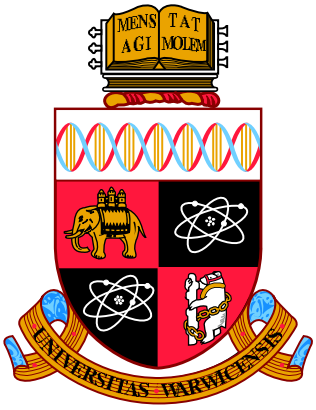

Control of Developmental Reprogramming During Somatic Cell Regeneration in *Arabidopsis thaliana*

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Abstract

Unlike most animals, plants can regenerate new tissues, organs or an entire organism by changing the developmentally committed program of individual cells - a phenomenon known as totipotency. This unique developmental plasticity of plants has been exploited in agriculture and horticulture to generate clonal plants in axenic culture through the induction of a somatic embryogenesis. However, some plant species are resistant to current regeneration protocols and the precise mechanisms responsible for totipotency in plant cells remain unknown. Using a novel system to induce somatic embryos from terminally differentiated somatic cells, we have investigated the importance of histone modifying proteins belonging to the Polycomb group (PcG) and Jumonji C-domain-containing class (JmJ-C), on cellular susceptibility to developmental reprogramming. We found that reprogramming success is influenced by cell type, developmental stage and that JmJ-C proteins play an important role in facilitating plant cellular plasticity. We also identified a protective role against epimutations during sexual reproduction in *Arabidopsis thaliana*. Furthermore, this system enabled the identification of key factors involved in the transition away from a differentiated state and into a new developmental fate, providing insights into the regulatory mechanism of totipotency in plants.

Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

Dr Julia Engelhorn (University of Warwick, United Kingdom) Assisting with the *rkd-1* gene/H3K27me3 intersection work (Chapter 4)

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List of Abbreviations

- **AGL15** AGAMOUS-LIKE 15
- **ALF4** ABERRANT LATERAL ROOT FORMATION 4
- **ARF** AUXIN RESPONSE FACTORS
- **BBM** BABY BOOM
- **bp** Base pair
- **C2H2-ZnF** Cys2His2 zinc finger
- **CAF1** CHROMATIN ASSEMBLY FACTOR 1
- **CaMV 35S** Cucumber mosaic virus 35S promotor
- **CenH3** Centromeric H3
- **CLF** CURLY LEAF
- **CMT1/2/3** CHROMOMETHYLASE 1/2/3
- **DDM1** DECREASE IN DNA METHYLATION 1
- **DEX** Dexamethasone
- **DME** DEMETER
- **DMR** Differentially Methylated Region
- **DNA** DeoxyriboNucleic Acid
- **DNMT1** DNA (cytosine-5)-methyltransferase 1
- **DRM1** DOMAINS REARRANGED METHYLTRANSFERASE 1
- **DRM2** DOMAINS REARRANGED METHYLTRANSFERASE 2
- **E(z)** ENHANCER OF ZESTE
- **easiRNA** Epigenetically activated small RNAs
- **ELF6** EARLY FLOWERING 6
- **EMF2** EM-BRYONIC FLOWER2
- **EMS** Ethyl MethaneSulphonate
- **epiRILs** Epigenetic Recombinant Inbred Lines
- **Esc** EXTRA SEX COMBS

- **FIE** FERTILIZATION INDEPENDENT ENDOSPERM
- **FIS2** FERTILIZATION INDEPENDENT SEED 2
- **FLC** FLOWERING LOCUS C
- **GFP** Green Fluorescent Protein
- **H2A** Histone 2A
- **H2AK119** Lysine 119 on histone 2A
- **H2B** Histone 2B
- **H3** Histone 3
- **H3K27me3** Tri-methylation at lysine 27 on histone H3
- **H3K4** Lysine 4 on histone 3
- **H3K9** Lysine 9 on histone 3
- **H3K9me3** Tri-methylation at lysine 9 on histone 3
- **H4** Histone 4
- **HDA6** HISTONE DEACETYLASE 6
- **HMTase** Histone Methyltransferase
- **inRKD4ox** *Arabidopsis* line with inducible RKD4 over expression
- **JMJ** JUMONJI
- **JmJ-C** Jumonji-C domain containing protein
- **K** Lysine
- **LBD** LATERAL ORGAN BOUNDARIES DOMAIN
- **LDL1/2** LYSINE-SPECIFIC DEMETHYLASE LIKE 1/LIKE 2
- **LEC2** LEAFY COTYLEDON 2
- **LHP1** LIKE HETEROCHROMATIN PROTEIN 1
- **LHY** LATE ELONGATED HYPOCOTYL
- **LRC** Lateral Root Cap
- **LSD1-4** LYSINE SPECIC DEMETHYLASE 1 -4
- **me1/me2/me3** Mono-/di-/trimethylated
- **MEA** MEDEA

- **MET1** METHYLTRANSFERASE 1
- **MR** Methylated Region
- **MSI1-5** MULTICOPY SUPRESSOR OF IRA 1-5
- **Pc** Chromodomain protein
- **PcG** Polycomb Group
- **PKL** PICKLE
- **PRC** Polycomb Repressive Complex
- **PRC1/2** Polycomb repressive complex 1/2
- **PREs** Polycomb Response Elements
- **R** Arginine
- **RAM** Root Apical Meristem
- **RBR** RETINOBLASTOMA-RELATED
- **RdDM** RNA-directed DNA methylation
- **REF6** RELATIVE OF EARLY FLOWERING 6
- **RKD4** RWP-RK DOMAIN-CONTAINING 4
- **RKD4ox** RKD4 over expression
- **RNA** RiboNucleic Acid
- **ROS1** REPRESSOR OF SILENCING 1
- **S** Synthesis
- **SAM** Shoot Apical Meristem
- **SE** Somatic Embryogenesis
- **SNP** Single Nucleotide Polymorphism
- **Su(z)12** Suppressor of Zeste
- **SWN** SWINGER
- **TAA1** TRYPTOPHAN AMINO-TRANSFERASE OF ARABIDOPSIS 1
- **TE** Transposable Element
- **TF** Transcription Factors
- **VIM1 and VIM2** VARIANT IN METHYLATION 1 and 2

- **VRN2** VERNALIZATION 2
- μ**l** Micro litre
- μ**m** Micro metre
- **QC** Quiescent Center
- **WOX** WUSCHEL-RELATED HOMEOBOX
- **WT** Wild-Type
- **WUS** WUSCHEL
- **WIND3** WOUND INDUCED DEDIFFERENTIATION 3

Chapter 1

Introduction

1.1 Cell Fate and Epigenetic Control of Gene Expression

Single cell organisms can respond dynamically to changes in the environment in order to survive (Mitchell et al., 2009). As organisms evolved in complexity into multicellular systems, the establishment of distinct cell linages allowed the cells to function collectively as an organism rather than as discrete entities. With the exception of gametes, all cells within multicellular organisms contain the same DNA content with cells able to express a large variation in patterns of gene expression; with consistent patterns used to identify distinct 'differentiated' cell types. These patterns are largely driven by functional regulatory networks and guided by key transcription factors such as homeotic genes (Drapek et al., 2017).

In order to ensure correct and proper development, the 'body plan' of multicellular organisms are typically set up early in development within small groups of cells, such as developing embryos or organ primordia (Ingham, 1988). As the organism's development progresses and the cells undergo further proliferation, the genetic developmental patterning is progressively elaborated and entrenched to give rise to a complete organism (Lawrence and Morata, 1994).

During development, the fate of a cell can be established intrinsically, by directly inheriting a developmental pathway from the parent cell through mitosis. Alternatively, cells may adopt fates dependent on or in response to extrinsic factors, in which case fate is controlled by environmental cues such as hormones or direct cell-to-cell signalling from neighbours (De Rybel et al., 2016). In animal systems, lineage-based mechanisms are thought to play a major role in organogenesis and dictating cell fate determination (Mercier and Scadden, 2015). However, in early development, animal cells remain sensitive to positional signal whereby cells are able to take the place of a cell that becomes damaged or destroyed, allowing the 'body plan' to be completed without defects. For example, in the zebrafish, whose body normally develops from an invariant pattern of cell lineage, laser ablation studies indicate that the identity of cells may change when their neighbours are destroyed (Xiao et al., 2016).

In plant systems, although parental linage can confer some inherited gene expression patterns and epigenetic modifications, the inherited lineage determinant can be overridden by positional information and external stimuli to give rise to new cell types (Yu et al., 2017). For example, in the root meristem, cortical initial cells undergo a periclinal division forming cells which become the cortex and the endodermal cell layers. Developmental lineage of these two cells are then determined by the positional regulation within the meristematic region of the root indicated by cell ablation studies (Van Den Berg et al., 1997; Berger et al., 1998) (Fig.1.1).

As a cell continues to progress down a particular developmental pathway they become more entrenched, and less responsive to extrinsic factors that may cause them to transition into a new cell type. This allows discrete organ structures to develop between neighbouring cell masses and prevents disruption of the organs function through loss of key gene expression patterns within differentiated cells (Trindade et al., 2017). For organisms like plants, this process allows patterns of cell fates to become established at actively proliferating regions which can then be maintained once the cell becomes more distant from the signalling environment

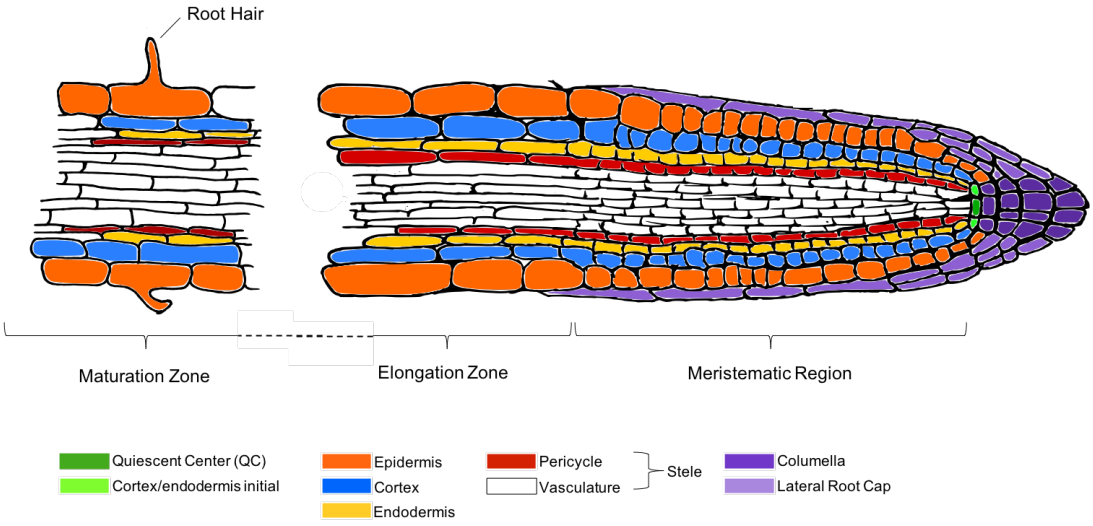


Figure 1.1: Cell Patterning in the Root Apical Meristem. Schematic diagram of cell patterning at the root apical meristem and the developmental zones marking the transition to fully differentiated cells indicated by root hairs. Cells forming the quiescent center (QC) maintain a stem cell niche. These quiescent cells maintain (by contact), a group of surrounding 'initals' cells that periodically divide giving rise to all the cell types along the root axis.

laying down the initial cell patterning.

In plants, most organs, including true leaves and flowers, are only generated after germination and not established during embryogenesis as in animals (Trindade et al., 2017). As a consequence, the adult body is composed of a sequence of organs that are formed during different developmental phases, a feature known as heteroblasty (Trindade et al., 2017). The continual growth is maintained through actively proliferating regions are called meristems, like the root apical meristem (RAM) and the shoot apical meristem (SAM) (Shani et al., 2006; Kyozuka, 2007; Dello Ioio et al., 2007). Meristems retain a niche of pluripotent stem cells which gives rise to the initial spatial patterning, determining organ formation (Fig.1.1). Within root development this leads to a range of developmental 'zones' as the cells mature and become more differentiated as the cell is isolated from the root meristem environment (Drapek et al., 2017). Morphologically this progress can be tracked through a meristematic to elongation phase, ending in a maturation

zone allowing the formation of terminally differentiated tissues such as root hairs (Fig.1.1). Over time, endogenous or environmental signals can lead to the identity of the SAM to change, resulting in formation of functionally and morphologically distinct organs, and often marks important developmental phase transitions in plants, such as transitions from vegetative to flowing development (reviewed in Barton 2010 and Trindade 2017).

Differentiation or developmental phase transitions require cellular changes at the molecular level, which often involve the switching on and off of entire developmental networks (Bassel, 2016). These transitions are often stable, so that cells or plants plants do not revert back to the previous developmental phases (Bassel, 2016), however, the molecular mechanisms underlining these transitions need to retain a degree of plasticity that allows for erasure of the gene networks at the onset of each generation.

Epigenetic modifications of chromatin provide a means of stabilising gene expression patterns in organisms over long periods of developmental time without affecting the DNA sequence. Chromatin modifications can include reversible chemical 'tagging' of DNA such as methylation, post translational modification of the histones, which form the nucleosomes around which the DNA is wrapped, or changes to the positions or densities of arrangement of these nucleosomes (Kouzarides, 2007); providing a dynamic structure that can respond in response to different environmental or developmental stimuli (Trindade et al., 2017).

1.2 Regulation of Gene Expression by Modification of Histones

In eukaryotes, the nucleus contain a large quantity of genetic material that must be effectively managed within every cell, to ensure appropriate temporal and spacial expression of genes. One major structural component of chromatin consists of histone complexes. Histones are conserved proteins with little variation of

amino acid sequence amongst eukaryotes and form the base unit around which DNA strands are wrapped, forming a unit of chromatin called the nucleosome. The nucleosome core is composed of an octamer of histones: two heterodimers of histone 2A (H2A) and histone 2B (H2B), and two heterodimers of histone 3 (H3) and histone 4 (H4) (Wolffe and Kurumizaka, 1998) around which approximately 147bp of DNA are wrapped (Gan et al., 2015).

