism in *sal1*-8 promoting the importance of performing sRNA-sequencing. Alternatively, an approach utilizing PAP

or XRN affinity chromatography, paired with mass spectrometry, may identify novel interacting partners in this pathway. Indeed, PAP affinity chromatography has also been performed to identify new potential targets, although none of which could readily explain the mCHH hypo-methylation observed here (Crisp, 2015). A caveat here, however, is that the chromatography was performed on extracts from healthy plant tissue where PAP, and its potential interactors, might be in low abundance. Therefore, repeating this strategy in the context of elevated PAP (*sal1*-8 or drought treated tissue Estavillo et al. 2011) may improve the detection of biologically relevant candidates.

The short TEs targeted by RdDM are predominantly located in euchromatic regions of the Arabidopsis genome and are, therefore, often adjacent to protein coding genes (Zemach et al., 2013). Furthermore, AGO4 and RNA Pol V binding, on which functioning RdDM is dependent, is enriched at promoter regions of genes adjacent to, or overlapping, these short euchromatic TEs (X. Zhong et al., 2012; Q. Zheng et al., 2013). Thus, it is unsurprising that aberrant RdDM can effect gene expression and plant development (Groth et al., 2014; Stroud et al., 2014; Rowley et al., 2017; R. Yang et al., 2017). Indeed, it was also reported that DRM2 and CMT3, but not CMT2, were key to maintaining methylation, and proper expression, of protein-coding genes (Stroud et al., 2014). Thus, irrespective of the interaction between the SAL1-PAP-XRN and RdDM pathways, there is the potential for a release of both gene and TE silencing. Indeed, there is prolific differential gene expression in *sal1*-8, a large extent of which can be explained through inhibition of the XRNs (Estavillo et al., 2011; Crisp, 2015). However, there is still a large portion of DEGs that are unique to *sal1*-8 that might potentially be explained by mCHH hypo-methylation.

Many of the mCHH hypo-DMRs in *sal1*-8 occurred adjacent to protein coding genes, particularly in the promoter containing upstream region of genes. Despite this, only a small proportion of the genes adjacent to detected hypo-DMRs showed differential expression. Furthermore, among these changes, there were approximately equal numbers of up- and down-regulated genes, contradictory to the initial expectation of increased gene expression as a result of lost methylation (Lister et al., 2008). Despite a lack of correlation between the hypo-methylation and up-regulation of adjacent genes, there is still the potential for individual cases of association. Certainly, given the complex nature of gene regulatory networks, including the effects of enhancer elements and post-transcriptional control, the contribution of differential methylation on gene expression is likely more nuanced and possibly lost in a complicated signal. Indeed, a handful of hypo-DMRs occur in the promoter region of strongly differentially expressed genes that may warrant further investigation. For instance, in the case of down-regulated genes, an exciting notion might be the loss of transcription factor occupancy as a result of differential methylation. Indeed, multiple reports suggest that DNA methylation may stabilize transcription factor occupancy, with up to 76% of all transcription factors showing methylation sensitivity (Maurano et al., 2015; O'Malley et al., 2016). Therefore, an initial strategy might be

to identify common transcription factor binding motifs, particularly at DEGs, including those heavily down-regulated; and overlap any enriched transcription factors with the epi-cistrome dataset (O'Malley et al., 2016) to test for methylation sensitivity. More intensively, the use of DNA affinity purification sequencing, using the identified candidates as baits, on *sal1*-8 and *xrn2xrn3* would validate any changes in transcription factor binding. This might reveal novel modes of gene regulation incorporating differential promoter methylation dictating altered transcription factor occupancy, although further validation may be required. The emerging prospect for artificially modifying methylation states also provides an exciting new tool to test for the contribution differential methylation on regulating the expression of the genome (Ford et al., 2017). Furthermore, the release of TE repression was also observed in a manner that appeared to mimic the *ddcc* quadruple mutant, which exhibits completely eliminated nonCG methylation resulting in prominent TE de-repression (Stroud et al., 2014). Interestingly, this was most prominent at longer TEs in *sal1*-8, rather than the more severely hypo-methylated shorter TEs. Regardless, identifying those TEs that are being actively transcribed and testing for TE activation or mobilization would implicate the SAL1-PAP-XRN pathway in the maintenance of genome stability, in additional to its canonical roles as a negative regulator of stress tolerance and RNA silencing (Gy et al., 2007; Wilson et al., 2009; Estavillo et al., 2011).

3.4 Conclusion

This chapter demonstrates the restoration of ABA signalling, in otherwise ABA insensitive mutants, through the SAL1-PAP-XRN pathway. Evidence suggests that this restoration occurs through a PAP-XRN mediated transcriptional up-regulation of CDPKs, which can activate the SLAC1 anion channel, thus, controlling guard cell turgor. However, PAP was also demonstrated to be, in and of itself, a signalling molecule that directly induces stomatal closure within 10 minutes. The nature of this mechanism is unlikely to be transcriptional, and the identification of this mechanism remains to be identified. Furthermore, cross-talk between the SAL1-PAP-XRN and RdDM pathways was presented, as *sal1*-8 exhibits the characteristics of a moderate RdDM mutant. The exact nature of the interaction requires further elucidation and likely involves factors beyond XRN2/3, which only accounts for a small proportion of the mCHH hypo-methylation.

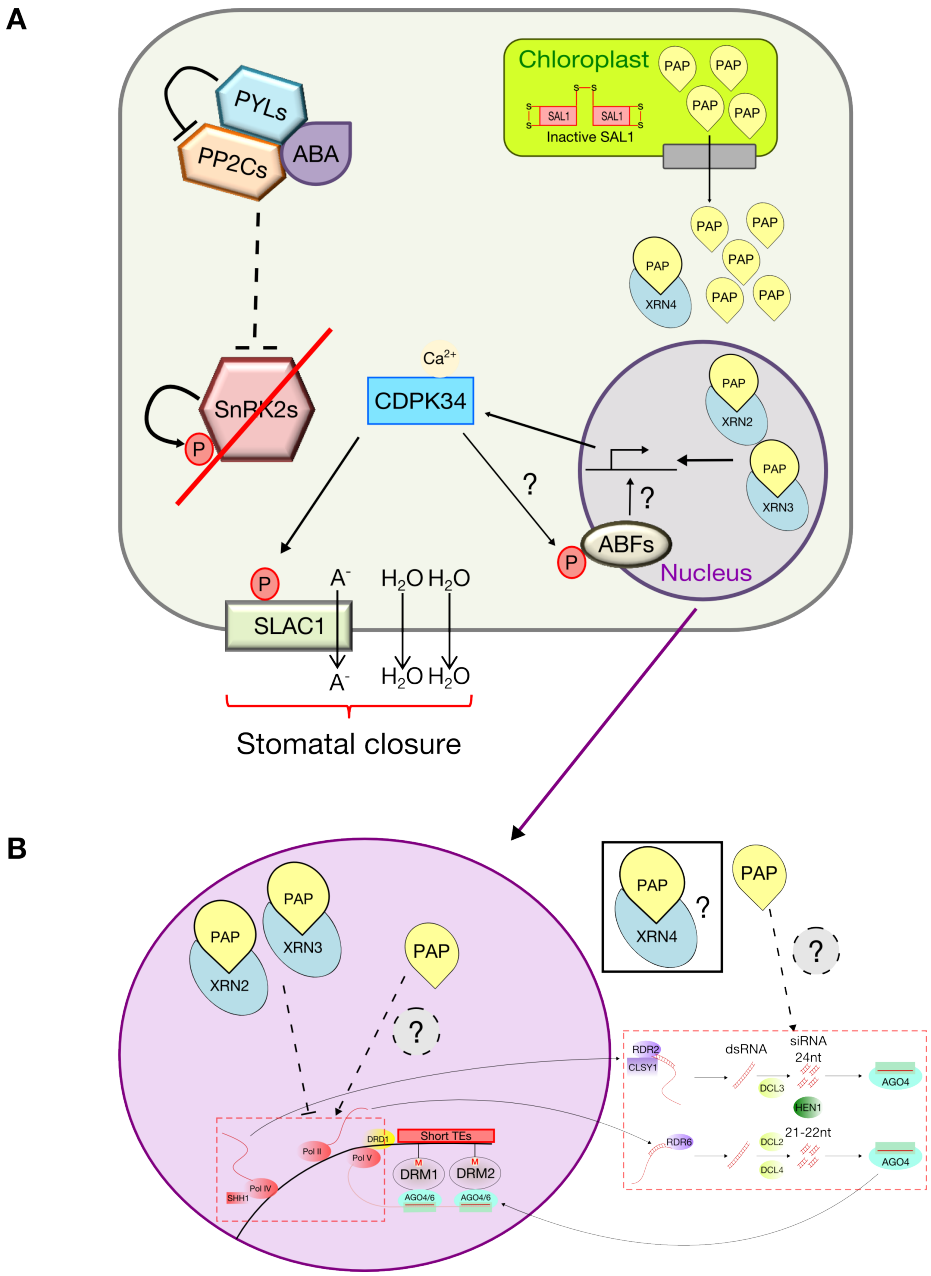

Figure 3.13: Model for the restoration of stomatal closure and interaction with the RdDM by the SAL1-PAP-XRN pathway

A Proposed model for the interaction of PAP and ABA signalling, in the context of inactive SnRK2.6/OST1. Inactivation of SAL1, either through genetic manipulation or redox regulation via oxidative stress, results in the accumulation of PAP in the chloroplast. Subsequently, PAP can be transported back into the cytosol and move into the nucleus, where it interacts with, and inhibits, the XRN enzymes. This leads to transcriptional reprogramming, including the up-regulation of *CDPK34*. CDPK34 can compensate for the lack of OST1/SnRK2.6 by phosphorylating the SLAC1 anion channel, inducing guard cell closure. It remains to be investigated whether up-regulation of additional CDPKs contributes towards this, or if this might feed back on the transcriptome by activating additional ABFs that could, in turn, up-regulate other components. PAP might also inhibit with the cytosolic XRN4, an interaction that also requires elucidation.

B PAP may play additional regulatory roles in the nucleus via an interaction with the RdDM pathway (red dashed boxes denote potential points of interaction) to affect transcriptional gene silencing. Genetic evidence suggests that *sal1*-8 is a moderate RdDM mutant, demonstrating prolific mCHH hypo-methylation predominantly at short TEs that was comparable to the methylome of *dcl2dcl3dcl4*. A canonical hypothesis is that PAP mediated inhibition of the XRNs might contribute towards aberrant siRNA biogenesis, however, *xrn2xrn3* showed an attenuated mCHH hypo-methylation that suggests there are additional factors that may facilitate this interaction. The contribution of PAP mediated XRN4 inhibition towards mCHH hypo-methylation also warrants further investigation, particularly due to the contribution of XRN4 towards suppressing transcriptional gene silencing and its sub-cellular localization alongside the factors required for siRNA biogenesis.

Chapter 4

The Arabidopsis DNA methylome is stable under transgenerational drought stress

---

This chapter presents, in full, results that have been published in Ganguly et al. 2017 and is available online at Plant Physiology.

4.1 Synopsis

Improving the responsiveness, acclimation, and memory of plants to abiotic stress holds substantive potential for improving agriculture. An unresolved question is the involvement of chromatin marks in the memory of agriculturally-relevant stresses. Such potential has spurred numerous investigations yielding both promising and conflicting results. Consequently, it remains unclear to what extent robust stress-induced DNA methylation variation can underpin stress memory. This chapter explores the potential for drought to induce potentially heritable epi-alleles and for examples of transgenerational memory. Using a slow onset water deprivation treatment (drought) in Arabidopsis, the malleability of the methylome was investigated in response to drought both within a generation; and under recurring drought over five successive generations along with evidence for memory in the descendants of drought-exposed lineages.

4.2 Results

4.2.1 Stress-associated variation in DNA methylation observed under a slow onset mild drought stress within a generation

A slow onset water deprivation treatment ('drought stress') was imposed on soil-grown plants by with-holding watering for nine days to assess the potential for drought stress to induce epi-alleles in the methylome. This caused a drop in RWC to around 60% (measured in representative plants) and visible leaf wilting (**Figure 4.1**). Whole rosettes were harvested from unstressed (*U*, n=3) and drought-treated plants (*D*, n=3). WGBS was performed on these samples to investigate the methylome at single base-pair resolution (Lister et al. 2008; Cokus et al. 2008; **Appendix A**-Dataset 2 Table 1).

To explore variations in the methylome between samples, pairwise comparisons of mean methylation levels, binned across 100bp tiles, was performed to capture the full extent of variation between all samples (Eichten & Springer, 2015). This revealed 2,141 mCG, 1,039 mCHG, and 718 mCHH DMRs across all samples; however, hierarchical clustering, based on methylation levels at these regions, did not cluster samples by treatment (**Figure 4.2**). Instead, clustering revealed the existence of two to three putative pre-existing methylome states, herein referred to as epi-types. This suggests that the predominant source of variation in the methylome between these samples arises from pre-existing differences. As the seed stock for this experiment was derived from bulk seed harvesting, as opposed to single seed descent, these differences are likely caused by distant relatedness between plants (Becker et al., 2011; Schmitz et al., 2011, 2013).

Notwithstanding the presence of epi-type DMRs, it was hypothesised that if the Arabidopsis methylome is truly malleable to abiotic stress then drought should induce conserved variations, between control and treated plants, amongst any epi-type variation. Despite the hierarchical clustering of 100bp tile-based DMRs showing negligible conservation of DMRs between treatments, any evidence for statistically significant treatment-conserved changes was tested through rank sum testing. However, upon correction for multiple testing, all of the observed changes were deemed to be insignificant. Whilst tile-based DMRs are a powerful tool for exploring broad-scale methylome variation, it is limited in its ability to appropriately attribute biological and technical information at single cytosines residues, thus losing statistical power. Therefore, an alternative approach to evaluate differential methylation was performed using *DSS* (H. Feng et al., 2014). This method employs Bayesian hierarchical modelling to incorporate the variation that exists both within and between biological replicates, at single cytosine resolution, to identify *bona fide* treatment-associated DMRs with greater statistical rigour, including *post-hoc* p-value adjustments.

To identify stress-associated differential methylation, DMR calling was performed in two stages. First, to account for the contribution of pre-existing methylome variation,

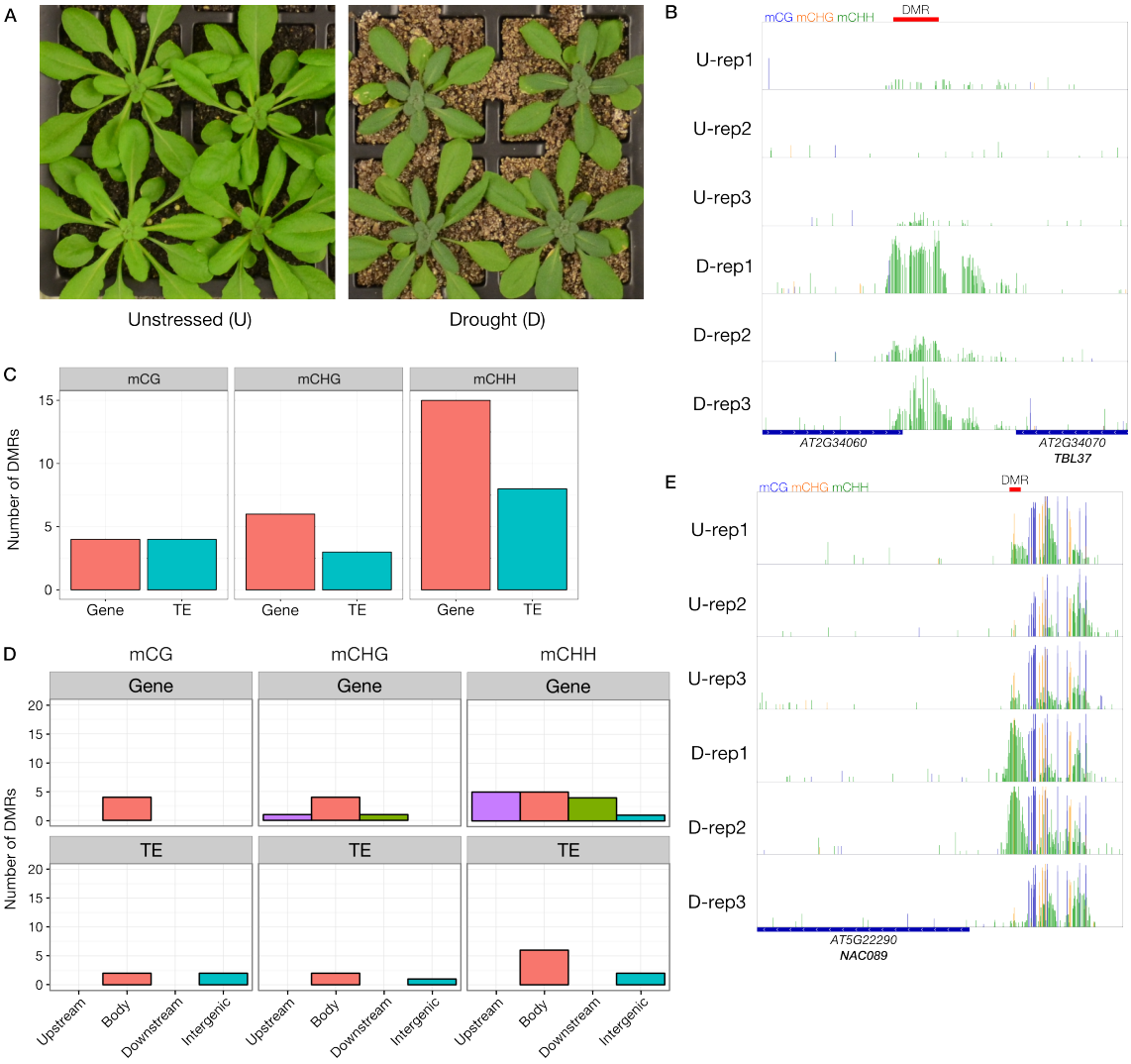

Figure 4.1: A mild drought stress is associated with variations in DNA methylation
A Representative plants that were either unstressed (*U*) or underwent a drought stress (*D*) involving nine days of withheld watering.
B Representative drought stress associated DMR identified by *DSS*. Rows represent individual samples. DNA methylation is shown at single cytosine resolution for mCG, mCHG, and mCHH as blue, orange, and green bars respectively. Underlying genomic elements are presented at the bottom and the exact region identified as a DMR is shown on top (red bar).
C Numbers of filtered stress-associated DMRs occurring near annotated protein coding genes and TEs for each methylation context.
D Detailed mapping of filtered stress-associated DMRs within, or near, annotated protein coding genes (upper panel) and TEs (lower panel) for each context of methylation. Body refers to DMRs within the genomic feature, upstream refers to DMRs within 1kb near the 5′ end of the feature, downstream refers to DMRs within 1kb of the 3′ end of the feature, and intergenic refers to DMRs that are further than 1kb away from the nearest genomic feature.
E Drought stress-associated DMR located upstream of *NAC DOMAIN CONTAINING PROTEIN 89 (NAC089)*, previously identified as a locus exhibiting transcriptional memory to repeated dehydration stress. Presented as in **A**.

Table 4.1: Numbers of DMRs between epi-type groups and treatments identified by *DSS*. The numbers of drought stress DMRs exclude those that were also identified between epi-type groups

DMR Class	Contrast	Sequence context		
		mCG	mCHG	mCHH
Epi-type	*U*-1,*U*-3,*D*-2 vs *U*-2,*D*-1,*D*-3	144	41	33
Drought stress	*U*-1,*U*-2,*U*-3 vs *D*-1,*D*-2,*D*-3	8	9	23

Table 4.2: Numbers of drought stress DMRs mapping to protein-coding genes either directly (gene body), within than 1kb (upstream/downstream) or greater than 1kb from the nearest gene (intergenic)

Location	Sequence context		
	mCG	mCHG	mCHH
Gene body	4	4	5
Upstream region ($<$ 1kb from nearest gene)	0	1	5
Downstream region ($<$ 1kb from nearest gene)	0	1	4
Intergenic ($>$ 1kb from nearest gene)	0	0	1

DMRs were identified between epi-type groups (epi-type DMRs) using *DSS* (**Table 4.1**, **Figure 4.2B**). The locations of epi-type DMRs were mapped relative to genomic features (**Appendix A**-Dataset 2 Table 2) based on the Araport11 genome re-annotation (Cheng et al., 2017). Epi-type associated DMRs had comparable numbers mapping to annotated protein-coding genes and TEs; however, they were predominantly in the mCG context within gene and TE bodies (**Figure 4.2C-D**).

Second, 49 stress-associated DMRs were identified using *DSS*, nine of which overlapped with pre-existing epi-type DMRs that were filtered from further analyses to produce a final list of 40 drought-associated DMRs (**Table 4.1**, **Figure 4.1B**, **Appendix A**-Dataset 2 Table 3). Positional mapping of drought and epi-type associated DMRs relative to protein coding genes and TEs were compared to explore whether they exhibited similar characteristics. Drought DMRs were more likely to be found within 1kb of genes (24/40; 60%) compared to epi-type associated DMRs (91/218; 42%). Interestingly, there were proportionally fewer mCG stress associated DMRs (8 of 40 DMRs, 20%), than epi-type associated (144/218, 66%), with the majority in the mCHH context (**Figure 4.1C**). Stress associated DMRs located near genes were predominantly non-mCG (20 of 24, 83%; **Figure 4.1D**,**Table 4.2**). These results potentiate the involvement of the RdDM pathway as a source of stress-induced methylome variation near genes (M. Matzke et al., 2009; Schmitz et al., 2013). The exact mechanism underpinning mCG-DMRs remains elusive, however, they have been suggested to act in a truly epigenetic (independent of underlying genetic variation) (Schmitz et al., 2013).

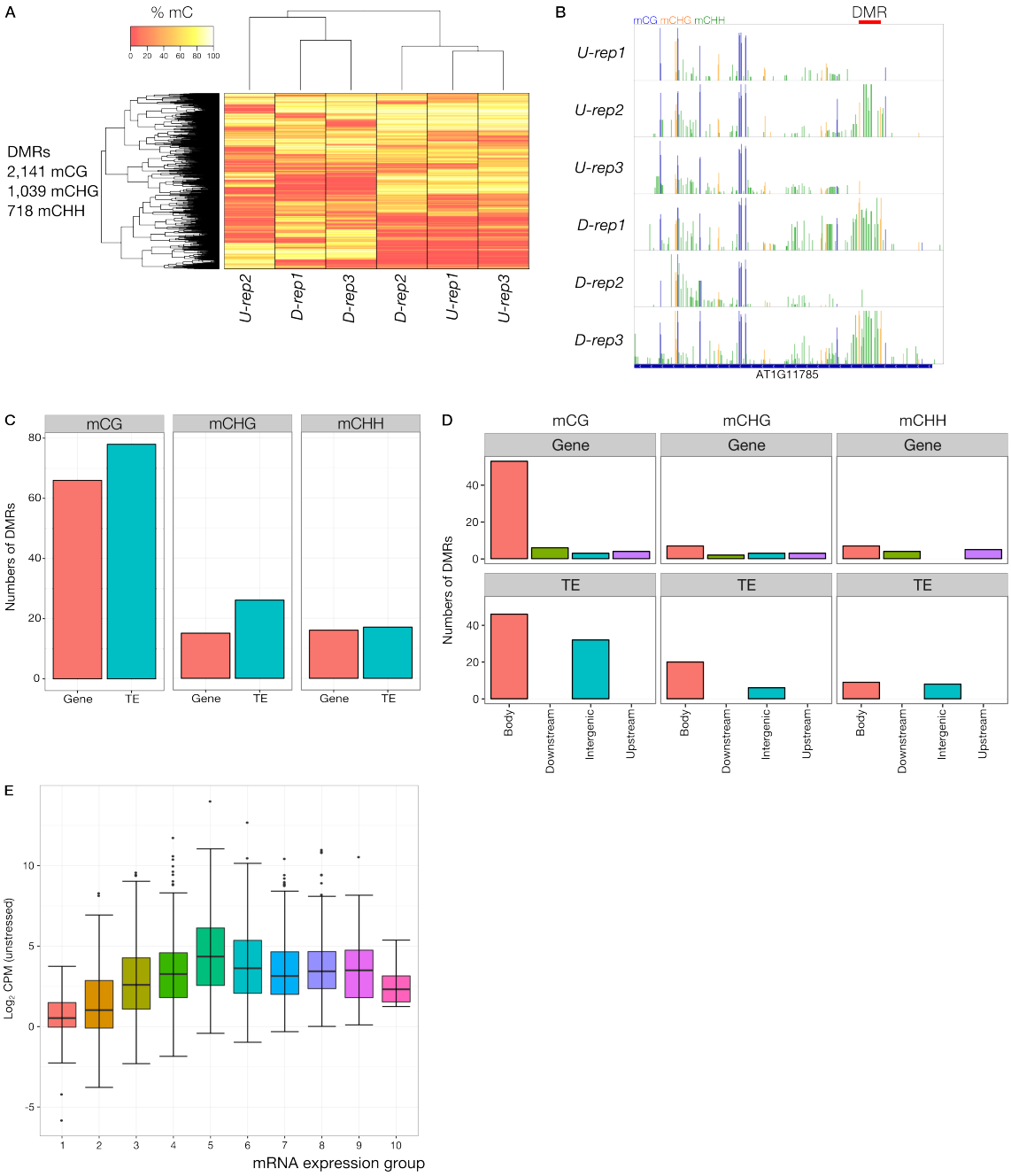

Figure 4.2: Pre-existing differences in the methylome define multiple epi-types
A Heatmap representing two-dimensional hierarchical clustering of methylation levels across all 100bp tile-based DMRs in all samples.
B Browser shot of DNA methylation levels, in each sample, surrounding a representative *DSS* identified epi-type DMR (red bar). Blue, orange, and green bars represent mean methylation in mCG, mCHG and mCHH contexts, respectively, at single cytosine resolution.
C Numbers of epi-type DMRs occurring near annotated protein coding genes and TEs for each context of methylation.
D Detailed mapping of epi-type DMRs within, or near, annotated protein coding genes (upper panel) and TEs (lower panel) for each context of methylation. Body refers to DMRs occurring within the genomic feature, upstream refers to DMRs within 1kb near the 5′ end of the feature, downstream refers to DMRs within 1kb near the 3′ end of the feature, and intergenic refers to DMRs that are further than 1kb away from the nearest genomic feature.
E Standard boxplots of $\log_2$ CPM of transcripts in each mRNA expression group defined using k-means clustering.

Table 4.3: Lineage-associated and spontaneous *DSS*-based DMRs identified in the transgenerational drought experiment

DMR Class	Contrast	Sequence context			
		mCG	mCHG	mCHH	Total
Lineage	G_5P_1 Unstressed vs G_5P_1 Drought	1	2	1	4
Spontaneous	G_0P_1 vs G_5P_1 Unstressed	1	10	12	23
	G_0P_1 vs G_5P_1 Drought	1	6	8	15

4.2.2 mRNA Sequencing and Promoter Methylation Profiling of Single Drought

Next, the 24 stress associated DMRs mapping near protein coding genes were further investigated for effects on the expression of neighbouring genes. There were 4,369 differentially expressed genes under this drought treatment compared to unstressed controls (Crisp et al., 2017). This comparison to this mRNA sequencing dataset revealed only four significant differentially regulated genes correlating with drought-associated DMRs (**Appendix A**-Dataset 2 Table 4). Not only is there a negligible relationship of drought DMRs to drought-responsive genes (hypergeometric test; $P_{X\geq4}$=0.54) but three of the correlating genes (*ENDOPLASMIC RETICULUM RETENTION DEFECTIVE 2 (ERD2)*, *AT2G20920*, *AT2G34060*) exhibited only weak gene expression changes. Interestingly, *NAC089*, which showed the strongest transcriptional response under these conditions (approximately 7-fold up-regulated), has been reported to demonstrate transcriptional memory in response to repeated dehydration stress (Ding et al., 2013). While there is an observable increase in mCHH, the hyper-methylated state is not conserved across drought stressed samples and similar levels of methylation remain in the adjacent downstream region from the identified DMR (**Figure 4.1E**). Therefore, while this methylation difference may have biological significance it is unclear whether this is truly associated with drought stress.

This profiling of the methylome suggests that it is relatively unresponsive to drought stress. Yet this does not rule out an association, it is possible that the methylation profile of drought responsive genes could distinguish them from other non-responsive genes. For instance, given that most up-regulated genes do not display a change in DNA methylation, their promoters may be un-methylated allowing for enhanced responsiveness. To investigate this possibility, the methylation state, in all contexts, across the promoter region (considered as 1kb upstream from gene annotation) of drought responsive genes was profiled in unstressed and drought treated plants. Methylation levels were averaged across genes clustered, using a k-means method, based on their $\log_2$ FC in mRNA expression (**Figure 4.3A**). There was no clear relationship between promoter methylation levels and the fold-change in mRNA observed, although either

strongly up- or down-regulated genes appeared to show lower levels of DNA methylation compared to other groups (**Figure 4.3B**). There also appeared to be a slight, yet general, increase in promoter mCHG and mCHH levels in drought treated samples. To test whether this increase reflected any characteristic of the promoters of drought-responsive loci, promoter methylation levels were averaged across 437 (mean group size from **Figure 4.3A**) randomly selected loci that did not respond to drought stress (**Figure 4.3C**). These loci, whilst generally having higher promoter methylation levels, also showed an increase in non-CG methylation providing further evidence that this methylation difference was not reflective of gene expression changes. It was also apparent that some expression groups, with transcripts showing relatively small changes in expression, had higher levels of promoter methylation possibly reflecting mRNA abundance under unstressed conditions. However, further inspection of transcripts in each expression group suggested that promoter methylation levels were not reflective of mRNA abundance under unstressed conditions (**Figure 4.2E**). Promoter non-CG methylation levels were explored further to test whether there were a subset of drought responsive genes driving this difference. However, these regions were found to be largely devoid of methylation with the exception of a subset of loci (**Figure 4.3D**). Despite the lack of association with drought-responsive mRNA expression, these findings implicate altered RdDM function under drought stress leading to elevated non-CG methylation upstream at a subset of genes (M. A. Matzke & Mosher, 2014).

4.2.3 Transgenerational recurring drought stress

The experiment above highlights that DMRs do appear after the onset of a drought within a single generation. Although DMRs are present, the experimental design limits the ability to examine their biological relevance in a number of ways. First, the seed stock used contained existing epi-types that may interfere with stress-responsive changes to DNA methylation and/or prohibit detailed analyses by diluting any signal from stress-induced variation. Second, a single generation experiment does not provide any insight into the heritable nature of methylation changes (M.-T. Hauser et al., 2011; Gutzat & Mittelsten Scheid, 2012). Third, biologically relevant DMRs may increase in number and persist over time if the stress is experienced repeatedly at different developmental stages both within a generation and across generations. Therefore, a single-seed descent, recurring, and transgenerational drought stress experiment was performed to directly address these experimental challenges.

Multiple independent lineages originating from a single inbred progenitor were propagated by single seed descent, akin to previous mutant accumulation line experiments (Shaw et al., 2000; Becker et al., 2011; Schmitz et al., 2011). Plant lineages were subjected to either control conditions (unstressed lineages) or repeated drought stress (drought stress lineages) comprised of a 14 day drought, 5 day recovery, followed by a second 12 day drought (**Figure 4.4A**, **Figure 4.5A**). The first stress occurred dur-

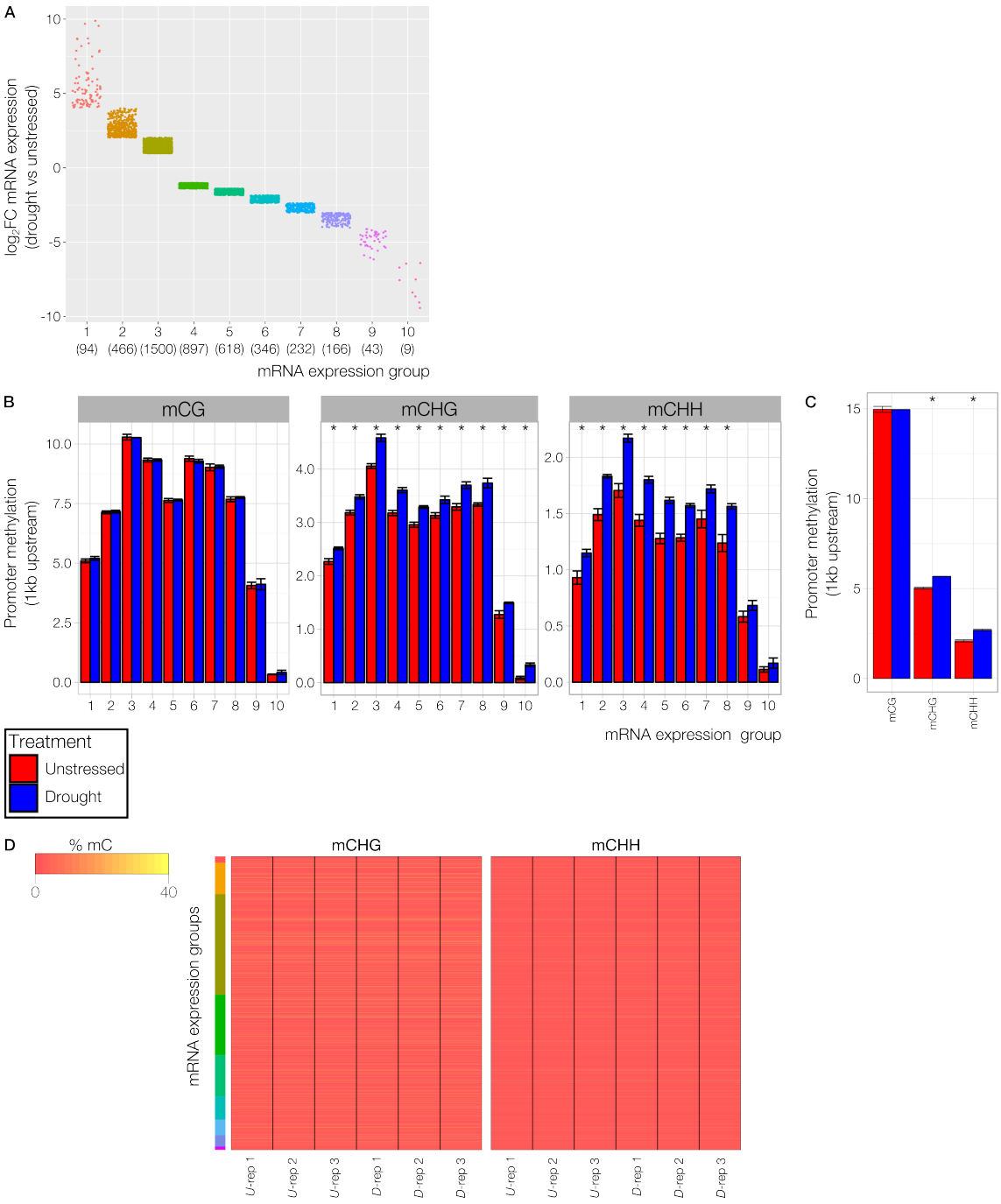

Figure 4.3: Promoter methylation levels at drought responsive genes
A Stripchart depicting $\log_2$ FC in mRNA expression of all drought responsive genes grouped based on k-means clustering. Dots represent individual drought-responsive loci. Numbers of genes in each cluster presented in brackets.
B Summarised methylation levels in the 1kb region directly upstream of drought-responsive loci averaged across all genes in each expression group as defined in **A**. Bars denote mean, error bars denote standard error of the mean (n=3), * denotes significant differences ($p < 0.05$) between treatments as determined by a Kruskal-Wallis rank sum test.
C Summarised methylation levels in the 1kb region directly upstream of 437 randomly selected non-drought responsive loci. Bars denote mean, error bars denote standard error of the mean (n=3), * denotes significant differences ($p < 0.05$) between treatments as determined by a Kruskal-Wallis rank sum test.
D Heatmaps of mCHG and mCHH levels summarised 1kb directly upstream of drought-responsive loci for individual transcripts ordered by expression group (as defined in **A**), and subsequently by $\log_2$FC (top = highest; bottom = lowest).

ing vegetative growth ("*D1*") and the second during flowering ("*D2*"). This repeated drought treatment was performed through successive generations starting from founding plants (G_0) through to 5th generation plants (G_5) (**Figure 4.4B**). Direct progeny (P_1) and progeny one generation removed (P_2) of G_4 and G_5 plants, from independent lineages per treatment, were compared for altered growth and resilience. The methylomes of six G_5 P_1 progeny per lineage condition, each from an independently propagated lineage, were assayed using WGBS. G_0 P_1 progeny were also assayed for a representation of initial methylome patterns prior to generations of experimental treatment.

PSII performance was monitored using measures of chlorophyll fluorescence, allowing for non-destructive assaying of plant stress and vitality under drought, to maximise survival rate (Haitz & Lichtenthaler, 1988; Woo et al., 2008). Representative traces of various PSII parameters (see **Table 2.1**) are shown for plants under control conditions, at the end of *D1*, and at the end of *D2* (**Figure 4.5B-F**). *D1* and *D2* plants demonstrated a corresponding reduction in both PSII quantum efficiency (ϕ_{PSII}) and photochemical quenching capacity (qP), a reduction in the estimated fraction of 'open' PSII centres (qL) and some reduction in the maximal potential efficiency of PSII (F_v'/F_m'). For all these measures, *D1* and *D2* plants largely demonstrated similar trends though the severity appeared greater after *D2*. For example, *D2* plants showed a severely impaired NPQ profile suggesting plants post-*D2* were severely stressed to the point that they could not sufficiently activate photoprotective mechanisms. This suggests greater impact of drought in mature plants undergoing the transition to reproduction.