There are two major classes of histones. Canonical histones are mainly expressed during the synthesis (S) phase of the cell cycle, incorporated during DNA replication, and assembled into the newly synthesized DNA strand (Henikoff and Ahmad, 2005). The second major class are expressed during all phases of the cell cycle and can be incorporated into nucleosomes without replication. This can occur within differentiated cells by chromatin re-modelers; H2A, H2B and H3 families contain variants that are included in this class (Malik and Henikoff, 2003; Bernstein and Hake, 2006). The incorporation of different variants of these histones can affect the levels of compaction within the genome, enhancing or inhibiting cellular processes involving DNA, such as transcription, replication, recombination and repair. Two distinct forms of chromatin have been identified in plants and animals: euchromatin is usually constituted by transcribed loci; and heterochromatin is enriched with TEs (transposable elements) and is typically transcriptionally silenced through the vegetative phase by DNA methylation and histone modifications (Bernatavichute et al., 2008; Law and Jacobsen, 2010).

Histones contain a N-terminal amino acid tail which extends out of the DNA-histone core which can be post translationally modified; with these modifications also affecting chromatin structure (Kouzarides, 2007). Histone variants have different residues within these N- terminal tails which can determine the modifications that can be made. For example, the H3 family contains a number of variants; Centromeric H3 (CenH3) variants that are essential for chromosome segregation during mitosis (Dalal et al., 2007; Houben et al., 2011) and differ from the other H3 proteins by an extension of the N-terminal tail, that is poorly conserved among eukaryotes (Malik and Henikoff, 2003). Other variants such as

H3.3 and H3.1 are similar in length and vary in amino acid sequence at four key positions at sites 31, 41, 87 and 90 (Waterborg and Robertson, 1996; Malik and Henikoff, 2003).

In plants four types of post-translational histone modification have been identified: ubiquitination, methylation, acetylation and phosphorilation (Pfluger and Wagner, 2007). Certain modifications are highly conserved between plants animals and fungi such as methylation of histone 3 at lysine 4 (H3K4) and lysine 9 (H3K9). These correlate well with gene activation and gene silencing, respectively (Pfluger and Wagner, 2007). Early studies proposed a 'histone code' which could be read by proteins, with some modifications (such as acetylation and phosphorylation), proposed to alter the charge of the histone, thereby controlling the degree of condensation of the chromatin fibre (Strahl and Allis, 2000). However, more recent evidence indicate that histone modifications (such as, methylation and acetylation) serve as binding platforms to recruit other protein complexes onto the chromatin (Gan et al., 2013).

Histone methylation is the most well studied forms of histone modifications, and occurs on the lysine (K) and arginine (R) residues on histone tails. Depending on the location of the amino acid residue and its degree of methylation, it can confer an activating or repressing status to chromatin (Gan et al., 2013, 2015). Modification of lysines are catalyzed by histone methyltransferases (HMTases), and in Arabidopsis there are 37 of these HMTases which can add one, two or three methyl groups to a lysine residues, occurring on at least five residues of H3 (H3-K4, -K9, -K27, -K36, and -K79) and a single lysine of H4 (H4-K20) (Thorstensen et al., 2011).

1.3 Polycomb Group Proteins

The trimethylation of lysine 27 of histone H3 (H3K27me3) is correlated with gene repression, and is deposited on at least 25% of genes in seedlings but is

dynamically regulated during a plant's growth and development (Zhang et al., 2007; Roudier et al., 2011; Lafos et al., 2011). In Drosophila and Arabidopsis, Polycomb Group (PcG) proteins have been shown to have a role in establishing, and maintaining, H3K27me3 as an epigenetic mark (Margueron and Reinberg, 2011).

PcG genes were initially identified from genetic screens in *Drosophila melanogaster* as regulators of Hox gene expression (Lewis, 1978), and represent a conserved system of long-term gene inactivation. PcG proteins form large complexes, several of which are conserved in evolution, and can directly repress target genes or indirectly promote gene expression through repression of microRNA encoding genes (Lafos et al., 2011). Homologues of members of this PcG have been identified in a number of organisms, including vertebrates, nematodes, and plants, and function to regulate developmental patterning (Schwartz and Pirrotta, 2007; Bemer and Grossniklaus, 2012).

In plants two conserved forms of PcG complexes have been described: polycomb repressive complex 2 (PRC2), which catalyzes the trimethylation of H3K27 (Cao et al., 2002; Müller et al., 2002; Schubert et al., 2006), and PRC1, which is associated with H2A lysine 119 mono-ubiquitination (Bratzel et al., 2010; Yang et al., 2013).

In Drosophila PRC2 is composed of the four subunits Enhancer of Zeste (E(z)), Suppressor of Zeste (Su(z)12), Extra sex combs (Esc), and p55 (Czermin et al., 2002; Müller et al., 2002), with E(z) being the catalytic HMTase (Margueron and Reinberg, 2011). Genetic studies have revealed that Arabidopsis has a single Esc homologue FERTILIZATION INDEPENDENT ENDOSPERM (FIE), three Su(z)12 homologues EMBRYONIC FLOWER2 (EMF2), VERNALIZATION2 (VRN2), and FERTILIZATION INDEPENDENT SEED2 (FIS2), three E(z) homologues CURLY LEAF (CLF), SWIN-GER (SWN), and MEDEA (MEA), and five p55 homologues MULTICOPY SUPRESSOR OF IRA 1–5 (MSI1–5) (Goodrich et al., 1997; Grossniklaus et al., 1998; Kiyosue et al., 1999; Chanvivat-

tana et al., 2004; Hennig et al., 2005).

Furthermore, in contrast to Drosophila, Arabidopsis has three distinct PRC2-like complexes, which function at specific phases throughout development (Trindade et al., 2017). The EMF complex regulates vegetative development and is involved in the transition to flowering (Yoshida, 2001; Schönrock et al., 2006; Jiang et al., 2008; Derkacheva et al., 2013). The VRN complex regulates the vernalization response, involving the accumulation of H3K27me3 at the *FLOWERING LOCUS C* (*FLC*) gene locus, facilitating flowing after a period of cold (Gendall et al., 2001; Wood et al., 2006; De Lucia et al., 2008; Derkacheva et al., 2013). The FIS complex regulates female gametophyte and seed development, preventing initiation of endosperm and seed development in the absence of fertilization (Spillane et al., 2000; Yadegari et al., 2000; Köhler et al., 2003; Wang et al., 2006). Each complex contains FIE as a core component but vary in homologes of the other subunits identified in Drosophila. CLF and SWN are known to function in both the EMF and VRN complexes in plants fulfilling a similar methyltransferases function to E(z). CLF and SWN are partially redundant but the strong developmental phenotype of *clf* and the absence of any obvious defects in *swn* has led to the idea that CLF is the most important E(z) homologue in these complexes (Chanvivattana et al., 2004; Jiang et al., 2008; Derkacheva and Hennig, 2014).

In animals, there is a prevailing hierarchical structure to the recruitment of PRC1 and PRC2, allowing the stable repression of genes (Gan et al., 2015). In this model, PRC2 is recruited by the cis-acting Polycomb response elements (PREs) and deposits the H3K27me3 mark onto specific locations to establish the repressive chromatin state (Margueron and Reinberg, 2011). PRC1 is then recruited to the region via interaction of a chromodomain protein (Pc) and H3K27me3, allowing monoubiquitination of H2AK119, further reinforcing the repressive status (Margueron and Reinberg, 2011). Similarly, in Arabidopsis, LIKE HETEROCHROMATIN PROTEIN 1/TERMINAL FLOWER 2 (LHP1/TFL2) acts as the H3K27me3-binding protein and recruits putative complexes, such as the PRC1-like complex, containing five PRC1- RING finger proteins that catalyze

the deposition of H2AK119ub (Turck et al., 2007; Calonje, 2014; Molitor and Shen, 2013). However, this hierarchical structure is being questioned with recent studies placing PRC1 either upstream, downstream or acting independently of PRC2 (reviewed in Merini and Calonje 2015).

1.4 Jumonji-C Proteins

Throughout the plant life cycle and in response to certain environmental or developmental cues, the established developmental programs have to be erased, such as during gametogenesis (Kawashima and Berger, 2014). Hence, genes networks repressed by PRC2 complexes and H3K27me3 may have to become reactivated. Currently, there are two known classes of histone demethylases in plants, the first are a family of lysine specific demethylase (LSD1-4), which catalyse the demethylation of mono-/dimethylated lysines, particularly those found on histone H3 (H3K4) (Jiang et al., 2007). The second class are Jumonji-C (JmjC) domain-containing proteins, which have the ability to demethylate all mono-/di-/trimethylated (me1/me2/me3) lysine residues, including those at lysine 27 on histone H3 (H3K27) (Gan et al., 2015).

In Arabidopsis, 21 JUMONJI (JmJ) genes have been identified (Lu et al., 2008; Hong et al., 2009), which are categorised into five groups based on similarities of the JmjC domain sequences (KDM5/JARID1, KDM4/JHDM3/JMJD2, KDM3/JHDM2, JMJD6 and JmjC domain-only group) and have been shown to regulated a number of chromatin remodelling function in response to biotic and abiotic stresses (Gan et al., 2015). From this family, five proteins have been described to have specific H3K27me3 demethylase activity (Fig.1.2), and are responsible for de-repressing gene expression in a time and tissue-appropriate manner during development (Hong et al., 2009).

JMJ30 has a role in circadian systems, acting in concert with TIMING OF CAB EXPRESSION 1 to promote temporal gene expression of targets such as LATE

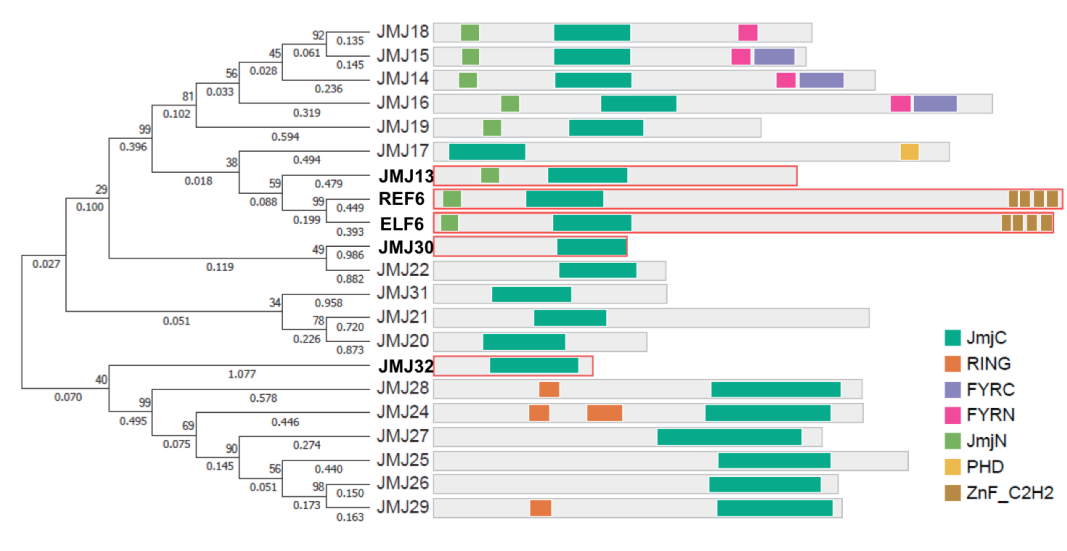


Figure 1.2: Phylogeny of Jumonji-C Domain Containing Proteins in *Arabidopsis thaliana*. A Phylogenetic tree constructed with MEGA (version 6.06) using the neighbour-joining method. Protein sequences and domain annotation were obtained from TAIR10. JmJ-C proteins identified with H3K27me3 demethylase activity (REF6, ELF6, JMJ13, JMJ30 and JMJ32) are highlighted in red. Phylogeny adapted from Yu et al., (2018)

ELONGATED HYPOCOTYL (LHY) (Lu et al., 2011b), while JMJ32 contributes to the thermosensory pathway of flowering control (Gan et al., 2014). These two proteins were shown to act together by delaying the H3K27me3 mediated repression of *FLC* locus, with the double mutant of *jmj30 jmj32* producing an early-flowering phenotype when grown at elevated temperatures (Lu et al., 2011b; Gan et al., 2014). Furthermore, JMJ30 has a wider role, and has recently been reported to also function to demethylate another repressive histone modification H3K9me3 (Lee et al., 2018). Two JmJ-C domain-containing proteins, JMJ11 and JMJ12, also known as EARLY FLOWERING 6 (ELF6) and RELATIVE OF EARLY FLOWERING 6 (REF6) are closely related H3K27me3 specific demethylases. They were initially identified by genetic screens to identify regulators of flowering time, and later shown to have a wider role in brassinosteroid signalling (Noh et al., 2004; Yu et al., 2008). More recently, these proteins were described to be acting, in a partially redundant fashion, in the removal of trimethylation

at lysine 27 on histone 3 with a third member of the family JMJ13 (Lu et al., 2011a; Crevillén et al., 2014; Yan et al., 2018).

REF6 was originally shown to function as a repressor of *FLC*, the expression of which is increased in the *ref6* mutant, delaying floweing (Noh et al., 2004). More recent genome-wide mapping of REF6 binding sites identified thousands of putative targets, interacting directly via a Cys2His2 zinc finger (C2H2-ZnF) DNA binding domain (Li et al., 2016a; Cui et al., 2016). ELF6 was initially observed to repress the floral integrators in the photoperiod pathway, based on finding that mutation of *elf6* resulted in increased *FLOWERING LOCUS T* and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* expression and had an early-flowering phenotype (Noh et al., 2004). However, more recently it has been shown that ELF6 participates in the reprogramming and resetting of the epigenetic state of FLC during gametogenesis (Crevillén et al., 2014). Thus, revealing a potential role of these proteins on the erasure of H3K27me3 and developmental memory between generations.

1.5 DNA Methylation in Plant Genomes

In Eukaryotes, DNA methylation serves as a mechanism to regulate gene expresssion, suppress transposable elements and can act as an epigenetic memory between generations (Du et al., 2015). DNA methylation corresponds to the enzymatic addition of a methyl group to the fifth carbon of the nucleotide cyctosine, catalysed by DNA methyltransferases. In plants, DNA methylation has been demonstrated to have important roles in development including the regulation of flowering time (Soppe et al., 2000), seed development (Gehring et al., 2006), and fruit ripening (Liu et al., 2015; Gallusci et al., 2016). This occurs in three contexts: CG, CHG, and CHH (with H standing for any nucleotide except G) and with average methylation levels varying between plant species (Becker et al., 2011; Zhang et al., 2006; Zilberman et al., 2007; Gent et al., 2013). In animals, DNA methylation is reset during gametogenesis and early embryogenesis (Feng

et al., 2010), while in plants DNA methylation can be a stable epigenetic modification, conserved through cell divisions and across generations, primarily in a CG context (Feng et al., 2010). However, plants are able to actively reset some methylation induced by environmental cues and this has been found to occur in a CHH context in the gametes (Wibowo et al., 2016).