4.2.4 Drought exposed lineages exhibit enhanced seed dormancy

Progeny of G_4 and G_5 plants from unstressed and drought treated lineages were compared to test whether sustained and repeated drought exposure over successive generations could lead to the formation of drought stress memory that might be evidenced as altered plant behaviour or enhanced drought tolerance in drought treated lineages.

Growth of three week old descendants, from unstressed and drought exposed lineages, were compared under control growth conditions for G_5 P_1 and P_2 progeny. There were no intra-generational differences in plant size, using either green pixel count or fresh biomass, between descendants of watered and drought-treated parents (**Figure 4.7A-B**). Growth rate in G_5 P_1 progeny, using green pixel counts of plant area over three weeks, showed that progenies from unstressed and drought lineages had equivalent growth rates (**Figure 4.7C**) and flowering times (**Figure 4.7D**). Thus, gross plant growth and development appears unaltered after experiencing repeated drought stress over previous generations.

Seed provisioning is considered to be a significant mechanism for the transmission of adaptive transgenerational effects. For instance, seeds developed during periods of stress often have altered nutrient or hormone profiles, which holds important biological consequences such as propensity to germinate (Herman & Sultan, 2011). Indeed, pre-

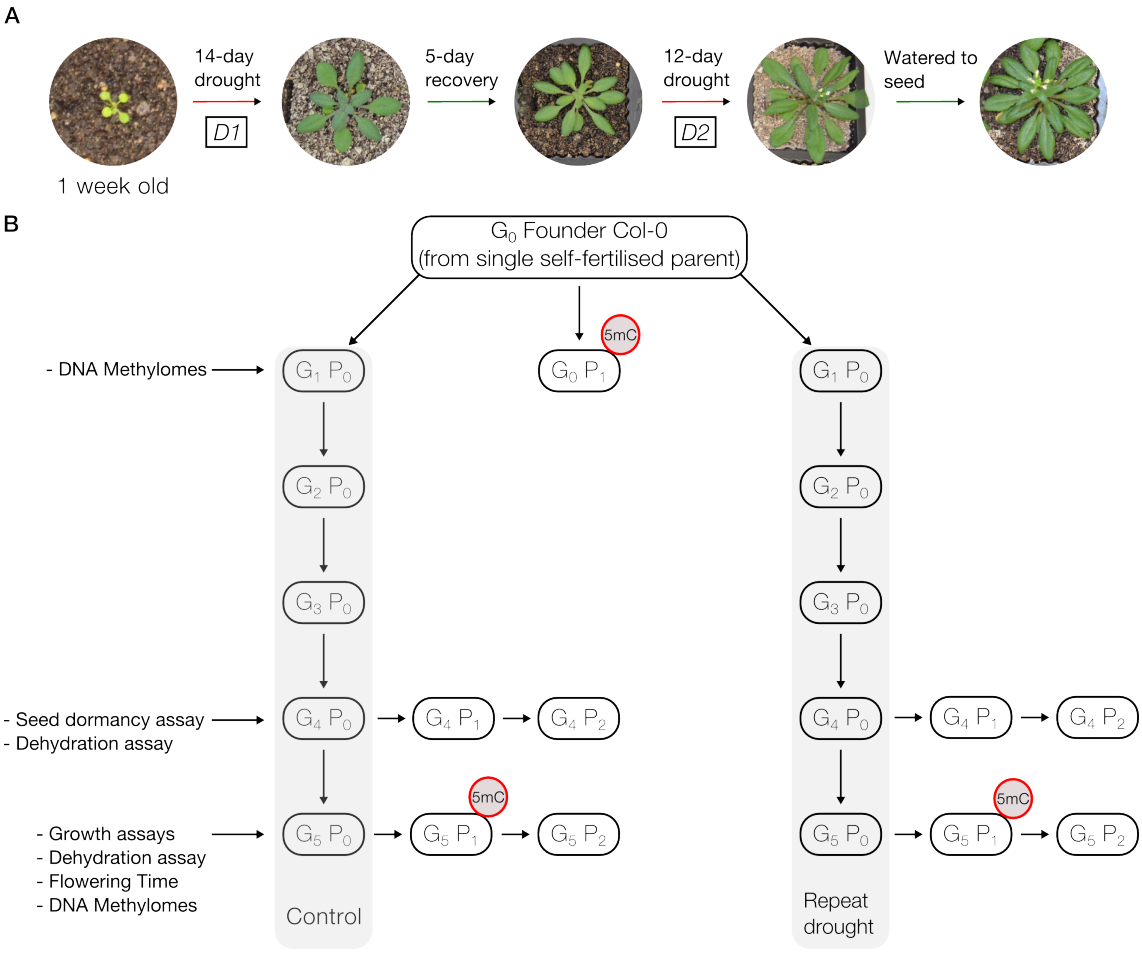

Figure 4.4: Transgenerational repeated drought stress experiment
A Scheme of repeated drought stress treatment performed every generation. Initial water deprivation ("*D1*") began on one week old seedlings and lasted for 14 days. After a recovery period of five days, a second treatment was performed ("*D2*") that lasted for 12 days.
B Multiple independent lineages were propagated by single seed descent for five generations, from a founding inbred progenitor, with half of the lines being exposed to the repeated drought stress treatment every generation. Testing for transgenerational stress memory was performed on the P_1 and P_2 progeny of G_4 and G_5 plants with plants from each independent lineage. WGBS was performed on P_1 progeny, each from an independent lineage, of G_0 and G_5 plants (red circle).

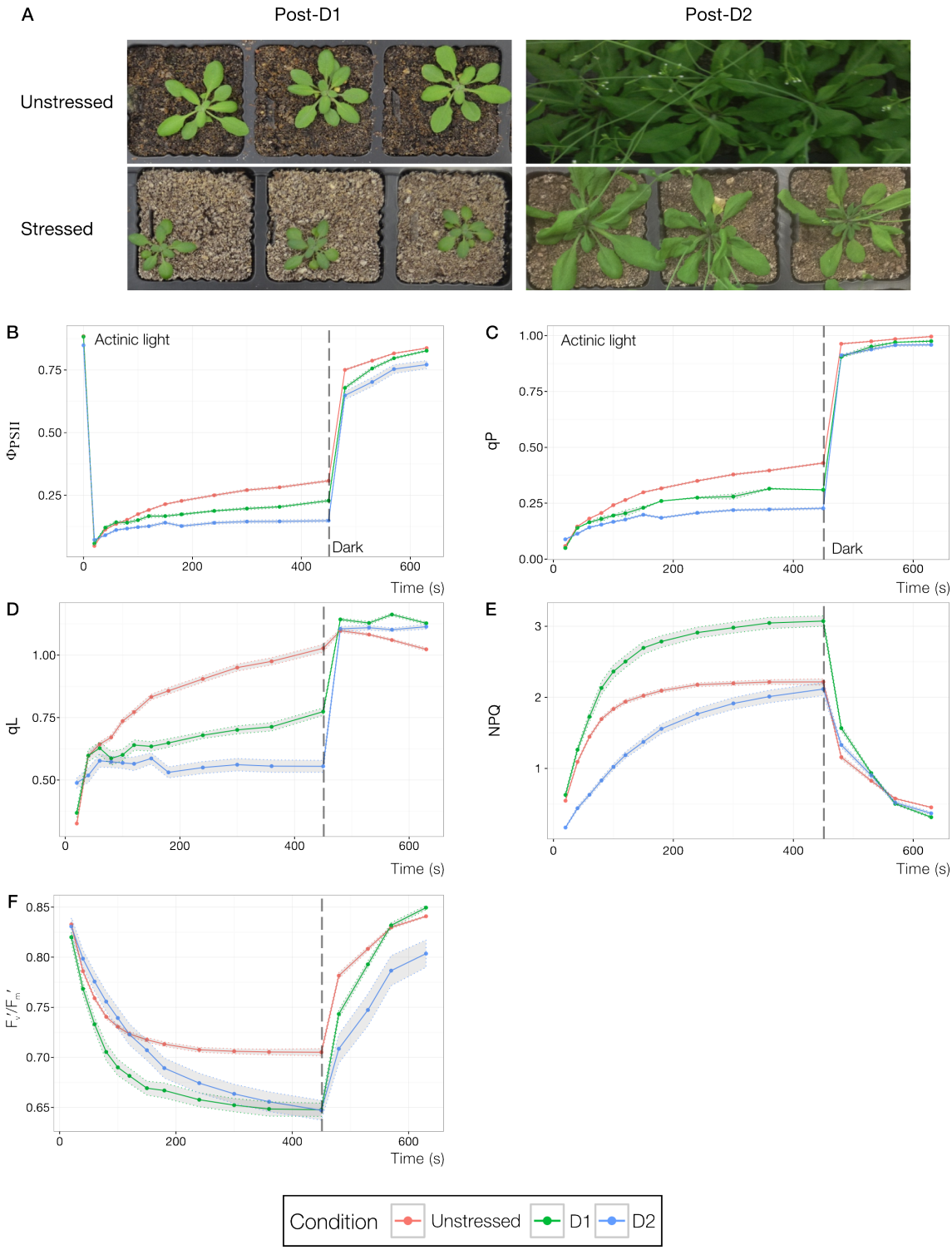

Figure 4.5: Representative plants and chlorophyll fluorescence profiles characterizing the impacts of D1 and D2

A Additional representative plants at the end of *D1* and *D2* treatments showing stunted growth and wilting.

B-F Representative traces for parameters monitoring PSII performance in unstressed (n=41), *D1* (n=13), and *D2* (n=14) plants: PSII quantum efficiency (ϕ_{PSII}), photochemical quenching (qP), fraction of open PSII centres (qL), NPQ non-photochemical quenching, and maximum quantum efficiency (F_v'/F_m'). Points denote mean, shaded regions denote standard error of the mean.

vious transgenerational studies have reported phenotypes reliant on maternal exposure to stress (Murgia et al., 2015; Nosalewicz et al., 2016). Therefore, altered seed provisioning was tested by comparing dormancy in seed from P_0 and P_1 progeny from G_4 plants of both lineages (**Figure 4.6A**). Seed dormancy was compared by constructing a Cox proportional hazards model producing a comparative hazards ratio (HR) (McNair et al., 2012). Seed from G_5 P_0 drought were 72% less likely to germinate (HR_D=0.28, $p < 0.001$) than seeds from unstressed lineages. It is possible that this was conveyed through maternal effects, such as increased ABA synthesis under drought stress, particularly since *D2* occurred during early reproductive stages (Cutler et al., 2010). When seed dormancy was further tested in *P1* seed, one generation removed from stress, the size of this effect was reduced but still statistically significant (HR_D=0.69, $p < 0.001$). While these observations are consistent with observations of maternal effects, in the form of altered seed provisioning, some dormancy is still retained in the seed of P_1 progeny a generation removed from experimental drought.

It is possible that any form of transgenerational memory might only be observable in conditions of water limitation. One of the key responses to drought stress is stomatal closure (Verslues et al., 2006) and recent investigations have found that environmentally-induced variation in stomatal development and index is, at least in part, regulated by DNA methylation with some evidence for transgenerational transmission (Tricker et al., 2012, 2013). Greater stomatal control to prevent dehydration would be beneficial under water limitation therefore stomatal responsiveness was compared between lineages. A detached rosette dehydration experiment was performed on G_4 and G_5 P_1 progeny. Independent experiments revealed that progeny from each lineage had very comparable rates of water loss with lineage holding a very weak effect (α_2 = 1.30-1.71, $p > 0.05$, **Figure 4.6B**). Ultimately, if any form of drought stress memory was conveyed to the progeny of drought-stressed plants, then these progeny would be expected to exhibit improved survivability under drought. However, G_5 P_1 progeny from both lineages demonstrated near identical survivability under a longer term drought measured using the fluorescence decline ratio (R_{fd}) as a vitality index (**Figure 4.6C**; Haitz & Lichtenthaler 1988). In total, phenotypic assessment of transgenerational drought lineages revealed enhanced seed dormancy to be the only form of drought stress memory, which was partially retained in seeds one generation removed from stress.

4.2.5 Negligible epi-alleles in the methylome associated with transgenerational drought stress

Beyond phenotypic measures of memory, the extent of DNA methylation variation between these lineages associated with the transgenerational repeated drought stress was also investigated. Methylomes were produced from whole rosettes of approximately three week old G_0 progeny (G_0 P_1) and G_5 progeny from six independent lineages per condition (G_5 P_1 unstressed, G_5 P_1 drought), grown under control growth conditions

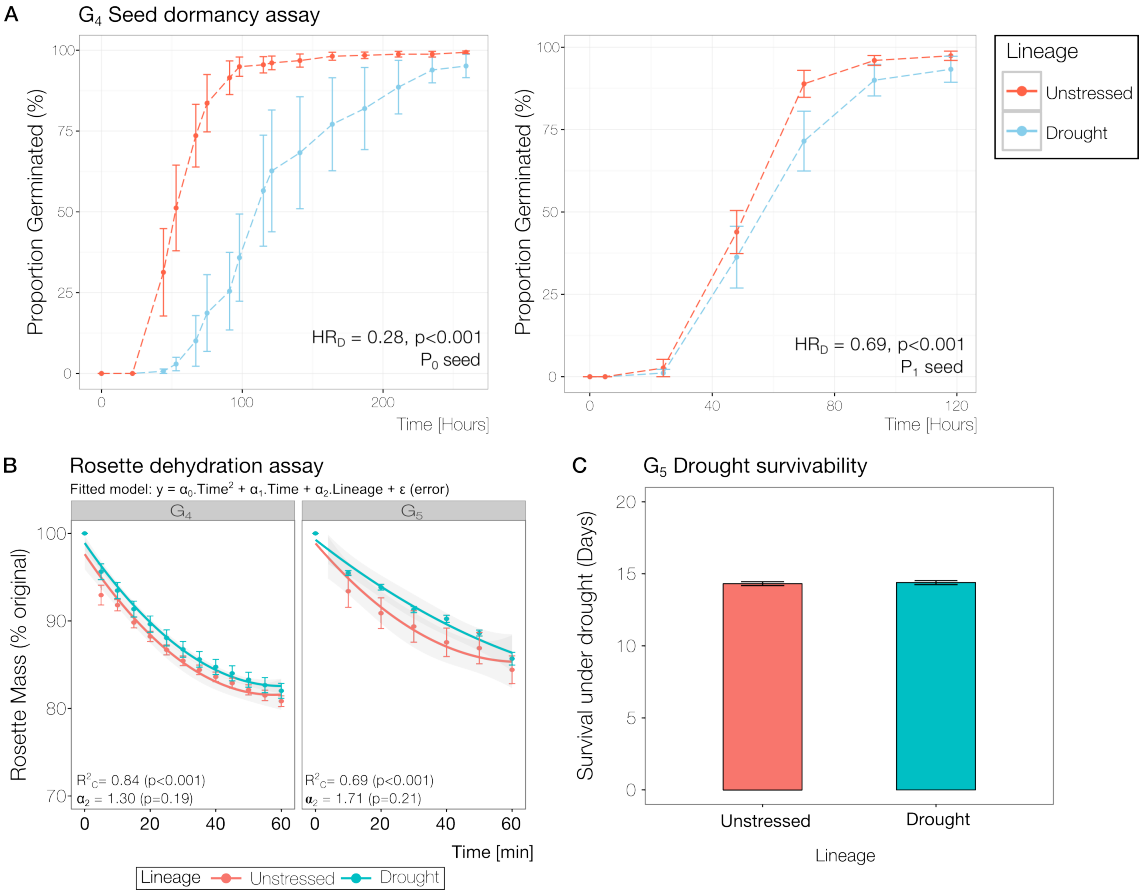

Figure 4.6: Progeny from drought exposed lineages show enhanced seed dormancy
A Independent dormancy assays performed on seed from P_0 (n=6; >25 seeds per plate) and P_1 (n=9, >25 seeds per plate) progeny of G_4 plants (P_0 and P_1 seed, respectively). Points denote mean proportion of seeds germinated; error bars denote standard error of the mean. HR denotes the calculated hazard ratio from a fitted Cox proportional hazards model, representing the likelihood of germination between groups (HR_D = drought vs unstressed lineage).
B Dehydration assay performed on detached rosettes of P_1 progeny of G_4 (n=12) and G_5 (n=11) plants (independent experiments). A second order polynomial regression, with a 95% confidence interval (shading), was performed to determine the coefficient for the lineage predictor term (α_2). R^2_C denotes the conditional R^2 calculated to assess model fit.
C Survival under terminal drought experiment on transgenerational descendants (unstressed, n=44; drought, n=51). Bars denote means and error bars denote standard error of the mean across two independent experiments.

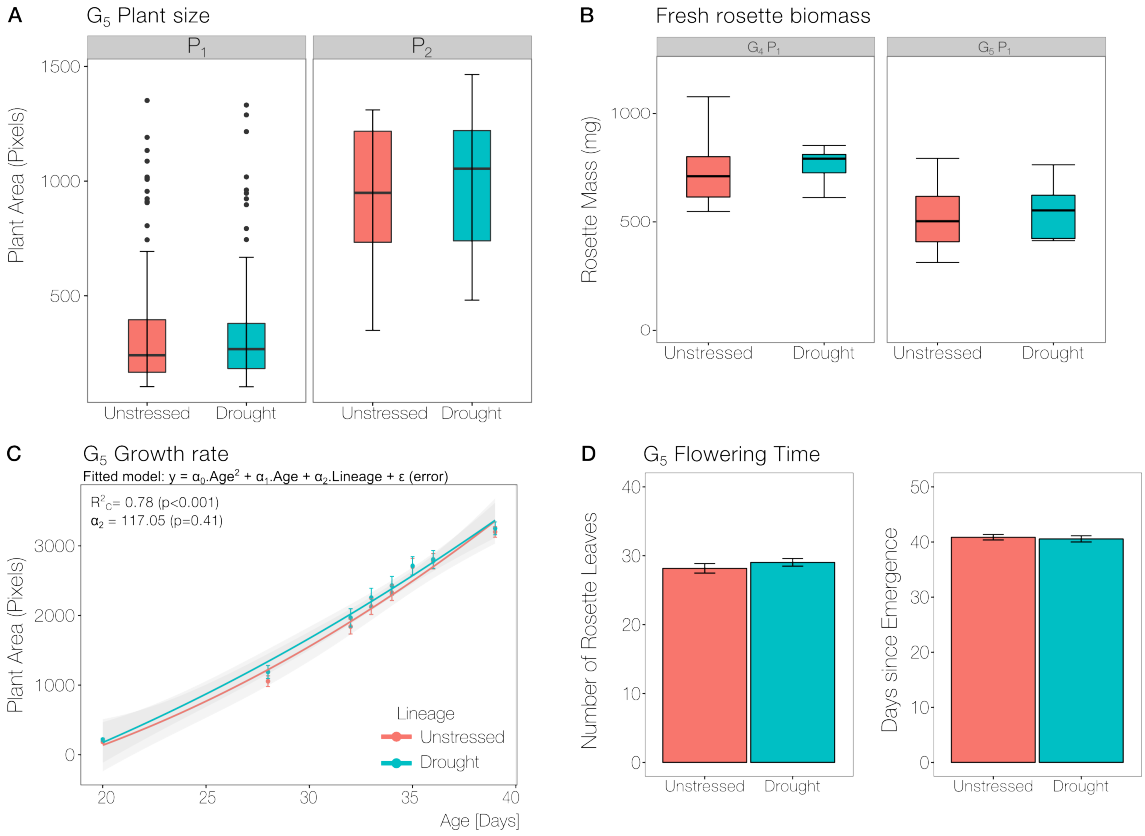

Figure 4.7: Characterising growth of G_4 and G_5 progeny from unstressed and drought exposed lineages

A Standard boxplots of plant area of three week old progeny of G_5 unstressed (n=65 P_1; 12 P_2) and drought (n=67 P_1; 14 P_2) descendants from independent experiments.

B Standard boxplots of fresh rosette mass of three week old P_1 progeny of both G_4 and G_5 plants (n=12).

C Growth rate of G_5 P_1 progeny was estimated using a second order polynomial regression on measures of plant area measured over time. Points denote means, error bars denote standard error of the mean, and the fitted polynomial is shown for each lineage (n=28) with a 95% confidence interval (shading). R^2_C denotes the conditional R^2 calculated to assess model fit.

D Flowering time of G_5 P_1 progeny, from unstressed and drought lineages (n=28), expressed as days since emergence and rosette leaf number at floral bud emergence. Bars denote means, error bars denote standard error of the mean.

(**Appendix A**-Dataset 2 Table 1). Each progeny plant that was sequenced came from an independently propagated lineage. Hierarchical clustering of all G_5 P_1 samples by genome-wide methylation levels, binned into 100bp regions, confirmed that broad methylome patterns were highly similar amongst all progeny excluding the possibility of genetic contamination, such as seed stock contamination or outcrossing, which could affect the methylome patterns observed (**Figure 4.8A**). In contrast to the previous experiment (**Figure 4.2A**), no clear epi-types were detected in the profiled G_5 P_1 progeny despite being derived from independent lineages, confirming the importance of comparing relatively closely related plants. To identify conserved drought-induced heritable changes in the methylome, *DSS* was utilized to call DMRs between progeny of G_5 control and G_5 drought lineages. This yielded just four transgenerational drought stress-associated DMRs (**Table 4.3**, **Appendix A**-Dataset 2 Table 5). None of these overlapped with the epi-type or stress associated DMRs that were identified in the previous within-generation drought stress experiment. This lack of variation was unexpected since 40 DMRs were observed within a generation from a single drought stress; however, this reinforces the notion that heritable stress-induced variations in the methylome are rare. Despite this conservative approach, none of the identified DMRs demonstrated complete conservation within treatment groups, and three of the DMRs mapped to repetitive regions of the genome (**Figure 4.8B**). The fourth DMR was in intergenic space, 800bp upstream of *CHOLINE/ETHANOLAMINE KINASE (CEK)3* (*AT4G09760*), and was only present in four of the six drought lineage progenies that were profiled.

4.2.6 Core ABA signalling and documented memory loci remain stable under transgenerational recurring drought stress

Given the negligible detection of transgenerational drought-associated DMRs using unbiased approaches, a targeted analyses was undertaken. The rationale for this strategy relates to the hypothesized biological relevance of methylation as a regulatory mechanism near, or within, annotated genes related to drought response and tolerance (Gutzat & Mittelsten Scheid, 2012). The directed approaches were used to examine DNA methylation levels at loci encoding the core components in the ABA signalling pathway crucial for drought response and previously characterised loci described to have stress-induced, transgenerational methylome variation.

ABA induces a signalling cascade, involving both transcriptional and post-transcriptional changes, which activates drought-tolerance mechanisms (Verslues et al., 2006; Cutler et al., 2010). Given its importance it was postulated that loci encoding key components of the ABA signalling pathway (F. Hauser et al., 2011) could be targets for memory formation. However, when differences in DNA methylation levels were assayed at these loci in G_5 P_1 progeny, between unstressed and drought-treated lineages, they were found to be near identical with the largest difference being a 4.45% decrease in mCG (**Appendix A**-Dataset 2 Table 6).

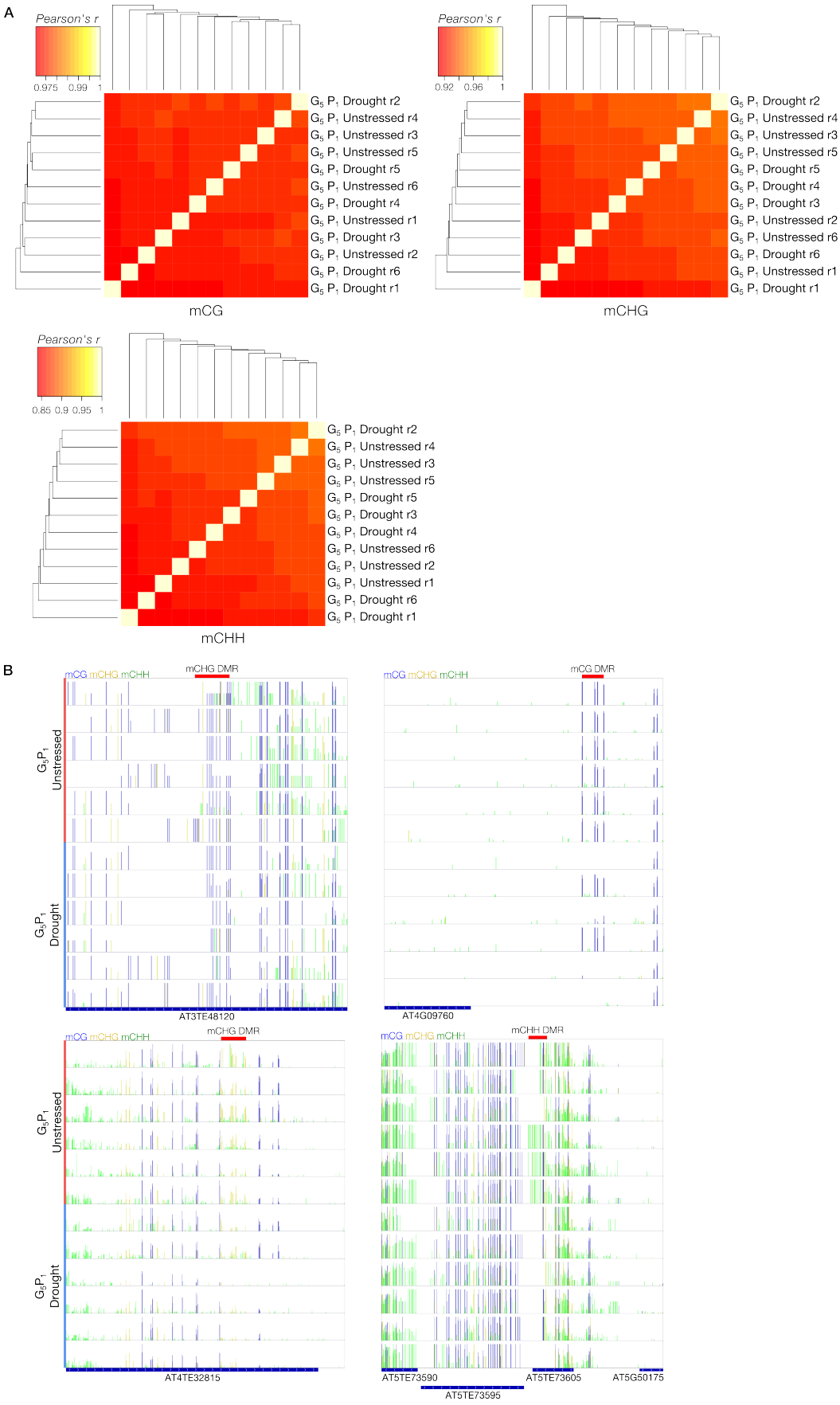

Figure 4.8: Limited methylome variation associated with transgenerational drought stress
A Heatmaps representing two-dimensional hierarchical clustering of correlations (Pearson's *r*) in genome-wide DNA methylation levels, in all sequence contexts, averaged across 100bp bins confirms similar broad methylome patterns between all G_5 descendants.
B *IGV* visualization of lineage-associated DMRs identified by *DSS* (red bar). Vertical blue, yellow, and green bars denote mean mCG, mCHG, and mCHH, respectively, at single cytosine resolution.

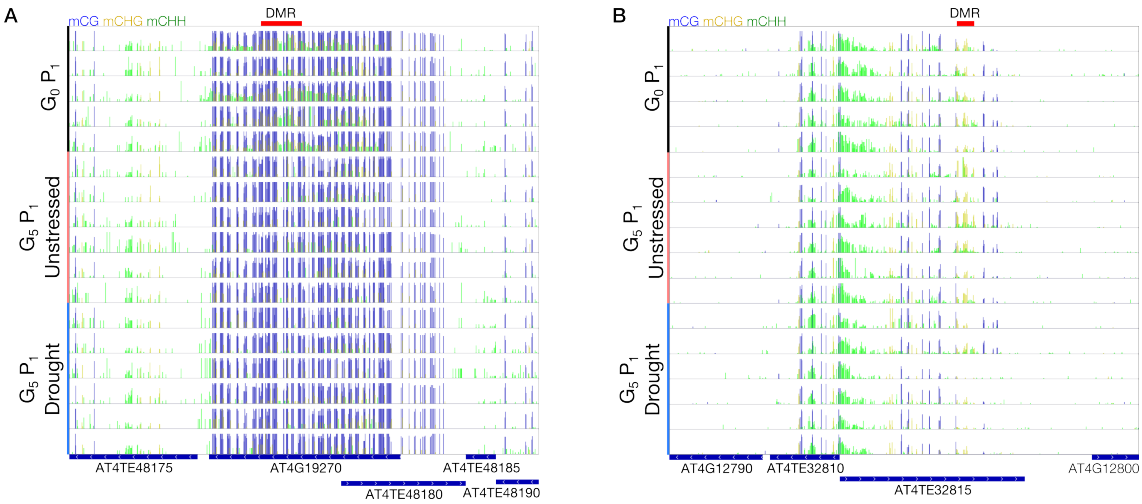

Figure 4.9: Labile regions in the methylome identified in transgenerational drought stress experiment
A *IGV* visualization of DNA methylation across the putative DNA methylation-labile locus *AT4G19270*. Blue, yellow, and green bars denote mean mCG, mCHG, and mCHH levels, respectively, at single cytosine resolution. Note the mCHH hypo-methylation that occurs in all G_5 P_1 descendants. Red horizontal bar denotes *DSS* identified DMR.
B *IGV* visualization of DNA methylation across *AT4TE32815*, the site of a drought-associated DMR in G_5 P_1 progeny that overlaps with the site of a previously published spontaneous DMR. Blue, yellow, and green bars denote mean mCG, mCHG, and mCHH levels, respectively, at single cytosine resolution. Red horizontal bar denotes *DSS* identified DMR.

Low humidity-induced hyper-methylation at the *FAMA (FAMA)* and *SPEECHLESS (SPCH)* loci was described to be transmitted to progeny in Ler (Tricker et al., 2012, 2013). These loci were profiled across P_1 progeny of G_0 and G_5 plants to look for evidence of hyper-methylation (**Figure 4.10A-B**). Interestingly, both loci were found to be largely devoid of DNA methylation across all experimental samples, comparable to the un-methylated non-stressed plants previously reported (Tricker et al., 2012). However, there was no evidence of any transmissible hyper-methylation at these loci, neither lineage-dependent nor drought-dependent. Notably, there was a region of stochastic differences, in all three sequence contexts of DNA methylation, downstream from the protein coding region of *FAMA*. This observation raises the following possibilities: (I) regulation of the methylome can be stress-type specific, and (II) different ecotypes, within a species, may have altered stress-induced regulation of the methylome.

Transgenerational hyper-osmotic stress was recently reported to induce enhanced salt tolerance in P_1 progeny of lineages exposed to salt stress for at least two generations (Wibowo et al., 2016). This enhanced tolerance was correlated with stress-associated DMRs, two of which occurred at TEs adjacent to *MYB DOMAIN PROTEIN 20 (MYB20)* and *CARBON/NITROGEN INSENSITIVE 1 (CNI1)*. In the case of *MYB20*, hyper-methylation across an upstream TE correlated with persistently down-regulated *MYB20* expression. In the case of *CNI1*, hypo-methylation across a downstream TE correlated with increased stress-responsive expression in the P_1. Thus, these loci were investigated in the context of transgenerational drought stress to see if any hyper- or

hypo-methylation was evident in G_5 P_1 progeny. The DNA methylation pattern across and upstream of *MYB20*, in all samples assayed in this study, was similar to unstressed G_0 P_1 progeny (**Figure 4.10C**). Interestingly, select drought lineages did appear to show hypo-methylation in an upstream TE akin to the P_2 progeny, one generation removed from 75mM salt stress, which did not exhibit enhanced salt tolerance (Wibowo et al., 2016). The *CNI1* locus was also largely devoid of DNA methylation; although, the downstream TE was partly methylated in all sequence contexts (**Figure 4.10D**). However, there was no transgenerational drought induced hypo-methylation at this downstream TE, as was observed across P_0, P_1 and P_2 progeny of 75mM salt stressed parents. This supports the stochastic nature of methylome variation in that the changes observed are not always universal and/or stable. Single studies may only be capturing a portion of this and potentiate the possibility that different abiotic stresses induce changes in the methylome to differing efficacies.

4.2.7 Greater stochastic variation and appearance of spontaneous DNA methylome epi-alleles in transgenerational lineages

Having observed a limited number of transgenerational drought stress-associated DMRs, the extent of stochastic variation in the methylome was explored. Using the aforementioned 100bp tile-based analysis revealed extensive variation using pairwise comparisons of all G_5 P_1 progeny across lineages (2,871 mCG, 2,284 mCHG, and 1,292 mCHH DMRs; **Appendix A**-Dataset 2 Table 7). Almost all changes appeared to be unique to individual lineages with negligible conservation within treatment groups (**Figure 4.11A**). Rank sum testing was repeated on these 100bp tile-based DMRs to test for association with treatment, however, none were significant after p-value correction for multiple comparisons. This suggests that predominant source of methylome variation is stochastic differences between lineages.

The spontaneous nature of epi-allele appearance in the Arabidopsis methylome has been well-characterised and has also been documented to increase in frequency under environmental stress (Becker et al., 2011; Schmitz et al., 2011; Jiang et al., 2014). The appearance of spontaneous DMRs in the lineages generated in this study was explored by comparing P_1 progeny from G_0 and G_5 plants using *DSS* (**Table 4.3**, **Appendix A**-Dataset 2 Tables 8-9). Interestingly, more *DSS*-based DMRs were identified between G_0 and G_5 progeny regardless of lineage (**Table 4.3**) at a magnitude comparable to previous observations of epi-allele accumulation (in the form of DMRs; Becker et al. 2011). Indeed, G_0 siblings were found to have more similar genome-wide DNA methylation patterns to each other than to G_5 descendants, particularly in the mCG context (**Figure 4.11B**). Exposure to repeated drought stress for five successive generations did not lead to a greater number of DMRs; in fact progeny from stressed lineages had fewer

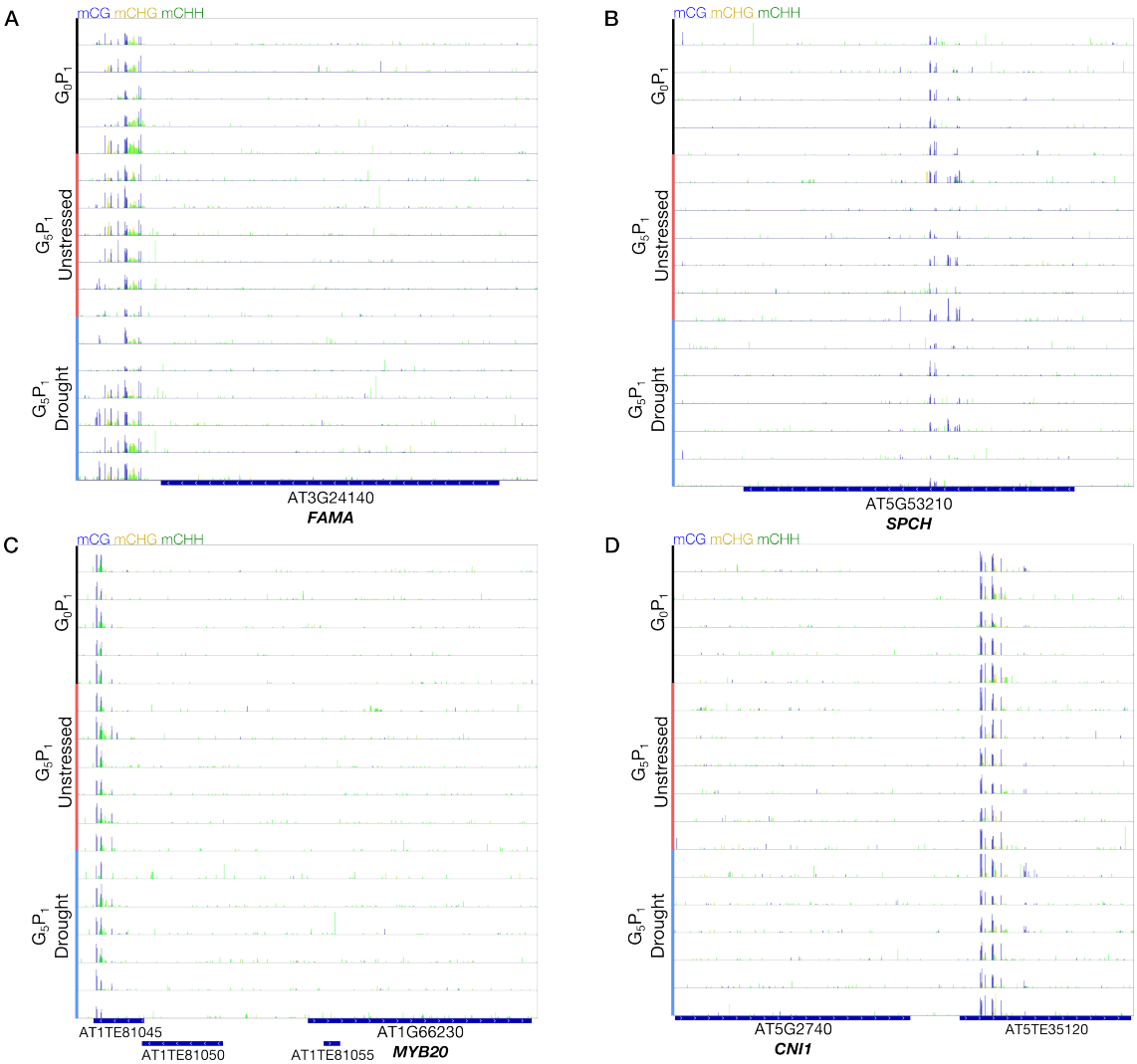

Figure 4.10: DNA methylation levels at loci reported to exhibit transgenerational stress-induced methylation variation

IGV visualization of DNA methylation, in G_0 P_1 control plants and G_5 P_1 plants of both unstressed and drought treated lineages, across loci documented to exhibit transgenerational memory of stress-induced changes in DNA methylation:

A *FAMA* (low-humidity hyper-methylation),

B *SPCH* (low-humidity hyper-methylation),

C *MYB20* (hyper-osmotic hyper-methylation), and

D *CNI1* (hyper-osmotic hypo-methylation).