In Arabidopsis, the mechanisms underlining DNA methylation are well described. Maintenance of symmetrical methylation (CG and CHG) is operated by two main enzymes: a DNMT1 type enzyme DNA methyltransferase 1 (MET1), is responsible for maintenance of cytosine methylation in a CG context, and requires VARIANT IN METHYLATION 1 and 2 (VIM1 and VIM2) for its activity (Fig.1.3 A) (Kim et al., 2014; Shook and Richards, 2014; Woo et al., 2008), while methylation in the CHG context is maintained by the plant-specific DNA methyltransferase CHROMOMETHYLASE 3 (CMT3) (Lindroth et al., 2001)(Fig.1.3 B). Non symmetrical CHH requires maintenance using *de novo* machinery, as there is no corresponding guiding methylated cytosine on the mother strand after replication. Therefore, methylation needs to be newly established in one of the two daughter DNA nucleotides and in Arabidopsis, *de novo* DNA methylation is established by two pathways (Law and Jacobsen, 2010). In the first, the RNA-directed DNA methylation (RdDM) pathway, small 21 or 24 nucleotide RNAs guide the targeting of two methyltransferases; DOMAIN REARRANGED 1 and 2 (DRM1 and DRM2) to CHH sites which sees the active methylation of the target regions (Matzke et al., 2015) (Fig.1.3 C). The second mechanism, relies on the chromatin remodeler DECREASE IN DNA METHYLATION 1 (DDM1) acting together with CHROMOMETHYLASE 2 or CHROMOMETHYLASE 3 which act to add de novo methylation in non-CG contexts (Stroud et al., 2014; Zemach et al., 2013) (Fig.1.3 C). Interestingly, these mechanisms for targeted methylation of a region are dependent on the histone environment, as the presence of H3K9me1 and/or H3K9me2 which have recently been shown to be vital for the binding of CMT2 and 3 to target sites. This histone modification also been linked to the targeting of the RdDM machinery responsible for the generation of the small RNAs used

to guide DRM1 and 2 to target sites (Du et al., 2015).

While DNA methylation can be added or maintained at a specific site, methylation can also be removed. This process can be passive DNA demethylation, where maintenance mechanisms (as described above) are not active during DNA replication. Alternatively, DNA regions may become specifically and actively demethylated through guided enzymatic activities. In plants, active demethylation relies on a family of methylcytosine DNA glycosylase–lyases, (DEMETER-like proteins), and in Arabidopsis, DNA glycosylases have been identified REPRESSOR OF SILENCING 1 (ROS1) and DEMETER (DME) (Chan et al., 2005). These proteins remove the methylated cytosine in DNA, which is subsequently repaired with an unmodified cytosine nucleotide through a base-excision repair process (Chan et al., 2005). ROS1 is found to be broadly expressed throughout developmental process, but DME is only expressed and functions during female gametogenesis (Zhu, 2009).

Therefore, DNA methylation provides a dynamic mechanism for the control of gene expression, with recent analysis showing distinctions in patterning between tissues and even between cell types (Widman et al., 2014; Kawakatsu et al., 2016). Mis-regulation of DNA methylation results in a range of aberrant developmental phenotypes (Saze et al., 2003).

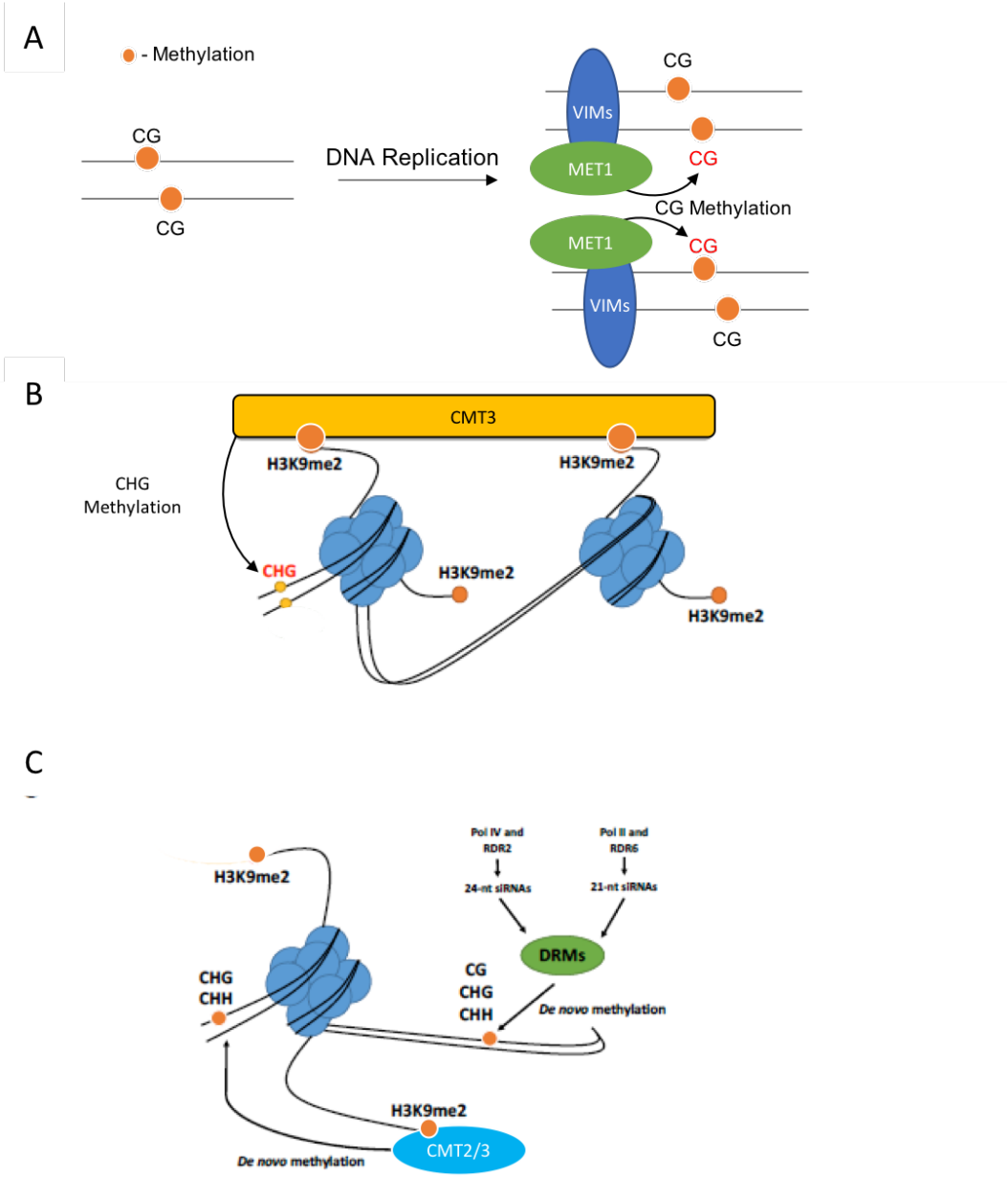


Figure 1.3: Models of DNA Methylation and Maintenance in *Arabidopsis thaliana*. DNA methylation in CG context is faithfully maintained on daughter strands of DNA through the action of VIM1/2 and MET1 proteins (A), CHG context is maintained after replication by CMT3 (B), and *de novo* DNA methylation can be established through two mechanisms: RNA-directed DNA methylation pathways via DRM1/2, and through CMT2/3 association with H3K9me2 (C). Adapted from Kawashima and Berger., (2014).

1.6 Developmental Programming and Regeneration

Changes in DNA methylation and histone modifications allow the dynamic regulation of gene expression in a spacial and temporal manner, allowing cells to respond to intrinsic and extrinsic developmental signals (Jaenisch and Bird, 2003). Plants and animals have almost certainly evolved multicellularity independently (Hedges et al., 2004), however, any multicellular organism with a relatively long lifespan is faced with dynamic, diverse threats to their survival, so both evolutionary models require the developmental plasticity to repair organs or cellular structures. As plants and animals evolved separately, it was hypothesised that there would be critical differences between the two kingdoms in terms of repair mechanism. However, at a fundamental level they are similar (Birnbaum and Sánchez Alvarado, 2008). Achieving regeneration involves 'turning back the clock' on differentiation or reactivating cells that have been frozen in developmental youth, while at the same time invoking ways to pattern the new tissue (Birnbaum and Sánchez Alvarado, 2008). Regeneration is often split into two main types; pluripotent - where a stem cell is capable of giving rise to several different cell types within a body, and totipotent - where cells are able to give rise to any cell type including embryos (Verdeil et al., 2007).

In animals, the regenerative capability ranges across species, organs and tissues but often involves the maintenance of regenerative stem cells within the body, which can be recruited to the site of damage, establishing repair or regeneration (Birnbaum and Sánchez Alvarado, 2008). However, as could be expected, the array of regenerative strategies can vary dramatically between organisms or even within neighbouring tissues. A number of model systems are well established and used to study regeneration in animals, ranging from the tail and limb of amphibians such as salamanders, axolotls and Xenopus tadpoles, heart and lens models in newts, fin and heart of zebrafish, gut and germ cells of Drosophila, blood, skin and gut in mice and the whole body of planarians and hydra (reviewed in Poss,

2010). The lack of cell migration in plants, which is precluded by rigid cell walls, prevents the movement of dispersed stem cells toward injured sites, necessitating an alternative approach to regeneration (Verdeil et al., 2007). Therefore, plants must repair damaged tissue by de-differentiation and re-specification of cells at the wound site (Asahina et al., 2011).

The regeneration potential of plants has been exploited in agriculture and horticulture with grafting and plantlet regeneration techniques used for centuries (Sang et al., 2018) and the regenerative potential of individual cells has fascinated scientists for over 100 years (Birnbaum and Sánchez Alvarado, 2008).

In plants, it was demonstrated that differentiated cells could be coaxed back into totipotency under a specific regiment of endogenous hormones (Skoog and Miller, 1957). This hormone combination could plausibly mimic an internal environment under regenerative conditions, and could initiate embryo production from some somatic tissue (Ikeuchi et al., 2016). This process can be used to regenerate clonal plants and *in vitro* regeneration is used widely as a plant propagation tool (Leelavathi et al., 2004). Protocols for tissue culture and somatic embryogenesis (SE) have been established in several crop species, such as soybean, potato, and cotton (Sharma et al., 2008; Thibaud-Nissen et al., 2003; Yang et al., 2012). However, elite lines of important crops such as maize and rice remain resistant to current culturing techniques, or resultant offspring becoming infertile, accumulating genetic disruptors such as transposable elements, thus inhibiting the wider application of these techniques (Zeven, 1973; Lowe et al., 2018). Additionally, closely related genotypes in *Medicago truncatula*, *Gossypium hirsutum* and *Zea mays* have been observed to have dramatically different regenerative potential, indicating the presence of epigenetic components to the regeneration pathway that are not currently understood (Orłowska et al., 2017; Li et al., 2018; Lowe et al., 2018). It has been established that cellular reprogramming relies on the erasure or application of a number of epigenetic modifications including DNA and histone methylation, and histone acetylation (Tanaka et al., 2008; Li et al., 2011; Ikeuchi et al., 2016). However, many of the molecular mechanisms underpinning

the removal of these modifications throughout these regeneration transitions are unclear.

1.7 Rationale and Aims for Research

In summary, epigenetic modifications within a cell provide an additional layer for the control of gene transcription. Combined, these factors allow for correct deployment of programs crucial for correct growth and development. Misregulation of these modifications can lead to severe developmental phenotypes so must be tightly maintained during cell differentiation. However, in order to respond to damage, plant cells must maintain a degree of plasticity, enabling targeted reactivation of gene networks allowing regeneration and re-specification of new tissues. However, the epigenetic regulation of plant cell regeneration and developmental reprogramming is currently unclear. This thesis aims to identify novel genetic components involved in induced regeneration pathways, and develop knowledge of the wider role of histone modification in somatic cell reprogramming.

Chapter 2

Materials and Methods

Plant Material and Growth Conditions

All plant material used in this study was derived from *Arabidopsis thaliana* (Col-0 accession). Induced regeneration experiments were carried out using an inducible construct for the expression of *RKD4* (Waki et al., 2011). The *ref6-5* mutant (GABI 705E03) was obtained from the GABI-Kat collection (Kleinboelting et al., 2012) (Fig.4.1). The *elf6-C* mutant was generated using CRISPR-Cas9 directed mutagenesis (Durr et al., 2018) (see below). Previously reported T-DNA insertion lines *ref6-1* (insertion at amino acid 1082), *elf6-3* (insertion at amino acid 169) (Noh et al., 2004; Kim et al., 1998) and a previously described miss-sense mutation *clf-81* (Schubert et al., 2006) were used in this study. Double mutant *ref6-5/elf6-C* was created by genetic crossing *ref6-5* and *elf6-C*.

Seeds for plate-grown experiments were surface sterilized using 10% of Sodium hypochlorite (VWR) with shaking for 10 minutes, then washed with sterile H_2O six times, dispersed in sterile 0.1% Agarose, before being placed on a base media of 1 x Murashige and Skoog (MS) salts (Duchefa Biochemie), pH 5.7 and 1% sucrose (Sigma-Aldrich) solidified with 0.8% phytoagar (Duchefa Biochemie) unless otherwise stated. Seeds grown in soil (John Innes and Perlite mix) were dispersed in 0.1% Agarose before being sown individually on the soil surface.

For all experiments in this study *Arabidopsis* seeds were stratified for two days at 4^{o}C for stratification. Seeds were germinated and grown in a light cabinet (Sigma), growth chamber (Conviron) or glasshouse under 16 h day, 8 h night photoperiod, at 22 ^{o}C, light intensity 100 μmol/sec/m2.

Table 2.1: List of Primers Used in This Study.