Blue, yellow, and green bars denote mean mCG, mCHG, and mCHH, respectively, at single cytosine resolution.

DMRs, than unstressed lineages, when compared to G_0 P_1 (**Table 4.3**). Nine regions were in common between variations accumulated in unstressed and drought exposed lineages, which may reflect truly labile DNA methylation sites from this dataset. None of these nine regions were in common with previously identified labile regions. From the total 38 spontaneous DMRs identified here, only three were found to overlap with regions previously associated with spontaneous variation (Becker et al., 2011; Schmitz et al., 2011). Curiously, a handful of the overlapping sites occur across a hypothetical protein surrounded by TEs on chromosome 4 (*AT4G19270*) where there has been extensive non-mCG hypo-methylation, yet unaffected mCG, in G_5 progeny (**Figure 4.9A**).

Labile regions of the Arabidopsis methylome have previously been identified, whether spontaneous, stress-induced, or driven by genetic divergence across diverse environments (Becker et al., 2011; Schmitz et al., 2011; Jiang et al., 2014; Hagmann et al., 2015; Wibowo et al., 2016). The four transgenerational drought stress-associated DMRs, identified in this study, were overlapped with regions identified across the aforementioned datasets to test whether any of the four regions were in common with previously reported stress-induced regions. One of these transgenerational drought-associated DMRs overlapped, however this region was not associated with a stress-induced change (**Figure 4.9B**, **Appendix A**-Dataset 2 Table 10).

Stochastic DMRs, using the 100bp tile-based method in this study, were also overlapped with previously published DMRs to look for conservation across methylation-labile regions. An overlap of the stochastic DMRs identified in this study showed that 617/6,447 (9.5%) occurred at regions previously identified. Overlaps with specific studies remained low ranging from 0.2% - 4.4% of regions from this study being previously detected. Of particular interest was to compare stochastic transgenerational DMRs identified here with previously identified transgenerational spontaneous DMRs across 30 generations of single-seed descent (Becker et al., 2011; Schmitz et al., 2011). 24/72 regions characterised as a site hosting a spontaneous transgenerational epi-allele (Schmitz et al., 2011), unlinked from cis-genetic variation, were identified out of 6,447 stochastic transgenerational DMRs (**Appendix A**-Dataset 2 Table 11). These were predominantly changes in mCG, occurring largely at intergenic or repetitive regions, including TEs and pseudogenes.

4.3 Discussion

Notions of transgenerational plant stress memory are often discussed alongside DNA and chromatin alterations, as a potential mechanism underpinning their storage and transmission (Herman & Sultan, 2011; Tricker, 2015; Crisp et al., 2016). In particular, DNA methylation is considered a key epigenetic mechanism for which there is now growing evidence (Luo et al., 1996; Boyko et al., 2010; Boyko & Kovalchuk, 2010; Tricker et al., 2013; Herman & Sultan, 2016; Wibowo et al., 2016; X. Zheng et al., 2017). The

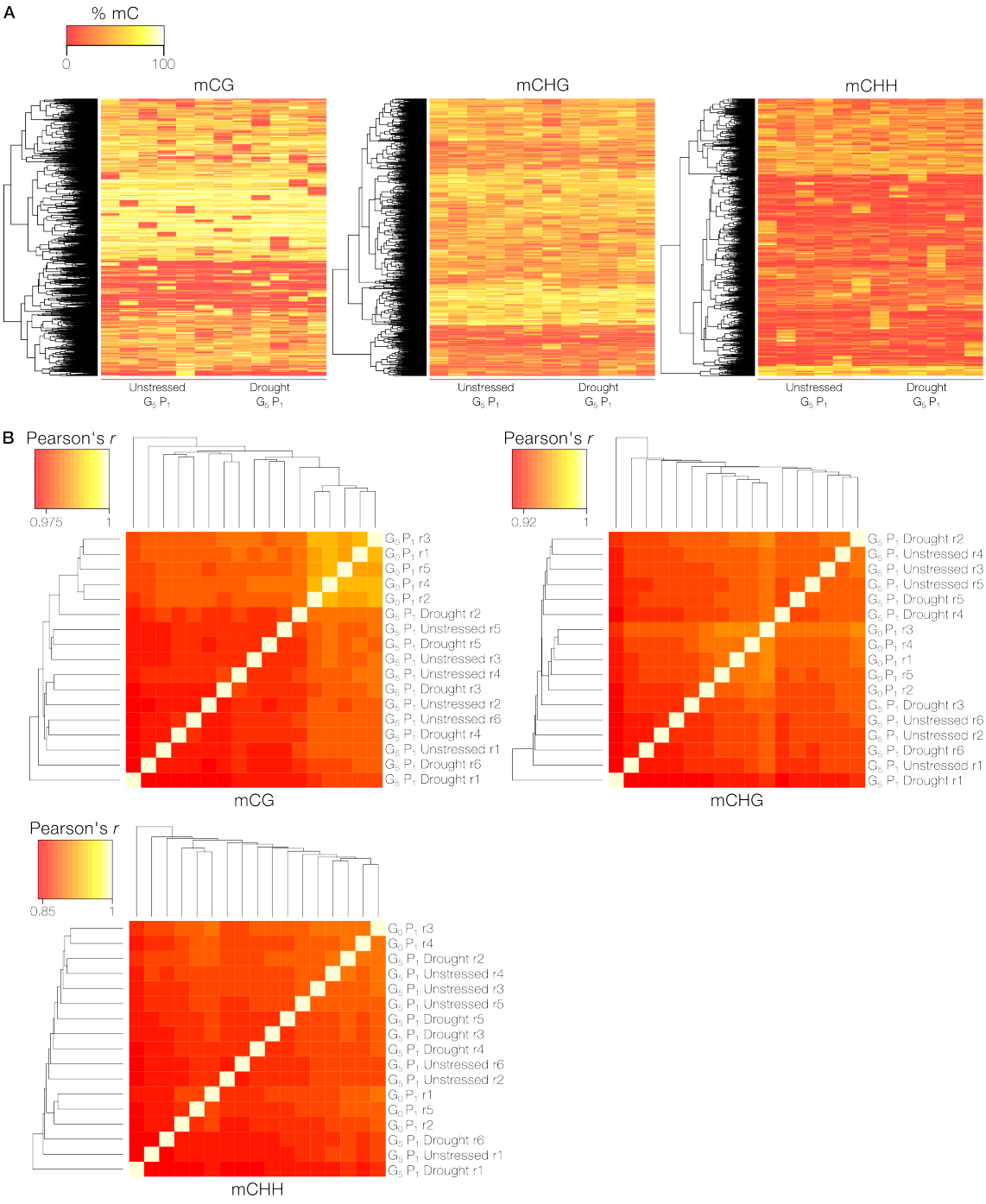

Figure 4.11: Stochastic and spontaneous methylome variation across transgenerational lineages

A Heatmaps of average methylation across 100bp tile-based DMRs identified from pairwise comparisons, with one-dimensional clustering of rows, between all samples.

B Heatmaps representing two-dimensional hierarchical clustering of correlations (Pearson's r) of genome-wide DNA methylation, in all sequence contexts, averaged across 100bp tiles for all G_0 and G_5 progeny.

specific contribution of DNA methylation at, or near, protein coding genes towards basal plant growth and endurance remains unknown, albeit essential for proper development (Finnegan et al., 1996; Henderson & Jacobsen, 2008; Zemach et al., 2013; Yamamuro et al., 2014). Despite documentation of the stable inheritance of spontaneously occurring epi-alleles (Becker et al., 2011; Schmitz et al., 2011), there still remains uncertainty for the malleability of the methylome to stress-induced variation (Seymour et al., 2014; Eichten & Springer, 2015; Secco et al., 2015, 2017). Whether or not such DNA methylation changes are necessary for transgenerational stress memory is still unclear, with various memory traits not always aligning with changes in the methylome (Ding et al., 2012; Sani et al., 2013; Murgia et al., 2015; Nosalewicz et al., 2016; Lämke et al., 2016). This chapter examined and compared the effect of drought stress on the Arabidopsis methylome both within and between generations.

Within generation methylation profiles in response to drought stress
Within a generation, plants experiencing a mild drought stress that induced a substantial transcriptional response exhibited 40 stress-associated DNA methylation epi-alleles. However, these did not appear to correlate with drought-responsive gene expression changes. Further investigation of promoter methylation status at drought-responsive genes did not reveal any methylation features that distinguish drought responsive genes. This did, however, reveal widespread non-CG hyper-methylation in gene promoter regions in drought treated plants implicating altered RdDMs performance under drought stress. Such observations are comparable to the non-CG hyper-methylation, predominantly in the mCHH context, that occurred in the root tissue of rice under phosphate-starvation (Secco et al., 2015). The use of a *DCL3* knock-down line suggested the phosphate-induced non-CG DMRs were largely RdDM-independent, and a similar approach would be beneficial to address the putative involvement of RdDM here. In both cases, however, there was minimal evidence of such methylation changes affecting gene expression. It is worth noting that the effects of DNA methylation changes could be confounded by the complexity of interactions between all the chemical marks that contribute to chromatin state (Eichten et al., 2014; Crisp et al., 2016). It is also possible that pre-existing epi-type differences could be influencing stress-inducible transcriptional changes. To systematically uncouple such effects would require a much larger scale sequencing effort, which may become a viable option in the future.

The identification of multiple epi-types within a seed stock derived from bulk harvesting further demonstrates the importance of appropriate experimental design when testing for DNA methylation mediated stress memory. Furthermore, as the epi-type DMRs were predominantly mCG-DMRs, it is possible that these epi-type DMRs represent true epigenetic differences arising between distantly related plants rather than being reflective of genetic differences (Schmitz et al., 2013). Regardless, the relative lack of drought stress-associated epi-alleles observed within a generation aligns with other studies using

phosphate, temperature, or UV radiation that all present a stoic methylome unperturbed by abiotic stress (Seymour et al., 2014; Eichten & Springer, 2015; Secco et al., 2015; Meng et al., 2016).

Transgenerational inheritance and methylome profiling

Between generations, descendants of lineages exposed to successive generations of recurring drought exhibited only four transgenerational drought stress-associated DMRs compared to unstressed lineages. Significantly, none of these were in common with the 40 drought-associated epi-alleles detected within a generation; reinforcing the notion that transgenerational adaptive DNA methylation is a rare occurrence, even under conditions of abiotic stress (Pecinka et al., 2009; Seymour et al., 2014; Eichten & Springer, 2015; Hagmann et al., 2015; Secco et al., 2015; Crisp et al., 2016). Three of the four mapped to repetitive and already heavily methylated regions of the genome. The fourth DMR was also in intergenic space, albeit 800bp upstream from *CEK3*. *CEK3* encodes a protein, most highly transcribed in the hypocotyl, that is a part of the Choline/Ethanolamine Kinase family for which *CEK4* has been implicated in phospholipid biosynthesis and embryo development, however, mutation of *CEK3* did not lead to the same phenotypes (Lin et al., 2015). It is unclear whether this DMR upstream of *CEK3* would be of biological significance; however, it does not appear to be required for transgenerational drought stress memory as it was only evident in four of the six drought lineage progeny profiled. One possibility, since each progeny plant was derived from an independent lineage, is this DMR is only weakly induced by drought stress; however, this would require further elucidation.

Targeted analyses of ABA-responsive genes were undertaken as they are critical for drought responses. A recent study also reported that key ABA signalling kinases regulate the activity of a chromatin-remodelling ATPase (Peirats-Llobet et al., 2016). This regulation allowed for the fine tuning of downstream components of the ABA pathway, in particular *ABA INSENSITIVE 5 (ABI5)*, further potentiating ABA-mediated chromatin variation that feeds back onto the ABA signalling pathway itself. However, this targeted analysis did not reveal treatment-specific methylation changes at any of the test loci.

When compared to published datasets studying methylome variation, one transgenerational drought stress DMR overlapped with a previously identified spontaneous locus (Schmitz et al., 2011). Certainly, the nature of methylome variation at all identified DMRs (stress-associated and stochastic) is reminiscent of the spontaneous changes previously characterised comparing plants separated by approximately 30 generations (Becker et al., 2011; Schmitz et al., 2011). Whether those stochastic variations in the methylome are tied to a particular lineage with biological consequence may warrant further investigation despite not being tied to the experimental treatment. Furthermore, a vast majority of DMRs identified in this study mapped to TEs or unannotated genomic regions. This is unsurprising given the expected relationship between DNA methylation

and TEs. TE movement is considered to be a driving force in the appearance of epi-alleles ("facilitated" or "obligatory" epi-alleles Richards, 2006) and, indeed, documented environmentally-induced "epigenetic" changes correlate with, though are not always necessary for, TE activity (Ito et al., 2011; Eichten et al., 2013; Ong-Abdullah et al., 2015; Ito et al., 2016; Stuart et al., 2016). Future studies should take into consideration the impact of TE regulation, under conditions of abiotic stress particularly in species with greater TE content, which possibly underpins at least a subset of the stochastic or spontaneous epi-alleles observed in this study.

There is evidence building for the possibility of transgenerational plant stress memory irrespective of chromatin variation (Agrawal, 2002; Rasmann et al., 2012; Murgia et al., 2015; Nosalewicz et al., 2016; X. Zheng et al., 2017). Indeed a distinction has been made between transgenerational epigenetic effects, referring to non-genetically determined transgenerational phenotypes, and transgenerational epigenetic inheritance, referring to non-genetically determined transgenerational phenotypes attributable to heritable chromatin modifications (Youngson & Whitelaw, 2008). Thus, evidence for the formation of transgenerational drought stress memory was investigated in drought exposed Arabidopsis lineages propagated by single seed descent. Despite successive generations of repeated drought stress, during both vegetative and reproductive growth stages, no altered above ground morphological growth phenotypes were observed. This was also surprising given the recent reports of transgenerational memory phenotypes observed in Arabidopsis for salinity and low humidity experiments (Sani et al., 2013; Tricker et al., 2013; Wibowo et al., 2016). A caveat of this study was that root phenotypes were not investigated, as previously *Polygonum persicaria* and barley roots demonstrated transgenerational memory phenotypes in response to drought (Herman & Sultan, 2016; Nosalewicz et al., 2016). However, root memory phenotypes are not a general occurrence as demonstrated in studies of phosphate starvation in rice (Secco et al., 2015). Recently, propagation of rice under drought stress lead to above-ground differences in generation 11 plants compared to the first generation (X. Zheng et al., 2017). However, in this study, critically there were no unstressed lineages incorporated to enable analysis of the phenotypic changes to be considered alongside associated DMRs, as opposed to the stochastic methylome variability observed herein that can arise over such a long-term experiment.

Here, the only evidence of transgenerational memory was in the form of increased seed dormancy (72% enhanced dormancy), which persisted, to some extent, beyond a generation of drought stress exposure (31% enhanced dormancy). This seed-specific memory might be expected of a rapid-cycling annual species whose success is dependent on seed behaviour (Grime et al., 1981; Thompson, 1994; Springthorpe & Penfield, 2015). Any effect of enhanced seed dormancy on other developmental phenotypes, in this study, would have been masked by the seed stratification treatment performed prior to experimentation. Though the potential adaptive advantage of increased seed

dormancy was not directly tested in this study, it would not be inconsequential as seed dormancy dictates the environment that progeny plants would germinate in; thus, having a potentially critical impact on early growth (Finch-Savage & Leubner-Metzger, 2006; Shu et al., 2016). Such a trait has also been suggested to be an advantage for progeny whose parents were affected by herbivory (Agrawal, 2002; Rasmann et al., 2012).

Increased seed dormancy is a classic form of maternal imprinting, whereby environmental conditions experienced by the maternal plant can influence seed development, altering seed properties including propensity to germinate. For example, seeds that develop under conditions of stress induce maternal ABA production, which can increase seed ABA content thus enhancing dormancy (Finch-Savage & Leubner-Metzger, 2006). This altered seed provisioning would be the simplest explanation for the enhanced seed dormancy observed, especially since the *D2* treatment occurred during early reproductive development. Indeed, independent transgenerational studies on iron deficiency have also shown memory phenotypes to be carried through altered seed provisioning that were lost in the absence of stress (Murgia et al., 2015). Here, however, the enhanced seed dormancy persisted, for another generation, in seeds developed in the absence of stress (P_1 seed), albeit to a weaker magnitude. This persistent memory is more consistent with the notion of transgenerational memory. The mechanism conveying this memory is not resolved; however, it appears to be DNA methylation independent. Histone modifications were not assayed in this study but variations may also have been induced. Indeed, osmotic-stress induced variation in histone methylation has previously been reported to mediate stress priming to hyper-osmotic stress within a generation lending support to this hypothesis (Sani et al., 2013).

4.4 Conclusion

This chapter presents a systematic investigation of the possibility for DNA methylation variants to act as heritable stress-induced epi-alleles to convey transgenerational drought stress memory for multiple physiological traits that could be associated with drought responsiveness. Overall, Arabidopsis showed one specific memory trait: elevated seed dormancy in both the direct seed of drought stressed parents (72% enhanced dormancy) and in seed produced from P_1 progeny, from drought exposed lineages, grown in the absence of stress albeit to a lesser magnitude (31% enhanced dormancy). Whether this conveys an adaptive advantage remains unclear as seed stratification was done prior to experimentation for above ground memory traits. Furthermore, there are likely to be cell-type specific responses that contribute to the complexity of plant stress memory, which will be important to consider in future investigations. Despite the appearance of 40 drought-associated DMRs within a generation, transgenerational drought stress-induced epi-alleles were rare and are unlikely to act as a mechanism to convey any form of transgenerational stress memory. Rather, the majority of DNA methylation states are

highly stable and the variation observed here, within and across generations, appears to occur stochastically predominantly at repetitive regions of the Arabidopsis genome. In conclusion, despite evidence of transgenerational drought stress memory for one of the six traits examined the methylome was relatively impervious to stress-induced changes.

Chapter 5

Maintenance of pre-existing DNA methylation states through recurring excess-light stress

---

This chapter presents, in full, results that have been published in Ganguly et al. 2018 and is available online at Plant, Cell & Environment.

5.1 Synopsis

The previous chapter reports a lack of drought-induced methylome variation in Arabidopsis, of which a negligible extent persisted under transgenerational recurring drought. Such results of a robust methylome impervious to abiotic stress raises multiple questions: (I) is this a general principle of the methylome or was it specific of drought stress and, (II) did a the lack of variation in the methylome reflect a lack of physiological memory in the transgenerational lineages? To extend this work by clarifying these unknowns the resulting hypothesis, of a methylome that is largely impervious to abiotic stress, was tested. In contrast to the previous chapter, where minimal physiological priming was observed, the abiotic stress of EL was chosen based on its ability to reprogram new tissues. Indeed, plants utilize various photoprotective mechanisms to optimize photosynthetic performance in response to fluctuations in light intensity, which can be pre-emptively primed in leaves unexposed to EL by those exposed via SAA (Karpinski et al., 1999; Rossel et al., 2007; Szechyńska-Hebda et al., 2010), particularly in the case of recurring EL stresses (Gordon et al., 2013). While key signalling molecules and transcription factors are known to contribute towards this priming signal, an unexplored question is the potential involvement and significance of chromatin marks towards the establishment, maintenance, and memory of light-stress acclimation. An essential prerequisite is that a stress should, in and of itself, lead to chromatin changes that could be heritable through mitotic or meiotic cell divisions, thus acting in a truly epigenetic manner (Eichten et al.,

2014). Furthermore, these changes should be able to induce differential gene expression at biologically relevant loci. Thus, this chapter addresses these questions by evaluating the contribution of DNA methylation variation towards EL stress acclimation. To do this, a WLRS time-course experiment was designed based on previous work examining the effects of repeated targeted EL treatments on the expression of SAA outputs, in particular, showing that unexposed newly emerging tissue were primed against fluctuating light intensities compared to pre-existing exposed leaves (Gordon et al., 2013). This chapter extends such observations into a whole rosette EL system allowing a systematic evaluation on the ability of EL stress to induce differential methylation, the potential for this variation to contribute towards altered gene regulation, and whether such changes persist into newly emerging leaves developed in the absence of stress (memory).

5.2 Results

5.2.1 Recurring excess-light stress to test for mitotic light stress memory

To investigate the contribution of DNA methylation towards SAA or mitotic memory, a recurring EL stress time-course, termed WLRS, was designed and performed (**Figure 5.1**). The quality of light and the treatment scheme applied was derived from previous studies (Jung et al., 2013; Gordon et al., 2013; Crisp et al., 2017) and involved three daily one-hour exposures to 10X standard growth irradiance, using a source of 'warm light' that effectively induces oxidative stress, for one week. When subjected to this type of stress, plants are known to acclimate through improved photoprotection and transcriptional memory pathways without causing permanent photosystem damage (Crisp et al., 2017). Accordingly, physiological measurements and methylome profiles were made on plants after both a single one hour exposure (Control + EL) and following the complete WLRS exposure at the same developmental stage ('day eight'). In addition, further physiological measurements were performed after one week of recovery (absence of stress) allowing the sampling of multiple tissue types: newly emerging and developed naive tissue, and recovered pre-existing leaves exposed to the stress; to test for mitotic memory.

5.2.2 Week long recurring stress leads to excess-light stress priming

Plants subjected to WLRS were compared to unstressed counterparts, on day eight, to establish EL stress priming consistent with previous reports of photo-acclimatory reprogramming via SAA (Szechyńska-Hebda et al., 2010; Gordon et al., 2013). Plants exposed to WLRS exhibited minor morphological differences compared to control plants

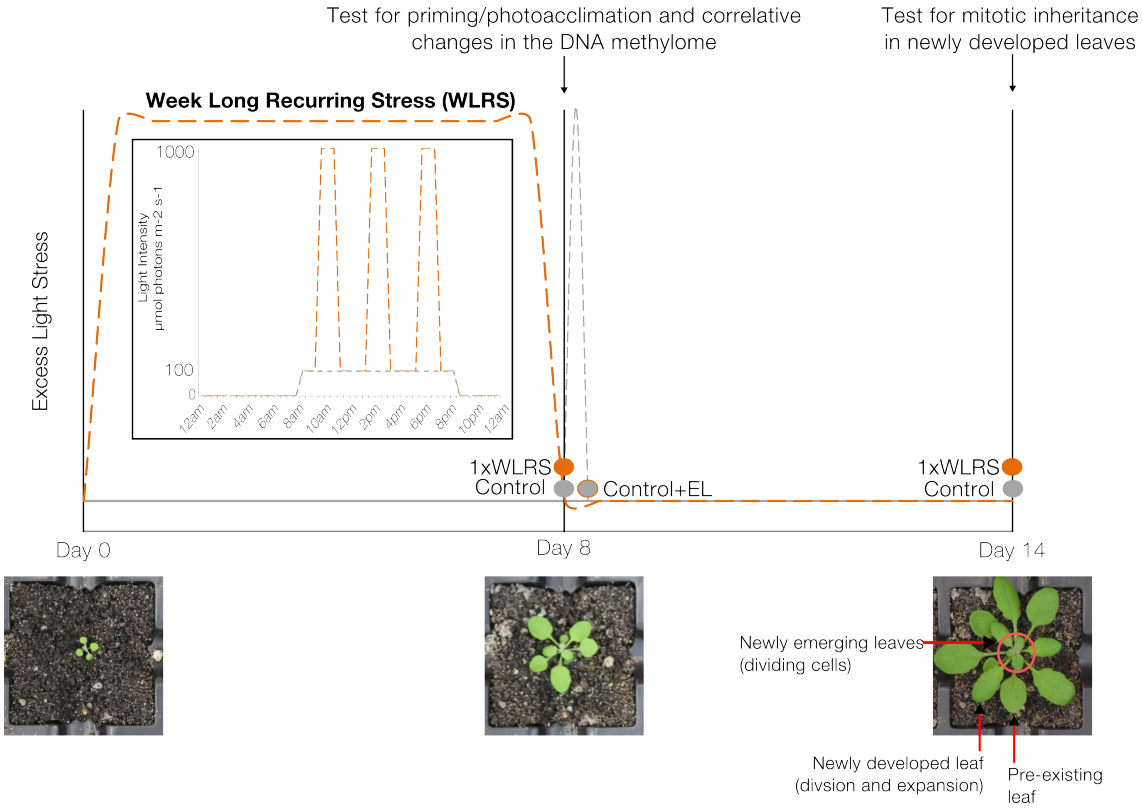

Figure 5.1: Week long recurring stress time-course
Schematic depicting the two week time-course employed to test for light stress priming and memory. This involved an initial week of recurring excess-light stress (WLRS, see inset for daily regime) followed by a one-week recovery period. Plants were exposed to 10X growth irradiance (1000μmol photons m^{-2} s^{-1}), for one hour, three times daily beginning at approximately two weeks of age (from planting). On day eight of the time-course, plants were subjected to only a single one-hour excess-light stress. Subsequently, plants continued to grow for one additional week under control light conditions (100μmol photons m^{-2} s^{-1}). Tissue sampling occurred on day eight of the time-course, immediately before and after the day eight one-hour light stress. PSII performance measurements were performed at noon of day eight, prior to the day eight stress treatment and seven days post WLRS. The latter involved distinguishing profiles of pre-existing exposed leaves, newly developed leaves, and newly emerging leaves where cell division is still occurring.

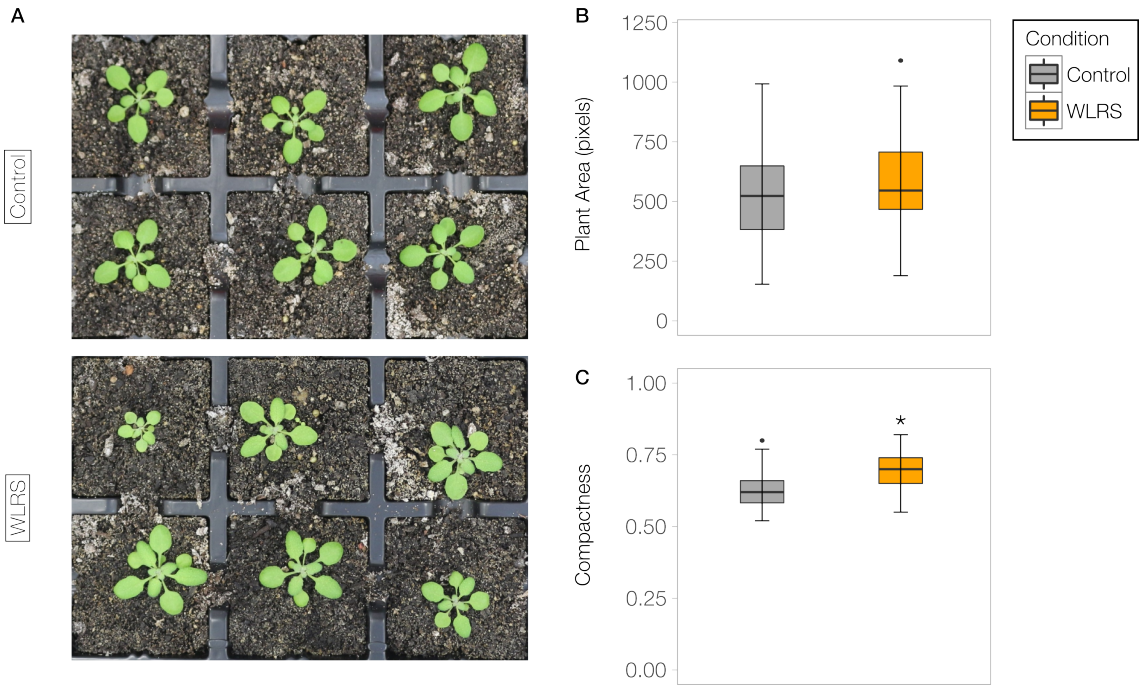

Figure 5.2: Plant growth on day eight of WLRS time-course
A Representative four week old plants on day eight of the WLRS time-course and unstressed controls. **B** Standard boxplots of plant area and compactness of four week old control (n=74) and WLRS treated plants (n=64). * denotes statistical significance ($p < 0.05$).

(**Figure 5.2A**). Although WLRS treated plants did not show altered plant leaf area, they did have a more compact rosette (**Figure 5.2B-C**). Previous reports of photoacclimatory reprogramming often report enhanced PSII photosynthetic performance, thus, chlorophyll fluorescence measurements were used to estimate various PSII parameters (see **Table 2.1**). These measurements were taken at 12pm on day eight of the time-course, at least 12 hours after the final stress treatment on day seven to allow for recovery and stabilisation of PSII behaviour. While a single one hour EL typically leads to temporary impairment of PSII photochemistry (Gordon et al., 2013; Crisp et al., 2017) the WLRS treatment led to ongoing changes (**Figure 5.3A-D**). This altered performance did not appear to be the result of permanent damage to PSII as measures of F_v/F_m and F_v'/F_m' indicated no impairment in the maximum potential quantum efficiency of PSII (**Figure 5.4A**). Despite this, WLRS treated plants exhibited increased PSII quantum yield (ϕ_{PSII}, **Figure 5.3A**) possibly due to greater photochemical capacity as a result of increased 'open' PSII centres (estimated by qP and qL; **Figure 5.3B-C**). Additionally, WLRS treated plants were faster to engage NPQ. However, this relaxed within minutes under actinic light as opposed to unstressed control plants that demonstrate greater NPQ activation towards the end of the light period (**Figure 5.3D**). Estimation of the fluorescence decline ratio (R_{fd}, **Figure 5.4B**) revealed a minor but significant difference, however, both groups of plants could be considered to have highly efficient carbon fixation (geq 3; Haitz & Lichtenthaler 1988).

Important components of photoacclimation include adjustments to the chlorophyll

a to chlorophyll *b* ratio, and the production of photoprotective pigments such as zeaxanthin, which contributes to NPQ (Anderson et al., 1988; Niyogi et al., 1998), and beta-carotene, which may also have a photoprotective role via quenching singlet oxygen (Telfer, 2002). Therefore the chlorophyll and carotenoid compositions of WLRS treated plants were examined. First, there was negligible variation in the levels of chlorophylls in WLRS treated plants when compared to control plants (**Figure 5.4C**), which mimics results in barley whereby improved PSII traits were not associated with an altered chlorophyll *a*:*b* ratio (Zivcak et al., 2014). Unexpectedly, there was a constitutive reduction in the proportion of lutein and altered ratios of xanthophyll cycle pigments, including reduced zeaxanthin and increased antheraxanthin under EL stress in WLRS treated plants (**Figure 5.3E**). No statistically significant changes in beta-carotene and neoxanthin were observed.

5.2.3 Evidence of photo-acclimatory reprogramming in newly developed and emerging leaves of WLRS treated plants

We next tested whether this primed state could be transmitted mitotically by profiling PSII performance after one week of recovery. A distinction was made, at this time-point, between three different leaf types: pre-existing, newly developed, and newly emerging leaves (**Figure 5.1**; Day 14). The latter two types were defined based on prior work (Donnelly et al., 1999). Newly developed leaves were considered to be derived from primordia or developing leaves present by the end of WLRS and having undergone subsequent cell division and expansion. Newly emerging leaves were considered to have developed post-WLRS thus, at the time of profiling, should be predominantly composed of newly dividing cells. Each of these tissue groups were assayed in WLRS exposed plants and compared to their unstressed counterparts.

The parameters ϕ_{PSII}, qP, and qL were observed to be consistent across the studied tissue types between control and WLRS treated plants suggesting that while these PSII parameters were primed by EL, this priming was lost in the absence of stress and were not mitotically transmissible (**Figure 5.6A-C**). Newly emerging leaves of WLRS treated plants displayed a slightly altered F_v'/F_m' profile (**Figure 5.5A**), although it is questionable whether this represents a physiologically relevant difference akin to the that observed on day eight (**Figure 5.3A**). More discernibly, newly developed and emerging leaves from WLRS treated plants exhibited altered R_{fd} and a distinct NPQ profile compared to corresponding leaves in unstressed controls and also to the pre-existing leaves in WLRS treated plants. This is consistent with the notion of mitotic memory, however, it does not appear to be the simple transmission of the priming on day eight (**Figure 5.5B-C**). In the case of NPQ, this profile did not resemble that of WLRS treated leaves on day eight (**Figure 5.3D**), however, R_{fd} values in new leaves were comparable to those profiled on stress-exposed tissue on day eight (**Figure 5.4B**). The pre-existing leaves of WLRS treated plants at day 14 also showed a comparatively relaxed NPQ profile, as

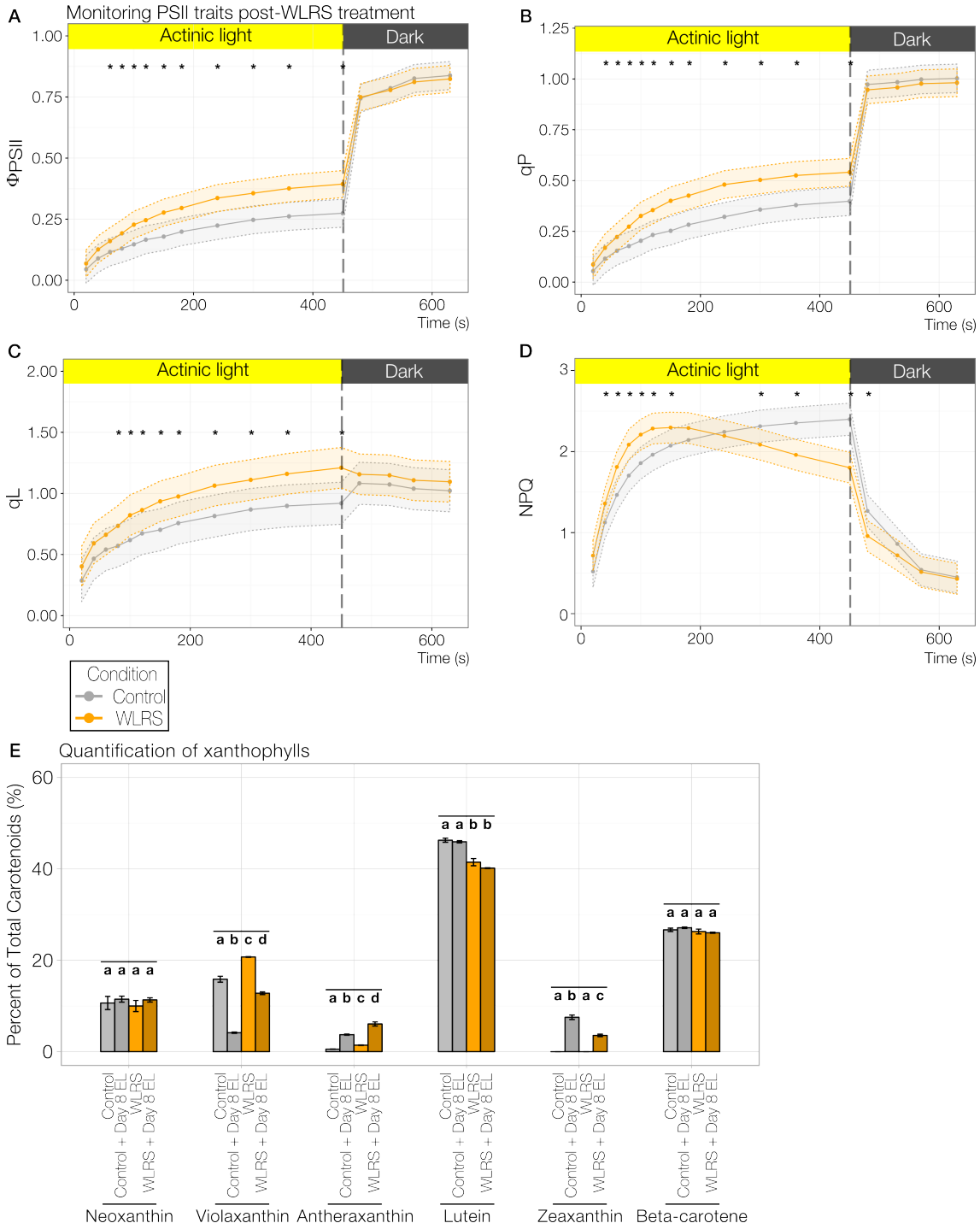

Figure 5.3: Photosynthetic priming post-WLRS

A - **D** PSII performance of control (n=200; grey) and WLRS exposed (n=170; orange) plants. Chlorophyll fluorescence measurements were taken 12 hours after the final day of WLRS and used to estimate: (**A**) PSII quantum efficiency (ϕ_{PSII}), (**B**) coefficient of photochemical quenching (qP), (**C**) fraction of open PSII Centres (qL), and (**D**) NPQ. Points denote computed least squares means; shaded regions denote 95% confidence interval; data aggregated across three independent experiments. * denotes statistical significance ($p < 0.05$) between conditions.