Primer	**Sequence (5'-3')**	**Used for**
GABI o8409	ATATTGACCATCATACTCATTGC	*ref6-5* genotyping
GABI_705E03.LP	AGATTGTAGCTTCTTCAATGATTGG	
GABI_705E03.RP	AACAACATATTTCCTCTGTCTGGAC	
CRISPR Elf6 for	CATTGGCACCTGTGTTTAGACCTAC	*elf6-C* genotyping
CRISPR ELF6 rev	TCCACGAACTGTTGGAGAGCTTCCA	
CRISPR A1	ATTGAAAAGGAAGCTAGTGCCTTT	Guide RNA CRISPR/cas9
CRISPR B1	ATTGGCCTAACTCTTGCTGCCTAG	
LEC1 for	TGGAGCTCCCTTCTCTCACTATC	qPCR primers
LEC1 rev	CTGCTGGACCACGATACCATTGT	
AtRKD4_F	ACGACGGTCTCATTTCCAAC	RKD4 genotyping
AtRKD4_R	CTAATTCCCTGCATCGCTTC	
CLF for	GTTCTCGCGATCGTATATCTTCGCG	Clf-81 genotyping
CLF rev	GATGTTTCTGGTTGGGGAGCT	
OLE1-RFP fw	AATTGTTAAACAGTAGGTATAGTAA	OLE1-RFP cloning
OLE1-RFP rv	AATTGTTTAAACTCTAGTAACATAGATGACAC	

Targeted Genetic Lesions by CRISPR/Cas9

The CRISPR-Cas9 targeted mutagenesis protocol was developed by Durr et al. 2018. Two protospacer sequences that act as guides, specific to a target sequence of the first exon of *ELF6* (Table:2.1 CRISPR A1 and B1) were designed using Breaking-Cas (Oliveros et al., 2016). Guide RNAs were sequentially cloned into a pEN-2xChimera vector and sequenced using Sanger sequencing.

To test the efficiency of the guide RNAs, transient expression of the CRISPR/Cas9

construct was performed in *Arabidopsis* mesophyll protoplasts (Yoo et al., 2007). Protoplasts were isolated from four week old Arabidopsis plants grown at 20^{o}C, under short day conditions (10h light 14h dark). Leaf tissue was isolated and incubated at 22^{o}C in an enzyme solution (20mM 4-Morpholineethanesulfonic acid (MES) pH 5.7, 0.4M mannitol, 20mM KCl, 1.5% (w/v) cellulase R10 (Duchefa Biochemie), 0.4% (w/v) macerozyme R10 (Duchefa Biochemie)) for 3 hours. Approximately 80,000 protoplasts were transformed with 16μg of plasmid (pUbi-CAS9 - Red) containing the guide RNAs. Transformation was initiated by addition of a Polyethylene glycol (PEG) solution (0.4% (w/v) PEG 4000 (Sigma 81240), 0.8M mannitol, 1M CaCl2) and stopped with addition of W5 solution (2mM MES pH5.7, 154mM NaCl, 125mM CaCl2 and 5mM KCl). Transformed protoplasts were incubated for two days under long day conditions at 22^{o}C. DNA was extracted, normalised for concentration, before the target region was amplified by semi-quantitative PCR using oligonucleotides flanking the target region (CRISPR ELF6 for and ELF6 rev). Guides showing high efficiency in creating deletions when in cells were selected for transformation into plants. Guide RNAs were cloned into a pDE-CAS9 vector, which contained a RFP seed selection reporter (OLE1-RFP) and transformed into plants using *Agrobacterium tumefaciens* (Clough and Bent, 1998).

Primary transformants were selected using the seed specific RFP reporter under a Leica MZ-FL III stereomicroscope (Leica Camera AG) and checked for a 3:1 segregation to ensure single copy transformation. RFP positive plants were grown on soil for approximately 4 weeks before genomic DNA was extracted. For genotyping of the mutation, oligos (Table:2.1, ELF6 for and ELF6 rev) were used to amplify the target region by PCR and fragment size was determined by gel electrophoresis (2% agarose gel with ethidioum bromide). Once a mutation was detected RFP negative seeds of single lines were sown on soil and genotyped for deletion events. Deletions were then confirmed using Sanger sequencing.

Arabidopsis Crosses by Manual Pollination

Crosses were performed by hand on 4-6 week old *Arabidopsis* plants. Petals, sepals and anthers were carefully removed from inflorescences using fine forceps. The stigma were left to mature for two days before crosses were carried out by manually touching anthers from mature flowers onto the stigma. Successful crosses were marked after elongation of the pistil was apparent. The inflorescence meristem was then removed and the silique bagged until mature.

Agrobacterium Transformation by the Floral Dip Method

An electrocompetent strain of *Agrobacterium tumefaciens* (Gv3101) with helper plasmid mp90 was used as the vector for *Agrobacterium* transformation. Before the transformation, the plasmid vector was diluted 1:100 in water, 1μl of the vector was mixed with competent cells and incubated on ice. Next cells were transferred into a pre chilled electrophoresis cuvette and put into Gene Pulser Xcell Electroporation system (Biorad). The cells were transferred into low salt LB media (10 g/l tryptone, 5 g/l NaCl, 5 g/l yeast extract) and incubated for 1 hour at 28^{o}C. Finally cells were centrifuged at 5000 rpm, the supernatant removed before the pelleted cells were re-suspended using 150 μl of low salt LB media and streaked on low salt LB agar plate containing selection antibiotics gentamicin, rifampicin and spectinomycin.

Arabidopsis plants were transformed via the floral dip method previously described (Clough and Bent, 1998). A single colony was picked from a transformed *Agrobacterium* culture and used to inoculate 5ml of SOC media (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose); with appropriate antibiotics to select for the plasmid to be introduced to the plant. The colony was grown at 28^{o}C overnight before

1ml was added to 500ml of LB media (with appropriate antibiotics) and cultured overnight. The culture was then centrifuged at 8000 rpm. The supernatant was removed and the cells were re-suspended in 500-700ml of 10% sucrose solution. 20 μl of silwet (L-77) per 100 ml was added just before dipping to the Agrobacterium suspension. *Arabidopsis* inflorescences were dipped and repeatedly agitated within the liquid for 1 minute before being transferred to a tray and covered with a bag overnight. Plants were allowed to grow and develop for another 4-6 weeks before seed was harvested and the primary transformants identified using the seed specific RFP reporter under a Leica MZ-FL III stereomicroscope (Leica Camera AG).

DNA Extraction

Leaf material was collected from five week old *Arabidopsis* plants and flash frozen using liquid nitrogen. Samples were pulverized to powder using glass beads. Sample extraction buffer (100mM Tris/Cl pH8.0, 50mM EDTA pH 8.0, 500mM NaCl, 10mM beta-mercaptoethanol) and 80μl 10% SDS was added then samples homogenised. Samples were incubated at 65^{o}C for 20 minutes, before moving to ice for 5 minutes. 200μl of 5M potassium acetate was added, tubes homogenised and incubated on ice for 20 minutes. The samples were centrifuged, supernatant isolated and the DNA was precipitated using 100% isopropanol at -20^{o}C overnight. Samples were centrifuged and the DNA pelleted before being washed with 70% ethanol, spun down then the supernatant was removed and the pellet left to air dry. DNA was re-suspended in TE buffer containing RNase (10mM Tris pH 8, 0.1 mM EDTA, RNase - 100μg/ml Thermo Scientific) and extracted DNA was stored at -20^{o}C. The quality and quantity of genomic DNA was checked using agarose gel electrophoresis and NanoDrop analysis (Thermo Scientific).

For whole genome bisulfite sequencing, rosette leaves were collected when the plants were five weeks old. Leaf material was flash-frozen in liquid nitrogen and pulverised to powder using shaking with glass beads. The genomic DNA was

extracted using Qiagen Plant DNesay kit (Qiagen) as per the manufacturers instructions. The quality and quantity of genomic DNA was checked using agarose gel electrophoresis and NanoDrop analysis (Thermo Scientific).

Bisulfite Sequencing

A library was generated using the Illumina TruSeq Nano kit (Illumina, CA, U.S.A) according to the manufacturer's instructions. The DNA was sheared to 350 bp before the adapter sequence was ligated into the sheared-DNA. The adapter-ligated DNA then underwent bisulfite treatment by using Epitect Plus DNA Bisulfite Conversion Kit (Qiagen, Hilden, Germany) as per the manufactuer's instructions. The treated DNA was cleaned-up before the library was enriched using Kapa Hifi Uracil+DNA polymerase (Kapa Biosystem, MA, U.S.A). Bisulphite sequencing was performed on an Illumina HiSeq2000 instrument. Bisulphite converted libraries were sequenced with 2x101-bp paired-end reads. Conventional *A. thaliana* DNA genomic libraries were analysed in control lanes.

Methylation Analysis

Raw reads were assessed for quality using FastQC analysis (Andrews, 2010). Reads were then trimmed using Trimmomatic (v0.36) with parameters: Illuminaclip: adapters.fa:2:30:10, Headcrop: 6, Leading: 3, Trailing: 3, Sliding Window: 4:15, minlen:3 (Bolger et al., 2014). The duplicate reads were removed and the trimmed reads were then re-assessed for quality using FastQC. Processed reads were mapped to the Arabidopsis reference genome (TAIR10) using bwa-meth using default parameters (Pedersen et al., 2014). Aligned reads were then used to call methylated and differentially methylated reads via a Hidden Markov Model and K means clustering using 'methyScore' with default parameters (Unpublished, Computomics Tubingen). Custom R-Scripts were then used to process

and visualise the differentially methylated regions and methylated regions.

RNA Extraction

Samples were collected, flash-frozen in liquid nitrogen, and pulverized using a pestle in a mortar. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) according to manufacturer instructions. The extracted RNA was stored at -80^{o}C until further use. RNA concentration and purity were estimated using a NanoDrop spectrophotometer.

Quantitative PCR Analysis

Extracted RNA was treated with TURBO DNA-freeTM (Promega, Madison, WI) following the manufacturer's instructions. cDNA was synthesized from 3μg of extracted RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. All RT-qPCR analyses were performed using a MyiQ System (BIO-RAD) with the MESA Blue qPCR MasterMix Plus reagent (Eurogentec Headquarters). Primers were designed using Primer3 software (Rozen and Skaletsky, 1999).

PCR fragments were analysed using a dissociation protocol to ensure that each amplicon was a single product. All RT-qPCRs were performed using three biological replicates in a final volume of 25 μl containing 5μl of cDNA template (diluted beforehand 1:10), 0.2μM of each primer, and 12.5 μl of 35μl 2xMESA Blue qPCR MasterMix (Eurogentec Headquarters) according to the manufacturer's instructions.

Each reaction was run in triplicate (technical replicates). Negative controls included in each run were a reaction conducted in the absence of reverse transcriptase and a reaction with no template (2 μl of nuclease-free water instead of 2 μl of cDNA). Analysis of expression data was performed according to the $\Delta\Delta$CT

method (Livak and Schmittgen, 2001) and normalised using AtPPA2 as reference gene (AT2G18230).

RKD4 Reprogramming Assays

Callus and Somatic Embryo Induction Assays

For the germination assay, age-matched seeds were surface sterilised and dispersed in agarose. 50 seeds of each line were plated on MS media in 9cm diameter Petri dishes. Seeds were stratified before being transferred into a growth cabinet. Seed germination was scored daily for six days post stratification, with a germination score based on emergence of the radical tip through the seed coat as observed under a dissecting microscope.

For the callus induction assay, age-matched seeds were surface sterilised and dispersed in agarose. 50 seeds from an inRKD4ox transgenic line were plated in 9cm diameter Petri dishes on MS media supplemented with 20μM Dexamethasone (DEX) to induce *RKD4* over expression in somatic tissue. Seeds were stratified before being transferred to a growth cabinet. *RKD4* over-expression was maintained for 14 days before callus induction was scored. Successful induction of callus state was scored based on the halt of normal developmental growth, swelling of the root and shoot and bleaching of cotyledons. Calluses were pooled into groups of 10 collected in pre-weighed eppendorf tubes and fresh weight measurements determined. Samples were then dried at 65^{o}C until there was no further reduction in mass before dry weight measurements were taken. Average callus mass was calculated by dividing pooled weight by the number of calluses within the pool.

Differentiated Tissue Response to Ectopic *RKD4* Expression

For the somatic tissue response assay seeds were surface sterilised and dispersed in agarose. 25 seeds of each line were plated in 12cm square Petri dishes on MS media. Seeds were stratified before being transferred to a growth cabinet. Seeds were germinated and grown vertically for 5 days before being transferred to plates containing MS media supplemented with 20μM DEX to induce *RKD4* over expression. *RKD4* over expression was maintained for 14 days before callus induction type was scored. The induction response was grouped into four categories: Type 1 - 'Insensitive' where no callus develops and the plant continues to develop normally; Type 2 - 'Root tip only" where the primary root meristem regenerate to callus while the shoot meristem develops normally; Type 3 'root and shoot' where the root and shoot meristems regenerates to callus but where lateral root develop normally; and Type 4 - 'complete induction' where all normal plant development ceases and calluses develop in root and shoot meristems and the lateral root primordia.

Cellular Responses to Ectopic *RKD4* Expression

For the cellular response assay seeds were surface sterilised and dispersed in agarose. Seeds of each line were plated in 12cm square Petri dishes on MS media. Seeds were stratified before being transferred to a growth cabinet. Seeds were germinated and grown vertically for 5 days before being transferred to plates containing MS media supplemented with 20μM DEX to induce *RKD4* over expression. *RKD4* over expression was maintained and plants were harvested and fixed (detailed below) over a time-course (timepoints 0, 3, 6, 12, 18, 24, 48, 72 hours).

Chloral Hydrate Root Clearing and Confocal Imaging

After 0 - 72 hours of *RKD4* induction time seedlings were carefully transferred to fixative (ethanol:acetic acid (3:1), Tween 20 1%(v/v), Dimethyl Sulfoxide 2%(v/v), Nonidet P-40 2%(v/v)) until chlorophyll had completely cleared. The fixative was sequentially replaced by clearing solution (0.2M NaOH, 1% SDS) and incubated at room temperature until the cotyledons become translucent. Seedlings were washed 3 times with water and once with amylase buffer (20mM sodium phosphate pH 7.0, 2mM NaCl, 0.25mM Ca_2Cl) before being treated with amylase (10mg/ml α-amylase + 0.1% sodium azide) at 37^{o}C overnight. Seedlings were washed twice with water, which was then replaced with 1% periodic acid and incubated at room temperature for 40 minutes. Seedlings were washed 3 times with water and once with Schiff's Reagent (100mM sodium metabisulphite (Sigma Aldrich), 0.15M HCl). Propidium iodide (PI) was added in ratio 1:10 (PI : Solution volume) and incubated at room temperature for 2 hours.

Seedlings were washed in water twice to remove remaining PI stain and the water replaced with chloral hydrate clearing solution (4g chloral hydrate : 1ml glycerol : 2ml water) and incubated at room temperature. Cleared seedlings were imaged after 2-3 days of chloral hydrate incubation using confocal microscopy (Zeiss LSM 880). Root stacks were assembled to create a surface mesh of the cell walls and segmented using MorphographX (de Reuille et al., 2015).

ClearSee Root Clearing and Confocal Imaging

After allocated *RKD4* induction time seedlings were transferred into Renaissance staining solution (4% para-formaldehyde in phosphate buffered saline (PBS), pH 7.4, 0.1% SCRI Renaissance 2200 (Renaissance Chemicals SR2200)). The solution was vacuum infiltrated into the sample and samples incubated at 4^{o}C overnight. Samples were washed twice with PBS before being exchanged for ClearSee solution (Xylitol (10% (w/v)), sodium deoxycholate (15% (w/v)), urea (25% (w/v)) in water) (Kurihara et al., 2015). ClearSee was vacuum infiltrated into samples

and incubated at room temperature for six hours for clearing to complete. Cleared seedlings were imaged using confocal microscopy (Zeiss LSM 880, 405nm).

Induced *de novo* Root Organogenesis in Leaf Explants

The first-pair of rosette leaves from 12 day old seedlings were cut at the position between petiole and blade in control and the mutant lines. The excised blade was transferred to B5 medium (Gamborg B5 basal medium with 0.5 g/l MES, and 0.8% agar, pH 5.7 Duchefa Biochemie). Adventitious root production at the cut site was scored by root meristem generation after 20 days (Chen et al., 2014).

EMS Mutagenesis

Approximately 100,000 pre-stratified *Arabidopsis* seeds (Col-0 (inRKD4ox), determined by weight) were divided into two tubes and treated with freshly prepared EMS/100 mM phosphate buffer (KH_2PO_4, K_2HPO_4, pH 7.5) - with a final EMS concentration of either 0.4% or 0.8% respectively. EMS Treatment was left for 8 hours with agitation, before residual EMS was removed by passing the seeds over a Buchner vacuum flask before washing 20 times with water. Seeds from both populations (referred to as $0.4M_1$ and $0.8M_1$) were dried overnight on filter paper before each treatment population was split into 20 equal sub-populations and sown directly onto soil in individual P1 trays, and germinated in glasshouse conditions at 20^oC. Upon flowering, trays were sub-divided into equal groups. Plants were bulk harvested and threshed before individual sub-groups were sown on in P1 trays with an approximate seed density of 1 seed per cm^2.

EMS High Throughput Liquid Screening of Bulk EMS populations

Approximately 300 seeds from each M3 sub-population were sampled using a micro-spoon spatula (18/10 Bochem 3340), surface sterilised with chlorine gas (25ml 14% Hypochlorate solution, 3ml HCl), then transferred to individual wells using 24 well plates (Thermo Fisher Scientific - 142485). 2ml of sterile liquid MS media with 50μM DEX was then added to the wells and plates sealed with micropore tape (Fisher Scientific) before stratification at 4^{o}C for 48hrs. Plates were transferred to a light cabinet (Sigma) with gentle agitation (60rpm) for five days days. Populations selected for further analysis contained plants producing leaf and root structures after germination.

Anthocyanin Accumulation Assay

Seeds were surface sterilized as above but placed on agar plates containing 1/2 x Murashige and Skoog (MS) salts (Duchefa Biochemie), pH 5.7 and 3% sucrose (Sigma-Aldrich) solidified with 0.8% phytoagar (Duchefa Biochemie). Seeds were sown on 9cm round plates and allowed to germinate for 5 days before anthocyanin pigmentation was observed in the leaves and hypocotol Solfanelli (2006) under a Leica MZ-FL III stereomicroscope (Leica Camera AG)..

Statistical Analysis

In this study, all statistical analysis was performed using statistical packages in R or Prism 7.

Chapter 3

Screening for Factors Affecting Developmental Reprogramming by *RKD4*

3.1 Introduction

Plants and animals diverged *c.*1.6 billion years ago, and evolution has equipped both with different survival techniques (Meyerowitz, 2002). Plants, living a sessile lifestyle, cannot escape from a deleterious environment. As a result, they are more likely to experience biotic and abiotic stresses, such as herbivory, mechanical damage or adverse weather conditions (Greb and Lohmann, 2016). Therefore, evolution has equipped plants to be able to minimise losses from these events.

One such adaptation is that, unlike most animal cells, plant cells retain the ability to re-differentiate into new cell types even after developmental pathways have been established. Regeneration in plant tissues can be achieved through two pathways; either reactivation of latent undifferentiated cells, such as an auxiliary bud, or through the reprogramming of differentiated cells by reactivating repressed developmental pathways (Shimizu-Sato and Mori, 2001; Asahina et al., 2011; Bellini et al., 2014). This gives plants a greater developmental plasticity

compared to animals, which has been exploited in plant breeding for hundreds of years through the use of propagation via cuttings or grafting from one variety to another (Melnyk and Meyerowitz, 2015). In 1902, Haberlandt first proposed the concept of a tissue culture from which artificial embryos could be generated, which kick-started attempts to regenerate whole individual plants from small tissues or single cells *in vitro* (Ikeuchi et al., 2016). The breakthrough came in 1957 with Skoog and Miller's discovery that the ratio of two exogenously applied plant hormones (auxin and cytokinin) could determine the fate of regenerating tissue (Skoog and Miller, 1957). High ratios of auxin to cytokinin generally led to root regeneration, and high ratios of cytokinin to auxin tended to promote shoot regeneration (Skoog and Miller, 1957). This was followed a year later by the report that single cells derived from carrot phloem retain the capacity to regenerate fully into whole plants (Steward, 1958). Subsequently, it has been found that *de novo* organogenesis can be initiated from most somatic tissues within a plant such as root, shoot, leaves, pollen and it is also possible to regenerate whole plants from individual cell-wall free cells (protoplasts) into fully functional plants (Zhu et al., 1997; Maraschin et al., 2005; Che et al., 2007; Atta et al., 2009).

Somatic embryogenesis (SE) is a process by which embryos can be produced from tissue other than reproductive cells and it is one of the best examples of a plant cell totipotency, as it truly demonstrates a single cell's ability to regenerate into all the tissues within the organism. SE has been demonstrated to be triggered in numerous natural situations including in response to wounding, environmental stresses or part of normal development. It has been reported in a number of plant species of *Citrus* and *Paeonia*(Koltunow et al., 1996; Von Arnold et al., 2002). More recently, induced SE has been observed through the over-expression of transcription factors. Ectopic expression of embryonic transcription factors have proven a good source of TFs capable of this transformation. For example, LEAFY COTYLEDON2 (LEC2) leads to the formation of somatic embryos in the hypocotyl, while over expression of BABY BOOM (BBM) was able to induce somatic embryo formation in the cotyledons (Boutilier et al., 2002; Braybrook

et al., 2006). To date a number of TFs from several different classes, with different roles during plant development have also been shown able to induce SE in *Arabidopsis* (summarised in Table 3.1).

Table 3.1: Transcription Factors Capable of Inducing Somatic Embryogenesis.

Gene	Protein Family	Ectopic Expression Phenotype	References
WUSCHEL (WUS)	Homeodomain TF	Induction of direct somatic embryogenesis in zygotic embryos without auxin	Chatfield et al., (2013); Gallois, et al., (2004)
WOUND INDUCED DEDIFFERENTIATION 1 (WIND1)	AP2/ERF TF	Callus formation from shoots, hypocotyls, and roots leading to indirect SE	Iwase et al., (2011); Ikeuchi et al., (2013)
WOUND INDUCED DEDIFFERENTIATION 3 (WIND3)	AP2/ERF TF	Callus formation leading to indirect SE	Ikeuchi et al., (2015)
AINTEGUMENTA-LIKE 5 (EMK/AIL5/PLT5)	AP2/ERF TF	Embryo-like structures form on cotyledons	Tsuwamoto et al., (2010)
BABY BOOM (BBM)	AP2/ERF TFs	Direct and indirect SE on cotyledons	Boutilier et al., (2002)
LEAFY COTYLEDON 1 (LEC1)	HAP3 domain TF	Induction of direct somatic embryogenesis at the position of leaves	Lotan et al. (1998)
LEAFY COTYLEDON 2 (LEC2)	B3 domain TF	Induction of direct somatic embryogenesis in seedlings without auxin	Stone et al. (2001)
AGAMOUS-LIKE 15 (AGL15)	MADS box TF	Direct SE from zygotic embryos and shoot apical meristem	Harding et al. (2003)
RWP-RK DOMAIN-CONTAINING 4 (RKD4)	RWP-RK domain TF	Short-term expression promotes indirect somatic embryogenesis at root and shoot meristems without auxin	Waki et al., (2011)

A family of transcription factors (RWP-RK) were recently identified to be key regulators of egg cell gene expression required for embryo specification and differentiation (Köszegi et al., 2011; Waki et al., 2011; Tedeschi et al., 2017). One member of this gene family, RKD4 is expressed very early in embryo development and is involved in embryo patterning (Waki et al., 2011). RWP-RK proteins work cooperatively with WUSCHEL-LIKE HOMEOBOX (WOX) homeodomain transcription factors to establish apical and basal fates of the embryo, by facilitating a YODA mitogen-associated protein (MAP) kinase cascade (Jeong et al., 2011). Interestingly, when *RKD4* is over-expressed in somatic tissue, formation of a callus at the root and shoot meristem is induced, from which somatic embryos develop once *RKD4* induction is stopped (Waki et al., 2011) (Fig.3.1)

One of the major bottlenecks facing widespread application of *in vitro* embryogenesis as a plant propagation tool is the low responsiveness of many economically

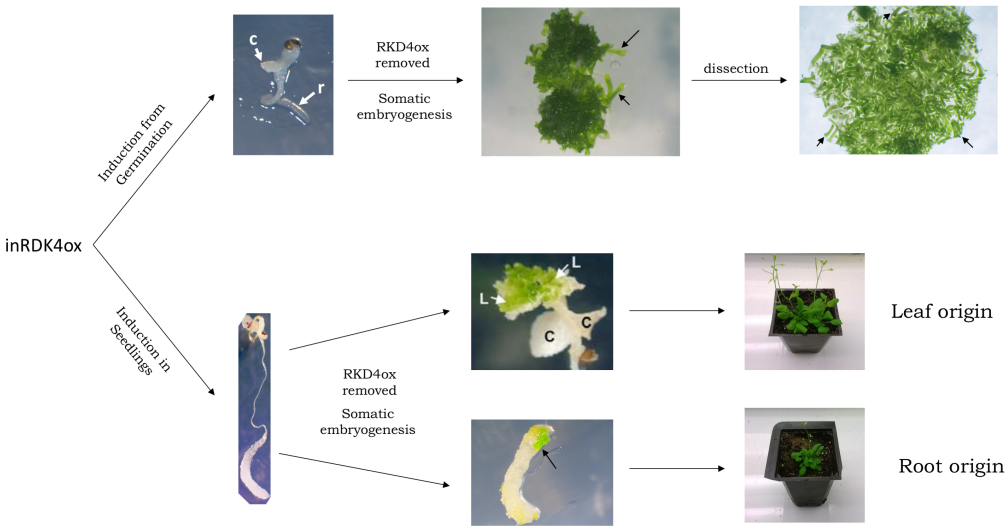


Figure 3.1: Somatic Embryo Induction by Ectopic *RKD4* Expression. The ectopic expression of *RKD4* in Arabidopsis induces developmental reprogramming leading to callus formation. Induction from germination leads to whole plant callus induction, while reduction from seedlings induces callus in root and shoot meristems. Somatic embryos develop after the removal of *RKD4* over expression which are viable to develop fertile plants.'r' = root, 'c' = cotyledons, 'L' = Leaf, black arrows highlight somatic embryos produced.

important species and genotypes (Horstman et al., 2017a). In addition, the reprogramming still relies on different plant growth regulators, mainly cytokinin and auxin and these hormones are known to be associated with mutagenesis and aberrant DNA methylation changes in cell and tissue cultures (Joyce et al., 2003). One example was the appearance of a floral malformation preventing fruit formation called the mantled abnormality, affecting oil palms produced by *in vitro* cloning (Zeven, 1973). In these plantations, 5% of the palms produced by hormone induced regeneration were affected, and the cause was recently linked to the activation of a transposable element during the culturing process (Ong-Abdullah et al., 2015).

More recently, plant breeders have been looking to SE initiated by transcription factors as a means to avoid these aberrant effects observed in hormone culture (Li et al., 2018). However, TF mediated reprogramming is often limited to specific tissues, cellular responses are relatively slow, and the culturing process can introduce point mutations, transposable element activation or epigenetic changes to the resulting offspring (Horstman et al., 2017b). Leading to the progeny produced having developmental or fertility impairments making them unsuitable for use in industry (Li et al., 2018; Lowe et al., 2018). However, some recent developments are being made, allowing regeneration to occur more independently of genotype, such as a recently demonstrated in *Zea mays* L. by the induction of BBM and WUSCHEL 2 (WUS2) (Lowe et al., 2018). However, the expression timing is still be limited to particular developmental stages and it is not clear whether the same programs will be effective in many agriculturally important species (Kim et al., 2012; Li et al., 2018; Lowe et al., 2018). These studies illustrate that the mechanisms controlling the regenerative capacity of plant cells are not yet well understood.

Mutagenesis by Ethyl Methanesulphonate

Genetic variation allows plants to respond differently to biotic and abiotic stresses by changing the gene networks and associations available within the plant. This enables a variable phenotypic response to the environment within the population (Grini et al., 1999). Natural or artificial selection of this variation can be used to enable selection of traits that are adventitious to a particular environment or market for example increased grain yield in wheat. That allows for generation of elite lines generated through multiple rounds of selection and inbreeding (Collard et al., 2005). In industry this variation can be introduced through out-breeding to wild relatives, but this introduces a lot of undesirable DNA that could impact the crop performance, so alternate methods were devised to introduce genetic variation within the elite lines without out-breading (Lai et al., 2004).

Ethyl methanesulphonate (EMS) is a chemical mutagen introducing mismatches in DNA by donating an alkyl residue to guanine or thymine nucleotides causing a base exchange. This induces changes in the genetic sequence from G/C to T/A or A/T to G/C (Lai et al., 2004); while both changes are possible it has been shown that in 99% of cases EMS induces C-to-T changes resulting in conversion from C/G to T/A (Kim et al., 2006). The aim of this treatment is to generate random point mutations across the entire genome of an organism, giving a high mutation load, generating a series of allelic mutations in all genes, from which beneficial mutations can be screened against a phenotype of interest (Till et al., 2003). EMS is commonly used as a more effective mutagen due to this alkylating ability, as other methods such as radiation have a higher frequency of strand breaks leading to inversion mutants at the doses required to generate the same mutation load achieved with EMS (Brock, 1976; Koornneef et al., 1982).