E Quantification of beta-carotene and xanthophylls expressed as a percentage of the total carotenoid pool. Bars denote mean, error bars denote standard error of the mean. Letters denote significant differences between conditions ($p < 0.05$, n=3) for each pigment.

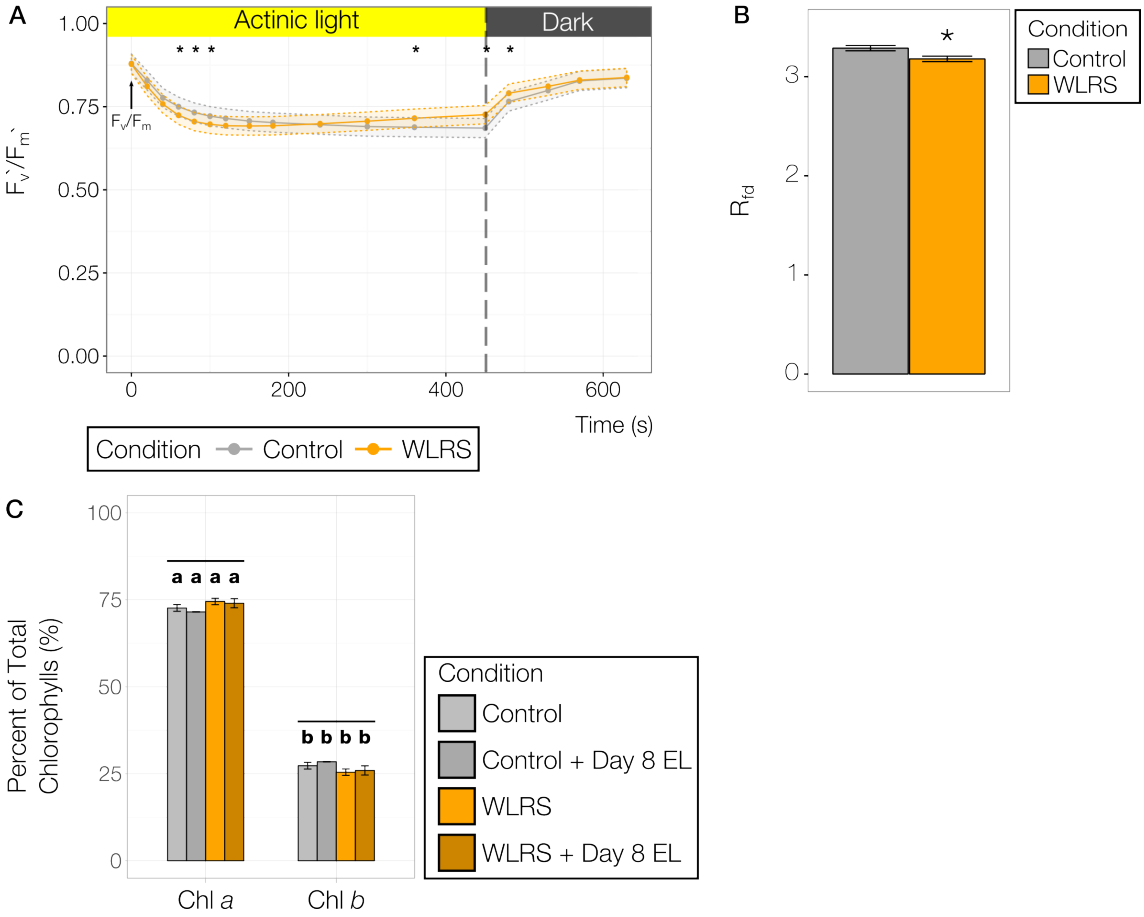

Figure 5.4: Further characterisation of PSII performance on day eight of WLRS
A F_v'/F_m' measured on control (n=200; grey) and WLRS exposed (n=170; orange) plants at 12pm. Points denote computed least squares means; shaded regions denote 95% confidence interval; data aggregated across three independent experiments. * denotes statistical significance ($p < 0.05$) between conditions.
B R_{fd} measured on control (n=200; grey) and WLRS exposed (n=170; orange) plants at 12pm. Bars denote raw means; error bars denote standard error of the mean; data aggregated across three independent experiments. * denotes statistical significance ($p < 0.05$) between conditions.
C Percentage of chlorophyll *a* and chlorophyll *b* of the total chlorophyll pool. Bars denote mean, error bars denote standard error of the mean. Letters denote significance groups for each pigment between conditions ($p < 0.05$, n=3).

compared to their profile after WLRS (day eight, **Figure 5.3D**), indicative of some form of recovery.

5.2.4 Gene expression of epigenetic components under excess light-stress

A possible mechanism for stress-induced differential methylation is through altered transcriptional control of chromatin modifying factors under abiotic stress. To test this, an EL stress time-course mRNA-sequencing dataset was utilized to explore whether such components may be responsive to a single, or repeated, EL stress (Crisp et al., 2017). A range of epigenomic factors including those involved in DNA (de)methylation, histone (de)methylation, chromatin remodelling, and small RNA biogenesis were collated to investigate if any such components were responsive to EL and thus potentially underpin any such epigenomic change (M. Matzke et al., 2009; Law & Jacobsen, 2010; Kurihara et al., 2012; Stroud et al., 2013, 2014; M. A. Matzke & Mosher, 2014; Ye et al., 2016). In total, 109 of these loci were detectable across four time-points including 24 hours recovery and repeated EL stress (**Figure 5.7A**, **Appendix A**-Dataset 3 Table 1). The majority of transcripts remained relatively unchanged across the observed time-points with *AGO2* showing the strongest up-regulation ($\approx$ 8-fold after 30 minutes EL). From the 109 detected epigenomic loci, 22 showed a significant response ($>$ 1.5 fold change vs time = 0, FDR $<$ 0.05) to either 30 or 60 minutes EL, or repeated 60 minutes EL (**Figure 5.7B**). Loci were also categorised based on broad epigenomic function, however, this did not reveal any enrichment in responsiveness from particular pathways or processes. Extensive transcriptional memory was previously observed across all transcripts (Crisp et al., 2017), so it was tested whether any epigenomic loci displayed such behaviour that might contribute to greater chromatin variation upon repeated stress. However, only *DMS4* showed a significantly altered response (FDR $<$ 0.05) after a subsequent EL stress compared to the initial stress. Thus, there was a general lack of transcriptional responsiveness of loci encoding for chromatin factors against single and recurring EL stress.

5.2.5 Limited variation in the DNA methylome associated with excess-light stress

Transcriptional stability of epigenomic encoding loci, under EL stress, does not preclude alterations in the methylome. To test whether potentially heritable changes in DNA methylation might be induced by EL stress, with the potential to contribute towards the EL priming or memory observed herein, WGBS was performed (**Appendix A**-Dataset 3 Table 2). Whole rosettes of unstressed (Control, n=3), single hour EL stress (Control+EL, n=3), and WLRS treated (WLRS, n=3) plants were harvested on day eight of the time-course. Broad methylome similarity was first compared by correlating genome-

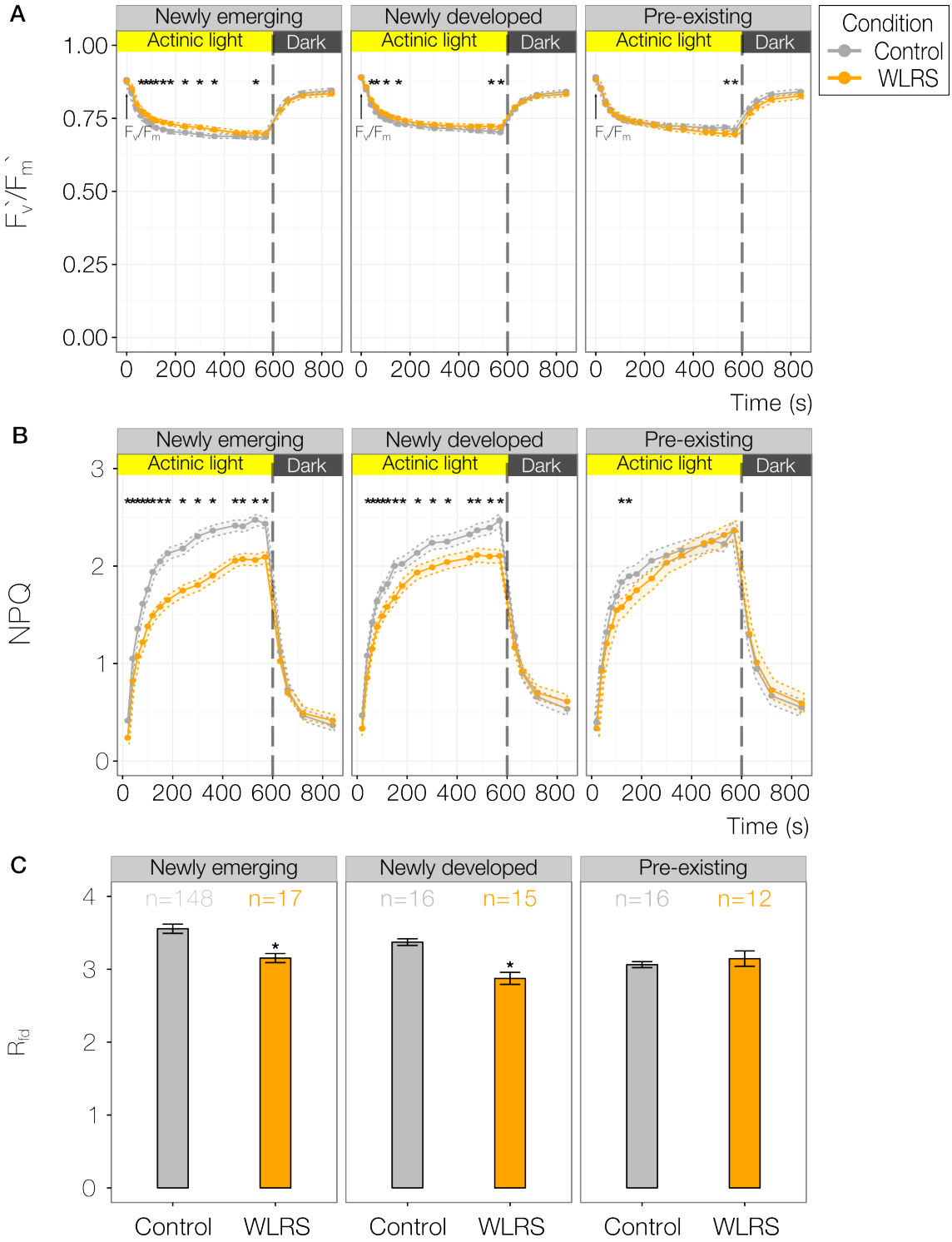

Figure 5.5: PSII parameters showing altered profiles in newly emerging leaves of WLRS treated plants

A - **B** F_v'/F_m' and NPQ measured on pre-existing leaves (n=16, n=12), newly developed leaves (n=16, n=15) and newly emerging leaves (n=18, n=17) seven days post-stress for control and WLRS exposed plants, respectively. Points denote computed least squares means; shaded regions denote 95% confidence interval; data aggregated across three independent experiments. * denotes statistical significance ($p < 0.05$) between conditions.

C R_{fd} measured on pre-existing leaves (n=16, n=12), newly developed leaves (n=16, n=15) and newly emerging leaves (n=18, n=17) seven days post-stress for control and WLRS exposed plants, respectively. Bars denote raw means; error bars denote standard error of the mean. * denotes statistical significance ($p < 0.05$) between conditions.

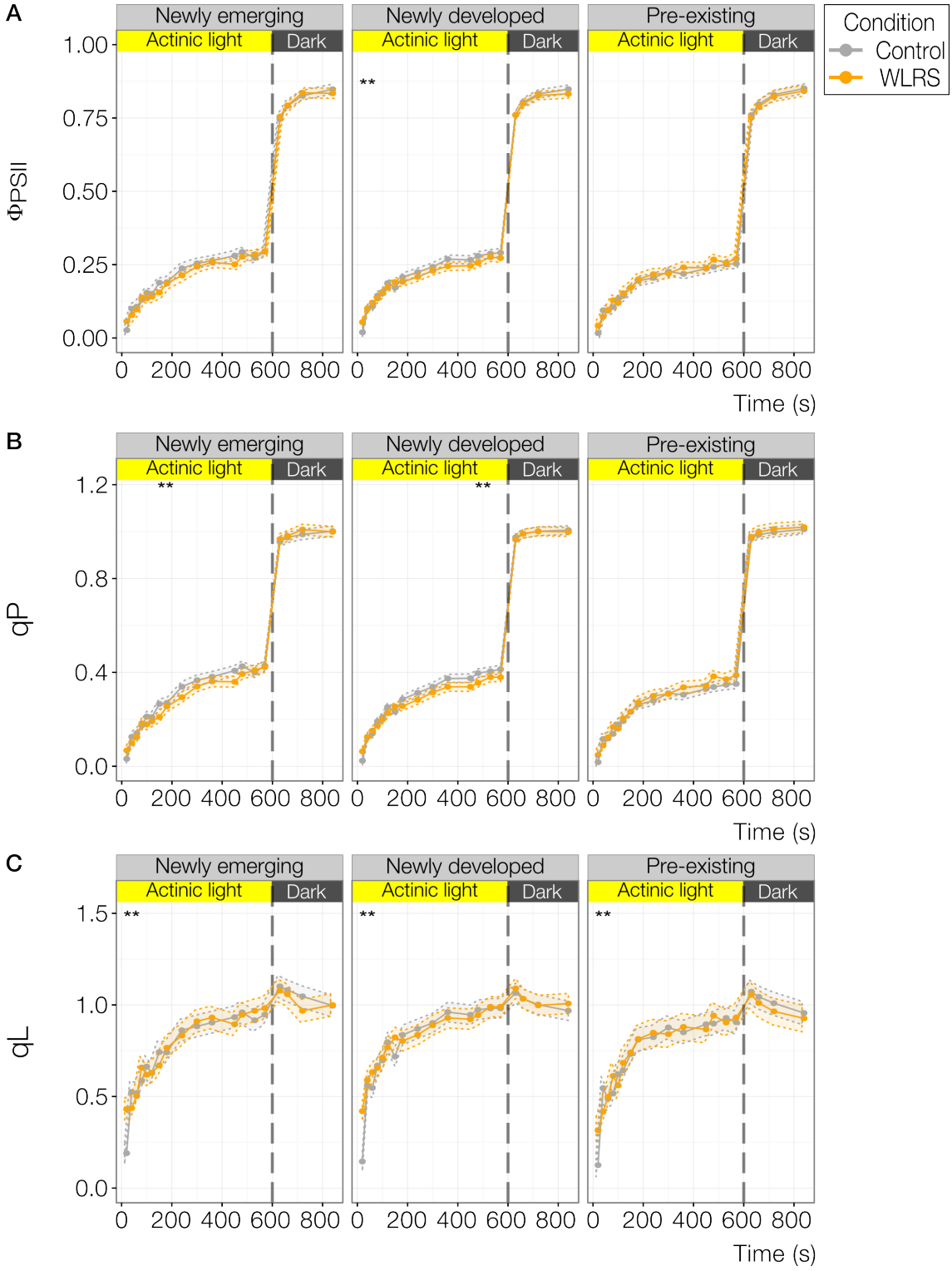

Figure 5.6: PSII traits with consistent profiles across tissue types
A - **C** ϕ_{PSII}, qP, and qL measured on pre-existing leaves (n=16, n=12), newly developed leaves (n=16, n=15) and newly emerging leaves (n=18, n=17) seven days post-stress for control and WLRS exposed plants, respectively. Points denote computed least squares means; shaded regions denote 95% confidence interval. * denotes statistical significance between conditions ($p < 0.05$).

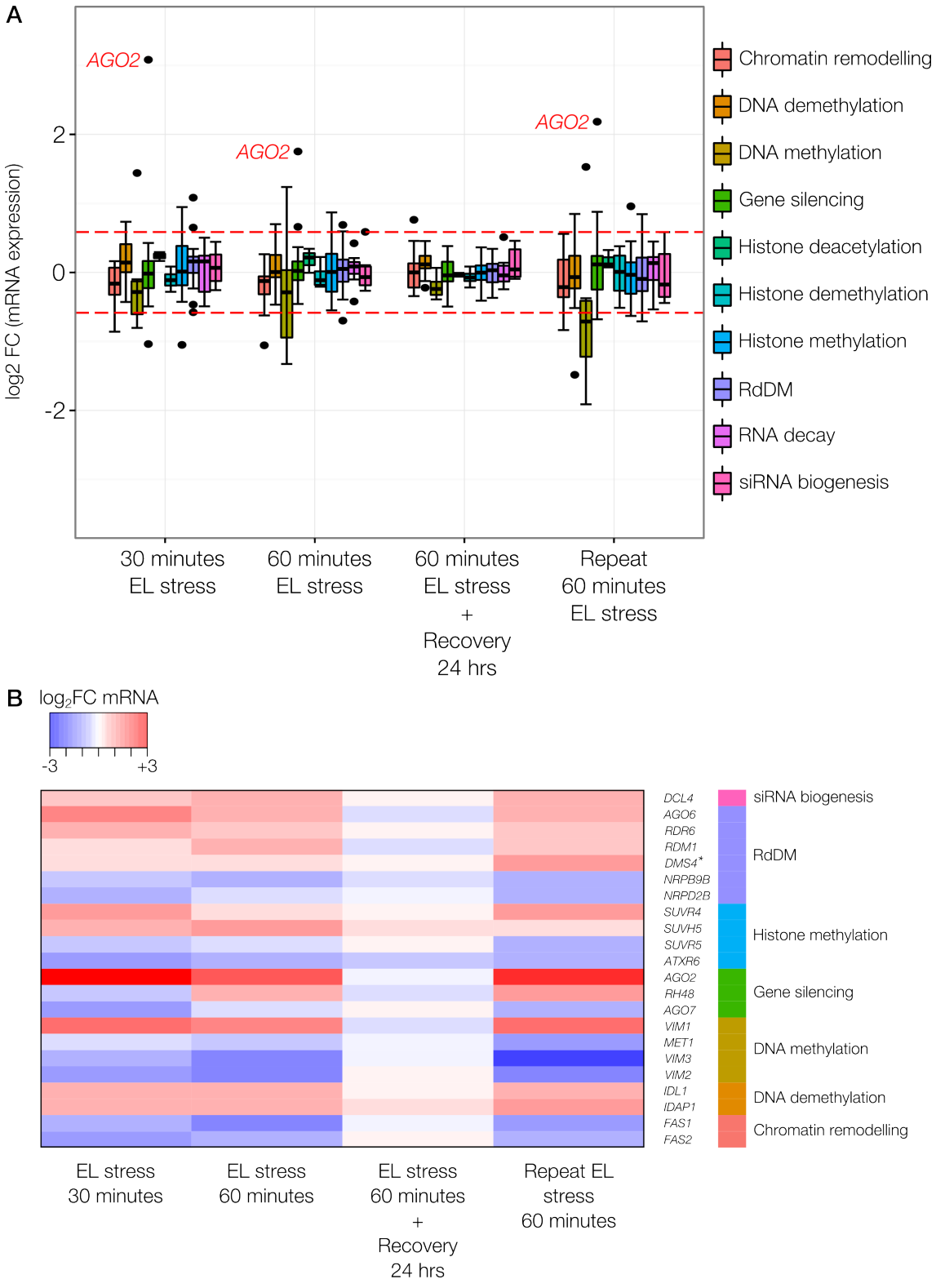

Figure 5.7: Expression changes in epigenomic component encoding loci under EL
A Standard boxplots of $\log_2$ transformed FC in mRNA abundance across four time-points in an EL stress and recovery time-course from published mRNA-sequencing data. Box colours denote broad epigenomic functions used to categorise loci. Dashed horizontal lines denote 1.5x up- and down-regulation.
B Heatmap of $\log_2$FC in mRNA abundance of epigenomic loci that were significantly (FC $>$ 1.5, FDR $<$ 0.05) responsive to EL stress across four time points: 30 minutes EL stress, 60 minutes EL stress, 60 minutes EL stress + 24 hrs recovery, and repeated 60 minutes EL stress. Side colours denote functional grouping. * denotes genes that had significantly altered response (FDR $<$ 0.05) upon a subsequent 60 minutes EL compared to the initial 60 minutes EL stress.

Table 5.1: *DSS*-based DMRs associated with single (Control + EL) or recurring (WLRS) EL stress

Contrast	Sequence context			
	mCG	mCHG	mCHH	Total
Control vs Control + EL	0	4	7	11
Control vs WLRS	0	3	3	6

wide methylation levels binned into 100bp tiles that revealed high correlation between all samples in the mCG (Pearson's $r > 0.98$), mCHG (Pearson's $r > 0.94$), and mCHH (Pearson's $r > 0.89$) sequence contexts.

To identify EL stress associated changes in the methylome, DMR calling was performed using *DSS* (H. Feng et al., 2014). This yielded no mCG-DMRs and a total of 17 non-mCG DMRs displaying differences in DNA methylation between unstressed and EL stressed plants (**Table 5.1**). Despite this conservative calling and statistical significance, the detected DMRs still displayed qualitative variation in methylation state within treatment groups (**Figure 5.8B**). Surprisingly, there were comparable numbers of DMRs after a single EL treatment (11 DMRs) and one week of recurring EL (6 DMRs). There was only one DMR in common between both comparisons; however, it also showed inconsistency within treatments groups (**Figure 5.8A**). Furthermore, the majority (13/17) of *DSS*-based DMRs were located at TEs (**Appendix A**-Dataset 3 Tables 3 - 4).

Having observed limited EL stress-associated DMRs, the extent of stochastic variation between samples, irrespective of treatment, was explored using pairwise comparisons of weighted methylation levels binned into 100bp tiles across the genome (Eichten & Springer, 2015). Here, pairwise comparisons between all samples revealed greater variation in all three sequence contexts (132 mCG, 815 mCHG, and 659 mCHH DMRs; **Figure 5.8C-D**; **Appendix A**-Dataset 3 Table 5). Thus, while negligible conserved variation was observed with EL stress, stochastic variation is evident in the methylome between sibling plants derived from an inbred parent.

5.3 Discussion

In the previous chapter a lack of drought-induced methylome variation was established; however, this was tied to only minor observations of physiological memory. There are numerous independent reports demonstrating that photoprotective mechanisms, induced by EL, can lead to robust programmable changes in newly developing leaf tissues (Karpinski et al., 1999; Thomas et al., 2003; Szechyńska-Hebda et al., 2010; Gordon et al., 2013). This provided a unique system with which to systematically test the hypothesis, of a methylome that is impervious to stress, using a recurring EL stress that reprograms new tissues, across a rosette, to be primed for altered light intensities. Using an experimental design to minimize genetic variation in Arabidopsis siblings, from an inbred parent,

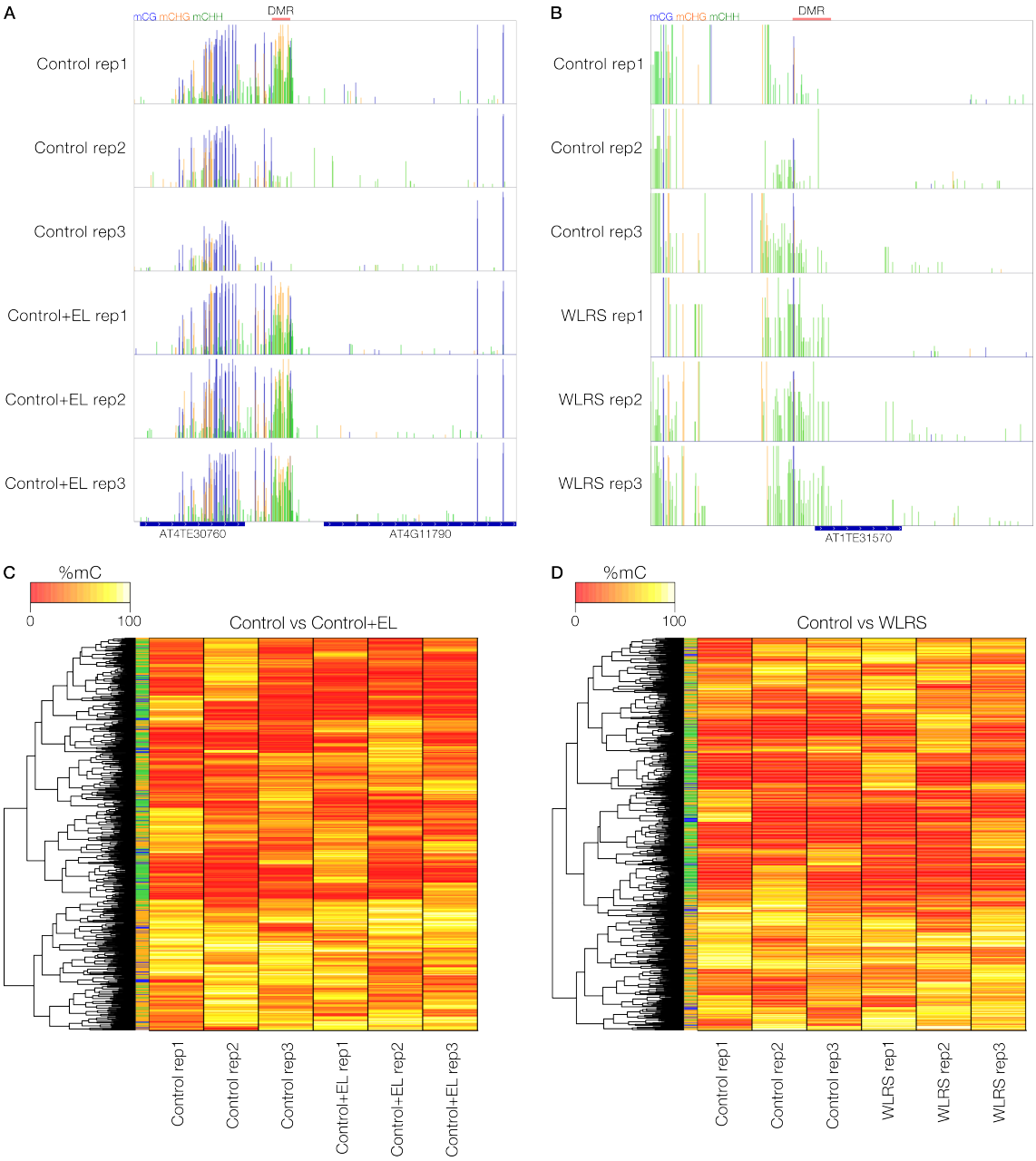

Figure 5.8: DNA methylation profiles of control and EL stressed plants
DSS based DMRs (n=3) visualized in *IGV* comparing: **A** control and one-hour excess-light stressed plants (Control vs Control + EL) and **B** control and WLRS-treated plants (Control vs WLRS). Bars denote mean methylation at individual cytosines. Blue, orange, and green bars denote mCG, mCHG, and mCHH sequence contexts, respectively.
C Heatmaps with one dimensional hierarchical clustering of average methylation across 100bp tile based DMRs between pairwise comparisons of Control and Control+EL samples. Blue, orange, and green side row colours denote mCG, mCHG, and mCHH DMRs, respectively.
D Heatmaps with one dimensional hierarchical clustering of average methylation across 100bp tile based DMRs between pairwise comparisons of Control and WLRS samples. Blue, orange, and green side row colours denote mCG, mCHG, and mCHH DMRs, respectively.

physiological measurements were coupled to in-depth methylome profiling to establish the contribution of stable DNA methylation changes towards photo-acclimatory reprogramming, in the context of SAA, priming, or memory.

WLRS induces EL stress priming

There is now ample evidence for the phenomenon of stress priming in plants, of a variety of species, whereby prior exposure to stress conveys a state of enhanced responsiveness (Conrath et al., 2006; Bruce et al., 2007; Crisp et al., 2016; Lämke & Bäurle, 2017). Indeed, priming has been documented in response to a variety of biotic and abiotic factors including heat, osmotic stress (drought and salt), insect herbivory, fungivory, and temperature (Cayuela et al., 1996; Jakab et al., 2005; Gorsuch et al., 2010; Ding et al., 2012; Rasmann et al., 2012; Sani et al., 2013; Ding et al., 2014; X. Wang et al., 2014; Firtzlaff et al., 2016; Hilker et al., 2016; Lämke et al., 2016). Furthermore, plants also demonstrate molecular, biochemical, and structural priming, or photoacclimation, to fluctuations in light intensity that can be relayed to unexposed plant organs via SAA (Karpinski et al., 1999; Yano & Terashima, 2001; Thomas et al., 2003; Rossel et al., 2007; Szechyńska-Hebda et al., 2010; Gordon et al., 2013).

Using a recurring EL stress treatment applied across a whole rosette, this chapter re-establishes and extends on prior work showing that EL exposure leads to priming, or photoacclimation, via persistent changes in PSII activity (Rossel et al., 2007; Szechyńska-Hebda et al., 2010; Gordon et al., 2013). The WLRS treatment led to distinct changes in numerous PSII traits that persisted after at least 12 hours of recovery. Collectively, these changes indicate a primed plant that has an increased capacity for photochemical quenching along with a reduced reliance on photoprotective mechanisms evident in a fast relaxing NPQ profile. The contribution of SAA in this system is difficult to dissect as a whole rosette EL stress was utilized as opposed to targeted (Gordon et al., 2013) or partial rosette treatments (Rossel et al., 2007), however, this was not the objective herein. A difficult point to reconcile using the current data is the minor, yet significant, reduction of R_{fd} in WLRS treated plants that is considered to reflect carbon fixation efficiency and is typically higher in sun-grown plants compared to shade-grown plants (Brestic & Zivcak, 2013). Accompanying these were changes in the EL-induced accumulation of xanthophylls. These observations contradicted expectations as WLRS treated plants showed reduced accumulation of the photoprotective pigments zeaxanthin and lutein, considered necessary for qE and commonly found at elevated levels in plants grown at higher light intensities (Demmig-Adams & Adams, 1996; Müller et al., 2001; Horton et al., 2005), which is, however, consistent with the notion of an EL resistant plant with a reduced reliance on photoprotection. Additionally, WLRS treated plants also exhibited constitutively reduced levels of lutein, also considered to play a photoprotective role (Jahns & Holzwarth, 2012). This observation aligns with work suggesting that lutein contributes to a higher NPQ capacity without the slower relaxation

associated with zeaxanthin in shade grown plants that have to respond to sun flecks (X. Zhu et al., 2004; Förster et al., 2011). Furthermore, a recent study identified that initial energy dissipation, during the transition to EL, is modulated by monomeric light-harvesting complexes (Dall'Osto et al., 2017), which could potentially explain, in part, the EL priming in WLRS treated plants. Indeed, mutants lacking these factors show impaired NPQ activation during the period time of rapid NPQ activation observed in WLRS treated plants. Examining these mutants under the WLRS treatment, to test for a lack of priming, would identify whether there is any contribution from these complexes. Regardless of mechanism, it is also unknown whether this priming truly leads to enhanced resilience. While it is clear that WLRS treated plants exhibited an improved response to an additional EL stress, evidence that these plants could better endure a more severe EL, compared to naive plants, would provide compelling evidence for truly enhanced light-stress tolerance. For example, observing an increased survival rate in WLRS treated under a constitutive 1500μmol photons m^{-2} s^{-1}.

Transmission of select PSII traits indicative of mitotic inheritance

The notion of stress priming has been extended to considerations of plant stress memory (Bruce et al., 2007), herein defined as the transmission of altered characteristics across cell divisions in the absence of continued stimulus (Eichten et al., 2014; Crisp et al., 2016). Indeed, a distinction must be made between stress priming and stress memory, the latter referring to phenomena persisting temporally across cell divisions. Earlier studies have reported how old and young leaves respond differently to EL stress, concluding that mature leaves can influence the structure of developing leaves, and stressed mature leaves can promote responsiveness in younger leaves through SAA (Karpinski et al., 1999; Yano & Terashima, 2001; Thomas et al., 2003; Gordon et al., 2013). While prior studies have examined leaf development during stress, there is yet to be an analysis of new leaves that emerged post-stress and thus testing the hypothesis of mitotic memory. Here, assays were performed on 'newly developed' leaves that have undergone both cell division and elongation post-stress and 'newly emerging' leaves that are largely composed of newly dividing cells (Donnelly et al., 1999). When assayed seven days post-stress, newly developed and newly emerging leaves showed alterations in NPQ and R_{fd} that were not observed in pre-existing exposed leaves or in corresponding control leaves. Furthermore, these altered profiles do not match the changes observed in exposed leaves post-WLRS treatment, suggesting this is not a simple inheritance of the initial primed state. This is most evident in the NPQ profiles of newly developed and emerging leaves, seven days post-WLRS, that do not show the sharp activation and fast relaxation observed in WLRS treated leaves (compare **Figure 5.3D** and **Figure 5.5B**). This suggests that this tissue has an enhanced capacity to deal with EL energy, consistent with the notion of mitotic inheritance.

Although the preferred hypothesis is for the observation of mitotic inheritance, sev-

eral alternative hypotheses are also possible and may warrant further investigation in the future. A simplistic model of retained alterations in leaf structure is difficult to reconcile with a leaf developed largely in the absence of stress, with newly developed chloroplasts displaying an altered requirement for NPQ. Contributions of systemic signals emanating from the exposed tissue to the newly developed or emerging tissue, such as carotenoid oxidation products, ROS, or mobile sRNAs (Ramel et al., 2012; Carmody et al., 2016; Lewsey et al., 2016), or the direct exposure of the apical meristem to EL stress might underpin the observations in this study. However, even if there was a contribution from these, cell division would still have occurred during the seven days post-stress interval in the newly emerged tissue. To confirm whether this is an SAA-mediated phenomenon may involve confirming the transmission of these traits in SAA-impaired mutants such as *RbohD* (Miller et al., 2009; Suzuki et al., 2013). A second caveat is the possibility that the initiating primordia of newly emerged leaves was present during the WLRS treatment, even if the newly emerged leaf was not macroscopically observable post treatment, possibly contributing to the observation of memory. If this was the case it is a reasonable assumption that the newly emerged leaf might display characteristics comparable to pre-existing leaves, but this was not observed. Regardless, future investigations would benefit from the confirmation of the absence of the primordia, for the leaf in which memory is tested, at the end of the stress. Regardless, determining the exact mechanism for this transmission was beyond scope here, instead it was to systematically test whether the methylome could contribute towards maintained photo-acclimatory reprogramming in a rapidly cycling species.

The methylome is unperturbed by single and recurring EL stress

An attractive notion is that a primed state, such as observed here, might be associated with chromatin variation. Indeed, the spread of systemic acquired resistance to biotic stresses has been reported to be accompanied by histone modifications including methylation, acetylation, and histone replacement (March-Díaz et al., 2008; Jaskiewicz et al., 2011). However, a systematic evaluation of variation in the methylome in the context of sustained photoacclimation in newly developed tissue (memory) is lacking. The system used herein enabled an evaluation for the ability of EL to induce potentially heritable methylome variation, which in turn could be propagated by, or contribute towards, SAA. Yet, no substantive EL-associated changes in the methylome were detected that could have had the potential to be propagated spatially, temporally, or mitotically. This was surprising given numerous reports of stress-responsive methylome variation within a generation (Herman & Sultan, 2011; Tricker et al., 2012; Yong-Villalobos et al., 2015; Wibowo et al., 2016), and altered stress responses upon genetic or chemical perturbation of epigenomic machinery (Kant et al., 2007; Le et al., 2014; Brzezinka et al., 2016; Herman & Sultan, 2016; Wibowo et al., 2016). The observations here align with the previous chapter and documented reports presenting an Arabidopsis methylome

that is relatively unperturbed by abiotic factors (Seymour et al., 2014; Hagmann et al., 2015; Eichten & Springer, 2015; Secco et al., 2015).

An accompanying study has also shown that the Arabidopsis methylome is unperturbed by EL stress. However, this prior study was complicated by extensive methylome variation attributed to pre-existing and stochastic differences in the epigenome, previously referred to as "epi-types" (Crisp et al., 2017). This might be considered unsurprising given the lack of a strong transcriptional response in loci encoding epigenomic components under EL. The experiments performed here utilized a fastidiously propagated population of plants by single seed descent, comparing siblings from a single inbred parent to remove as much pre-existing differences as possible (Ganguly et al., 2017). Surprisingly, only a relatively modest level of stochastic difference was observed between siblings that is comparable to previous reports on plants separated by 30 generations of inbreeding (Schmitz et al., 2011; Becker et al., 2011).

Having identified and accounted for stochastic differences between the plants, using *DSS* to model variance within treatment groups, it was found that the recurring WLRS treatment did not lead to a greater induction of DMRs compared to a single EL stress. Furthermore, the majority of *DSS*-based EL associated DMRs were non-mCG DMRs occurring at TEs, rather than at functionally-relevant genes, reinforcing the notion that TEs may be drivers of epigenomic change in conjunction with the RdDM pathway ("facilitated" or "obligatory epi-alleles"; Richards 2006; Eichten et al. 2013; Schmitz et al. 2013; Eichten et al. 2014; S. Li et al. 2015; Secco et al. 2015). This might suggest that species with greater TE content may present greater capacity for epigenomic changes. The few DMRs occurring within or adjacent to protein-coding genes did not show any appreciable differences or conservation within treatment groups. Indeed, the vast majority of detectable differential methylation were found to be stochastic differences across all samples. Thus, the Arabidopsis methylome appears to be impervious to EL stress regardless of frequency (single or recurring). This suggests that DNA methylation is unlikely to contribute towards EL stress priming, including in the context of SAA. Post-transcriptional, metabolic, and signalling factors likely underpin the EL stress priming and memory that will require further elucidation. This does not rule out other genomic or chromatin factors, it is also possible that the aforementioned histone modifications could be involved in this phenomenon. Indeed, multiple investigations have reported transcriptional memory independently of DNA methylation changes where the mechanisms involved pertained to histone methylation (H3K4me2, H3K4me3, H3K27me2), stalled polymerase II levels (Ser5P polymerase II), and RNA stability (Ding et al., 2012; Sani et al., 2013; Lämke et al., 2016; Crisp et al., 2017), which warrant further investigation in the context of WLRS-induced priming and memory.