Chemical mutagenesis has proved to be a versatile tool, as not only can it be used to generate loss or gain of function mutants but also can be used to understand specific amino acid residues in protein function, although more recently targeted genetic approaches such as the CRISPR-cas9 system is proving to be increasingly

popular for targeted genetic work (Durr et al., 2018). However, EMS is lethal in high concentrations or can cause sterility preventing further propagation of the population, so dose must be varied to offset the negative effects of a high mutation load (Stephenson et al., 2010). The mutation rate of EMS varies with the concentration, exposure time and variability between batches making it difficult to standardise the procedure. In industry, EMS-based mutagenesis is a well established technique used to generate breeding lines in a number of plant species. This popularity is driven mainly because it is not classed as a transgenic approach, removing the need for additional regulation associated with genetically modified crops (Till et al., 2006). In research laboratories, EMS-based mutagenesis coupled with advances in high throughput sequencing techniques provide a relatively straightforward unbiased approach to finding previously unknown genes involved in a pathway of interest (Friedman and Perrimon, 2007). This began as a means to identify previously unknown gene functions but more recently has been used to identifying gene interactions by creating mutagenic population within an already mutant background and looking for enhancer or suppressor screens.

3.1.1 Experimental Rationale

Protocols for the regeneration of plant tissues are well established, but can introduce severe developmental defects within the resulting progeny or are not viable in some economically important species. Recent developments in TF mediated SE are proving to be an alternate method that could be used to overcome some of the problems of hormone induced regeneration. However, these systems are often tissue or developmental stage dependent and the mechanisms unpinning these regeneration responses are not well understood. This project aimed to produce an EMS population of *Arabidopsis thaliana* (Col-0) containing a dexamethasone (DEX) inducible *RKD4* over expression construct (*inRKD4ox*) to identify potential factors regulating RKD4 mediated regeneration and wider plant control of regeneration.

3.2 Results

3.2.1 Generation of an EMS population

Unlike other transcription factor reprogramming, *RKD4* induces reprogramming consistently, relatively quickly (with callus formation visible within 72 hours) and has a distinctive spatial response pattern in the root and shoot meristems, once ectopic RKD4 expression begins (Waki et al., 2011). These traits provides an interesting opportunity to develop high throughput screens for factors involved in controlling somatic cell reprogramming and totipotency.

Seeds of *Arabidopsis thaliana* containing an inducible construct for the over-expression of *RKD4* (Waki et al., 2011) (inRKD4ox, M_1) were treated with EMS (Low concentration 0.4% and high concentration 0.8%). This treatment created two mutant M_1 populations (0.4 and 0.8) which then were germinated on soil. 0.8 M_1 although viable and germinated well, displayed severe impairment in fertility thus reducing seed yield, in the M_2 generation. This suggested that the mutation load was too high to effectively propagate the population, so this line was not taken forward. The population 0.4 M_1 germinated well, and had fewer instances of infertility, whist still displaying the recessive chlorophyll deficient sections within leaves associated with successful mutagenesis (Stephenson et al., 2010); providing a viable population to continue with in this study. The M_1 population was allowed to self propagate before being separated into bulk pools of approximately 200 plants and harvested. M_2 plants were re-sown and allowed to self propagate so that any recessive mutations that may have become homozygous within the M_2 generation were propagated before screening. M_2 plants were grouped in bulk pools of approximately 150 plants, generating a total of 1440 M_3 populations to be taken forward for screening.

3.2.2 Screening the M_3 Population

A multi-step suppressor screen was designed to identify potential candidates for further analysis (outlined in Figure 3.2 A). In control samples, *RKD4* induction upon germination resulted in the generation of an undifferentiated cell culture with no defined plant structures (Fig.3.2 B-control well). A high throughput system using liquid MS media supplemented with 50μM DEX was designed allowing for the easy identification of hypo-sensitive lines by the formation of green leaves and plant roots in hypo-sensitive seedlings (Fig.3.2 B - Black arrows). By screening 288 M_3 populations twice using this method, only those producing hypo-sensitive plants in both screens were taken forward to the next stage of selection. This screening identified 26 populations showing suppressed callus induction with leaf and root structure formation and these populations were taken forward for further investigation.

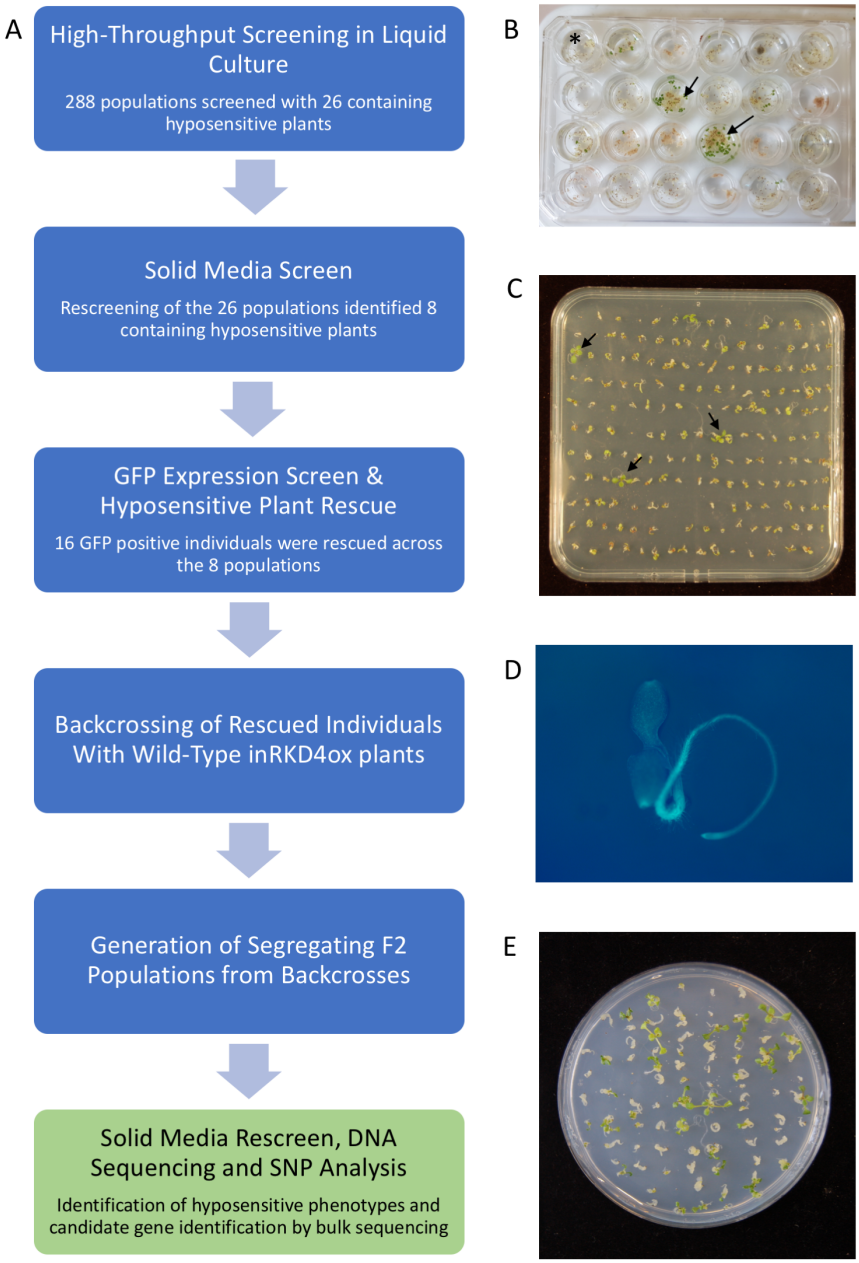


Figure 3.2: Protocol for the Screening of Hypo-sensitive Responses to Ectopic *RKD4* Expression. Flow chart outlining stages in screening for hypo-sensitive responses to RKD4 induced reprogramming (A). Representative pictures taken at each stage during the screen within this project: Liquid screen (B) Solid media (C) GFP positive plant (D) F2 population screen (E). Black arrows indicate hypo-sensitive plants. Blue colouring indicates work completed within this study, Green indicates work still to be completed. '*' indicates control well.

These 26 populations were then germinated on solid media (supplemented with 20μM DEX), which provided a more consistent induction treatment. In control plants, regeneration could be scored by identifying swelling of the root, hypocotyl and shoot meristem, while the cotyledons lose chrolophyll content turning white, resulting in an unstructured callus developing, while insensitive plants germinate and develop normally. From the original 26 populations, solid media screening revealed only 8 populations containing insensitive plants, from which 26 individuals were rescued and transferred to soil.

The *inRKD4ox* line used for the mutagenesis contains the GAL4/UAS system (Waki et al., 2011). It is possible that the EMS could have mutagenised components of the inducible *RKD4* construct, resulting in false positives in the repressive screen. The inRKD4ox line expresses green fluorescent protein (GFP) after DEX induction, so fluorescence microscopy was used to identify hypo-sensitive plants for expression of this reporter gene (Fig.3.2 A). This screen ended up with 16 positive and 10 negatives candidates for further screening. These candidates were individually back-crossed to control inRKD4ox plants and allowed to self pollinate; generating single origin F2 populations from each hypo-sensitive individual for genetic sequencing.

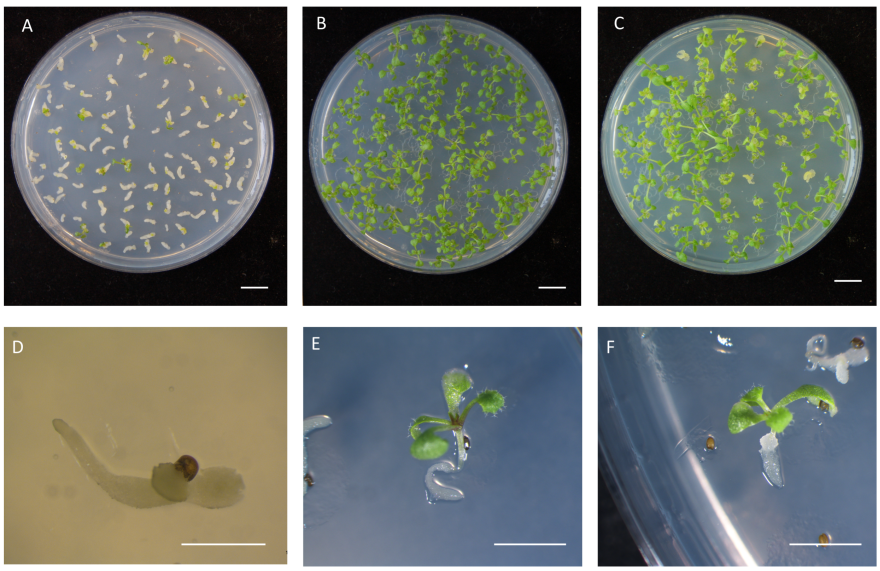


Figure 3.3: Somatic Regeneration by Ectopic Expression of *RKD4* in Mutagenised *A. thaliana*. The over expression of *RKD4* in Arabidopsis induce developmental reprogramming leading to callus formation in germinating seedlings. After 12 days of induction using dexamethasone normal root and shoot development had abated in WT plants, with chlorophyll bleaching observed in the cotyledons and tissue beginning to swell as callus begins to form (A,D). EMS line 3.3.5.1 shows plants fully resistant to RKD4 expression and displaying normal growth (B). EMS line 4.9.1.2 shows partial resistance to reprogramming (C). EMS line 8.1.2.1 shows individuals display a 'shoot insensitive' reprogramming phenotype, where plant hypocotyl, cotyledons and shoot meristem developed normally while the root produced callus (E and F). Bars = 1cm.

Progeny from the 16 GFP positive candidates were re-screened on solid media and a range of phenotypes were observed. In nine lines, the progeny were observed to have whole plant resistance, whereby plants germinated on media containing DEX and developed root and shoot structures indistinguishable from control plants. In six lines these whole plant hypo-sensitive responses were identified in all progeny plants from a single individual, indicating homozygous or dominant effects preventing induction in the parental plant (Fig.3.3 B). Alternatively, progeny of three individuals presented with segregating populations of susceptible, intermediary and resistant phenotypes (in a 1:2:1 ratio respectively, Fig.3.3 C) indicating a segregating recessive trait preventing induction.

In addition to whole plant effects, spatial phenotypes were observed in seven lines. In these cases, callus formation occurred in a tissue specific manner, including two lines which produced 'shoot insensitive' phenotypes (fig.3.3 E and F). Resistant and 'shoot insensitive' plants were rescued, transferred to soil and back-crossed to control inRKD4ox lines. F1 plants from these crosses are being grown to generate segregating F2 populations for analysis of the RKD4 over expression phenotype. These will be analysed by high throughput sequencing to identify single nucleotide polymorphisms within the populations (Wachsman et al., 2017), with the aim to identify candidates genes responsible for the observed phenotypes.

3.3 Discussion

3.3.1 An EMS Mutagenesis to Identify Mutants Hyposensitive to TF-Induced Reprogramming

EMS based mutagenesis is a variable technique depending on concentration and exposure time making it difficult to predict the best condition (Koornneef et al., 1982). Therefore, in this study a multi-concentration approach was taken to maximise the efficiency of mutagenesis. Previous studies have calculated that ≈45,000 seeds are needed in the M_1 population to give a ≥95% chance of finding a mutation in any given G:C base pair within the *Arabidopsis* genome; assuming an average mutation rate of $1.6 \times 10^{-}5$ mutations per locus per genome resulting in 700 mutations per M_1 plant (data from a combination of different studies) (Jander et al., 2003; Kim et al., 2006). Assuming the mutation frequency is a combination of EMS concentration and treatment time, a proportionally similar treatment protocol (0.2% (w/v), 16hrs) to the one used in this study produced on average 1,100 mutations in per plant in the M_1 generation in *Arabidopsis* 1(Jander et al., 2003). Therefore, it was reasoned that the 50,000 seed population and treatment protocol (0.4% and 0.8%, and 8hrs treatment) should be sufficient to produce a saturating population, against which the sensitivity of RKD4-induced reprogramming could be screened. However, the exact mutation rate within the M_1 generation could not be precisely determined, thus the frequency of albino plants and fertility in M_2 was used to select the best mutant population.

The successful identification of multiple candidate plants in the M_3 generation demonstrates that the mutagenesis treatment was sufficient to create phenotypic variation within the population for screening. Further screening of the remaining 80% of the M_3 populations should bring to light novel factors involved in the RKD4-induced reprogramming. This population could be further utilised to identify candidates that are hyper-sensitive to the RKD4 mediated reprogramming. In established plants regeneration is limited to meristematic regions of the root

and shoot, and absent from mature tissue. This cell specificity could be exploited to quickly screen for mutagenised plants responsive to *RKD4* over-expression in these mature cell areas, which indicates a hypersensitive response.

Another point of investigation could be the acquisition of competency within the RKD4 mediated callus. Plant cell regeneration is a muti-step process in which plant cells must de-differentiate from a previous cell fate, before re-establishing a new developmental program (Sang et al., 2018). The study undertaken during this project and the additional one proposed above only would identify mutants involved in the first step of the regeneration process.

Some genotypes have been found to competent to regenerate callus but are not capable of re-differentiation, indicating a mechanistic disconnect between the two steps. A resent investigation of *Medicago truncatula* cv. Jemalong revealed a regenerative disparity between a highly embryogenic line M9-10a and its non-embryogenic predecessor line, M9, in response to exogenous hormones (Orłowska et al., 2017). The cell mass generated by *RKD4ox* is embryonically competent and develops viable embryos after the removal of the chemical inducer. Further screens could reveal factors implicated in the rate at which, and proportion of cells within the callus that become regeneratively competent, and may provide further insight into the acquisition of competency within regeneration systems.

Using an EMS screen to identify factors affecting somatic cell reprogramming using the inRKD4ox system has some limitations. In *Arabidopsis*, RKD4 is necessary for pattern formation in early embryogenesis, being expressed from egg stage to the globular-stage embryo. In *rkd4* mutants this pattern formation in is severely disrupted (Waki et al., 2011). Therefore, it follows that mutations in some of the key gene targets of RKD4 could lead to defects in embryo structure or viability, reducing or eliminating their propagation within the test populations. This would result in an absence, or under representation of these mutants within the test populations. However, currently it is not clear whether the gene network activated during somatic tissue reprogramming is similar to that of natural em-

bryo development. Furthermore, in the context of somatic regeneration, it may be changes to the cellular or epigenomic environment, and not the natural pathway of RKD4 itself would be the most interesting targets to identify; allowing wider barriers regeneration to be identified.

3.3.2 Mutations Affecting Regeneration

Due to its initially unstructured appearance, callus is often referred to as "undifferentiated" or "dedifferentiated" but recent molecular findings suggest this may not be the case (Ikeuchi et al., 2018). It has been demonstrated that auxin induced callus occurs via a pathway similar to that of lateral root formation; specifically through 'pericycle-like' cells (Atta et al., 2009; Sugimoto et al., 2010). This finding is supported by evidence in the *ABERRANT LATERAL ROOT FORMATION 4* mutant (*alf4*) whereby the initial pericycle division is blocked, and in these lines callus formation is completely inhibited (Sugimoto et al., 2010; Che et al., 2007). More recently, it was shown that by comparing the transcriptome profiles of early embryogenic and callus cells to cells derived from different root tissues, callus tissue was similar to that of the proximal lateral root cap (LRC) (Magnani et al., 2017). This suggests that, rather than de-differentiating when callus is formed, cells are re-differentiating into a developmentally plastic state with some root-cell-like features. This indicates that perhaps roots are a more developmentally plastic tissue. Evidence for this was demonstrated by He et al., (2012, where double mutants in CURLY LEAF and SWINGER (*clf-50/swn-1*) were unable to generate callus from leaf explants while callus formation from root explants was unaffected. The authors hypothesised that this was due to the failure of PRC2 to repress leaf specific genes, needed to allow regeneration to occur. One of the mutant candidates identified in this screen (SHOOT INSENSITIVE 1) could be hypothesised to be similarly impaired in this developmental resetting within the shoot, while the root cells, that were already in a more 'developmentally plastic state' could be reprogrammed as normal.

Further work is needed to identify the genetic lesions responsible for the observed hypo-sensitive phenotypes. To this end segregating F2 populations were produced, in which the hypo-sensitive phenotype could be collected, and common single nucleotide polymorphisms could be identified. This could be achieved through established bioinformatic pipelines, such as those for isogenic mapping by sequencing that have previously been described (Hartwig et al., 2012; Wachsman et al., 2017), allowing the identification of gene candidates affecting RKD4 induction.

Compared to other transcription factors that mediate somatic embrogenesis programs, the mechanism underpinning reprogramming by RKD4 is not well understood. Over the last decade a number of studies have used chromatin immunoprecipitation and gene expression analysis to identify the pathways that control transcription factor-induced somatic embryo formation. These studies have shown that these transcription factors tend to regulate common pathways, often involving the hormones auxin and cytokinin in *Arabidopsis* (Braybrook et al., 2006; Stone et al., 2008; Horstman et al., 2017b). In the case of LEC2, ectopic expression activates key regulators in the auxin pathway such as the transcription factor AGAMOUS-LIKE 15 (AGL15); as well as genes encoding key enzymes in auxin biosynthesis such as *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*) and the *YUCCA 2 and 4* genes (Braybrook et al., 2006; Wójcikowska et al., 2013; Zhao, 2014). Recent developments have shown that there is transcriptional cross-talk between these different transcription factors able to induce SE in *Arabidopsis*, with BBM being shown to transcriptionally regulate both *LEC1* and *LEC2* (Horstman et al., 2017b), providing a potential framework into which RKD4 activity can be integrated. These interactions that should be illuminated by the continued screening of this population.

3.4 Summary

In this study, the treatment of inRKD4ox *Arabidopsis thaliana* plants with EMS successfully produced a viable mutant population. This population was propagated to M_3 and split into 1440 test populations against which hyper and hypo-sensitive responses to RKD4 induced regeneration were screened. Screening 288 of these populations for hypo-sensitivity to the RKD4 reprogramming signal resulted in 16 GFP positive candidates displaying whole plant, and shoot insensitive phenotypes. These candidates have been back-crossed to the wild type inRKD4ox line to generate F2 populations. Further investigations are required using whole genome sequencing to identify the causal genetic lesions responsible for the RKD4 related phenotypes.

Chapter 4

The Role of PcG and JmJ-C Proteins in Developmental Reprogramming

4.1 Introduction

Once the potential regenerative capacity of plant cells was identified (Skoog and Miller, 1957), it did not take long before *in vitro* embryogenesis was being used as a plant propagation tool in both industrial and academic laboratories (Horstman et al., 2017a). Somatic embryos retain the genotype of the original explant, making them ideal for the propagation of clonal plants. This has a number of uses in biotechnology, from scaling up of breeding material for testing, shortening the time needed for identification of desirable traits in heterozygous plants, or quickly cultivating plants with long life cycles (Noceda et al., 2009). These protocols have led to significant increases in production efficiency and uniformity, and in the quality of crop germplasm, especially in the forestry sector (Lelu-Walter et al., 2013; Georget et al., 2017; Chin and Tan, 2018). However, the use of somatic embryogenesis (SE) for propagation is hampered by the resistance of some plant species to culturing techniques and/or the production of aberrant phenotypes re-

sulting from somatic mutations or stable chromatin modifications incurred during the long culturing process (Joyce et al., 2003; Miguel and Marum, 2011).

After the initial regeneration experiments in the 1950s the critical question quickly shifted from whether cells were totipotent, to factors that limit cell totipotency. The early regeneration methods developed by Skoog and Miller used plant auxin to initiate regeneration, but the resultant callus required manipulation of the precise balance between cytokinin and auxin to allow the transition to new cell fates: high ratios of auxin to cytokinin generally led to root regeneration and high ratios of cytokinin to auxin tended to promote shoot regeneration (Skoog and Miller, 1957). Thus, in order to effectively regenerate, plant cells must first de-differentiate before re-differentiating into a new cell type, in a two-step mechanism (Sugimoto et al., 2010; Efroni et al., 2016).

Early studies indicated that these hormone balances mimicked those established early in developmental patterning of the resultant tissues suggesting that the application of these hormones were overriding the established hormone patterns, and that cell identity was determined via the immediate environmental conditions (Michalczuk et al., 1992b,a; Kopertekh and Butenko, 1995). Subsequent, microarray and imaging studies found that after hormone treatment, genes usually expressed in plant meristems became activated; for example genes required for normal shoot patterning *WUSCHEL* and *SHOOT MERISTEMLESS* were found to be expressed early in the shoot induction phase after hormone treatment (Cary et al., 2002; Gordon et al., 2007), supporting the view that exogenous hormone treatment was conferring a shoot apical meristem (SAM)-like environment, activating the appropriate developmental gene networks. Later studies demonstrated that these patterns of gene expression were being established early in hormone induced reprogramming, and mirrored the timing and gene networks observed during normal shoot formation. For example, in roots explants expression of *CUP SHAPED COTYLEDON 1 and 2* (*CUC1/2*), which act redundantly in embryonic shoot formation, were up-regulated while still on callus inducing media, before the formation of shoots (Cary et al., 2002; Gordon et al., 2007). While

this is consistent with the upstream role *CUC* genes play in the early formation of the SAM (Aida et al., 1999; Daimon et al., 2003; Hibara et al., 2003), expression of these genes were not ubiquitous within the generated callus indicating an internal establishment of cell fate between cells within a callus independently of the immediate hormone environment (Atta et al., 2009).

In addition, recent studies have cast doubt on the whether all plant cells share the same regenerative capacity. Early studies demonstrated that regeneration efficiency was found to be much higher in protoplasts from earlier stages of development as opposed to fully differentiated tissue (Binding, 1974, 1975; Vasil and Vasil, 1974). More recent work in Arabidopsis, demonstrated that callus originated from one specific tissue type, the pericycle (Sugimoto et al., 2010). In addition, cells within the root and shoot meristems required a shorter competency step to produce somatic embryos, indicating that these cells required less reprogramming to achieve regeneration (Liu et al., 2014; Reinhardt et al., 2003). Furthermore, Raghavan (2005) showed that regeneration using synthetic auxin 2,4-D was progressive, with *de novo* organogenesis of shoot, root and somatic embryo representing a developmental continuum (Raghavan, 2005). These studies raise the question of whether plant cells contain a cell fate memory, and whether this memory must be erased or bypassed to allow regeneration to occur (Sugimoto et al., 2011).

The nature and regulation of this cell memory is currently unknown. A number of studies have hypothesised that mechanisms that act on chromatin are prime candidates for stabilizing cell fate (Alabert et al., 2015; Barth and Imhof, 2010; Nashun et al., 2015; Birnbaum and Roudier, 2017). Gene expression can be regulated on a chromatin level by a number of mechanisms including DNA methylation, histone modifications, incorporation of histone variants, as well as modifying and ATP-dependent nucleosome remodeling enzymes (Feng and Jacobsen, 2011). Combinations of chromatin modifications have been shown to create distinct transcriptional outcomes (Baroux and Autran, 2015; She et al., 2013; She and Baroux, 2014). Chromatin modifications have also been shown to accompany

phase transitions in development such as during germ cell development, marking the transition from somatic to reproductive cells (She and Baroux, 2015; Baroux and Autran, 2015). These studies highlight the role that chromatin level modifications play in controlling cell developmental phase transitions under normal development, and so may play a role in the suppression of plant cell regeneration capacity to ensure correct development.

In contrast to DNA methylation, histone modifications have a better-defined role in controlling plant development (Goodrich and Tweedie, 2002; Schubert et al., 2005). In particular Histone H3 trimethylated on lysine 27 (H3K27me3) is dynamically deposited and removed throughout development and has been linked to the establishment and maintenance of a gene repressive state (Alabert et al., 2015; Barth and Imhof, 2010). This modification has recently been shown to be restored quickly in G2 phase after call division, assisted by CHROMATIN ASSEMBLY FACTOR 1 (CAF1) and incorporation of the H3.1 variant (Jiang and Berger, 2017) and actively maintained after passage of a DNA replication fork by chromodomain helicase DNA-binding chromatin remodeler PICKLE (PKL) (Carter et al., 2018). This illustrates that this modification can be inherited through cell division and therefore has the potential to act a an epigenetic mark and potential barrier to regeneration.

In Arabidopsis, Polycomb Repressive Complexes (PRCs) are responsible for the establishment and maintenance of repressive histone modifications, including H3K27me3 (Cao et al., 2002; Schubert et al., 2006). During hormone induced callus formation, He et al., (2012) demonstrated that PRC2 mediated H3K27me3 reprogramming was vital for leaf-to-callus transition; While double mutants in the PRC2 component genes *CURLY LEAF* (*CLF*) and *SWINGER* (*SWN*), exhibit loss of differentiated state, ectopic callus formation and somatic embryogenesis (Ikeuchi et al., 2015; He et al., 2012; Chanvivattana et al., 2004). These results demonstrate a role for PRC2 and H3K27me3 in suppressing developmental programs, controlling reprogramming and instilling cellular memory in adult plants.

The potential existence of cellular memory being an epigenetic barrier to induced regeneration implies that there must be a mechanism to remove, or bypass persistent chromatin states during cellular reprogramming (Birnbaum and Roudier, 2017). This could be achieved enzymatically, by actively removing the necessary chromatin modifications, or passively through replication-coupled dilution.

With regards to the former, while components of the H3K27me3 writer, Polycomb repressive complex 2 (PRC2), have been reported for over a decade, it is only comparatively recently that JUMONJI-C (JmJ-C) proteins have been identified as specific H3K27me3 demethylases (Lu et al., 2011a; Gan et al., 2015). EARLY FLOWERING 6 (ELF6) and RELATIVE OF EARLY FLOWERING 6 (REF6) are two closely related genes of the JmJ-C protein group. They were first discovered antagonistically regulating flowering time, and have subsequently been reported to specifically demethylate H3K27me3 and H3K27me2 (Noh et al., 2004; Yu et al., 2008; Lu et al., 2011a).The antagonistic action between the two groups (PcG and JmJ-C) could allows the dynamic targeting of genes by H3K27me3 throughout plant development, and could also be involved in the dynamic regulation during regeneration.

Many of the genes implicated in regeneration have been shown to be PcG targets, such as WOUND INDUCED DEDIFFERENTIATION3 (WIND3) and LEAFY COTYLEDON2 (LEC2) (Ikeuchi et al., 2015). While recent expression studies have shown components of JmJ-C and PcG systems are active in the tissue specific regions, responsive to RKD4 mediated reprogramming (de Lucas et al., 2016; Noh et al., 2004). Somatic regeneration induced by hormone treatment produces a raft of global changes in gene expression and chromatin environment, making the identification of key regulators within the mechanism difficult to identify. Furthermore, the long incubation times and amorphous nature of callus induction makes pinpointing initiation events or cellular tracking difficult. In recent decades, several transcription factors (TFs) such as RKD4 have been shown to induce somatic embryo formation when over-expressed in somatic tissue (Summerised in Chapter 3, table 3.1). These systems provide an interesting opportu-

nity to study the molecular framework underlying totipotency and regeneration competency of plant tissue. These TF mediated SE responses are often tissue specific (RKD4 over expression induces formation of a callus specifically at the root and shoot meristem) and this provides a cellular framework against which genetic/epigenetic effects can be screened.