5.4 Conclusion

The key aim of this chapter was to understand the nature of photo-acclimatory memory and changes in the methylome. Photoacclimation to recurring EL stress was evident through enhanced PSII performance in exposed tissues, as expected based on previously reported SAA phenotypes. Significantly, NPQ and R_{fd} showed evidence of mitotic transmission. Despite this, the DNA methylome showed negligible stress-associated variation, with the vast majority attributable to stochastic differences, confirming the hypothesis of a stoic methylome that is not tied to an acclimatory response.

Chapter 6

Thesis Summary and Discussion

This thesis makes several important contributions to our understanding of the molecular mechanisms plants utilize to contend with recurring stress and the role of DNA methylation, in the context of plant stress priming and memory, which will be summarised in this final section.

Novel roles for the SAL1-PAP-XRN pathway in ABA signalling and mCHH maintenance

Chapter three characterises the cross-talk between PAP and ABA signalling. The key finding was involvement of PAP in regulating ABA-mediated processes, namely stomatal closure. It was demonstrated that PAP was, in and of itself, a secondary messenger that is capable of inducing stomatal closure directly, through priming of down-stream ABA components. Through further investigation, as discussed in chapter three, the exact mechanism and targets of PAP in the ABA pathway can be verified. This may reveal novel targets in addition to, or independent of, the XRNs, potentially providing additional mechanisms to regulate, or manipulate stomatal movements, and ultimately drought tolerance in plants.

Chapter three also exposes a unique framework linking stress signalling and subsequent regulation of known RdDM pathways influencing chromatin structure. Indeed, further molecular characterisation of *sal1*-8 led to the unexpected discovery of an interaction between the SAL1-PAP-XRN and RdDM pathways, capable of inducing methylome variation. Comparative analyses revealed that *sal1*-8 had reduced levels of mCHH at sites regulated by the RdDM pathway, suggesting that *SAL1* is required for proper maintenance of mCHH, however, further investigation is needed to resolve the molecular connection between these pathways. These results aligns with work showing that the RdDM pathway acts as a link between abiotic stress responses and the methylome (Popova et al., 2013; Khan et al., 2014; M. A. Matzke & Mosher, 2014; Secco et al., 2015; R. Yang et al., 2017), as well as being crucial for maintaining meristematic identity and epigenomic reprogramming during gametogenesis (Calarco et al., 2012; Baubec et

al., 2014).

Re-evaluating the notion of plant stress priming and memory

Chapters four and five utilize independent experimental systems to examine the prevalence of plant stress priming and memory. This thesis makes a clear distinction between these two processes, as described in the introduction, whereby the latter involves the persistence of stress-induced traits across mitotic or meiotic cell divisions (Eichten et al., 2014; Crisp et al., 2016). These are evaluated in response to recurring drought and EL stress, and the potential contribution from stress-responsive DNA methylation changes (discussed below). Such scenarios, in particular in the case of stress memory, are prime candidates where DNA methylation, as a heritable chromatin mark, might contribute toward plant stress responses by providing an elegant mechanism for the transmission of acclimatory responses and improved resilience (Crisp et al., 2016; Lämke & Bäurle, 2017).

A recurring EL stress experiment (WLRS) showed that plants can form memories, within a generation, to pre-emptively adjust to fluctuating light intensities. Key evidence for this came in the form of persistent alterations of photosynthetic traits, indicative of enhanced EL tolerance, in newly developed leaves, from EL primed plants, even in the absence of stress. On the other hand, a transgenerational recurring drought experiment showed that descendants of drought-stressed lineages largely did not show examples of transgenerational memory. Indeed, for the majority of traits tested, including plant growth and drought tolerance, there was little difference between stressed and control lineages. However, transgenerational memory was demonstrated in the form of enhanced seed dormancy, in drought-stressed lineages, that persisted for one generation in the absence of stress, which could be considered advantageous for a rapid cycling species (Grime et al., 1981; Thompson, 1994; Springthorpe & Penfield, 2015). Although further investigation is required to elucidate the mechanism(s) for both cases of memory, these results suggests that plants can consolidate their defences against recurring stress, which is most evident within a generation. Whether the specific forms of memory observed herein relate to the type or severity of stresses applied, or the species examined, warrants further investigation. Regardless, these results suggest that the nature of plant memory appears to be subtle and selective, and is likely dependent on the circumstances of the regulatory mechanisms underpinning acclimation towards a specific stimulus or abiotic stress.

The potential for memory is juxtaposed by considerations of plant forgetfulness, an active process promoting stress recovery to return to maximising growth that would, theoretically, enable optimal seed set (Tricker, 2015; Crisp et al., 2016, 2017). Indeed, considerations of the costs of maladaptive memories may explain the subtle or specific nature of those observed. Collectively, plant stress memory appears to be most visible within a generation and finding ways to utilize such memory could be beneficial to

"prime" plants in preparation for incoming stresses. This could be paired with mechanisms to promote efficient stress recovery to minimize the duration of upheld plant defences (Crisp et al., 2016). Interestingly, one of the memory traits observed after WLRS is faster NPQ relaxation in trained plants, a trait which was recently linked to increased plant productivity using transgenic approaches in tobacco (Kromdijk et al., 2016).

The physiological observations of stress priming observed herein appear extremely modest in comparison to recent reports in Arabidopsis (Sani et al., 2013; Wibowo et al., 2016), where stark contrasts can be made between primed and naive plants. In light of these conflicting results, it is difficult to conclusively derive the requirements, capacity, and prevalence of priming. The immediate differences between these studies are the use of plate-based methods for priming as opposed to the soil-based treatments performed here. Whether or not this reflects different responses from plate- and soil-based stresses is unclear. One possibility is that plate-based treatments may be more severe, by nature, and it is the severity of the initial stress that is determinant of the priming. Indeed, a systematic comparison of the effects of the same priming stress under equivalent plate- and soil-based conditions may clarify this, for example, measuring photosynthetic traits and light-responsive gene expression in soil- and plate-grown plants under EL.

Furthermore, a systematic evaluation for the requirements of a stress to induce priming, for example its frequency, severity, the cell types effected, or the maturity of the stressed tissues, would greatly benefit attempts to characterise the mechanisms required. In the first instance, this might involve a detailed meta-analyse of published experiments performed thus far, or a time-course experiment under a constitutive stress paired with physiological measurements of known outputs of priming, for example, NPQ responses under recurring EL might provide a system to answer some of these unknowns. An added layer of complexity would then be to study the molecular responses of different cell-types per time-point, which would greatly improve the spatial resolution of such an experiment. An alternative strategy might also be to take a reverse genetics approach to screen for aberrant priming patterns, which is possible in the case of EL stress priming through the use of high-throughput chlorophyll fluorescence imaging (Humplík et al., 2015). The use of luciferase reporter gene constructs, using known transcriptional memory loci (Ding et al., 2012; Crisp et al., 2017), may also provide insight.

An important consideration when attempting to draw conclusions regarding stress memory from the results herein, is that the short lifespan of Arabidopsis may predispose its behaviour against consolidating transgenerational memories. Rather it may be more advantageous for a rapidly cycling species to set seed for the next generation when possible using the resources accumulated beforehand (stress escape). The transient transgenerational drought memory observed, in the form of enhanced seed dormancy, fits such a scenario to reduce the disposition of offspring, whose parents endured drought, to germinate at relatively unfavourable conditions. Whereas, consolidation of

memory within a generation to fluctuating light-stress may be advantageous to ensure older leaves are more resilient to a commonly occurring stress. It is feasible that plant species with longer life-spans, such as in Norway spruce, where a strategy of stress escape is not possible, could show a greater disposition to form stress memory. If stress severity is an important consideration for priming and inducing methylation differences, another approach may be to investigate extremophile plants, and their disposition to display stress priming and memory alongside, or independently of, environmentally-induced differential methylation. An interesting observation, which is contradictory to the notion of species-specific disposition of stress memory, was that structurally related genes in maize, from a set of memory loci defined in Arabidopsis, showed comparable memory responses (Ding et al., 2014). Indeed, this suggests that there is a wider spread ability to show stress memory. Whether this response is conserved beyond maize and Arabidopsis, and whether conserved transcriptional control translates to conserved physiology also requires systematic evaluation.

Re-evaluating the roles of DNA methylation in plant stress responses

Maintenance of the methylome is clearly important for genome stability and proper plant growth and development (Finnegan et al., 1996; Henderson & Jacobsen, 2008; Reinders et al., 2009; Groth et al., 2014; Stroud et al., 2014; Williams & Gehring, 2017). As a heritable chromatin mark, it provides a clear and elegant mechanism for phenotypic plasticity and plant memory and thus has been invoked as a mechanism for epigenetic inheritance of stress responses (Youngson & Whitelaw, 2008; Gutzat & Mittelsten Scheid, 2012). However, as discussed in the introduction, this thesis is not investigating epigenetic inheritance. Rather it presents a systematic evaluation for the capacity of a chromatin mark, DNA methylation, to show functional stress-responsive variation, which may potentiate the capacity for it to underpin truly *epigenetic* phenomena (Eichten et al., 2014). However, such a role appears unlikely based on the results herein. Indeed, the major contribution of this thesis was to extensively demonstrate that the Arabidopsis methylome is largely impervious to both drought and EL stress, regardless of frequency or time-scale, despite accompanying demonstrations of physiological memory in both circumstances. Rather, the predominant source of differential methylation was identified as being stochastic variation, reflective of distant relatedness rather than environmental experience (Schmitz et al., 2011; Crisp et al., 2017). This finding demonstrates the necessity to revise our expectations for the role of DNA methylation, and potentially other epigenetic process, towards stress responses. There are two key questions that remain unanswered: *(I)* to what extent does abiotic stress induce methylome variation and, *(II)* to what extent do these variations lead to altered gene expression and physiology.

There are a plethora of studies that report differential methylation in response to a variety of environmental sources (reviewed extensively Herman & Sultan 2011; Crisp et al. 2016), however, there are also increasing reports for the stability of the methylome in

spite of environmental differences and stressful exposures, with which the outcomes of this thesis align as discussed in chapter four (Seymour et al., 2014; Eichten & Springer, 2015; Dubin et al., 2015; Secco et al., 2015; Ganguly et al., 2017; Crisp et al., 2017). Even studies performed using the same stress (phosphate starvation) on the same species (Arabidopsis), but by different groups, lead to opposing conclusions (Secco et al., 2015; Yong-Villalobos et al., 2015). Such conflicting reports across this field may be due to a lack of experimental consistency and statistical rigour when attempting to measure differential methylation, which are often confounded by extensive stochastic variation reflected in the results of this thesis, despite the use of inbred lines from a common ancestor, and echoed by several other studies (Schmitz et al., 2011; Eichten & Springer, 2015; Crisp et al., 2017). Indeed, a recent transgenerational study in rice attempted to make conclusions about methylation variants induced over generations of drought, however, did not include a control unstressed lineage to account for the stochastic differences (X. Zheng et al., 2017). Furthermore, the ancestry of the plants compared across these studies remains unknown, which is likely the predominant source of variation in the methylome. Many of the issues faced with determining conditions promoting stress priming, are also echoed here, such as a poor or non-existent characterisation of the effects of a stress used to test for stress-induced differential methylation. Collectively, the vast array of conflicting results presents a methylome that truly acts akin to genetic polymorphisms, such as has been measured and compared in mutant accumulation lines (Becker et al., 2011; Schmitz et al., 2011), with sporadic studies finding evidence of conserved differences correlating with a phenotype, such as the *BAD KARMA* variant in oil palm (Ong-Abdullah et al., 2015). Disentangling the source of this variation, whether truly *epigenetic* or not, and the extent to which this is functional remains a future challenge.

Many of the assumptions regarding the repressive effects of methylation rely on correlative observations across natural populations that do not necessarily allow for a systematic assessment. However, when these are performed, a poor correlation between methylation and gene expression, or plant phenotype, is often revealed (Seymour et al., 2014; Dubin et al., 2015; Secco et al., 2015; Meng et al., 2016). A lack of a broad correlation should not preclude the potential for association, however, as various characteristics of DMRs, and the regulation of their associated gene(s), are often not taken into account. For example, tightly developmentally-regulated genes might show robust expression in the face of a gene body DMR, whereas genes showing greater variability in their expression may be more predisposed to promoter methylation levels. Thus, there are likely specific context dependent characteristics that are lost in a whole-genome correlative survey. The subsequent challenge is that once a potential association is found, the methods were not available to systematically test for this, as one might between germplasm with conserved polymorphisms. The recent advent of artificially inducing methylation differences, however, presents a strategy to be able to systematically test

these (Ford et al., 2017). An alternative possibility to traditionally expected repressive effects of DNA methylation are its potential ability to effect methylation-sensitive DNA binding proteins, including the vast proportion of transcription factors in Arabidopsis (Maurano et al., 2015; O'Malley et al., 2016; H. Zhu et al., 2016). This vastly exacerbates the possibilities of interactions that promoter-localized DMRs may have with an associated gene, although biologically relevant examples of such regulation are required. The *epi-cistrome* dataset, identifying methylation sensitive transcription factors, provides a starting point with which potential candidate DMRs can be linked through an association with the transcription factor's predicted binding motif, as was discussed in chapter three. Lastly, there are a suit of potentially complementary and antagonistic chromatin marks with which DNA methylation competes with to ultimately shape the expression of the underlying region. However, the effects of DNA methylation are often assessed in singularity, due to technical and financial considerations of adopting combinatorial strategies. Future studies may warrant adopting methodologies such as Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) (Buenrostro et al., 2015) to capture overall DNA accessibility in response to environmental perturbations. Indeed, the observations of physiological memory that is independent of large-scale conserved changes in DNA methylation, raises questions for the mechanism(s) responsible. Whilst numerous alternative candidates could be tested, including histone marks and variants, RNA stability, sRNAs, or RNA Pol II modifications (Crisp et al., 2016; Lämke & Bäurle, 2017), the use of ATAC-seq in the context of WLRS stress priming and memory may reveal novel modes of a plant stress acclimation.

It is also possible that the methylation maintenance machinery is effective enough to reverse any potential stress-induced methylation that may have been generated but not captured by the profiling performed. Thus, stressing mutants with impaired methylation machinery may warrant future investigation to observe for aberrant stress responses, such as in response to EL or drought. It would also be interesting if such impairments could feedback onto the regulation of stress signalling pathways, thus potentially revealing the hypothesized regulatory effects of DNA methylation. The capacity for priming and memory could also be investigated in these mutants, potentially based on the observations made herein, to further assess the contribution of regulating DNA methylation to such processes. However, future projects must be performed with caution and rigorous experimental design to identify a mechanism and demonstrate causality. Furthermore, greater care with respect to sampling (e.g. cell-specific profiling) might also reveal more intricate interactions between stress signalling and the methylome. There is evidence implicating the importance of regulating DNA methylation during reproduction, cell differentiation, and development; as methylome perturbations are observed over reproduction, seed and fruit development, germination, and across cell types in root tissues and embryos (Calarco et al., 2012; S. Zhong et al., 2013; Kawakatsu et al., 2016, 2017; Narsai et al., 2017). Additionally, testing stress responses across epiRILs, which are near isogenic lines

but have highly variables methylomes, might also provide great utility, as in past studies (Johannes et al., 2009; Reinders et al., 2009; Zhang et al., 2013; Cortijo et al., 2014), to reveal functional differential methylation linked to altered stress responses. Combining the use of ATAC-seq (Buenrostro et al., 2015) across such populations might also reveal the characteristics of DMRs, in the absence of genetic variation, that are predisposed to having functional impacts. Indeed, this would be an exciting technique to perform on *sal1*-8 to further investigate the extent to which the mCHH hypo-methylation might be impacting gene expression through facilitating DNA accessibility.

There are also additional considerations to be taken into account when drawing conclusions from this thesis. The experimental work was performed in Arabidopsis, which has a relatively small diploid genome. An emerging notion is the potential for TEs to be drivers of epigenomic change (facilitated epi-alleles, Richards 2006) that, despite not being strictly *epigenetic*, can effect both the chromatin landscape and provide unique regulatory elements (Eichten et al., 2014; Springer et al., 2016). Indeed, rice showed a greater capacity for stress-induced changes than Arabidopsis that were preferentially localized to TEs, although this was also considered to be a secondary effect of altered transcriptional activity (Secco et al., 2015). Building on this, the majority of stress-associated changes identified here were also localized at TEs, which, taken together, is congruent with recent studies suggesting TEs as major drivers of epigenomic change (Eichten et al., 2013; Schmitz et al., 2013; S. Li et al., 2015; Eichten et al., 2016; Stuart et al., 2016). Regardless, this suggests that performing such a systematic analyses in species with greater TE content, such as maize, may reveal a greater level of truly stress-associated variation. Although preliminary studies suggest prolific stochastic variation still exists (Eichten & Springer, 2015), this is likely confounded by the existence of multiple epi-types as has been identified herein and previously (Crisp et al., 2017). Future examinations of inbred populations of species with high TE content may reveal a greater predisposition for chromatin variation. Furthermore, in such a genome, methylome variation also has the potential to pose greater impact on a plant's phenotype as a result of TE de-repression. This importance may extend to genomes such as wheat, which has undergone extensive endoreduplication to result in hexaploidy, a significant proportion of which consists of pseudo-genes. It is feasible that DNA methylation variation may have greater impact and importance in such a context.

Reconciling SAL1-PAP signalling, during EL and drought, and mCHH regulation

The lack of stress-induced differential methylation is also surprising when taken into consideration with results from chapter three: if PAP accumulates under these conditions (drought and EL; Estavillo et al. 2011) then why was there no impairment in mCHH via its potential interaction with RdDM? Firstly, such a drastic aberration in the methylome is unlikely given the proper activity of the complete suit of methylation machinery. It

is also possible that PAP accumulation was not as effectively induced as previous experiments, thus pairing methylome data with PAP levels in the accompanying tissue may clarify any potential contribution. It is also possible that the stress, utilized herein, was too moderate as it was designed to ensure plant survival. Thus, a more severe, potentially combinatorial, stress may reveal greater epigenomic change, particularly for the operation SAL1-PAP-XRN signalling as one potential mechanism to induce changes via abiotic stress. For example, the drought-stress associated DMRs observed within a generation used only a modest drought ($\approx$ 60% RWC after 9 days of withheld watering), whereas, PAP tends to accumulate under severe drought ($\approx$ 35% RWC; Estavillo et al. 2011). Despite only rare examples of stress-associated changes in the methylome, these were almost exclusively in the nonCG context which fits an interaction with RdDM. Additionally, SAL1-PAP-XRN signalling holds physiological relevance in plant guard cells, therefore, using cell-specific strategies to test whether guard cells, or other cell types, demonstrate greater sensitivity to methylation changes may warrant further investigation. Finally, systematically testing for the ability of PAP, in and of itself, to induce differential methylation would provide further weight to a PAP-XRN interaction with the RdDM pathway, validating a model of stress-induced PAP levels inducing changes in the methylome. This could include re-sequencing of the *sal1*-6 allele alongside the complemented *sal1*-6/*35S::AHL* line (where over-expression of *AHL* reduces PAP to WT levels; Hirsch et al. 2011). Alternatively, the effect of exogenously applied PAP to a Col-0 methylome would confirm, and reveal the kinetics of, PAP-induced differential methylation. Furthermore, identifying the exact mechanism(s) contributing to the mCHH hypo-methylation in *sal1*-8 would also provide further clues for the conditions required to induce variable DNA methylation, for example observing the cell-types or environmental conditions where such factors are most prevalent or differentially regulated (transcriptionally or post-transcriptionally).

Conclusions

This thesis has furthered our understanding of the SAL1-PAP-XRN pathway and its effects on plant stress tolerance. Novel discoveries are presented with respect to the diverse cellular impacts of a chloroplast-to-nucleus retrograde signalling pathway, including regulating stomatal closure and mCHH levels. This provides a unique framework linking stress signalling and the chromatin landscape. Furthermore, despite some capacity for priming and memory in Arabidopsis, under varying time-scales and different stressors, the Arabidopsis methylome was found to be largely impervious to stress-induced variation. Thus, this thesis favours a structural role for DNA methylation, involved in maintaining genome stability, as opposed to an environmentally-flexible regulator. This does not preclude the contribution from other chromatin marks, nor should it diminish the importance of other post-transcriptional and biochemical factors, towards stress priming and memory. Significantly, extensive evidence is presented refuting the hyperbolic po-

tential of chromatin marks, or epigenetics, towards plant stress memory, reinforcing the need for unbiased systematic evaluations of contexts where DNA methylation may hold a significant functional relevance.

Appendices

Appendix A

Supplemental datasets

Supplemental dataset 1: Summary alignment metrics for all publicly accessed next-generation sequencing datasets

Supplemental dataset 2: Transgenerational drought stress analyses

Supplemental dataset 3: Excess-light stress analyses

Appendix B

Primer sequences and descriptions

Table B.1: Cycling conditions used for qPCR experiments

Temperature°C	Duration (s)	Cycles
95	30	1
95; 60[1]; 72	15; 15; 20	40

Table B.2: Cycling conditions used for *in situ* qPCR

Temperature°C	Duration (s)	Cycles
98	60	1
95; 60; 72	10; 25; 7	35
72	350	1

Table B.3: Gene specific primers used for qPCR

Gene	**AGI**	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**
ANAC019	AT1G52890	AAGCTTTGGTGTTTTACATCGG	CAATCATCCAACTTAGTGCTTCC
LBD1	AT1G07900	TCTCCTCCACCATCACTTTCACC	AACTTGGCCGGATCCGTTGG
HB-7	AT2G46680	TGGCTTCTCAGTTCGAGTCC	GCAACCACCGCTTGATCTCC
KAT2	AT4G18290	ACCAATCAACTAAGCTCCGTAA	CCAGCCACATCTCCCATC
NRT1.1	AT1G12110	TATCAGTCGCACAATCCG	AGACGGCGGTGGTTAGGAG
PYL4	AT2G38310	GATTCCGTTCAGATTCCGAT	CGGAGCAACACTGATTAGGA
PYL9	AT1G01360	TGGTCTTCCTGCAACAACAT	TGTGATCACCACCGATGATT
SNRK3.22	AT2G30360	GTGGTGAATCGGAGAGGTTT	TCAGATTCTCCTCTCTCGCA
CRK2	AT3G19100	GCTGCTTTGATGGCTCTGTCC	GTGATGAGGCCATTTTTGTTGGG
CDPK32	AT3G57530	CTCCCGAGGTGCTAAAACGG	ACTCCTTGTTCAGTTTCTGCC
CDPK34	AT5G19360	TGGGCTGAGTCAGAGAATGGG	TGCCTGTGGTGAGATGACTGG
CRK8	AT1G49580	CCCTTCTTGTCTTCTGACGCT	GGATGCATCAAGGCTTGGGA
PP2AA3	AT1G13320	CGACCAAGCGGTTGTGGAGA	CACAATTCGTTGCTGTCTTCTTT

Table B.4: Gene specific primers used for observing gene expression localisation via *in situ* qPCR

Gene	**AGI**	**Forward primer (5′-3′)**	**Reverse primer (5′-3′)**
CDPK34	AT5G19360	TGGGCTGAGTCAGAGAATGGG	TGCCTGTGGTGAGATGACTGG
18S rRNA	18S rRNA	GGTAATTCCAGCTCCAAT	GTTTATGGTTGAGACTAG

Glossary

$^1O_2$ singlet oxygen. 4, 9

ABA abscisic acid. 1, 6, 7, 11, 13–15, 19, 27, 31, 41–50, 63, 65–68, 73, 74, 86, 89, 97, 99, 119
ABA2 ABA DEFICIENT 2. 11
ABF ABA-RESPONSIVE ELEMENT BINDING FACTOR. 13, 67, 68, 74
ABI5 ABA INSENSITIVE 5. 97
ABRE ABA-RESPONSIVE ELEMENT. 13
AGO ARGONAUTE. 23, 52, 53, 69, 71, 72, 108
ANOVA analysis of variance. 31, 32, 34, 36, 39, 45, 48
APX2 ASCORBATE PEROXIDASE 2. 15
Arabidopsis *Arabidopsis thaliana*. 27, 29, 37, 63, 65, 69, 72, 75, 76, 92, 94, 96, 98–101, 112, 116, 117, 121–126
AS2 3′-ethylsulfanyl-ABA. 31, 46, 48, 66
ATAC-seq Assay for Transposase-Accessible Chromatin with high-throughput sequencing. 124, 125
ATP adenosine triphosphate. 3, 19, 31, 43–45, 65

BABA β-aminobutyric acid. 16, 19
bZIP basic-domain leucine zipper. 13

Ca^{2+} calcium. 11, 31, 44, 46, 66, 67
CDPK CALCIUM-DEPENDENT PROTEIN KINASE. 13, 50, 51, 67, 68, 73, 74
CEK CHOLINE/ETHANOLAMINE KINASE. 89, 97
CLSY1 CLASSY 1. 23
CMT CHROMOMETHYLASE. 10, 21, 22, 53, 55, 71, 72
CNI1 CARBON/NITROGEN INSENSITIVE 1. 91–93
CO_2 carbon dioxide. 3, 6, 11, 35
cordycepin 3′-deoxyadenosine. 31, 47, 48, 66
CPM counts per million. 37, 80
CRK CDPK-RELATED KINASE. 50, 51

dCAPS derived cleaved amplified polymorphic sequence. 29
DCL DICER-LIKE. 23, 52, 55, 71
DDM1 DECREASE IN DNA METHYLATION 1. 10, 21, 22, 53
DEG differentially expressed gene. 37, 62, 64, 72, 73
DEG7 DEGP PROTEASE 7. 5
DMR differentially methylated region. 25, 38–40, 57, 60–62, 64, 69, 72, 76–81, 89–92, 94–98, 100, 112, 113, 117, 123–126
DREB2A DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN 2A. 16
DRM DOMAINS REARRANGED METHYLTRANSFERASE. 21–24, 68, 71, 72

EL excess-light. 3–5, 7–9, 12, 14, 15, 19, 26, 27, 30, 46, 101, 102, 104, 105, 108, 111–118, 120–122, 124, 125
epiRILs epigenetic recombinant inbred lines. 25, 124
ERD2 ENDOPLASMIC RETICULUM RETENTION DEFECTIVE 2. 79

FC fold-change. 54, 64, 81, 82, 111
FLC FLOWERING LOCUS C. 17
FPKM fragments per kilobase per million reads. 37, 60, 62, 64

H_2O_2 hydrogen peroxide. 4, 9, 15
HEN1 HUA ENHANCER 1. 23
HR hazards ratio. 86, 87

KAT POTASSIUM CHANNEL IN ARABIDOPSIS THALIANA. 13, 47
KEA3 K^+ EFFLUX ANTIPORTER 3. 19

LHC light-harvesting complex. 3

MET1 METHYLTRANSFERASE 1. 10, 21, 22, 53
methylome genome-wide patterns in DNA methylation. 24–27, 40, 52, 53, 55–57, 68–72, 74–76, 78, 79, 83, 89–92, 94, 96, 97, 100, 108, 112, 116–119, 122–126
mRNA messenger RNA. 52, 62, 79, 81, 82, 111
MYB20 MYB DOMAIN PROTEIN 20. 91–93

NAC089 NAC DOMAIN CONTAINING PROTEIN 89. 77, 79
NAD nicotinamide adenine dinucleotide. 19
NADPH nicotinamide adenine dinucleotide phosphate. 3
NCED 9-*cis*-epoxycarotenoid dioxygenases. 11
NPQ non-photochemical quenching. 5, 9, 19, 35, 83, 85, 104–106, 109, 114–116, 118, 121
NRPD2B NUCLEAR RNA POLYMERASE D2B. 52, 71

PAP 3′-phosphoadenosine 5′-phosphate. 14–16, 27, 31, 41–48, 50, 52, 55, 63, 65–70, 72–74, 119, 125, 126
PET photosynthetic electron transport. 3, 4
PGR5 PROTON GRADIENT REGULATION 5. 5
PKL PICKLE. 68
PP2AA3 PROTEIN PHOSPHATASE 2A SUBUNIT A3. 33
PP2C PROTEIN PHOSPHATASE TYPE-2C. 1, 13
PsbS PHOTOSYSTEM II SUBUNIT S. 5, 19
PSI Photosystem I. 3–5
PSII Photosystem II. 3–5, 9, 30, 34, 35, 83, 85, 103–107, 109, 110, 114, 115, 118
PYL PYRABACTIN RESISTENCE1-LIKE. 1, 13, 46, 66, 67

qRT-PCR quantitative RT-PCR. 32, 33, 50

R_{fd} fluorescence decline ratio. 86
RdDM RNA-directed DNA methylation. xix, 21–24, 42, 52, 53, 55–58, 60, 62, 63, 65, 68–74, 78, 81, 96, 117, 119, 125, 126
RDR RNA-DIREECTED RNA POLYMERASE. 23, 52, 68
RNA ribonucleic acid. 1, 15–17, 22, 23, 25, 31, 32, 42, 52, 63, 68–71, 73, 124
RNA Pol RNA Polymerase. 22, 23, 52, 53, 68, 71, 72, 124
ROS reactive oxygen species. 3–5, 7, 9, 11, 13, 42, 44, 52, 53, 66, 116
ROS1 REPRESSOR OF SILENCING 1. 24, 52–55, 70, 71
RPKM reads per kilobase per million reads. 37
RWC relative water content. 30, 76, 126

SAA Systemic acquired acclimation. 5, 9, 12, 17, 101, 102, 114–118
SAL1 SAL1 PHOSPHATASE-LIKE PROTEIN. 15, 16, 27, 41–44, 52, 55, 63, 65, 66, 68, 69, 71–74, 119, 125, 126
SAM *S*-adenosylmethionine. 19, 21
SHH1 SAWADEE HOMEODOMAIN HOMOLOG 1. 23
siRNA small interfering RNA. 22–24, 52, 68–72, 74
SLAC1 SLOW ANION CHANNEL-ASSOCIATED 1. 13, 50, 67, 73, 74
SnRK2 SNF1-RELATED PROTEIN KINASE 2. 13, 50, 65–67, 74
SPCH SPEECHLESS. 91, 93
sRNA small RNA. 22, 52, 69, 70, 72, 116, 124
STN7 PROTEIN KINASE STN7. 5

TE transposable element. 22–24, 52–57, 59–61, 63, 64, 72–74, 77, 78, 80, 91, 92, 94, 98, 112, 117, 125
Tukey's HSD Tukey's honest significant difference test. 31, 32, 34, 36, 39, 45, 48

VDE VIOLAXANTHIN DE-EPOXIDASE. 19

WGBS Whole genome bisulfite sequencing. 52, 53, 69, 76, 83, 84, 108
WLRS Week Long Recurring Stress. 30, 34, 102–110, 114–117, 120, 121, 124

XRN 5′-3′ exoribonuclease. 15, 16, 19, 27, 41, 42, 44, 46, 50, 52, 53, 55, 57, 63, 66–74, 119, 126

ZAT10 ZINC FINGER PROTEIN ZAT10. 16
ZEP ZEAXANTHIN EPOXIDASE. 19

References

Agrawal, A. A. (2002, dec). Herbivory and Maternal Effects: Mechanisms and Consequences of Transgenerational Induced Plant Resistance. *Ecology*, *83*(12), 3408.
doi: 10.2307/3072089

Allahverdiyeva, Y., Suorsa, M., Tikkanen, M., & Aro, E.-M. (2015, may). Photoprotection of photosystems in fluctuating light intensities. *Journal of Experimental Botany*, *66*(9), 2427–2436.
doi: 10.1093/jxb/eru463

Al-Lawati, A., Al-Bahry, S., Victor, R., Al-Lawati, A. H., & Yaish, M. W. (2016, feb). Salt stress alters DNA methylation levels in alfalfa (Medicago spp). *Genetics and molecular research : GMR*, *15*(1), 15018299.
doi: 10.4238/gmr.15018299

Anderson, J. M., Chow, W. S., & Goodchild, D. J. (1988). Thylakoid Membrane Organisation in Sun/Shade Acclimation. *Australian Journal of Plant Physiology*, *15*(2), 11.
doi: 10.1071/PP9880011

Armbruster, U., Carrillo, L. R., Venema, K., Pavlovic, L., Schmidtmann, E., Kornfeld, A., ... Jonikas, M. C. (2014, nov). Ion antiport accelerates photosynthetic acclimation in fluctuating light environments. *Nature Communications*, 5439.
doi: 10.1038/ncomms6439

Armbruster, U., Leonelli, L., Correa Galvis, V., Strand, D., Quinn, E. H., Jonikas, M. C., & Niyogi, K. K. (2016, may). Regulation and Levels of the Thylakoid K + /H + Antiporter KEA3 Shape the Dynamic Response of Photosynthesis in Fluctuating Light. *Plant and Cell Physiology*, *57*(7), pcw085.
doi: 10.1093/pcp/pcw085

Armstrong, F., Leung, J., Grabov, A., Brearley, J., Giraudat, J., & Blatt, M. R. (1995, oct). Sensitivity to abscisic acid of guard-cell K+ channels is suppressed by abi1-1, a mutant Arabidopsis gene encoding a putative protein phosphatase. *Proceedings of the National Academy of Sciences*, *92*(21), 9520–9524.
doi: 10.1073/pnas.92.21.9520

Athman, A., Tanz, S. K., Conn, V. M., Jordans, C., Mayo, G. M., Ng, W. W., ... Gilliham, M. (2014). Protocol: a fast and simple in situ PCR method for localising gene expression in plant tissue. *Plant Methods*, *10*(1), 29.
doi: 10.1186/1746-4811-10-29

Atkin, O. K., Loveys, B. R., Atkinson, L. J., & Pons, T. L. (2006, sep). Phenotypic plasticity and growth temperature: understanding interspecific variability. *Journal of experimental botany*, *57*(2), 267–81.
doi: 10.1093/jxb/erj029

Bache, S. M., & Wickham, H. (2014). *magrittr: A Forward-Pipe Operator for R.*

Baker, N. R. (2008). Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annual review of plant biology*, *59*(1), 89–113.
doi: 10.1146/annurev.arplant.59.032607.092759

Barber, J., & Andersson, B. (1992, feb). Too much of a good thing: light can be bad for photosynthesis. *Trends in biochemical sciences*, *17*(2), 61–6.

Basbouss-Serhal, I., Pateyron, S., Cochet, F., Leymarie, J., & Bailly, C. (2017, mar). 5′ to 3′ mRNA Decay Contributes to the Regulation of Arabidopsis Seed Germination by Dormancy. *Plant Physiology*, *173*(3), 1709–1723.
doi: 10.1104/pp.16.01933

Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A., & Dean, C. (2004, jan). Vernalization requires epigenetic silencing of FLC by histone methylation. *Nature*, *427*(6970), 164–167.
doi: 10.1038/nature02269

Basu, S., Ramegowda, V., Kumar, A., & Pereira, A. (2016). Plant adaptation to drought stress. *F1000Research*, *5*.
doi: 10.12688/f1000research.7678.1

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software*, *67*(1), 1–48. doi: 10.18637/jss.v067.i01

Baubec, T., Finke, A., Mittelsten Scheid, O., & Pecinka, A. (2014, apr). Meristem-specific expression of epigenetic regulators safeguards transposon silencing in Arabidopsis. *EMBO reports*, *15*(4), 446–452.
doi: 10.1002/embr.201337915

Becker, C., Hagmann, J., Müller, J., Koenig, D., Stegle, O., Borgwardt, K., & Weigel, D. (2011, sep). Spontaneous epigenetic variation in the Arabidopsis thaliana methylome. *Nature*, *480*(7376), 245–249.
doi: 10.1038/nature10555

Bensaude, O. (2011, may). Inhibiting eukaryotic transcription. Which compound to choose? How to evaluate its activity? *Transcription*, *2*(3), 103–108.
doi: 10.4161/trns.2.3.16172

Bewick, A. J., Ji, L., Niederhuth, C. E., Willing, E.-M., Hofmeister, B. T., Shi, X., ... Schmitz, R. J. (2016, aug). On the origin and evolutionary consequences of gene body DNA methylation. *Proceedings of the National Academy of Sciences*, *113*(32), 9111–9116.
doi: 10.1073/pnas.1604666113

Bewick, A. J., Niederhuth, C. E., Ji, L., Rohr, N. A., Griffin, P. T., Leebens-Mack, J., & Schmitz, R. J. (2017, may). The evolution of CHROMOMETHYLASES and gene body DNA methylation in plants. *Genome biology*, *18*(1), 65.
doi: 10.1186/s13059-017-1195-1

Blatt, M. R., Thiel, G., & Trentham, D. R. (1990, aug). Reversible inactivation of K+ channels of Vcia stomatal guard cells following the photolysis of caged inositol 1,4,5-trisphosphate. *Nature*, *346*(6286), 766–769.
doi: 10.1038/346766a0

Blevins, T., Podicheti, R., Mishra, V., Marasco, M., Wang, J., Rusch, D., ... Pikaard, C. S. (2015, oct). Identification of Pol IV and RDR2-dependent precursors of 24 nt siRNAs guiding de novo DNA methylation in Arabidopsis. *eLife*, *4*(OCTOBER2015), e09591.
doi: 10.7554/eLife.09591

Blevins, T., Pontvianne, F., Cocklin, R., Podicheti, R., Chandrasekhara, C., Yerneni, S., ... Pikaard, C. S. (2014, apr). A Two-Step Process for Epigenetic Inheritance in Arabidopsis. *Molecular Cell*, *54*(1), 30–42.
doi: 10.1016/j.molcel.2014.02.019

Boavida, L. C., Becker, J. D., & Feijo, J. A. (2005). The making of gametes in higher plants. *The International Journal of Developmental Biology*, *49*(5-6), 595–614.
doi: 10.1387/ijdb.052019lb

Bornman, J. F., Barnes, P. W., Robinson, S. A., Ballaré, C. L., Flint, S. D., & Caldwell, M. M. (2015). Solar ultraviolet radiation and ozone depletion-driven climate change: effects on terrestrial ecosystems. *Photochem. Photobiol. Sci.*, *14*(1), 88–107.
doi: 10.1039/C4PP90034K

Boudsocq, M., Droillard, M.-J., Barbier-Brygoo, H., & Laurière, C. (2007, mar). Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant molecular biology*, *63*(4), 491–503.
doi: 10.1007/s11103-006-9103-1

Boudsocq, M., & Sheen, J. (2013, jan). CDPKs in immune and stress signaling. *Trends in Plant Science*, *18*(1), 30–40.
doi: 10.1016/j.tplants.2012.08.008

Boursiac, Y., Léran, S., Corratgé-Faillie, C., Gojon, A., Krouk, G., & Lacombe, B. (2013, jun). ABA transport and transporters. *Trends in Plant Science*, *18*(6), 325–333.
doi: 10.1016/j.tplants.2013.01.007

Boyer, J. S. (1982, oct). Plant productivity and environment. *Science (New York, N.Y.)*, *218*(4571), 443–8.
doi: 10.1126/science.218.4571.443

Boyko, A., Blevins, T., Yao, Y., Golubov, A., Bilichak, A., Ilnytskyy, Y., ... Kovalchuk, I. (2010, mar). Transgenerational Adaptation of Arabidopsis to Stress Requires DNA Methylation and the Function of Dicer-Like Proteins. *PLoS ONE*, *5*(3), e9514.
doi: 10.1371/journal.pone.0009514

Boyko, A., & Kovalchuk, I. (2010, aug). Transgenerational response to stress in Arabidopsis thaliana. *Plant signaling & behavior*, *5*(8), 995–8.
doi: 10.1371/journal.pone.0009514

Bradbeer, J. W., Atkinson, Y. E., Börner, T., & Hagemann, R. (1979, jun). Cytoplasmic synthesis of plastid polypeptides may be controlled by plastid-synthesised RNA. *Nature*, *279*(5716), 816–817.
doi: 10.1038/279816a0

Brestic, M., & Zivcak, M. (2013). PSII Fluorescence Techniques for Measurement of Drought and High Temperature Stress Signal in Crop Plants: Protocols and Applications. In G. R. Rout & A. B. Das (Eds.), *Molecular stress physiology of plants* (pp. 87–131). India: Springer India.
doi: 10.1007/978-81-322-0807-5_4

Bruce, T. J., Matthes, M. C., Napier, J. A., & Pickett, J. A. (2007, dec). Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science*, *173*(6), 603–608.
doi: 10.1016/j.plantsci.2007.09.002

Brzezinka, K., Altmann, S., Czesnick, H., Nicolas, P., Gorka, M., Benke, E., ... Bäurle, I. (2016, sep). Arabidopsis FORGETTER1 mediates stress-induced chromatin memory through nucleosome remodeling. *eLife*, *5*(September), 1–23.
doi: 10.7554/eLife.17061

Buenrostro, J. D., Wu, B., Chang, H. Y., & Greenleaf, W. J. (2015, jan). ATAC-seq: A Method for Assaying Chromatin Accessibility Genome-Wide. In *Current protocols*

in molecular biology (pp. 21.29.1–21.29.9). Hoboken, NJ, USA: John Wiley & Sons, Inc.
doi: 10.1002/0471142727.mb2129s109

Caffarri, S., Tibiletti, T., Jennings, R. C., & Santabarbara, S. (2014). A comparison between plant photosystem I and photosystem II architecture and functioning. *Current protein & peptide science*, *15*(4), 296–331.
doi: 10.2174/1389203715666140327102218

Cai, S., Chen, G., Wang, Y., Huang, Y., Marchant, D. B., Wang, Y., ... Chen, Z.-H. (2017, jun). Evolutionary Conservation of ABA Signaling for Stomatal Closure. *Plant Physiology*, *174*(2), 732–747.
doi: 10.1104/pp.16.01848

Calarco, J. P., Borges, F., Donoghue, M. T., Van Ex, F., Jullien, P. E., Lopes, T., ... Martienssen, R. A. (2012, sep). Reprogramming of DNA Methylation in Pollen Guides Epigenetic Inheritance via Small RNA. *Cell*, *151*(1), 194–205.
doi: 10.1016/j.cell.2012.09.001

Cao, X., & Jacobsen, S. E. (2002, jul). Role of the Arabidopsis DRM Methyltransferases in De Novo DNA Methylation and Gene Silencing. *Current Biology*, *12*(13), 1138–1144.
doi: 10.1016/S0960-9822(02)00925-9

Carmody, M., Crisp, P. A., D'Alessandro, S., Ganguly, D., Gordon, M., Havaux, M., ... Pogson, B. J. (2016). Uncoupling High Light Responses from Singlet Oxygen Retrograde Signaling and Spatial-Temporal Systemic Acquired Acclimation. *Plant Physiology*, *171*(3), 1734–1749.
doi: 10.1104/pp.16.00404

Cayuela, E., Perez-Alfocea, F., Caro, M., & Bolarin, M. C. (1996, feb). Priming of seeds with NaCl induces physiological changes in tomato plants grown under salt stress. *Physiologia Plantarum*, *96*(2), 231–236.
doi: 10.1111/j.1399-3054.1996.tb00207.x

Chan, K. X., Mabbitt, P. D., Phua, S. Y., Mueller, J. W., Nisar, N., Gigolashvili, T., ... Pogson, B. J. (2016, aug). Sensing and signaling of oxidative stress in chloroplasts by inactivation of the SAL1 phosphoadenosine phosphatase. *Proceedings of the National Academy of Sciences*, *113*(31), E4567–E4576.
doi: 10.1073/pnas.1604936113

Chan, K. X., Phua, S. Y., Crisp, P., McQuinn, R., & Pogson, B. J. (2016, apr). Learning the Languages of the Chloroplast: Retrograde Signaling and Beyond. *Annual Review of Plant Biology*, *67*(1), 25–53.
doi: 10.1146/annurev-arplant-043015-111854

Chan, K. X., Wirtz, M., Phua, S. Y., Estavillo, G. M., & Pogson, B. J. (2013, jan). Balancing metabolites in drought: the sulfur assimilation conundrum. *Trends in Plant Science*, *18*(1), 18–29.
doi: 10.1016/j.tplants.2012.07.005

Chantarachot, T., & Bailey-Serres, J. (2017, nov). Polysomes, stress granules and processing bodies: a dynamic triumvirate controlling cytoplasmic mRNA fate and function. *Plant Physiology*, pp.01468.2017.
doi: 10.1104/pp.17.01468

Chen, Z.-H., & Blatt, M. R. (2010, oct). Membrane Transport in Guard Cells. In *Encyclopedia of life sciences* (pp. 1–13). Chichester, UK: John Wiley & Sons, Ltd.
doi: 10.1002/9780470015902.a0021630

Chen, Z.-H., Eisenach, C., Xu, X.-Q., Hills, A., & Blatt, M. R. (2012). Protocol: optimised electrophyiological analysis of intact guard cells from Arabidopsis. *Plant Methods*, *8*(1), 15.
doi: 10.1186/1746-4811-8-15

Chen, Z.-H., Hills, A., Batz, U., Amtmann, A., Lew, V. L., & Blatt, M. R. (2012, jul). Systems Dynamic Modeling of the Stomatal Guard Cell Predicts Emergent Behaviors in Transport, Signaling, and Volume Control. *PLANT PHYSIOLOGY*, *159*(3), 1235–1251.
doi: 10.1104/pp.112.197350

Cheng, C.-Y., Krishnakumar, V., Chan, A. P., Thibaud-Nissen, F., Schobel, S., & Town, C. D. (2017, feb). Araport11: a complete reannotation of the Arabidopsis thaliana reference genome. *The Plant Journal*, *89*(4), 789–804.
doi: 10.1111/tpj.13415

Choi, H.-I., Hong, J.-H., Ha, J.-O., Kang, J.-y., & Kim, S. Y. (2000, jan). ABFs, a Family of ABA-responsive Element Binding Factors. *Journal of Biological Chemistry*, *275*(3), 1723–1730.
doi: 10.1074/jbc.275.3.1723

Ciszak, K., Kulasek, M., Barczak, A., Grzelak, J., Maćkowski, S., & Karpiński, S. (2015). PsbS is required for systemic acquired acclimation and post-excess-light-stress optimization of chlorophyll fluorescence decay times in Arabidopsis. *Plant signaling & behavior*, *10*(1), e982018.
doi: 10.4161/15592324.2014.982018

Cokus, S. J., Feng, S., Zhang, X., Chen, Z., Merriman, B., Haudenschild, C. D., ... Jacobsen, S. E. (2008, mar). Shotgun bisulphite sequencing of the Arabidopsis genome reveals DNA methylation patterning. *Nature*, *452*(7184), 215–219.
doi: 10.1038/nature06745

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., ... Mauch-Mani, B. (2006, oct). Priming: Getting Ready for Battle. *Molecular Plant-Microbe Interactions*, *19*(10), 1062–1071.
doi: 10.1094/MPMI-19-1062

Coombes, N. (2011). *DiGGer: DiGGer design generator under correlation and blocking.*

Cortijo, S., Wardenaar, R., Colome-Tatche, M., Gilly, A., Etcheverry, M., Labadie, K., ... Johannes, F. (2014, mar). Mapping the Epigenetic Basis of Complex Traits. *Science*, *343*(6175), 1145–1148.
doi: 10.1126/science.1248127

Cramer, G. R., Urano, K., Delrot, S., Pezzotti, M., & Shinozaki, K. (2011). Effects of abiotic stress on plants: a systems biology perspective. *BMC Plant Biology*, *11*(1), 163.
doi: 10.1186/1471-2229-11-163

Crisp, P. A. (2015). *Balancing the messages: RNA metabolism mediates stress-signaling and recovery in plants* (Unpublished doctoral dissertation). The Australian National University.

Crisp, P. A., Ganguly, D., Eichten, S. R., Borevitz, J. O., & Pogson, B. J. (2016). Reconsidering plant memory: Intersections between stress recovery, RNA turnover, and epigenetics. *Science Advances*, *2*(2), e1501340–e1501340.
doi: 10.1126/sciadv.1501340

Crisp, P. A., Ganguly, D. R., Smith, A. B., Murray, K. D., Estavillo, G. M., Searle, I., ... Pogson, B. J. (2017, aug). Rapid Recovery Gene Downregulation during Excess-Light Stress and Recovery in Arabidopsis. *The Plant Cell*, *29*(8), 1836–1863.
doi: 10.1105/tpc.16.00828

Cutler, S. R., Rodriguez, P. L., Finkelstein, R. R., & Abrams, S. R. (2010, jun). Abscisic Acid: Emergence of a Core Signaling Network. *Annual Review of Plant Biology*, *61*(1), 651–679.
doi: 10.1146/annurev-arplant-042809-112122

Daccord, N., Celton, J.-M., Linsmith, G., Becker, C., Choisne, N., Schijlen, E., ... Bucher, E. (2017, jun). High-quality de novo assembly of the apple genome and methylome dynamics of early fruit development. *Nature Genetics*, *49*(7), 1099–1106.
doi: 10.1038/ng.3886

Dall'Osto, L., Cazzaniga, S., Bressan, M., Paleček, D., Židek, K., Niyogi, K. K., ... Bassi, R. (2017, apr). Two mechanisms for dissipation of excess light in monomeric and trimeric light-harvesting complexes. *Nature Plants*, *3*(5), 17033.
doi: 10.1038/nplants.2017.33

De Lucia, F., Crevillen, P., Jones, A. M. E., Greb, T., & Dean, C. (2008, nov). A PHD-Polycomb Repressive Complex 2 triggers the epigenetic silencing of FLC during vernalization. *Proceedings of the National Academy of Sciences*, *105*(44), 16831–16836.
doi: 10.1073/pnas.0808687105

Demmig-Adams, B. (1990, oct). Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1020*(1), 1–24.
doi: 10.1016/0005-2728(90)90088-L

Demmig-Adams, B., Adams, W. I., Logan, B., & Verhoeven, A. (1995). Xanthophyll Cycle-Dependent Energy Dissipation and Flexible Photosystem II Efficiency in Plants Acclimated to Light Stress. *Australian Journal of Plant Physiology*, *22*(2), 249.
doi: 10.1071/PP9950249

Demmig-Adams, B., & Adams, W. W. (1996). Xanthophyll cycle and light stress in nature: uniform response to excess direct sunlight among higher plant species. *Planta*, *198*(3), 460–470.
doi: 10.1007/BF00620064

Demmig-Adams, B., Gilmore, A. M., & Adams, W. W. (1996, mar). Carotenoids 3: in vivo function of carotenoids in higher plants. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, *10*(4), 403–12.

Demmig-Adams, B., Winter, K., Krüger, A., & Czygan, F. C. (1989, jul). Light Response of CO(2) Assimilation, Dissipation of Excess Excitation Energy, and Zeaxanthin Content of Sun and Shade Leaves. *Plant physiology*, *90*(3), 881–6.
doi: 10.1104/PP.90.3.881

Dichtl, B., Stevens, A., & Tollervey, D. (1997, dec). Lithium toxicity in yeast is due to the inhibition of RNA processing enzymes. *The EMBO journal*, *16*(23), 7184–95.
doi: 10.1093/emboj/16.23.7184

Dietz, K.-J. (2015, may). Efficient high light acclimation involves rapid processes at multiple mechanistic levels. *Journal of experimental botany*, *66*(9), 2401–14.
doi: 10.1093/jxb/eru505

Ding, Y., Fromm, M., & Avramova, Z. (2012, mar). Multiple exposures to drought 'train' transcriptional responses in Arabidopsis. *Nature Communications*, *3*, 740.
doi: 10.1038/ncomms1732

Ding, Y., Liu, N., Virlouvet, L., Riethoven, J.-J., Fromm, M., & Avramova, Z. (2013, dec). Four distinct types of dehydration stress memory genes in Arabidopsis thaliana. *BMC plant biology*, *13*(1), 229.

doi: 10.1186/1471-2229-13-229

Ding, Y., Virlouvet, L., Liu, N., Riethoven, J.-J., Fromm, M., & Avramova, Z. (2014). Dehydration stress memory genes of Zea mays; comparison with Arabidopsis thaliana. *BMC Plant Biology*, *14*(1), 141.
doi: 10.1186/1471-2229-14-141

Dong, J., & Bergmann, D. C. (2010). Stomatal patterning and development. *Current topics in developmental biology*, *91*(C), 267–97.
doi: 10.1016/S0070-2153(10)91009-0

Donnelly, P. M., Bonetta, D., Tsukaya, H., Dengler, R. E., & Dengler, N. G. (1999, nov). Cell Cycling and Cell Enlargement in Developing Leaves of Arabidopsis. *Developmental Biology*, *215*(2), 407–419.
doi: 10.1006/dbio.1999.9443

Du, J., Zhong, X., Bernatavichute, Y. V., Stroud, H., Feng, S., Caro, E., ... Jacobsen, S. E. (2012, sep). Dual Binding of Chromomethylase Domains to H3K9me2-Containing Nucleosomes Directs DNA Methylation in Plants. *Cell*, *151*(1), 167–180.
doi: 10.1016/j.cell.2012.07.034

Duan, C.-G., Wang, X., Tang, K., Zhang, H., Mangrauthia, S. K., Lei, M., ... Zhu, J.-K. (2015, oct). MET18 Connects the Cytosolic Iron-Sulfur Cluster Assembly Pathway to Active DNA Demethylation in Arabidopsis. *PLOS Genetics*, *11*(10), e1005559.
doi: 10.1371/journal.pgen.1005559

Dubin, M. J., Zhang, P., Meng, D., Remigereau, M.-S., Osborne, E. J., Paolo Casale, F., ... Nordborg, M. (2015, may). DNA methylation in Arabidopsis has a genetic basis and shows evidence of local adaptation. *eLife*, *4*(MAY), 1–23.
doi: 10.7554/eLife.05255

Dyer, A. R., Brown, C. S., Espeland, E. K., McKay, J. K., Meimberg, H., & Rice, K. J. (2010, mar). The role of adaptive trans-generational plasticity in biological invasions of plants. *Evolutionary applications*, *3*(2), 179–92.
doi: 10.1111/j.1752-4571.2010.00118.x

Eichten, S. R., Briskine, R., Song, J., Li, Q., Swanson-Wagner, R., Hermanson, P. J., ... Springer, N. M. (2013, aug). Epigenetic and Genetic Influences on DNA Methylation Variation in Maize Populations. *The Plant Cell*, *25*(8), 2783–2797.
doi: 10.1105/tpc.113.114793

Eichten, S. R., Schmitz, R. J., & Springer, N. M. (2014, jul). Epigenetics: Beyond Chromatin Modifications and Complex Genetic Regulation. *Plant physiology*, *165*(3), 933–947.
doi: 10.1104/pp.113.234211

Eichten, S. R., & Springer, N. M. (2015, may). Minimal evidence for consistent changes in maize DNA methylation patterns following environmental stress. *Frontiers in Plant Science*, *6*(May), 1–10.
doi: 10.3389/fpls.2015.00308

Eichten, S. R., Stuart, T., Srivastava, A., Lister, R., & Borevitz, J. O. (2016, nov). DNA methylation profiles of diverse Brachypodium distachyon align with underlying genetic diversity. *Genome Research*, *26*(11), 1520–1531.
doi: 10.1101/gr.205468.116

Eisenach, C., Chen, Z.-H., Grefen, C., & Blatt, M. R. (2012, jan). The trafficking protein SYP121 of Arabidopsis connects programmed stomatal closure and K+ channel activity with vegetative growth. *The Plant Journal*, *69*(2), 241–251.
doi: 10.1111/j.1365-313X.2011.04786.x

Endo, A., Sawada, Y., Takahashi, H., Okamoto, M., Ikegami, K., Koiwai, H., ... Nambara, E. (2008, aug). Drought Induction of Arabidopsis 9-cis-Epoxycarotenoid Dioxygenase Occurs in Vascular Parenchyma Cells. *Plant Physiology*, *147*(4), 1984–1993.
doi: 10.1104/pp.108.116632

Estavillo, G. M., Crisp, P. A., Pornsiriwong, W., Wirtz, M., Collinge, D., Carrie, C., ... Pogson, B. J. (2011, nov). Evidence for a SAL1-PAP Chloroplast Retrograde Pathway That Functions in Drought and High Light Signaling in Arabidopsis. *The Plant Cell*, *23*(11), 3992–4012.
doi: 10.1105/tpc.111.091033

Exposito-Rodriguez, M., Laissue, P. P., Yvon-Durocher, G., Smirnoff, N., & Mullineaux, P. M. (2017, jun). Photosynthesis-dependent H2O2 transfer from chloroplasts to nuclei provides a high-light signalling mechanism. *Nature communications*, *8*(1), 49.
doi: 10.1038/s41467-017-00074-w

Farquhar, G. D., & Sharkey, T. D. (1982, jun). Stomatal Conductance and Photosynthesis. *Annual Review of Plant Physiology*, *33*(1), 317–345.
doi: 10.1146/annurev.pp.33.060182.001533

Feng, H., Conneely, K. N., & Wu, H. (2014, apr). A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data. *Nucleic acids research*, *42*(8), e69.
doi: 10.1093/nar/gku154

Feng, S., Cokus, S., Schubert, V., Zhai, J., Pellegrini, M., & Jacobsen, S. (2014, sep). Genome-wide Hi-C Analyses in Wild-Type and Mutants Reveal High-Resolution Chromatin Interactions in Arabidopsis. *Molecular Cell*, *55*(5), 694–707.
doi: 10.1016/j.molcel.2014.07.008

Fernández, A. P., & Strand, A. (2008, oct). Retrograde signaling and plant stress: plastid signals initiate cellular stress responses. *Current opinion in plant biology*, *11*(5), 509–13.
doi: 10.1016/j.pbi.2008.06.002

Finch-Savage, W. E., & Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. *The New phytologist*, *171*(3), 501–23.
doi: 10.1111/j.1469-8137.2006.01787.x

Finkelstein, R. R., Gampala, S. S. L., & Rock, C. D. (2002). Abscisic acid signaling in seeds and seedlings. *The Plant cell*, *14 Suppl*, S15–45.
doi: 10.1105/tpc.010441

Finnegan, E. J., Peacock, W. J., & Dennis, E. S. (1996, aug). Reduced DNA methylation in Arabidopsis thaliana results in abnormal plant development. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(16), 8449–54.
doi: 10.1073/pnas.93.16.8449

Firtzlaff, V., Oberländer, J., Geiselhardt, S., Hilker, M., & Kunze, R. (2016, sep). Pre-exposure of Arabidopsis to the abiotic or biotic environmental stimuli "chilling" or "insect eggs" exhibits different transcriptomic responses to herbivory. *Scientific Reports*, *6*(1), 28544.
doi: 10.1038/srep28544

Fleta-Soriano, E., & Munné-Bosch, S. (2016, feb). Stress Memory and the Inevitable Effects of Drought: A Physiological Perspective. *Frontiers in Plant Science*, *7*(February), 1–6.
doi: 10.3389/fpls.2016.00143

Ford, E. E., Grimmer, M. R., Stolzenburg, S., Bogdanovic, O., de Mendoza, A., Farnham, P. J., ... Lister, R. (2017). Frequent lack of repressive capacity of promoter DNA methylation identified through genome-wide epigenomic manipulation. *bioRxiv*, 170506.
doi: 10.1101/170506

Förster, B., Pogson, B. J., & Osmond, C. B. (2011, may). Lutein from deepoxidation of lutein epoxide replaces zeaxanthin to sustain an enhanced capacity for nonphotochemical chlorophyll fluorescence quenching in avocado shade leaves in the dark. *Plant physiology*, *156*(1), 393–403.
doi: 10.1104/pp.111.173369

Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2006, feb). Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(6), 1988–93.

doi: 10.1073/pnas.0505667103

Ganguly, D. R., Crisp, P. A., Eichten, S. R., & Pogson, B. J. (2017, dec). The Arabidopsis DNA Methylome Is Stable under Transgenerational Drought Stress. *Plant Physiology*, *175*(4), 1893–1912.
doi: 10.1104/pp.17.00744

Ganguly, D. R., Crisp, P. A., Eichten, S. R., & Pogson, B. J. (2018, apr). Maintenance of pre-existing DNA methylation states through recurring excess-light stress. *Plant, cell & environment*.
doi: 10.1111/pce.13324

Gazzani, S. (2004, nov). A Link Between mRNA Turnover and RNA Interference in Arabidopsis. *Science*, *306*(5698), 1046–1048.
doi: 10.1126/science.1101092

Geiger, D., Scherzer, S., Mumm, P., Marten, I., Ache, P., Matschi, S., ... Hedrich, R. (2010, apr). Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca2+ affinities. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(17), 8023–8.
doi: 10.1073/pnas.0912030107

Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., ... Hedrich, R. (2009, dec). Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. *Proceedings of the National Academy of Sciences*, *106*(50), 21425–21430.
doi: 10.1073/pnas.0912021106

Gigolashvili, T., Geier, M., Ashykhmina, N., Frerigmann, H., Wulfert, S., Krueger, S., ... Flügge, U.-I. (2012, oct). The Arabidopsis thylakoid ADP/ATP carrier TAAC has an additional role in supplying plastidic phosphoadenosine 5'-phosphosulfate to the cytosol. *The Plant cell*, *24*(10), 4187–204.
doi: 10.1105/tpc.112.101964

Goldberg, A. D., Allis, C. D., & Bernstein, E. (2007, feb). Epigenetics: a landscape takes shape. *Cell*, *128*(4), 635–8.
doi: 10.1016/j.cell.2007.02.006

Gong, Z., Morales-Ruiz, T., Ariza, R. R., Roldán-Arjona, T., David, L., & Zhu, J. K. (2002, dec). ROS1, a repressor of transcriptional gene silencing in Arabidopsis, encodes a DNA glycosylase/lyase. *Cell*, *111*(6), 803–14.

González-Guzmán, M., Apostolova, N., Bellés, J. M., Barrero, J. M., Piqueras, P., Ponce, M. R., ... Rodríguez, P. L. (2002, aug). The short-chain alcohol dehydrogenase

ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *The Plant cell*, *14*(8), 1833–46.
doi: 10.1105/tpc.002477.development

Goodger, J. Q., & Schachtman, D. P. (2010, oct). Re-examining the role of ABA as the primary long-distance signal produced by water-stressed roots. *Plant Signaling & Behavior*, *5*(10), 1298–1301.
doi: 10.4161/psb.5.10.13101

Gordon, M. J. (2012). *Sun Smart Plants: Leaf to Leaf Signalling in Response to High Light Stress* (Unpublished doctoral dissertation). The Australian National University.

Gordon, M. J., Carmody, M., Albrecht, V., & Pogson, B. (2013). Systemic and Local Responses to Repeated HL Stress-Induced Retrograde Signaling in Arabidopsis. *Frontiers in Plant Science*, *3*(January), 303.
doi: 10.3389/fpls.2012.00303

Gorsuch, P. A., Sargeant, A. W., Penfield, S. D., Quick, W. P., & Atkin, O. K. (2010, sep). Systemic low temperature signaling in Arabidopsis. *Plant and Cell Physiology*, *51*(9), 1488–1498.
doi: 10.1093/pcp/pcq112

Gouil, Q., & Baulcombe, D. C. (2016, dec). DNA Methylation Signatures of the Plant Chromomethyltransferases. *PLOS Genetics*, *12*(12), e1006526.
doi: 10.1371/journal.pgen.1006526

Gregory, B. D., O'Malley, R. C., Lister, R., Urich, M. A., Tonti-Filippini, J., Chen, H., ... Ecker, J. R. (2008, jun). A Link between RNA Metabolism and Silencing Affecting Arabidopsis Development. *Developmental Cell*, *14*(6), 854–866.
doi: 10.1016/j.devcel.2008.04.005

Grime, J. P., Mason, G., Curtis, A. V., Rodman, J., & Band, S. R. (1981, nov). A Comparative Study of Germination Characteristics in a Local Flora. *The Journal of Ecology*, *69*(3), 1017.
doi: 10.2307/2259651

Groth, M., Moissiard, G., Wirtz, M., Wang, H., Garcia-Salinas, C., Ramos-Parra, P. A., ... Jacobsen, S. E. (2016, jun). MTHFD1 controls DNA methylation in Arabidopsis. *Nature Communications*, *7*, 11640.
doi: 10.1038/ncomms11640

Groth, M., Stroud, H., Feng, S., Greenberg, M. V. C., Vashisht, A. A., Wohlschlegel, J. A., ... Ausin, I. (2014, dec). SNF2 chromatin remodeler-family proteins FRG1 and -2 are required for RNA-directed DNA methylation. *Proceedings of the National Academy of Sciences*, *111*(49), 17666–17671.

doi: 10.1073/pnas.1420515111

Gutierrez, R. A., Ewing, R. M., Cherry, J. M., & Green, P. J. (2002, aug). Identification of unstable transcripts in Arabidopsis by cDNA microarray analysis: Rapid decay is associated with a group of touch- and specific clock-controlled genes. *Proceedings of the National Academy of Sciences*, *99*(17), 11513–11518.
doi: 10.1073/pnas.152204099

Gutzat, R., & Mittelsten Scheid, O. (2012, nov). Epigenetic responses to stress: triple defense? *Current Opinion in Plant Biology*, *15*(5), 568–573.
doi: 10.1016/j.pbi.2012.08.007

Gy, I., Gasciolli, V., Lauressergues, D., Morel, J.-B., Gombert, J., Proux, F., ... Mallory, A. C. (2007, nov). Arabidopsis FIERY1, XRN2, and XRN3 are endogenous RNA silencing suppressors. *The Plant cell*, *19*(11), 3451–61.
doi: 10.1105/tpc.107.055319

Haag, J. R., & Pikaard, C. S. (2011, jul). Multisubunit RNA polymerases IV and V: purveyors of non-coding RNA for plant gene silencing. *Nature Reviews Molecular Cell Biology*, *12*(8), 483–492.
doi: 10.1038/nrm3152

Haag, J. R., Ream, T. S., Marasco, M., Nicora, C. D., Norbeck, A. D., Pasa-Tolic, L., & Pikaard, C. S. (2012, dec). In Vitro Transcription Activities of Pol IV, Pol V, and RDR2 Reveal Coupling of Pol IV and RDR2 for dsRNA Synthesis in Plant RNA Silencing. *Molecular Cell*, *48*(5), 811–818.
doi: 10.1016/j.molcel.2012.09.027

Hagmann, J., Becker, C., Müller, J., Stegle, O., Meyer, R. C., Wang, G., ... Weigel, D. (2015, jan). Century-scale Methylome Stability in a Recently Diverged Arabidopsis thaliana Lineage. *PLoS Genetics*, *11*(1), e1004920.
doi: 10.1371/journal.pgen.1004920

Haitz, M., & Lichtenthaler, H. K. (1988). The Measurement of Rfd-Values as Plant Vitality Indices with the Portable Field Chlorophyll Fluorometer and the Pam-Fluorometer. In *Applications of chlorophyll fluorescene in photosynthesis research, stress physiology, hydrobiology and remote sensing* (pp. 249–254). Dordrecht: Springer Netherlands.
doi: 10.1007/978-94-009-2823-7_31

Hauser, F., Waadt, R., & Schroeder, J. I. (2011, may). Evolution of Abscisic Acid Synthesis and Signaling Mechanisms. *Current Biology*, *21*(9), R346–R355.
doi: 10.1016/j.cub.2011.03.015

Hauser, M.-T., Aufsatz, W., Jonak, C., & Luschnig, C. (2011, aug). Transgenerational epigenetic inheritance in plants. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1809*(8), 459–468.
doi: 10.1016/j.bbagrm.2011.03.007

Havecker, E. R., Wallbridge, L. M., Hardcastle, T. J., Bush, M. S., Kelly, K. A., Dunn, R. M., ... Baulcombe, D. C. (2010, feb). The Arabidopsis RNA-Directed DNA Methylation Argonautes Functionally Diverge Based on Their Expression and Interaction with Target Loci. *The Plant Cell*, *22*(2), 321–334.
doi: 10.1105/tpc.109.072199

He, X.-J., Chen, T., & Zhu, J.-K. (2011, mar). Regulation and function of DNA methylation in plants and animals. *Cell Research*, *21*(3), 442–465.
doi: 10.1038/cr.2011.23

Heard, E., & Martienssen, R. A. (2014, mar). Transgenerational Epigenetic Inheritance: Myths and Mechanisms. *Cell*, *157*(1), 95–109.
doi: 10.1016/j.cell.2014.02.045

Henderson, I. R., & Jacobsen, S. E. (2008, jun). Tandem repeats upstream of the Arabidopsis endogene SDC recruit non-CG DNA methylation and initiate siRNA spreading. *Genes & Development*, *22*(12), 1597–1606.
doi: 10.1101/gad.1667808

Herman, J. J., & Sultan, S. E. (2011). Adaptive Transgenerational Plasticity in Plants: Case Studies, Mechanisms, and Implications for Natural Populations. *Frontiers in Plant Science*, *2*(December), 1–10.
doi: 10.3389/fpls.2011.00102

Herman, J. J., & Sultan, S. E. (2016, sep). DNA methylation mediates genetic variation for adaptive transgenerational plasticity. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1838), 20160988.
doi: 10.1098/rspb.2016.0988

Hess, W. R., Müller, A., Nagy, F., & Börner, T. (1994, feb). Ribosome-deficient plastids affect transcription of light-induced nuclear genes: genetic evidence for a plastid-derived signal. *MGG Molecular & General Genetics*, *242*(3), 305–12.
doi: 10.1007/BF00280420

Hetherington, A. M., & Woodward, F. I. (2003, aug). The role of stomata in sensing and driving environmental change. *Nature*, *424*(6951), 901–908.
doi: 10.1038/nature01843

Hilker, M., Schwachtje, J., Baier, M., Balazadeh, S., Bäurle, I., Geiselhardt, S., ... Kopka, J. (2016, nov). Priming and memory of stress responses in organisms lacking a nervous system. *Biological Reviews*, *91*(4), 1118–1133.
doi: 10.1111/brv.12215

Hirsch, J., Misson, J., Crisp, P. A., David, P., Bayle, V., Estavillo, G. M., ... Marin, E. (2011, feb). A Novel fry1 Allele Reveals the Existence of a Mutant Phenotype Unrelated to 5′->3′ Exoribonuclease (XRN) Activities in Arabidopsis thaliana Roots. *PLoS ONE*, *6*(2), e16724.
doi: 10.1371/journal.pone.0016724

Hirth, M., Dietzel, L., Steiner, S., Ludwig, R., Weidenbach, H., And, J. P., & Pfannschmidt, T. (2013). Photosynthetic acclimation responses of maize seedlings grown under artificial laboratory light gradients mimicking natural canopy conditions. *Frontiers in Plant Science*, *4*(September), 334.
doi: 10.3389/fpls.2013.00334

Horton, P., Johnson, M. P., Perez-Bueno, M. L., Kiss, A. Z., & Ruban, A. V. (2008, mar). Photosynthetic acclimation: Does the dynamic structure and macro-organisation of photosystem II in higher plant grana membranes regulate light harvesting states? *FEBS Journal*, *275*(6), 1069–1079.
doi: 10.1111/j.1742-4658.2008.06263.x

Horton, P., Wentworth, M., & Ruban, A. (2005, aug). Control of the light harvesting function of chloroplast membranes: The LHCII-aggregation model for non-photochemical quenching. *FEBS Letters*, *579*(20), 4201–4206.
doi: 10.1016/j.febslet.2005.07.003

Hosy, E., Vavasseur, A., Mouline, K., Dreyer, I., Gaymard, F., Poree, F., ... Sentenac, H. (2003, apr). The Arabidopsis outward K+ channel GORK is involved in regulation of stomatal movements and plant transpiration. *Proceedings of the National Academy of Sciences*, *100*(9), 5549–5554.
doi: 10.1073/pnas.0733970100

Hou, X., Rivers, J., León, P., McQuinn, R. P., & Pogson, B. J. (2016, sep). Synthesis and Function of Apocarotenoid Signals in Plants. *Trends in plant science*, *21*(9), 792–803.
doi: 10.1016/j.tplants.2016.06.001

Huettel, B., Kanno, T., Daxinger, L., Aufsatz, W., Matzke, A. J. M., & Matzke, M. (2006, jun). Endogenous targets of RNA-directed DNA methylation and Pol IV in Arabidopsis. *The EMBO Journal*, *25*(12), 2828–2836.
doi: 10.1038/sj.emboj.7601150

Humplík, J. F., Lazár, D., Husičková, A., & Spíchal, L. (2015, dec). Automated phenotyping of plant shoots using imaging methods for analysis of plant stress responses – a review. *Plant Methods*, *11*(1), 29.
doi: 10.1186/s13007-015-0072-8

Iqbal, M., & Ashraf, M. (2007, mar). Seed Preconditioning Modulates Growth, Ionic Relations, and Photosynthetic Capacity in Adult Plants of Hexaploid Wheat under Salt Stress. *Journal of Plant Nutrition*, *30*(3), 381–396.
doi: 10.1080/01904160601171330

Ito, H., Gaubert, H., Bucher, E., Mirouze, M., Vaillant, I., & Paszkowski, J. (2011, apr). An siRNA pathway prevents transgenerational retrotransposition in plants subjected to stress. *Nature*, *472*(7341), 115–119.
doi: 10.1038/nature09861

Ito, H., & Kakutani, T. (2014, jun). Control of transposable elements in Arabidopsis thaliana. *Chromosome Research*, *22*(2), 217–223.
doi: 10.1007/s10577-014-9417-9

Ito, H., Kim, J.-M., Matsunaga, W., Saze, H., Matsui, A., Endo, T. A., ... Seki, M. (2016, sep). A Stress-Activated Transposon in Arabidopsis Induces Transgenerational Abscisic Acid Insensitivity. *Scientific Reports*, *6*(1), 23181.
doi: 10.1038/srep23181

Jahns, P., & Holzwarth, A. R. (2012, jan). The role of the xanthophyll cycle and of lutein in photoprotection of photosystem II. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1817*(1), 182–193.
doi: 10.1016/j.bbabio.2011.04.012

Jakab, G., Cottier, V., Toquin, V., Rigoli, G., Zimmerli, L., Métraux, J.-P., & Mauch-Mani, B. (2001). β-Aminobutyric acid-induced resistance in plants. *European Journal of Plant Pathology*, *107*(1), 29–37.
doi: 10.1023/A:1008730721037

Jakab, G., Ton, J., Flors, V., Zimmerli, L., Métraux, J.-P., & Mauch-Mani, B. (2005, sep). Enhancing Arabidopsis salt and drought stress tolerance by chemical priming for its abscisic acid responses. *Plant physiology*, *139*(1), 267–74.
doi: 10.1104/pp.105.065698

Jaskiewicz, M., Conrath, U., & Peterhänsel, C. (2011, jan). Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO reports*, *12*(1), 50–55.
doi: 10.1038/embor.2010.186

Ji, L., Neumann, D. A., & Schmitz, R. J. (2015, jun). Crop Epigenomics: Identifying, Unlocking, and Harnessing Cryptic Variation in Crop Genomes. *Molecular Plant*, *8*(6), 860–870.
doi: 10.1016/j.molp.2015.01.021

Jiang, C., Mithani, A., Belfield, E. J., Mott, R., Hurst, L. D., & Harberd, N. P. (2014, nov). Environmentally responsive genome-wide accumulation of de novo Arabidopsis thaliana mutations and epimutations. *Genome Research*, *24*(11), 1821–1829.
doi: 10.1101/gr.177659.114

Johannes, F., Porcher, E., Teixeira, F. K., Saliba-Colombani, V., Simon, M., Agier, N., ... Colot, V. (2009, jun). Assessing the impact of transgenerational epigenetic variation on complex traits. *PLoS genetics*, *5*(6), e1000530.
doi: 10.1371/journal.pgen.1000530

Johnson, R. R., Wagner, R. L., Verhey, S. D., & Walker-Simmons, M. K. (2002, oct). The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. *Plant physiology*, *130*(2), 837–46.
doi: 10.1104/pp.001354

Jones, P. A. (2012, may). Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nature Reviews Genetics*, *13*(7), 484–492.
doi: 10.1038/nrg3230

Jung, H.-s., Crisp, P. A., Estavillo, G. M., Cole, B., Hong, F., Mockler, T. C., ... Chory, J. (2013, aug). Subset of heat-shock transcription factors required for the early response of Arabidopsis to excess light. *Proceedings of the National Academy of Sciences*, *110*(35), 14474–14479.
doi: 10.1073/pnas.1311632110

Jung, H.-s., & Niyogi, K. K. (2009, jun). Quantitative genetic analysis of thermal dissipation in Arabidopsis. *Plant physiology*, *150*(2), 977–86.
doi: 10.1104/pp.109.137828

Kant, P., Kant, S., Gordon, M., Shaked, R., & Barak, S. (2007, sep). STRESS RESPONSE SUPPRESSOR1 and STRESS RESPONSE SUPPRESSOR2, Two DEAD-Box RNA Helicases That Attenuate Arabidopsis Responses to Multiple Abiotic Stresses. *PLANT PHYSIOLOGY*, *145*(3), 814–830.
doi: 10.1104/pp.107.099895

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G., & Mullineaux, P. (1999, apr). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. *Science*, *284*(5414), 654–7.
doi: 10.1126/science.284.5414.654

Kawakatsu, T., Nery, J. R., Castanon, R., & Ecker, J. R. (2017, dec). Dynamic DNA methylation reconfiguration during seed development and germination. *Genome Biology*, *18*(1), 171.
doi: 10.1186/s13059-017-1251-x

Kawakatsu, T., Stuart, T., Valdes, M., Breakfield, N., Schmitz, R. J., Nery, J. R., ... Ecker, J. R. (2016, apr). Unique cell-type-specific patterns of DNA methylation in the root meristem. *Nature Plants*, *2*(5), 16058.
doi: 10.1038/nplants.2016.58

Khan, A., Garbelli, A., Grossi, S., Florentin, A., Batelli, G., Acuna, T., ... Barak, S. (2014, jul). The Arabidopsis STRESS RESPONSE SUPPRESSOR DEAD-box RNA helicases are nucleolar- and chromocenter-localized proteins that undergo stress-mediated relocalization and are involved in epigenetic gene silencing. *The Plant Journal*, *79*(1), 28–43.
doi: 10.1111/tpj.12533

Kim, J.-M., To, T. K., Matsui, A., Tanoi, K., Kobayashi, N. I., Matsuda, F., ... Seki, M. (2017, jun). Acetate-mediated novel survival strategy against drought in plants. *Nature Plants*, *3*(7), 17097.
doi: 10.1038/nplants.2017.97

Kim, T.-H., Böhmer, M., Hu, H., Nishimura, N., & Schroeder, J. I. (2010, jun). Guard Cell Signal Transduction Network: Advances in Understanding Abscisic Acid, CO 2 , and Ca 2+ Signaling. *Annual Review of Plant Biology*, *61*(1), 561–591.
doi: 10.1146/annurev-arplant-042809-112226

Kinoshita, T., Nishimura, M., & Shimazaki, K. (1995, aug). Cytosolic Concentration of Ca2+ Regulates the Plasma Membrane H+-ATPase in Guard Cells of Fava Bean. *The Plant cell*, *7*(8), 1333–1342.
doi: 10.1105/tpc.7.8.1333

Klein, M., & Papenbrock, J. (2004, aug). The multi-protein family of Arabidopsis sulphotransferases and their relatives in other plant species. *Journal of experimental botany*, *55*(404), 1809–20.
doi: 10.1093/jxb/erh183

Koiwai, H., Nakaminami, K., Seo, M., Mitsuhashi, W., Toyomasu, T., & Koshiba, T. (2004, apr). Tissue-specific localization of an abscisic acid biosynthetic enzyme, AAO3, in Arabidopsis. *Plant physiology*, *134*(4), 1697–707.
doi: 10.1104/pp.103.036970

Komatsu, K., Suzuki, N., Kuwamura, M., Nishikawa, Y., Nakatani, M., Ohtawa, H., ... Sakata, Y. (2013, jul). Group A PP2Cs evolved in land plants as key regulators of intrinsic desiccation tolerance. *Nature Communications*, *4*, 1–9.

doi: 10.1038/ncomms3219

Koornneef, M., Reuling, G., & Karssen, C. M. (1984, jul). The isolation and characterization of abscisic acid-insensitive mutants of Arabidopsis thaliana. *Physiologia Plantarum*, *61*(3), 377–383.
doi: 10.1111/j.1399-3054.1984.tb06343.x

Kouzarides, T. (2007, feb). Chromatin modifications and their function. *Cell*, *128*(4), 693–705.
doi: 10.1016/j.cell.2007.02.005

Kramer, P. J., & Boyer, J. S. (1995). *Water relations of plants and soils*. Academic Press 1995.

Kromdijk, J., Głowacka, K., Leonelli, L., Gabilly, S. T., Iwai, M., Niyogi, K. K., & Long, S. P. (2016). Improving photosynthesis and crop productivity by accelerating recovery from photoprotection. *Science (New York, N.Y.)*, *354*(6314), 857–861.
doi: 10.1126/science.aai8878

Krueger, F., & Andrews, S. R. (2011, jun). Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. *Bioinformatics*, *27*(11), 1571–1572.
doi: 10.1093/bioinformatics/btr167

Kurahashi, Y., Terashima, A., & Takumi, S. (2009, jun). Variation in dehydration tolerance, ABA sensitivity and related gene expression patterns in D-genome progenitor and synthetic hexaploid wheat lines. *International journal of molecular sciences*, *10*(6), 2733–51.
doi: 10.3390/ijms10062733

Kurihara, Y. (2017, jan). Activity and roles of Arabidopsis thaliana XRN family exoribonucleases in noncoding RNA pathways. *Journal of Plant Research*, *130*(1), 25–31.
doi: 10.1007/s10265-016-0887-z

Kurihara, Y., Schmitz, R. J., Nery, J. R., Schultz, M. D., Okubo-Kurihara, E., Morosawa, T., ... Ecker, J. R. (2012, apr). Surveillance of 3' Noncoding Transcripts Requires FIERY1 and XRN3 in Arabidopsis. *G3 (Bethesda, Md.)*, *2*(4), 487–98.
doi: 10.1534/g3.111.001362

Lämke, J., & Bäurle, I. (2017, dec). Epigenetic and chromatin-based mechanisms in environmental stress adaptation and stress memory in plants. *Genome Biology*, *18*(1), 124.
doi: 10.1186/s13059-017-1263-6

Lämke, J., Brzezinka, K., Altmann, S., & Bäurle, I. (2016, jan). A hit-and-run heat shock factor governs sustained histone methylation and transcriptional stress memory. *The EMBO journal*, *35*(2), 162–75.

doi: 10.15252/embj.201592593

Langmead, B., & Salzberg, S. L. (2012, mar). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, *9*(4), 357–359.
doi: 10.1038/nmeth.1923

Law, J. A., Du, J., Hale, C. J., Feng, S., Krajewski, K., Palanca, A. M. S., ... Jacobsen, S. E. (2013, may). Polymerase IV occupancy at RNA-directed DNA methylation sites requires SHH1. *Nature*, *498*(7454), 385–389.
doi: 10.1038/nature12178

Law, J. A., & Jacobsen, S. E. (2010, mar). Establishing, maintaining and modifying DNA methylation patterns in plants and animals. *Nature Reviews Genetics*, *11*(3), 204–220.
doi: 10.1038/nrg2719

Le, T.-N., Schumann, U., Smith, N. A., Tiwari, S., Au, P. C. K., Zhu, Q.-H., ... Wang, M.-B. (2014, sep). DNA demethylases target promoter transposable elements to positively regulate stress responsive genes in Arabidopsis. *Genome Biology*, *15*(9), 458.
doi: 10.1186/s13059-014-0458-3

Lee, B.-R., Huseby, S., Koprivova, A., Chételat, A., Wirtz, M., Mugford, S. T., ... Kopriva, S. (2012, jun). Effects of fou8/fry1 Mutation on Sulfur Metabolism: Is Decreased Internal Sulfate the Trigger of Sulfate Starvation Response? *PLoS ONE*, *7*(6), e39425.
doi: 10.1371/journal.pone.0039425

Lee, S. C., Lan, W., Buchanan, B. B., & Luan, S. (2009, dec). A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. *Proceedings of the National Academy of Sciences*, *106*(50), 21419–21424.
doi: 10.1073/pnas.0910601106

Lefcheck, J. S. (2016, may). piecewiseSEM: Piecewise structural equation modelling in R for ecology, evolution, and systematics. *Methods in Ecology and Evolution*, *7*(5), 573–579.
doi: 10.1111/2041-210X.12512

Lei, M., Zhang, H., Julian, R., Tang, K., Xie, S., & Zhu, J.-K. (2015, mar). Regulatory link between DNA methylation and active demethylation in Arabidopsis. *Proceedings of the National Academy of Sciences*, *112*(11), 3553–3557.
doi: 10.1073/pnas.1502279112

Lenth, R. V. (2016). Least-Squares Means: The R Package lsmeans. *Journal of Statistical Software*, *69*(1), 1–33. doi: 10.18637/jss.v069.i01

Lewsey, M. G., Hardcastle, T. J., Melnyk, C. W., Molnar, A., Valli, A., Urich, M. A., ... Ecker, J. R. (2016, feb). Mobile small RNAs regulate genome-wide DNA methylation. *Proceedings of the National Academy of Sciences*, *113*(6), E801–E810. doi: 10.1073/pnas.1515072113

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... Durbin, R. (2009, aug). The Sequence Alignment/Map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079. doi: 10.1093/bioinformatics/btp352

Li, J., Yang, Z., Yu, B., Liu, J., & Chen, X. (2005, aug). Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis. *Current biology : CB*, *15*(16), 1501–7. doi: 10.1016/j.cub.2005.07.029

Li, Q., Eichten, S. R., Hermanson, P. J., & Springer, N. M. (2014, mar). Inheritance Patterns and Stability of DNA Methylation Variation in Maize Near-Isogenic Lines. *Genetics*, *196*(3), 667–676. doi: 10.1534/genetics.113.158980

Li, Q., Gent, J. I., Zynda, G., Song, J., Makarevitch, I., Hirsch, C. D., ... Springer, N. M. (2015, nov). RNA-directed DNA methylation enforces boundaries between heterochromatin and euchromatin in the maize genome. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(47), 14728–33. doi: 10.1073/pnas.1514680112

Li, Q., Song, J., West, P. T., Zynda, G., Eichten, S. R., Vaughn, M. W., & Springer, N. M. (2015, aug). Examining the Causes and Consequences of Context-Specific Differential DNA Methylation in Maize. *Plant Physiology*, *168*(4), 1262–1274. doi: 10.1104/pp.15.00052

Li, S., Vandivier, L. E., Tu, B., Gao, L., Won, S. Y., Li, S., ... Chen, X. (2015, feb). Detection of Pol IV/RDR2-dependent transcripts at the genomic scale in Arabidopsis reveals features and regulation of siRNA biogenesis. *Genome Research*, *25*(2), 235–245. doi: 10.1101/gr.182238.114

Li, X.-P., Björkman, O., Shih, C., Grossman, A. R., Rosenquist, M., Jansson, S., & Niyogi, K. K. (2000, jan). A pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature*, *403*(6768), 391–395. doi: 10.1038/35000131

Li, X.-P., Gilmore, A. M., Caffarri, S., Bassi, R., Golan, T., Kramer, D., & Niyogi, K. K. (2004, may). Regulation of Photosynthetic Light Harvesting Involves Intrathylakoid Lumen pH Sensing by the PsbS Protein. *Journal of Biological Chemistry*, *279*(22), 22866–22874.
doi: 10.1074/jbc.M402461200

Li, X.-P., Phippard, A., Pasari, J., & Niyogi, K. K. (2002). Structure–function analysis of photosystem II subunit S (PsbS) in vivo. *Functional Plant Biology*, *29*(10), 1131.
doi: 10.1071/FP02065

Li, Z., Wakao, S., Fischer, B. B., & Niyogi, K. K. (2009). Sensing and Responding to Excess Light. *Annual Review of Plant Biology*, *60*(1), 239–260.
doi: 10.1146/annurev.arplant.58.032806.103844

Liao, Y., Smyth, G. K., & Shi, W. (2013, may). The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Research*, *41*(10), e108–e108.
doi: 10.1093/nar/gkt214

Liao, Y., Smyth, G. K., & Shi, W. (2014, apr). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923–930.
doi: 10.1093/bioinformatics/btt656

Lichtenthaler, H. K., Buschmann, C., & Knapp, M. (2005, sep). How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll fluorescence decrease ratio RFd of leaves with the PAM fluorometer. *Photosynthetica*, *43*(3), 379–393.
doi: 10.1007/s11099-005-0062-6

Lichtenthaler, H. K., & Miehé, J. A. (1997, aug). Fluorescence imaging as a diagnostic tool for plant stress. *Trends in Plant Science*, *2*(8), 316–320.
doi: 10.1016/S1360-1385(97)89954-2

Lin, Y.-C., Liu, Y.-c., & Nakamura, Y. (2015, may). The Choline/Ethanolamine Kinase Family in Arabidopsis: Essential Role of CEK4 in Phospholipid Biosynthesis and Embryo Development. *The Plant Cell*, *27*(5), 1497–1511.
doi: 10.1105/tpc.15.00207

Lindbo, J. A., Silva-Rosales, L., Proebsting, W. M., & Dougherty, W. G. (1993, dec). Induction of a Highly Specific Antiviral State in Transgenic Plants: Implications for Regulation of Gene Expression and Virus Resistance. *The Plant cell*, *5*(12), 1749–1759.
doi: 10.1105/tpc.5.12.1749

Lister, R., O'Malley, R. C., Tonti-Filippini, J., Gregory, B. D., Berry, C. C., Millar, A. H., & Ecker, J. R. (2008, may). Highly Integrated Single-Base Resolution Maps of the Epigenome in Arabidopsis. *Cell*, *133*(3), 523–536.
doi: 10.1016/j.cell.2008.03.029

Liu, R., How-Kit, A., Stammitti, L., Teyssier, E., Rolin, D., Mortain-Bertrand, A., ... Gallusci, P. (2015, aug). A DEMETER-like DNA demethylase governs tomato fruit ripening. *Proceedings of the National Academy of Sciences*, *112*(34), 10804–10809.
doi: 10.1073/pnas.1503362112

Luo, D., Carpenter, R., Vincent, C., Copsey, L., & Coen, E. (1996, oct). Origin of floral asymmetry in Antirrhinum. *Nature*, *383*(6603), 794–799.
doi: 10.1038/383794a0

Lynch, T., Erickson, B. J., & Finkelstein, R. R. (2012, dec). Direct interactions of ABA-insensitive(ABI)-clade protein phosphatase(PP)2Cs with calcium-dependent protein kinases and ABA response element-binding bZIPs may contribute to turning off ABA response. *Plant Molecular Biology*, *80*(6), 647–658.
doi: 10.1007/s11103-012-9973-3

Lyons, D. B., & Zilberman, D. (2017, nov). DDM1 and Lsh remodelers allow methylation of DNA wrapped in nucleosomes. *eLife*, *6*, e30674.
doi: 10.7554/eLife.30674

Malcheska, F., Ahmad, A., Batool, S., Müller, H. M., Ludwig-Müller, J., Kreuzwieser, J., ... Rennenberg, H. (2017, jun). Drought-Enhanced Xylem Sap Sulfate Closes Stomata by Affecting ALMT12 and Guard Cell ABA Synthesis. *Plant physiology*, *174*(2), 798–814.
doi: 10.1104/pp.16.01784

Manning, K., Tör, M., Poole, M., Hong, Y., Thompson, A. J., King, G. J., ... Seymour, G. B. (2006, aug). A naturally occurring epigenetic mutation in a gene encoding an SBP-box transcription factor inhibits tomato fruit ripening. *Nature Genetics*, *38*(8), 948–952.
doi: 10.1038/ng1841

March-Díaz, R., García-Domínguez, M., Lozano-Juste, J., León, J., Florencio, F. J., & Reyes, J. C. (2008, nov). Histone H2A.Z and homologues of components of the SWR1 complex are required to control immunity in Arabidopsis. *The Plant Journal*, *53*(3), 475–487.
doi: 10.1111/j.1365-313X.2007.03361.x

Martín, G., Leivar, P., Ludevid, D., Tepperman, J. M., Quail, P. H., & Monte, E. (2016, may). Phytochrome and retrograde signalling pathways converge to antagonistically regulate a light-induced transcriptional network. *Nature Communications*, *7*, 11431.

doi: 10.1038/ncomms11431

Martínez de Alba, A. E., Moreno, A. B., Gabriel, M., Mallory, A. C., Christ, A., Bounon, R., ... Maizel, A. (2015, mar). In plants, decapping prevents RDR6-dependent production of small interfering RNAs from endogenous mRNAs. *Nucleic Acids Research*, *43*(5), 2902–2913.
doi: 10.1093/nar/gkv119

Matzke, M., Kanno, T., Daxinger, L., Huettel, B., & Matzke, A. J. M. (2009, jun). RNA-mediated chromatin-based silencing in plants. *Current opinion in cell biology*, *21*(3), 367–76.
doi: 10.1016/j.ceb.2009.01.025

Matzke, M. A., Kanno, T., & Matzke, A. J. (2015, apr). RNA-Directed DNA Methylation: The Evolution of a Complex Epigenetic Pathway in Flowering Plants. *Annual Review of Plant Biology*, *66*(1), 243–267.
doi: 10.1146/annurev-arplant-043014-114633

Matzke, M. A., & Mosher, R. A. (2014, may). RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nature Reviews Genetics*, *15*(6), 394–408.
doi: 10.1038/nrg3683

Maurano, M. T., Wang, H., John, S., Shafer, A., Canfield, T., Lee, K., & Stamatoyannopoulos, J. A. (2015, aug). Role of DNA Methylation in Modulating Transcription Factor Occupancy. *Cell Reports*, *12*(7), 1184–1195.
doi: 10.1016/j.celrep.2015.07.024

Maxwell, K., & Johnson, G. N. (2000, apr). Chlorophyll fluorescence–a practical guide. *Journal of experimental botany*, *51*(345), 659–68.
doi: 10.1093/jxb/51.345.659

Mazzucotelli, E., Mastrangelo, A. M., Crosatti, C., Guerra, D., Stanca, A. M., & Cattivelli, L. (2008, apr). Abiotic stress response in plants: When post-transcriptional and post-translational regulations control transcription. *Plant Science*, *174*(4), 420–431.
doi: 10.1016/j.plantsci.2008.02.005

McCarthy, D. J., Chen, Y., & Smyth, G. K. (2012, may). Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Research*, *40*(10), 4288–4297.
doi: 10.1093/nar/gks042

McCue, A. D., Nuthikattu, S., Reeder, S. H., & Slotkin, R. K. (2012, feb). Gene Expression and Stress Response Mediated by the Epigenetic Regulation of a Transposable Element Small RNA. *PLoS Genetics*, *8*(2), e1002474.

doi: 10.1371/journal.pgen.1002474

McCue, A. D., Panda, K., Nuthikattu, S., Choudury, S. G., Thomas, E. N., & Slotkin, R. K. (2015, jan). ARGONAUTE 6 bridges transposable element mRNA-derived siRNAs to the establishment of DNA methylation. *The EMBO Journal*, *34*(1), 20–35.
doi: 10.15252/embj.201489499

McKinlay, A., Podicheti, R., Wendte, J. M., Cocklin, R., & Rusch, D. B. (2017, dec). RNA polymerases IV and V influence the 3′ boundaries of Polymerase II transcription units in Arabidopsis. *RNA Biology*, 1–11.
doi: 10.1080/15476286.2017.1409930

McNair, J. N., Sunkara, A., & Frobish, D. (2012, jun). How to analyse seed germination data using statistical time-to-event analysis: non-parametric and semi-parametric methods. *Seed Science Research*, *22*(02), 77–95.
doi: 10.1017/S0960258511000547

Meng, D., Dubin, M., Zhang, P., Osborne, E. J., Stegle, O., Clark, R. M., & Nordborg, M. (2016, jul). Limited Contribution of DNA Methylation Variation to Expression Regulation in Arabidopsis thaliana. *PLOS Genetics*, *12*(7), e1006141.
doi: 10.1371/journal.pgen.1006141

Merret, R., Descombin, J., Juan, Y.-t., Favory, J.-J., Carpentier, M.-C., Chaparro, C., ... Bousquet-Antonelli, C. (2013, dec). XRN4 and LARP1 Are Required for a Heat-Triggered mRNA Decay Pathway Involved in Plant Acclimation and Survival during Thermal Stress. *Cell Reports*, *5*(5), 1279–1293.
doi: 10.1016/j.celrep.2013.11.019

Mickelbart, M. V., Hasegawa, P. M., & Bailey-Serres, J. (2015, mar). Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nature Reviews Genetics*, *16*(4), 237–251.
doi: 10.1038/nrg3901

Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M. A., Shulaev, V., ... Mittler, R. (2009, aug). The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. *Science signaling*, *2*(84), ra45.
doi: 10.1126/scisignal.2000448

Milo, R., & Phillips, R. (2015). *Cell Biology by the Numbers* (1st ed.). Garland Science (Taylor & Francis Group).

Minagawa, J. (2011, aug). State transitions—The molecular remodeling of photosynthetic supercomplexes that controls energy flow in the chloroplast. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1807*(8), 897–905.

doi: 10.1016/j.bbabio.2010.11.005

Mirouze, M., Lieberman-Lazarovich, M., Aversano, R., Bucher, E., Nicolet, J., Reinders, J., & Paszkowski, J. (2012, apr). Loss of DNA methylation affects the recombination landscape in Arabidopsis. *Proceedings of the National Academy of Sciences*, *109*(15), 5880–5885.
doi: 10.1073/pnas.1120841109

Mittler, R., & Blumwald, E. (2015, jan). The roles of ROS and ABA in systemic acquired acclimation. *The Plant cell*, *27*(1), 64–70.
doi: 10.1105/tpc.114.133090

Molinier, J., Ries, G., Zipfel, C., & Hohn, B. (2006, aug). Transgeneration memory of stress in plants. *Nature*, *442*(7106), 1046–1049.
doi: 10.1038/nature05022

Müller, P., Li, X. P., & Niyogi, K. K. (2001, apr). Non-photochemical quenching. A response to excess light energy. *Plant physiology*, *125*(4), 1558–66.
doi: 10.1104/pp.125.4.1558

Müller-Xing, R., Xing, Q., & Goodrich, J. (2014). Footprints of the sun: memory of UV and light stress in plants. *Frontiers in plant science*, *5*(September), 474.
doi: 10.3389/fpls.2014.00474

Munekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M., & Shikanai, T. (2002, aug). PGR5 Is Involved in Cyclic Electron Flow around Photosystem I and Is Essential for Photoprotection in Arabidopsis. *Cell*, *110*(3), 361–371.
doi: 10.1016/S0092-8674(02)00867-X

Murata, Y., Mori, I. C., & Munemasa, S. (2015, apr). Diverse Stomatal Signaling and the Signal Integration Mechanism. *Annual Review of Plant Biology*, *66*(1), 369–392.
doi: 10.1146/annurev-arplant-043014-114707

Murchie, E. H., & Lawson, T. (2013, oct). Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of Experimental Botany*, *64*(13), 3983–3998.
doi: 10.1093/jxb/ert208

Murchie, E. H., Pinto, M., & Horton, P. (2009, feb). Agriculture and the new challenges for photosynthesis research. *New Phytologist*, *181*(3), 532–552.
doi: 10.1111/j.1469-8137.2008.02705.x

Murgia, I., Giacometti, S., Balestrazzi, A., Paparella, S., Pagliano, C., & Morandini, P. (2015, sep). Analysis of the transgenerational iron deficiency stress memory in Arabidopsis thaliana plants. *Frontiers in Plant Science*, *6*(September), 1–14.
doi: 10.3389/fpls.2015.00745

Mustilli, A.-C., Merlot, S., Vavasseur, A., Fenzi, F., & Giraudat, J. (2002, dec). Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *The Plant cell*, *14*(12), 3089–99.

Mustroph, A., Zanetti, M. E., Jang, C. J. H., Holtan, H. E., Repetti, P. P., Galbraith, D. W., ... Bailey-Serres, J. (2009, nov). Profiling translatomes of discrete cell populations resolves altered cellular priorities during hypoxia in Arabidopsis. *Proceedings of the National Academy of Sciences*, *106*(44), 18843–18848.
doi: 10.1073/pnas.0906131106

Nagarajan, V. K., Jones, C. I., Newbury, S. F., & Green, P. J. (2013, jun). XRN 5′→3′ exoribonucleases: Structure, mechanisms and functions. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1829*(6-7), 590–603.
doi: 10.1016/j.bbagrm.2013.03.005

Nambara, E., & Marion-Poll, A. (2005, jun). Abscisic acid biosynthesis and catabolism. *Annual review of plant biology*, *56*(1), 165–85.
doi: 10.1146/annurev.arplant.56.032604.144046

Narsai, R., Gouil, Q., Secco, D., Srivastava, A., Karpievitch, Y. V., Liew, L. C., ... Whelan, J. (2017, dec). Extensive transcriptomic and epigenomic remodelling occurs during Arabidopsis thaliana germination. *Genome Biology*, *18*(1), 172.
doi: 10.1186/s13059-017-1302-3

Negi, J., Matsuda, O., Nagasawa, T., Oba, Y., Takahashi, H., Kawai-Yamada, M., ... Iba, K. (2008, mar). CO2 regulator SLAC1 and its homologues are essential for anion homeostasis in plant cells. *Nature*, *452*(7186), 483–486.
doi: 10.1038/nature06720

Neuhierl, B., Thanbichler, M., Lottspeich, F., & Böck, A. (1999, feb). A Family of S-Methylmethionine-dependent Thiol/Selenol Methyltransferases. *Journal of Biological Chemistry*, *274*(9), 5407–5414.
doi: 10.1074/jbc.274.9.5407

Nguyen, A. H., Matsui, A., Tanaka, M., Mizunashi, K., Nakaminami, K., Hayashi, M., ... Seki, M. (2015, sep). Loss of Arabidopsis 5'-3' Exoribonuclease AtXRN4 Function Enhances Heat Stress Tolerance of Plants Subjected to Severe Heat Stress. *Plant & cell physiology*, *56*(9), 1762–72.
doi: 10.1093/pcp/pcv096

Niederhuth, C. E., & Schmitz, R. J. (2017, jan). Putting DNA methylation in context: from genomes to gene expression in plants. *Biochimica et biophysica acta*, *1860*(1), 149–156.
doi: 10.1016/j.bbagrm.2016.08.009

Nilkens, M., Kress, E., Lambrev, P., Miloslavina, Y., Müller, M., Holzwarth, A. R., & Jahns, P. (2010, apr). Identification of a slowly inducible zeaxanthin-dependent component of non-photochemical quenching of chlorophyll fluorescence generated under steady-state conditions in Arabidopsis. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, *1797*(4), 466–475.
doi: 10.1016/j.bbabio.2010.01.001

Niyogi, K. K. (1999, jun). PHOTOPROTECTION REVISITED: Genetic and Molecular Approaches. *Annual review of plant physiology and plant molecular biology*, *50*(1), 333–359.
doi: 10.1146/annurev.arplant.50.1.333

Niyogi, K. K., Grossman, A. R., & Björkman, O. (1998, jul). Arabidopsis mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *The Plant cell*, *10*(7), 1121–34.
doi: 10.1105/tpc.10.7.1121

Nosalewicz, A., Siecińska, J., Śmiech, M., Nosalewicz, M., Wiącek, D., Pecio, A., & Wach, D. (2016, nov). Transgenerational effects of temporal drought stress on spring barley morphology and functioning. *Environmental and Experimental Botany*, *131*, 120–127.
doi: 10.1016/j.envexpbot.2016.07.006

Nuthikattu, S., McCue, A. D., Panda, K., Fultz, D., DeFraia, C., Thomas, E. N., & Slotkin, R. K. (2013, may). The initiation of epigenetic silencing of active transposable elements is triggered by RDR6 and 21-22 nucleotide small interfering RNAs. *Plant physiology*, *162*(1), 116–31.
doi: 10.1104/pp.113.216481

Obayashi, T., Aoki, Y., Tadaka, S., Kagaya, Y., & Kinoshita, K. (2017, dec). ATTED-II in 2018: A Plant Coexpression Database Based on Investigation of the Statistical Property of the Mutual Rank Index. *Plant and Cell Physiology*.
doi: 10.1093/pcp/pcx191

Oelmüller, R., Levitan, I., Bergfeld, R., Rajasekhar, V. K., & Mohr, H. (1986, sep). Expression of nuclear genes as affected by treatments acting on the plastids. *Planta*, *168*(4), 482–92.
doi: 10.1007/BF00392267

Oelmüller, R., & Mohr, H. (1986, jan). Photooxidative destruction of chloroplasts and its consequences for expression of nuclear genes. *Planta*, *167*(1), 106–13.
doi: 10.1007/BF00446376

Ogle, D. H. (2017). *FSA: Fisheries Stock Analysis.*

O'Malley, R. C., Huang, S.-s. C., Song, L., Lewsey, M. G., Bartlett, A., Nery, J. R., ... Ecker, J. R. (2016, may). Cistrome and Epicistrome Features Shape the Regulatory DNA Landscape. *Cell*, *165*(5), 1280–1292.
doi: 10.1016/j.cell.2016.04.038

Ong-Abdullah, M., Ordway, J. M., Jiang, N., Ooi, S.-E., Kok, S.-Y., Sarpan, N., ... Martienssen, R. A. (2015, sep). Loss of Karma transposon methylation underlies the mantled somaclonal variant of oil palm. *Nature*, *525*(7570), 533–537.
doi: 10.1038/nature15365

Osakabe, Y., Osakabe, K., Shinozaki, K., & Tran, L.-S. P. (2014, mar). Response of plants to water stress. *Frontiers in Plant Science*, *5*(March), 1–8.
doi: 10.3389/fpls.2014.00086

Otagaki, S., Kasai, M., Masuta, C., & Kanazawa, A. (2013, feb). Enhancement of RNA-directed DNA methylation of a transgene by simultaneously downregulating a ROS1 ortholog using a virus vector in Nicotiana benthamiana. *Frontiers in Genetics*, *4*(FEB), 1–10.
doi: 10.3389/fgene.2013.00044

Panda, K., Ji, L., Neumann, D. A., Daron, J., Schmitz, R. J., & Slotkin, R. K. (2016, aug). Full-length autonomous transposable elements are preferentially targeted by expression-dependent forms of RNA-directed DNA methylation. *Genome biology*, *17*(1), 170.
doi: 10.1186/s13059-016-1032-y

Park, S.-Y., Fung, P., Nishimura, N., Jensen, D. R., Fujii, H., Zhao, Y., ... Cutler, S. R. (2009, may). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science (New York, N.Y.)*, *324*(5930), 1068–71.
doi: 10.1126/science.1173041

Pastori, G. M., & Foyer, C. H. (2002, jun). Common components, networks, and pathways of cross-tolerance to stress. The central role of "redox" and abscisic acid-mediated controls. *Plant physiology*, *129*(2), 460–8.
doi: 10.1104/pp.011021

Pecinka, A., Dinh, H. Q., Baubec, T., Rosa, M., Lettner, N., & Scheid, O. M. (2010, sep). Epigenetic Regulation of Repetitive Elements Is Attenuated by Prolonged Heat Stress in Arabidopsis. *The Plant Cell*, *22*(9), 3118–3129.
doi: 10.1105/tpc.110.078493

Pecinka, A., Rosa, M., Schikora, A., Berlinger, M., Hirt, H., Luschnig, C., & Scheid, O. M. (2009, apr). Transgenerational Stress Memory Is Not a General Response in Arabidopsis. *PLoS ONE*, *4*(4), e5202.
doi: 10.1371/journal.pone.0005202

Peirats-Llobet, M., Han, S.-K., Gonzalez-Guzman, M., Jeong, C. W., Rodriguez, L., Belda-Palazon, B., ... Rodriguez, P. L. (2016, jan). A Direct Link between Abscisic Acid Sensing and the Chromatin-Remodeling ATPase BRAHMA via Core ABA Signaling Pathway Components. *Molecular Plant*, *9*(1), 136–147.
doi: 10.1016/j.molp.2015.10.003

Pélissier, T., Thalmeir, S., Kempe, D., Sänger, H. L., & Wassenegger, M. (1999, apr). Heavy de novo methylation at symmetrical and non-symmetrical sites is a hallmark of RNA-directed DNA methylation. *Nucleic acids research*, *27*(7), 1625–34.
doi: 10.1093/nar/27.7.1625

Penterman, J., Uzawa, R., & Fischer, R. L. (2007, dec). Genetic interactions between DNA demethylation and methylation in Arabidopsis. *Plant physiology*, *145*(4), 1549–57.
doi: 10.1104/pp.107.107730

Pesaresi, P., Hertle, A., Pribil, M., Kleine, T., Wagner, R., Strissel, H., ... Leister, D. (2009, aug). Arabidopsis STN7 kinase provides a link between short- and long-term photosynthetic acclimation. *The Plant cell*, *21*(8), 2402–23.
doi: 10.1105/tpc.108.064964

Piskurewicz, U., Iwasaki, M., Susaki, D., Megies, C., Kinoshita, T., & Lopez-Molina, L. (2016, dec). Dormancy-specific imprinting underlies maternal inheritance of seed dormancy in Arabidopsis thaliana. *eLife*, *5*(DECEMBER2016), 1–23.
doi: 10.7554/eLife.19573

Pogson, B. J., Niyogi, K. K., Björkman, O., & DellaPenna, D. (1998, oct). Altered xanthophyll compositions adversely affect chlorophyll accumulation and nonphotochemical quenching in Arabidopsis mutants. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(22), 13324–9.

Pogson, B. J., Woo, N. S., Förster, B., & Small, I. D. (2008, nov). Plastid signalling to the nucleus and beyond. *Trends in Plant Science*, *13*(11), 602–609.
doi: 10.1016/j.tplants.2008.08.008

Popova, O. V., Dinh, H. Q., Aufsatz, W., & Jonak, C. (2013, mar). The RdDM pathway is required for basal heat tolerance in Arabidopsis. *Molecular plant*, *6*(2), 396–410.
doi: 10.1093/mp/sst023

Pornsiriwong, W., Estavillo, G. M., Chan, K. X., Tee, E. E., Ganguly, D., Crisp, P. A., ... Pogson, B. J. (2017, mar). A chloroplast retrograde signal, 3'-phosphoadenosine 5'-phosphate, acts as a secondary messenger in abscisic acid signaling in stomatal closure and germination. *eLife*, *6*, 1–34.
doi: 10.7554/eLife.23361

Pradhan, G. P., Prasad, P. V. V., Fritz, A. K., Kirkham, M. B., & Gill, B. S. (2012). Effects of drought and high temperature stress on synthetic hexaploid wheat. *Functional Plant Biology*, *39*(3), 190.
doi: 10.1071/FP11245

Probst, A. V., Dunleavy, E., & Almouzni, G. (2009, mar). Epigenetic inheritance during the cell cycle. *Nature Reviews Molecular Cell Biology*, *10*(3), 192–206.
doi: 10.1038/nrm2640

Probst, A. V., & Mittelsten Scheid, O. (2015, oct). Stress-induced structural changes in plant chromatin. *Current Opinion in Plant Biology*, *27*, 8–16.
doi: 10.1016/j.pbi.2015.05.011

Qi, Y., He, X., Wang, X.-J., Kohany, O., Jurka, J., & Hannon, G. J. (2006, sep). Distinct catalytic and non-catalytic roles of ARGONAUTE4 in RNA-directed DNA methylation. *Nature*, *443*(7114), 1008–1012.
doi: 10.1038/nature05198

Qian, W., Miki, D., Zhang, H., Liu, Y., Zhang, X., Tang, K., ... Zhu, J.-K. (2012, jun). A Histone Acetyltransferase Regulates Active DNA Demethylation in Arabidopsis. *Science*, *336*(6087), 1445–1448.
doi: 10.1126/science.1219416

Quadrana, L., & Colot, V. (2016, nov). Plant Transgenerational Epigenetics. *Annual Review of Genetics*, *50*(1), 467–491.
doi: 10.1146/annurev-genet-120215-035254

Quinlan, A. R., & Hall, I. M. (2010, mar). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842.
doi: 10.1093/bioinformatics/btq033

R Core Team. (2016). *R: A Language and Environment for Statistical Computing.* Vienna, Austria.

Ramakers, C., Ruijter, J. M., Deprez, R. H. L., & Moorman, A. F. M. (2003, mar). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience letters*, *339*(1), 62–6.

Ramakrishna, A., & Ravishankar, G. A. (2011, nov). Influence of abiotic stress signals on secondary metabolites in plants. *Plant signaling & behavior*, *6*(11), 1720–31.
doi: 10.4161/psb.6.11.17613

Ramel, F., Birtic, S., Ginies, C., Soubigou-Taconnat, L., Triantaphylides, C., & Havaux, M. (2012, apr). Carotenoid oxidation products are stress signals that mediate gene responses to singlet oxygen in plants. *Proceedings of the National Academy of Sciences*, *109*(14), 5535–5540.

doi: 10.1073/pnas.1115982109

Rasmann, S., De Vos, M., Casteel, C. L., Tian, D., Halitschke, R., Sun, J. Y., ... Jander, G. (2012, feb). Herbivory in the previous generation primes plants for enhanced insect resistance. *Plant physiology*, *158*(2), 854–63.
doi: 10.1104/pp.111.187831

Reinders, J., Wulff, B. B., Mirouze, M., Mari-Ordonez, A., Dapp, M., Rozhon, W., ... Paszkowski, J. (2009, apr). Compromised stability of DNA methylation and transposon immobilization in mosaic Arabidopsis epigenomes. *Genes & Development*, *23*(8), 939–950.
doi: 10.1101/gad.524609

Richards, E. J. (2006, may). Inherited epigenetic variation — revisiting soft inheritance. *Nature Reviews Genetics*, *7*(5), 395–401.
doi: 10.1038/nrg1834

Robinson, J. T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2011, jan). Integrative genomics viewer. *Nature Biotechnology*, *29*(1), 24–26.
doi: 10.1038/nbt.1754

Rocha, P. S. C. F., Sheikh, M., Melchiorre, R., Fagard, M., Boutet, S., Loach, R., ... Furner, I. (2005, feb). The Arabidopsis HOMOLOGY-DEPENDENT GENE SILENCING1 gene codes for an S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing. *The Plant cell*, *17*(2), 404–17.
doi: 10.1105/tpc.104.028332

Rodriguez-Granados, N. Y., Ramirez-Prado, J. S., Veluchamy, A., Latrasse, D., Raynaud, C., Crespi, M., ... Benhamed, M. (2016, may). Put your 3D glasses on: plant chromatin is on show. *Journal of Experimental Botany*, *67*(11), 3205–3221.
doi: 10.1093/jxb/erw168

Rodríguez-Leal, D., Lemmon, Z. H., Man, J., Bartlett, M. E., & Lippman, Z. B. (2017, oct). Engineering Quantitative Trait Variation for Crop Improvement by Genome Editing. *Cell*, *171*(2), 470–480.e8.
doi: 10.1016/j.cell.2017.08.030

Rossel, J. B., Walter, P. B., Hendrickson, L., Chow, W. S., Poole, A., Mullineaux, P. M., & Pogson, B. J. (2006, feb). A mutation affecting ASCORBATE PEROXIDASE 2 gene expression reveals a link between responses to high light and drought tolerance. *Plant, cell & environment*, *29*(2), 269–81.
doi: 10.1111/j.1365-3040.2005.01419.x

Rossel, J. B., Wilson, P. B., Hussain, D., Woo, N. S., Gordon, M. J., Mewett, O. P., ... Pogson, B. J. (2007, dec). Systemic and intracellular responses to photooxidative stress in Arabidopsis. *The Plant cell*, *19*(12), 4091–110.
doi: 10.1105/tpc.106.045898

Rowley, M. J., Rothi, M. H., Böhmdorfer, G., Kuciński, J., & Wierzbicki, A. T. (2017, may). Long-range control of gene expression via RNA-directed DNA methylation. *PLOS Genetics*, *13*(5), e1006749.
doi: 10.1371/journal.pgen.1006749

Ruban, A. V., Berera, R., Ilioaia, C., van Stokkum, I. H. M., Kennis, J. T. M., Pascal, A. A., ... van Grondelle, R. (2007, nov). Identification of a mechanism of photoprotective energy dissipation in higher plants. *Nature*, *450*(7169), 575–578.
doi: 10.1038/nature06262

Ruelland, E., Vaultier, M.-N., Zachowski, A., & Hurry, V. (2009). Chapter 2 Cold Signalling and Cold Acclimation in Plants. In *Advances in botanical research* (1st ed., Vol. 49, pp. 35–150). Elesvier Ltd.
doi: 10.1016/S0065-2296(08)00602-2

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., & Moorman, A. F. M. (2009, apr). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Research*, *37*(6), e45–e45.
doi: 10.1093/nar/gkp045

Rungrat, T., Awlia, M., Brown, T., Cheng, R., Sirault, X., Fajkus, J., ... Wilson, P. (2016, jan). Using Phenomic Analysis of Photosynthetic Function for Abiotic Stress Response Gene Discovery. *The Arabidopsis Book*, *14*, e0185.
doi: 10.1199/tab.0185

Sani, E., Herzyk, P., Perrella, G., Colot, V., & Amtmann, A. (2013, jun). Hyperosmotic priming of Arabidopsis seedlings establishes a long-term somatic memory accompanied by specific changes of the epigenome. *Genome Biology*, *14*(6), R59.
doi: 10.1186/gb-2013-14-6-r59

Sauter, A., Davies, W. J., & Hartung, W. (2001, oct). The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *Journal of experimental botany*, *52*(363), 1991–7.
doi: 10.1093/jexbot/52.363.1991

Saze, H., Mittelsten Scheid, O., & Paszkowski, J. (2003, may). Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. *Nature genetics*, *34*(1), 65–9.
doi: 10.1038/ng1138

Schmitz, R. J., Schultz, M. D., Lewsey, M. G., O'Malley, R. C., Urich, M. A., Libiger, O., ... Ecker, J. R. (2011, oct). Transgenerational Epigenetic Instability Is a Source of Novel Methylation Variants. *Science*, *334*(6054), 369–373.
doi: 10.1126/science.1212959

Schmitz, R. J., Schultz, M. D., Urich, M. A., Nery, J. R., Pelizzola, M., Libiger, O., ... Ecker, J. R. (2013, mar). Patterns of population epigenomic diversity. *Nature*, *495*(7440), 193–198.
doi: 10.1038/nature11968

Schöttler, M. A., & Tóth, S. Z. (2014). Photosynthetic complex stoichiometry dynamics in higher plants: environmental acclimation and photosynthetic flux control. *Frontiers in plant science*, *5*(May), 188.
doi: 10.3389/fpls.2014.00188

Schultz, M. D., Schmitz, R. J., & Ecker, J. R. (2012, dec). 'Leveling' the playing field for analyses of single-base resolution DNA methylomes. *Trends in Genetics*, *28*(12), 583–585.
doi: 10.1016/j.tig.2012.10.012

Schumann, T., Paul, S., Melzer, M., Dörmann, P., & Jahns, P. (2017, may). Plant Growth under Natural Light Conditions Provides Highly Flexible Short-Term Acclimation Properties toward High Light Stress. *Frontiers in Plant Science*, *8*.
doi: 10.3389/fpls.2017.00681

Schwartz, S. H., Tan, B. C., Gage, D. A., Zeevaart, J. A., & McCarty, D. R. (1997, jun). Specific oxidative cleavage of carotenoids by VP14 of maize. *Science (New York, N.Y.)*, *276*(5320), 1872–4.

Secco, D., Wang, C., Shou, H., Schultz, M. D., Chiarenza, S., Nussaume, L., ... Lister, R. (2015, jul). Stress induced gene expression drives transient DNA methylation changes at adjacent repetitive elements. *eLife*, *4*(JULY2015), 1–26.
doi: 10.7554/eLife.09343

Secco, D., Whelan, J., Rouached, H., & Lister, R. (2017, oct). Nutrient stress-induced chromatin changes in plants. *Current Opinion in Plant Biology*, *39*, 1–7.
doi: 10.1016/j.pbi.2017.04.001

Seeley, K. A., Byrne, D. H., & Colbert, J. T. (1992, jan). Red Light-Independent Instability of Oat Phytochrome mRNA in Vivo. *The Plant cell*, *4*(1), 29–38.
doi: 10.1105/tpc.4.1.29

Seki, M., Ishida, J., Narusaka, M., Fujita, M., Nanjo, T., Umezawa, T., ... Shinozaki, K. (2002, nov). Monitoring the expression pattern of around 7,000 Arabidopsis genes

under ABA treatments using a full-length cDNA microarray. *Functional & integrative genomics*, *2*(6), 282–91.
doi: 10.1007/s10142-002-0070-6

Seymour, D. K., Koenig, D., Hagmann, J., Becker, C., & Weigel, D. (2014, nov). Evolution of DNA methylation patterns in the Brassicaceae is driven by differences in genome organization. *PLoS genetics*, *10*(11), e1004785.
doi: 10.1371/journal.pgen.1004785

Shaw, R. G. G., Byers, D. L. L., & Darmo, E. (2000). Spontaneous Mutational Effects on Reproductive Traits of Arabidopsis thaliana. *Genetics*, *155*, 369–378.

Sheldon, C. C., Rouse, D. T., Finnegan, E. J., Peacock, W. J., & Dennis, E. S. (2000, mar). The molecular basis of vernalization: The central role of FLOWERING LOCUS C (FLC). *Proceedings of the National Academy of Sciences*, *97*(7), 3753–3758.
doi: 10.1073/pnas.97.7.3753

Shinozaki, K., Yamaguchi-Shinozaki, K., & Seki, M. (2003, oct). Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology*, *6*(5), 410–417.
doi: 10.1016/S1369-5266(03)00092-X

Shu, K., Liu, X.-d., Xie, Q., & He, Z.-h. (2016, jan). Two Faces of One Seed: Hormonal Regulation of Dormancy and Germination. *Molecular Plant*, *9*(1), 34–45.
doi: 10.1016/j.molp.2015.08.010

Sierla, M., Waszczak, C., Vahisalu, T., & Kangasjärvi, J. (2016, jul). Reactive Oxygen Species in the Regulation of Stomatal Movements. *Plant Physiology*, *171*(3), 1569–1580.
doi: 10.1104/pp.16.00328

Simeunovic, A., Mair, A., Wurzinger, B., & Teige, M. (2016, jun). Know where your clients are: subcellular localization and targets of calcium-dependent protein kinases. *Journal of Experimental Botany*, *67*(13), 3855–3872.
doi: 10.1093/jxb/erw157

Skirycz, A., & Inzé, D. (2010, apr). More from less: plant growth under limited water. *Current Opinion in Biotechnology*, *21*(2), 197–203.
doi: 10.1016/j.copbio.2010.03.002

Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B., & Mauch-Mani, B. (2012, feb). Descendants of primed Arabidopsis plants exhibit resistance to biotic stress. *Plant physiology*, *158*(2), 835–43.
doi: 10.1104/pp.111.191593

Slesak, I., Libik, M., Karpinska, B., Karpinski, S., & Miszalski, Z. (2007). The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. *Acta biochimica Polonica*, *54*(1), 39–50.

Slotkin, R. K., Vaughn, M., Borges, F., Tanurdžić, M., Becker, J. D., Feijó, J. A., & Martienssen, R. A. (2009, feb). Epigenetic Reprogramming and Small RNA Silencing of Transposable Elements in Pollen. *Cell*, *136*(3), 461–472.
doi: 10.1016/j.cell.2008.12.038

Smith, L. M., Pontes, O., Searle, I., Yelina, N., Yousafzai, F. K., Herr, A. J., ... Baulcombe, D. C. (2007, may). An SNF2 protein associated with nuclear RNA silencing and the spread of a silencing signal between cells in Arabidopsis. *The Plant Cell*, *19*(5), 1507–21.
doi: 10.1105/tpc.107.051540

Spinoni, J., Naumann, G., Carrao, H., Barbosa, P., & Vogt, J. (2014, jun). World drought frequency, duration, and severity for 1951-2010. *International Journal of Climatology*, *34*(8), 2792–2804.
doi: 10.1002/joc.3875

Springer, N. M. (2013, apr). Epigenetics and crop improvement. *Trends in Genetics*, *29*(4), 241–247.
doi: 10.1016/j.tig.2012.10.009

Springer, N. M., Lisch, D., & Li, Q. (2016, feb). Creating Order from Chaos: Epigenome Dynamics in Plants with Complex Genomes. *The Plant Cell*, *28*(2), 314–325.
doi: 10.1105/tpc.15.00911

Springthorpe, V., & Penfield, S. (2015, mar). Flowering time and seed dormancy control use external coincidence to generate life history strategy. *eLife*, *4*(4), 1–17.
doi: 10.7554/eLife.05557

Strand, Å., Asami, T., Alonso, J., Ecker, J. R., & Chory, J. (2003, jan). Chloroplast to nucleus communication triggered by accumulation of Mg-protoporphyrinIX. *Nature*, *421*(6918), 79–83.
doi: 10.1038/nature01204

Stroud, H., Do, T., Du, J., Zhong, X., Feng, S., Johnson, L., ... Jacobsen, S. E. (2014, dec). Non-CG methylation patterns shape the epigenetic landscape in Arabidopsis. *Nature Structural & Molecular Biology*, *21*(1), 64–72.
doi: 10.1038/nsmb.2735

Stroud, H., Greenberg, M. V., Feng, S., Bernatavichute, Y. V., & Jacobsen, S. E. (2013, jan). Comprehensive Analysis of Silencing Mutants Reveals Complex Regulation of the Arabidopsis Methylome. *Cell*, *152*(1-2), 352–364.

doi: 10.1016/j.cell.2012.10.054

Stuart, T., Eichten, S. R., Cahn, J., Karpievitch, Y. V., Borevitz, J. O., & Lister, R. (2016, dec). Population scale mapping of transposable element diversity reveals links to gene regulation and epigenomic variation. *eLife*, *5*(DECEMBER2016), 1–27. doi: 10.7554/eLife.20777

Sun, X., Fu, T., Chen, N., Guo, J., Ma, J., Zou, M., ... Zhang, L. (2010, mar). The stromal chloroplast Deg7 protease participates in the repair of photosystem II after photoinhibition in Arabidopsis. *Plant physiology*, *152*(3), 1263–73. doi: 10.1104/pp.109.150722

Sung, S., & Amasino, R. M. (2004, jan). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. *Nature*, *427*(6970), 159–64. doi: 10.1038/nature02195

Susek, R. E., Ausubel, F. M., & Chory, J. (1993, sep). Signal transduction mutants of Arabidopsis uncouple nuclear CAB and RBCS gene expression from chloroplast development. *Cell*, *74*(5), 787–99. doi: 10.1016/0092-8674(93)90459-4

Suzuki, N., Devireddy, A. R., Inupakutika, M. A., Baxter, A., Miller, G., Song, L., ... Mittler, R. (2015, nov). Ultra-fast alterations in mRNA levels uncover multiple players in light stress acclimation in plants. *The Plant Journal*, *84*(4), 760–772. doi: 10.1111/tpj.13039

Suzuki, N., Miller, G., Salazar, C., Mondal, H. A., Shulaev, E., Cortes, D. F., ... Mittler, R. (2013, sep). Temporal-Spatial Interaction between Reactive Oxygen Species and Abscisic Acid Regulates Rapid Systemic Acclimation in Plants. *The Plant Cell*, *25*(9), 3553–3569. doi: 10.1105/tpc.113.114595

Szechyńska-Hebda, M., Kruk, J., Górecka, M., Karpińska, B., & Karpiński, S. (2010, jul). Evidence for light wavelength-specific photoelectrophysiological signaling and memory of excess light episodes in Arabidopsis. *The Plant cell*, *22*(7), 2201–18. doi: 10.1105/tpc.109.069302

Takahashi, S., & Badger, M. R. (2011, jan). Photoprotection in plants: a new light on photosystem II damage. *Trends in Plant Science*, *16*(1), 53–60. doi: 10.1016/j.tplants.2010.10.001

Takahashi, S., Seki, M., Ishida, J., Satou, M., Sakurai, T., Narusaka, M., ... Shinozaki, K. (2004, sep). Monitoring the expression profiles of genes induced by hyperosmotic, high salinity, and oxidative stress and abscisic acid treatment in Arabidopsis cell culture using a full-length cDNA microarray. *Plant Molecular Biology*, *56*(1), 29–55.

doi: 10.1007/s11103-004-2200-0

Takeuchi, J., Okamoto, M., Akiyama, T., Muto, T., Yajima, S., Sue, M., ... Todoroki, Y. (2014, jun). Designed abscisic acid analogs as antagonists of PYL-PP2C receptor interactions. *Nature chemical biology*, *10*(6), 477–82.
doi: 10.1038/nchembio.1524

Tan, B.-C., Joseph, L. M., Deng, W.-T., Liu, L., Li, Q.-B., Cline, K., & McCarty, D. R. (2003, jul). Molecular characterization of the Arabidopsis 9-cis epoxycarotenoid dioxygenase gene family. *The Plant journal : for cell and molecular biology*, *35*(1), 44–56.

Tang, K., Lang, Z., Zhang, H., & Zhu, J.-K. (2016, oct). The DNA demethylase ROS1 targets genomic regions with distinct chromatin modifications. *Nature Plants*, *2*(11), 16169.
doi: 10.1038/nplants.2016.169

Taudt, A., Colomé-Tatché, M., & Johannes, F. (2016, may). Genetic sources of population epigenomic variation. *Nature Reviews Genetics*, *17*(6), 319–332.
doi: 10.1038/nrg.2016.45

Telfer, A. (2002, oct). What is beta-carotene doing in the photosystem II reaction centre? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *357*(1426), 1431–70.
doi: 10.1098/rstb.2002.1139

Therneau, T. M. (2015). *A Package for Survival Analysis in S*.

Thomas, P. W., Woodward, F. I., & Quick, W. P. (2003, nov). Systemic irradiance signalling in tobacco. *New Phytologist*, *161*(1), 193–198.
doi: 10.1046/j.1469-8137.2003.00954.x

Thompson, L. (1994). The Spatiotemporal Effects of Nitrogen and Litter on the Population Dynamics of Arabidopsis Thaliana. *Journal of Ecology*, *82*(1), 63–68.

Ton, J., Jakab, G., Toquin, V., Flors, V., Iavicoli, A., Maeder, M. N., ... Mauch-Mani, B. (2005, mar). Dissecting the beta-aminobutyric acid-induced priming phenomenon in Arabidopsis. *The Plant cell*, *17*(3), 987–99.
doi: 10.1105/tpc.104.029728

Tran, R. K., Zilberman, D., de Bustos, C., Ditt, R. F., Henikoff, J. G., Lindroth, A. M., ... Henikoff, S. (2005). Chromatin and siRNA pathways cooperate to maintain DNA methylation of small transposable elements in Arabidopsis. *Genome biology*, *6*(11), R90.
doi: 10.1186/gb-2005-6-11-r90

Triantaphylidès, C., & Havaux, M. (2009, apr). Singlet oxygen in plants: production, detoxification and signaling. *Trends in Plant Science*, *14*(4), 219–228.
doi: 10.1016/j.tplants.2009.01.008

Tricker, P. J. (2015, sep). Transgenerational inheritance or resetting of stress-induced epigenetic modifications: two sides of the same coin. *Frontiers in Plant Science*, *6*(September), 1–6.
doi: 10.3389/fpls.2015.00699

Tricker, P. J., Gibbings, J. G., Rodríguez López, C. M., Hadley, P., & Wilkinson, M. J. (2012, jun). Low relative humidity triggers RNA-directed de novo DNA methylation and suppression of genes controlling stomatal development. *Journal of Experimental Botany*, *63*(10), 3799–3813.
doi: 10.1093/jxb/ers076

Tricker, P. J., López, C. M., Gibbings, G., Hadley, P., & Wilkinson, M. J. (2013, mar). Transgenerational, Dynamic Methylation of Stomata Genes in Response to Low Relative Humidity. *International Journal of Molecular Sciences*, *14*(4), 6674–6689.
doi: 10.3390/ijms14046674

Tsuzuki, M., Motomura, K., Kumakura, N., & Takeda, A. (2017, mar). Interconnections between mRNA degradation and RDR-dependent siRNA production in mRNA turnover in plants. *Journal of Plant Research*, *130*(2), 211–226.
doi: 10.1007/s10265-017-0906-8

Tyystjärvi, E. (2008, feb). Photoinhibition of Photosystem II and photodamage of the oxygen evolving manganese cluster. *Coordination Chemistry Reviews*, *252*(3-4), 361–376.
doi: 10.1016/j.ccr.2007.08.021

van Loon, L. C. (2016, apr). The Intelligent Behavior of Plants. *Trends in plant science*, *21*(4), 286–294.
doi: 10.1016/j.tplants.2015.11.009

Varriale, A. (2014, jan). DNA methylation, epigenetics, and evolution in vertebrates: facts and challenges. *International journal of evolutionary biology*, *2014*, 475981.
doi: 10.1155/2014/475981

Verslues, P. E., Agarwal, M., Katiyar-Agarwal, S., Zhu, J., & Zhu, J.-K. (2006, feb). Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *The Plant Journal*, *45*(4), 523–539.
doi: 10.1111/j.1365-313X.2005.02593.x

Virlouvet, L., Ding, Y., Fujii, H., Avramova, Z., & Fromm, M. (2014, jul). ABA signaling is necessary but not sufficient for RD29B transcriptional memory during

successive dehydration stresses in Arabidopsis thaliana. *The Plant Journal*, *79*(1), 150–161.
doi: 10.1111/tpj.12548

Virlouvet, L., & Fromm, M. (2015, jan). Physiological and transcriptional memory in guard cells during repetitive dehydration stress. *New Phytologist*, *205*(2), 596–607.
doi: 10.1111/nph.13080

Vriet, C., Hennig, L., & Laloi, C. (2015, apr). Stress-induced chromatin changes in plants: of memories, metabolites and crop improvement. *Cellular and Molecular Life Sciences*, *72*(7), 1261–1273.
doi: 10.1007/s00018-014-1792-z

Waddington, C. H. (2012, feb). The Epigenotype. *International Journal of Epidemiology*, *41*(1), 10–13.
doi: 10.1093/ije/dyr184

Walter, J., Nagy, L., Hein, R., Rascher, U., Beierkuhnlein, C., Willner, E., & Jentsch, A. (2011, apr). Do plants remember drought? Hints towards a drought-memory in grasses. *Environmental and Experimental Botany*, *71*(1), 34–40.
doi: 10.1016/j.envexpbot.2010.10.020

Wang, R.-S., Pandey, S., Li, S., Gookin, T. E., Zhao, Z., Albert, R., & Assmann, S. M. (2011, dec). Common and unique elements of the ABA-regulated transcriptome of Arabidopsis guard cells. *BMC Genomics*, *12*(1), 216.
doi: 10.1186/1471-2164-12-216

Wang, X., Vignjevic, M., Jiang, D., Jacobsen, S., & Wollenweber, B. (2014, dec). Improved tolerance to drought stress after anthesis due to priming before anthesis in wheat (Triticum aestivum L.) var. Vinjett. *Journal of Experimental Botany*, *65*(22), 6441–6456.
doi: 10.1093/jxb/eru362

Wang, X.-B., Wu, Q., Ito, T., Cillo, F., Li, W.-X., Chen, X., ... Ding, S.-W. (2010, jan). RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in Arabidopsis thaliana. *Proceedings of the National Academy of Sciences*, *107*(1), 484–489.
doi: 10.1073/pnas.0904086107

Wang, Y., Chen, Z.-H., Zhang, B., Hills, A., & Blatt, M. R. (2013, oct). PYR/PYL/RCAR abscisic acid receptors regulate K+ and Cl- channels through reactive oxygen species-mediated activation of Ca2+ channels at the plasma membrane of intact Arabidopsis guard cells. *Plant physiology*, *163*(2), 566–77.
doi: 10.1104/pp.113.219758

Warnes, G. R., Bolker, B., Bonebakker, L., Gentleman, R., Liaw, W. H. A., Lumley, T., ... Venables, B. (2016). *gplots: Various R Programming Tools for Plotting Data.*

Wassenegger, M., Heimes, S., Riedel, L., & Sänger, H. L. (1994, feb). RNA-directed de novo methylation of genomic sequences in plants. *Cell*, *76*(3), 567–576.
doi: 10.1016/0092-8674(94)90119-8

Westgate, M., Passioura, J., & Munns, R. (1996). Water Status and ABA Content of Floral Organs in Drought-Stressed Wheat. *Australian Journal of Plant Physiology*, *23*(6), 763.
doi: 10.1071/PP9960763

Wibowo, A., Becker, C., Marconi, G., Durr, J., Price, J., Hagmann, J., ... Gutierrez-Marcos, J. (2016, may). Hyperosmotic stress memory in Arabidopsis is mediated by distinct epigenetically labile sites in the genome and is restricted in the male germline by DNA glycosylase activity. *eLife*, *5*(MAY2016), 1–27.
doi: 10.7554/eLife.13546

Wickham, H. (2007). Reshaping Data with the reshape Package. *Journal of Statistical Software*, *21*(12), 1–20.

Wickham, H. (2009). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.

Wickham, H. (2011). The Split-Apply-Combine Strategy for Data Analysis. *Journal of Statistical Software*, *40*(1), 1–29.

Wierzbicki, A. T., Ream, T. S., Haag, J. R., & Pikaard, C. S. (2009, may). RNA polymerase V transcription guides ARGONAUTE4 to chromatin. *Nature Genetics*, *41*(5), 630–634.
doi: 10.1038/ng.365

Williams, B. P., & Gehring, M. (2017, dec). Stable transgenerational epigenetic inheritance requires a DNA methylation-sensing circuit. *Nature Communications*, *8*(1), 2124.
doi: 10.1038/s41467-017-02219-3

Williams, B. P., Pignatta, D., Henikoff, S., & Gehring, M. (2015, mar). Methylation-Sensitive Expression of a DNA Demethylase Gene Serves As an Epigenetic Rheostat. *PLOS Genetics*, *11*(3), e1005142.
doi: 10.1371/journal.pgen.1005142

Wilson, P. B., Estavillo, G. M., Field, K. J., Pornsiriwong, W., Carroll, A. J., Howell, K. A., ... Pogson, B. J. (2009, apr). The nucleotidase/phosphatase SAL1 is a

negative regulator of drought tolerance in Arabidopsis. *The Plant Journal*, *58*(2), 299–317.
doi: 10.1111/j.1365-313X.2008.03780.x

Woo, N. S., Badger, M. R., & Pogson, B. J. (2008). A rapid, non-invasive procedure for quantitative assessment of drought survival using chlorophyll fluorescence. *Plant Methods*, *4*(1), 27.
doi: 10.1186/1746-4811-4-27

Woodson, J. D., Perez-Ruiz, J. M., & Chory, J. (2011, may). Heme Synthesis by Plastid Ferrochelatase I Regulates Nuclear Gene Expression in Plants. *Current Biology*, *21*(10), 897–903.
doi: 10.1016/j.cub.2011.04.004

Xiao, Y., Savchenko, T., Baidoo, E. E. K., Chehab, W. E., Hayden, D. M., Tolstikov, V., ... Dehesh, K. (2012, jun). Retrograde signaling by the plastidial metabolite MEcPP regulates expression of nuclear stress-response genes. *Cell*, *149*(7), 1525–35.
doi: 10.1016/j.cell.2012.04.038

Xie, Z., Johansen, L. K., Gustafson, A. M., Kasschau, K. D., Lellis, A. D., Zilberman, D., ... Carrington, J. C. (2004, feb). Genetic and Functional Diversification of Small RNA Pathways in Plants. *PLoS Biology*, *2*(5), e104.
doi: 10.1371/journal.pbio.0020104

Xiong, L., Lee Bh, Ishitani, M., Lee, H., Zhang, C., & Zhu, J.-K. (2001, aug). FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis. *Genes & development*, *15*(15), 1971–84.
doi: 10.1101/gad.891901

Xiong, L., & Zhu, J.-K. (2003, sep). Regulation of abscisic acid biosynthesis. *Plant physiology*, *133*(1), 29–36.
doi: 10.1104/pp.103.025395

Yamamuro, C., Miki, D., Zheng, Z., Ma, J., Wang, J., Yang, Z., ... Zhu, J.-K. (2014, jun). Overproduction of stomatal lineage cells in Arabidopsis mutants defective in active DNA demethylation. *Nature Communications*, *5*.
doi: 10.1038/ncomms5062

Yang, R., Zheng, Z., Chen, Q., Yang, L., Huang, H., Miki, D., ... Zhang, H. (2017, dec). The developmental regulator PKL is required to maintain correct DNA methylation patterns at RNA-directed DNA methylation loci. *Genome Biology*, *18*(1), 103.
doi: 10.1186/s13059-017-1226-y

Yang, Y., Costa, A., Leonhardt, N., Siegel, R. S., & Schroeder, J. I. (2008, feb). Isolation of a strong Arabidopsis guard cell promoter and its potential as a research tool. *Plant Methods*, *4*(1), 6.
doi: 10.1186/1746-4811-4-6

Yang, Z., Liu, J., Tischer, S. V., Christmann, A., Windisch, W., Schnyder, H., & Grill, E. (2016, jun). Leveraging abscisic acid receptors for efficient water use in Arabidopsis. *Proceedings of the National Academy of Sciences*, *113*(24), 6791–6796.
doi: 10.1073/pnas.1601954113

Yano, S., & Terashima, I. (2001, dec). Separate Localization of Light Signal Perception for Sun or Shade Type Chloroplast and Palisade Tissue Differentiation in Chenopodium album. *Plant and Cell Physiology*, *42*(12), 1303–1310.
doi: 10.1093/pcp/pce183

Ye, R., Chen, Z., Lian, B., Rowley, M. J., Xia, N., Chai, J., ... Qi, Y. (2016, jan). A Dicer-Independent Route for Biogenesis of siRNAs that Direct DNA Methylation in Arabidopsis. *Molecular cell*, *61*(2), 222–35.
doi: 10.1016/j.molcel.2015.11.015

Yelina, N. E., Choi, K., Chelysheva, L., Macaulay, M., de Snoo, B., Wijnker, E., ... Henderson, I. R. (2012, aug). Epigenetic Remodeling of Meiotic Crossover Frequency in Arabidopsis thaliana DNA Methyltransferase Mutants. *PLoS Genetics*, *8*(8), e1002844.
doi: 10.1371/journal.pgen.1002844

Yong-Villalobos, L., González-Morales, S. I., Wrobel, K., Gutiérrez-Alanis, D., Cervantes-Peréz, S. A., Hayano-Kanashiro, C., ... Herrera-Estrella, L. (2015, dec). Methylome analysis reveals an important role for epigenetic changes in the regulation of the Arabidopsis response to phosphate starvation. *Proceedings of the National Academy of Sciences*, *112*(52), E7293–E7302.
doi: 10.1073/pnas.1522301112

Yoshida, T., Fujita, Y., Maruyama, K., Mogami, J., Todaka, D., Shinozaki, K., & Yamaguchi-Shinozaki, K. (2015, jan). Four Arabidopsis AREB/ABF transcription factors function predominantly in gene expression downstream of SnRK2 kinases in abscisic acid signalling in response to osmotic stress. *Plant, cell & environment*, *38*(1), 35–49.
doi: 10.1111/pce.12351

Youngson, N. A., & Whitelaw, E. (2008, sep). Transgenerational Epigenetic Effects. *Annual Review of Genomics and Human Genetics*, *9*(1), 233–257.
doi: 10.1146/annurev.genom.9.081307.164445

Zeevaart, J. A., & Boyer, G. L. (1984, apr). Accumulation and transport of Abscisic Acid and its metabolites in ricinus and xanthium. *Plant physiology*, *74*(4), 934–9.
doi: 10.1104/pp.74.4.934

Zemach, A., Kim, M. Y., Hsieh, P.-H., Coleman-Derr, D., Eshed-Williams, L., Thao, K., ... Zilberman, D. (2013, mar). The Arabidopsis Nucleosome Remodeler DDM1 Allows DNA Methyltransferases to Access H1-Containing Heterochromatin. *Cell*, *153*(1), 193–205.
doi: 10.1016/j.cell.2013.02.033

Zhang, Y.-Y., Fischer, M., Colot, V., & Bossdorf, O. (2013, jan). Epigenetic variation creates potential for evolution of plant phenotypic plasticity. *New Phytologist*, *197*(1), 314–322.
doi: 10.1111/nph.12010

Zhao, Z., Zhang, W., Stanley, B. A., & Assmann, S. M. (2008, dec). Functional proteomics of Arabidopsis thaliana guard cells uncovers new stomatal signaling pathways. *The Plant cell*, *20*(12), 3210–26.
doi: 10.1105/tpc.108.063263

Zheng, B., Wang, Z., Li, S., Yu, B., Liu, J.-Y., & Chen, X. (2009, dec). Intergenic transcription by RNA polymerase II coordinates Pol IV and Pol V in siRNA-directed transcriptional gene silencing in Arabidopsis. *Genes & development*, *23*(24), 2850–60.
doi: 10.1101/gad.1868009

Zheng, Q., Rowley, M. J., Böhmdorfer, G., Sandhu, D., Gregory, B. D., & Wierzbicki, A. T. (2013, jan). RNA polymerase V targets transcriptional silencing components to promoters of protein-coding genes. *The Plant Journal*, *73*(2), 179–189.
doi: 10.1111/tpj.12034

Zheng, X., Chen, L., Xia, H., Wei, H., Lou, Q., Li, M., ... Luo, L. (2017, jan). Transgenerational epimutations induced by multi-generation drought imposition mediate rice plant's adaptation to drought condition. *Scientific Reports*, *7*(January), 39843.
doi: 10.1038/srep39843

Zheng, X., Zhu, J., Kapoor, A., & Zhu, J.-K. (2007, mar). Role of Arabidopsis AGO6 in siRNA accumulation, DNA methylation and transcriptional gene silencing. *The EMBO Journal*, *26*(6), 1691–1701.
doi: 10.1038/sj.emboj.7601603

Zhong, S., Fei, Z., Chen, Y.-R., Zheng, Y., Huang, M., Vrebalov, J., ... Giovannoni, J. J. (2013, jan). Single-base resolution methylomes of tomato fruit development reveal epigenome modifications associated with ripening. *Nature Biotechnology*, *31*(2), 154–159.
doi: 10.1038/nbt.2462

Zhong, X., Du, J., Hale, C. J., Gallego-Bartolome, J., Feng, S., Vashisht, A. A., ... Jacobsen, S. E. (2014, may). Molecular Mechanism of Action of Plant DRM De Novo DNA Methyltransferases. *Cell*, *157*(5), 1050–1060.
doi: 10.1016/j.cell.2014.03.056

Zhong, X., Hale, C. J., Law, J. A., Johnson, L. M., Feng, S., Tu, A., & Jacobsen, S. E. (2012, sep). DDR complex facilitates global association of RNA polymerase V to promoters and evolutionarily young transposons. *Nature structural & molecular biology*, *19*(9), 870–5.
doi: 10.1038/nsmb.2354

Zhong, X., Hale, C. J., Nguyen, M., Ausin, I., Groth, M., Hetzel, J., ... Jacobsen, S. E. (2015, jan). DOMAINS REARRANGED METHYLTRANSFERASE3 controls DNA methylation and regulates RNA polymerase V transcript abundance in Arabidopsis. *Proceedings of the National Academy of Sciences*, *112*(3), 911–916.
doi: 10.1073/pnas.1423603112

Zhu, H., Wang, G., & Qian, J. (2016, aug). Transcription factors as readers and effectors of DNA methylation. *Nature Reviews Genetics*, *17*(9), 551–565.
doi: 10.1038/nrg.2016.83

Zhu, S.-Y., Yu, X.-C., Wang, X.-J., Zhao, R., Li, Y., Fan, R.-C., ... Zhang, D.-P. (2007, oct). Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. *The Plant cell*, *19*(10), 3019–36.
doi: 10.1105/tpc.107.050666

Zhu, X., Ort, D. R., Whitmarsh, J., & Long, S. P. (2004, may). The slow reversibility of photosystem II thermal energy dissipation on transfer from high to low light may cause large losses in carbon gain by crop canopies: a theoretical analysis. *Journal of Experimental Botany*, *55*(400), 1167–1175.
doi: 10.1093/jxb/erh141

Zilberman, D., Cao, X., & Jacobsen, S. E. (2003, jan). ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science (New York, N.Y.)*, *299*(5607), 716–9.
doi: 10.1126/science.1079695

Zivcak, M., Brestic, M., Kalaji, H. M., & Govindjee. (2014, mar). Photosynthetic responses of sun- and shade-grown barley leaves to high light: is the lower PSII connectivity in shade leaves associated with protection against excess of light? *Photosynthesis Research*, *119*(3), 339–354.
doi: 10.1007/s11120-014-9969-8