4.1.1 Experimental Rationale

In summary, during regeneration plant cells must transition from one specialized cell identity to another, redefining their fate (Efroni et al., 2016). Therefore, plant cells must effectively erase or bypass existing cell fate memory, or simply activate cells exhibiting cellular specialisation but lacking an epigenetic restriction on the alteration of cell fate (Sugimoto et al., 2010). However, which mechanism is responsible for plant cell plasticity is currently unclear. This project aimed to address this question by using the controlled over-expression of the zygotic transcription factor *RKD4*, as a model system for somatic cell regeneration. This system will be used to investigate the role of histone methyltransferase (CLF) and histone demethylases (REF6 and ELF6) in removing or maintaining barriers to cellular reprogramming.

4.2 Results

4.2.1 Phenotypic Characterisation of *ref6/elf6* in the *inRKD4ox* Background

A genetic approach was selected to investigate the role of PcG and JmJ-C proteins on RKD4 mediated regeneration. However, the construct used to induce RKD4 over-expression contains a CaMV 35S promoter. Most T-DNA insertion mutant lines also contain this promoter, thus it could trigger transcriptional silencing of RKD4 (Mlotshwa et al., 2010). A novel T-DNA mutant line was isolated for *REF6* which does not contain sequences from the CaMV 35S promoter, and this allele was named *ref6-5*.

T-DNA insertion lines for mutants in *ELF6* lacking the CaMV 35S promoter were unavailable, so CRISPR/Cas9 was used to introduce a targeted mutation in this gene; the new allele was named (*elf6-C*). Two protospacer sequences that act as guides, specific to the *ELF6* gene at bp sites 501-520 and 665-684 were used (Oliveros et al., 2016). Efficiency of the guides were tested on *Arabidopsis* mesophyll protoplasts before being cloned into a pDE-CAS9 vector (Durr et at., 2018), which contained a RFP seed selection reporter (OLE1-RFP) and transformed into plants using *Agrobacterium tumefaciens* (Clough and Bent, 1998). Primary transformants were selected using the seed specific RFP reporter and sown on soil. Once a mutation was identified using PCR, RFP negative seeds of single lines were sown on soil and genotyped for deletion events. Sanger sequencing of the amplified *ELF6* gene fragment from *elf6-C* lines revealed a 173bp deletion between bp sites 516 and 690 in the *ELF6* gene (Fig4.1). *elf6-C* lines were backcrosssed to wild-type (*inRKD4ox*) and re-selected in F2 to minimise off target mutation events induced by CRISPR/Cas9 expression.

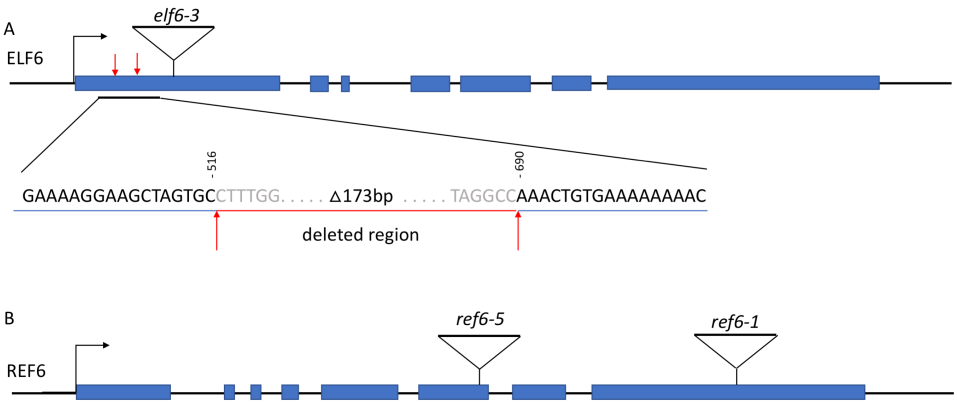


Figure 4.1: Mutant Alleles of JmJ-C Histone Demethylases. Schematic drawing of T-DNA insertions (triangles) in *REF6* and *ELF6* genes (A and B respectively). Target region for RNA-guided Cas9 editing of *ELF6* and 173bp deletion introduced in *elf6-C*.

Phenotypic analysis of *ref6-5* revealed an apparent delay in flowering time compared to control plants, similar to previously described mutants for this gene (Noh et al., 2004). Interestingly, *ref6-5* delayed flowering was greater than that previously described for *ref6-1*, suggesting that this new allele was stronger than others previously reported (Fig.4.2). Phenotypic analysis of *elf6-C* showed a similar early flowering time phenotype to the previously described T-DNA insertion mutant *elf6-3* (Fig.4.2) (Noh et al., 2004).

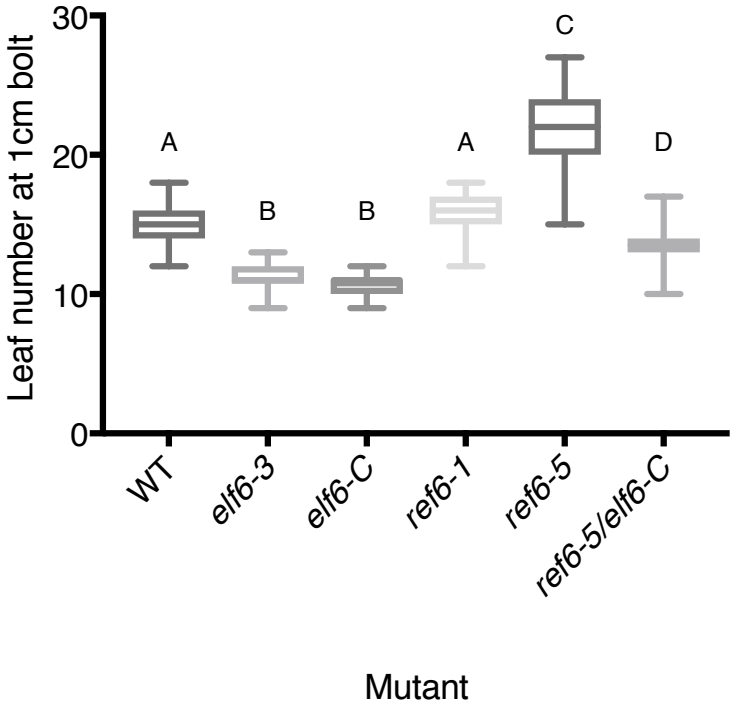


Figure 4.2: Flowering Time Phenotype in JmJ-C Mutants Under Long Day Conditions. Box plot of wild-type (inRKD4ox, Col-0), *elf6-3*, *elf6-C*, *ref6-1*, *ref6-5*, and *ref6-5/elf6-C* double mutants grown in soil until bolting. Flowering time was advanced in *elf6* mutants and inhibited in *ref6-5* mutant. Significant differences in the data were supported by ANOVA and indicated with letters ($p \leq 0.0015$), $n \geq 26$.

Surprisingly, when *elf6-C* and *ref6-5* mutations were combined by genetic cross, the double mutant (*ref6-5/elf6-C*) displayed severe pleiotropic developmental defects (Fig. 4.3). Double mutant plants had a dwarf phenotype, increased petals in some inflorescences, decreased silique length and distortion of leaf morphology, affecting leaf margin development, serration and causing downward curled leaves (Fig. 4.3).

This phenotype co-segregated with *ref6-5/elf6-C*, after backcrosses to both *inRKD4ox* and Col-0 lines, indicating the phenotype was not due to off target mutations of the CRISPR-cas9 targeting of *ELF6*. The phenotype was consistent between mutants with and without the inRKD4ox construct, thus demonstrating that these phenotypes were independent of the inRKD4ox construct (Fig.4.2). In addition, this phenotype has subsequently been observed in crosses between T-DNA insertion mutants *ref6-5* and *elf6-3*, while double mutants *ref6-1/elf6-3* do not present with the phenotype (data not shown). This suggests that the *ref6-1* and previously described *ref6-1/elf6-3* mutants are of poor penetrance or weaker alleles (Yu et al., 2008), and suggests that *ref6-5/elf6-C* mutant are likely true null alleles.

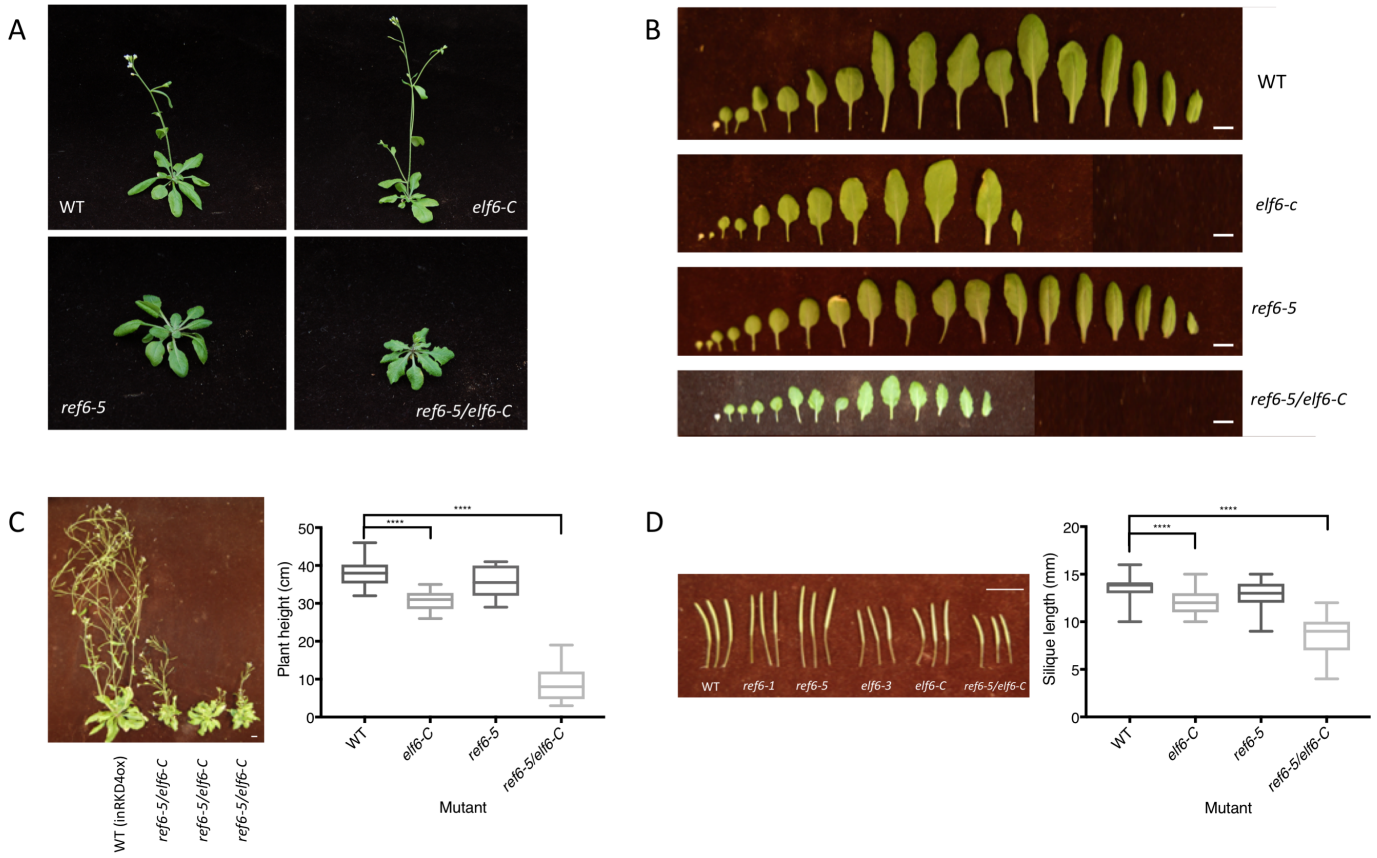


Figure 4.3: Novel phenotypes present in the JmJ-C mutants. Wild-type, *elf6-C*, *ref6-5*, and *ref6-5/elf6-C* double mutants were grown until bolting (photos were all taken at the same timepoint) (A). Leaf morphology of the mutants compared to WT at point of bolting (B). *ref6-5/elf6-C* mutants displayed dwarf phenotype (C) and silique length was significantly reduced compared to wild-type plants (D). Significant differences supported by Students t-test indicated by '****' $p \leq 0.001$, n=30, White bars = 1cm.

4.2.2 Initiation of RKD4 Induced Reprogramming

In post-embryonic development, somatic cells become progressively more differentiated and only a restricted number of cell types remain competent to form new tissues or organs (Chen et al., 2014). Explants from juvenile plants have been shown to regenerate shoots more effectively than those from mature plants (Dong and Jia, 1991; Baker and Bhatia, 1993; Becerra et al., 2004; Zhang et al., 2015), indicating that as differentiation progresses, additional barriers to regeneration are established. To assess the innate potential of the mutant lines to regenerate, RKD4ox was induced from germination while the plants were in a juvenile state.

When *RKD4* is ectopically expressed in germinating WT embryos, seedlings develop preferentially to callus rather than following the normal developmental program. This leads to the arrest of normal development, and cell proliferation is evident across all tissues types as well as bleaching of cotyledons. Callus induction was unaffected by *elf6-C* and *clf-81*, which responded similarly to control plants. However, callus induction was inhibited by 20% in *ref6-5* and further repressed in the double mutant *ref6-5/elf6-C*, with 40% of plants escaping callus formation going on to develop normal root and shoot structures (Fig.4.4 A, ANOVA, $p \leq 0.0125$ and $p \leq 0.001$ respectively).

Of the plants that did develop calluses it was clear that callus growth was inhibited in both *ref6-5* and *ref6-5/elf6-C* lines causing a reduction of 41% and 62% respectively in accumulated dry mass after 14 days (ANOVA, $p \leq 0.0001$). Although callus initiation was not effected in *clf-81* and *elf6-C* mutants, callus growth showed an increase of 246% and 25% in dry mass accumulation respectively compared to that of control (Fig.4.4 B ANOVA, $p \leq 0.0001$ and $p \leq 0.006$ respectively).

These results suggest that there is partial functional redundancy between *REF6* and *ELF6*, as the induction of callus is further repressed in double mutants rather than being similar to *ref6* alone. In addition, the differences in callus mass suggest potentially opposing roles for *REF6* and *ELF6* after callus is initiated within this

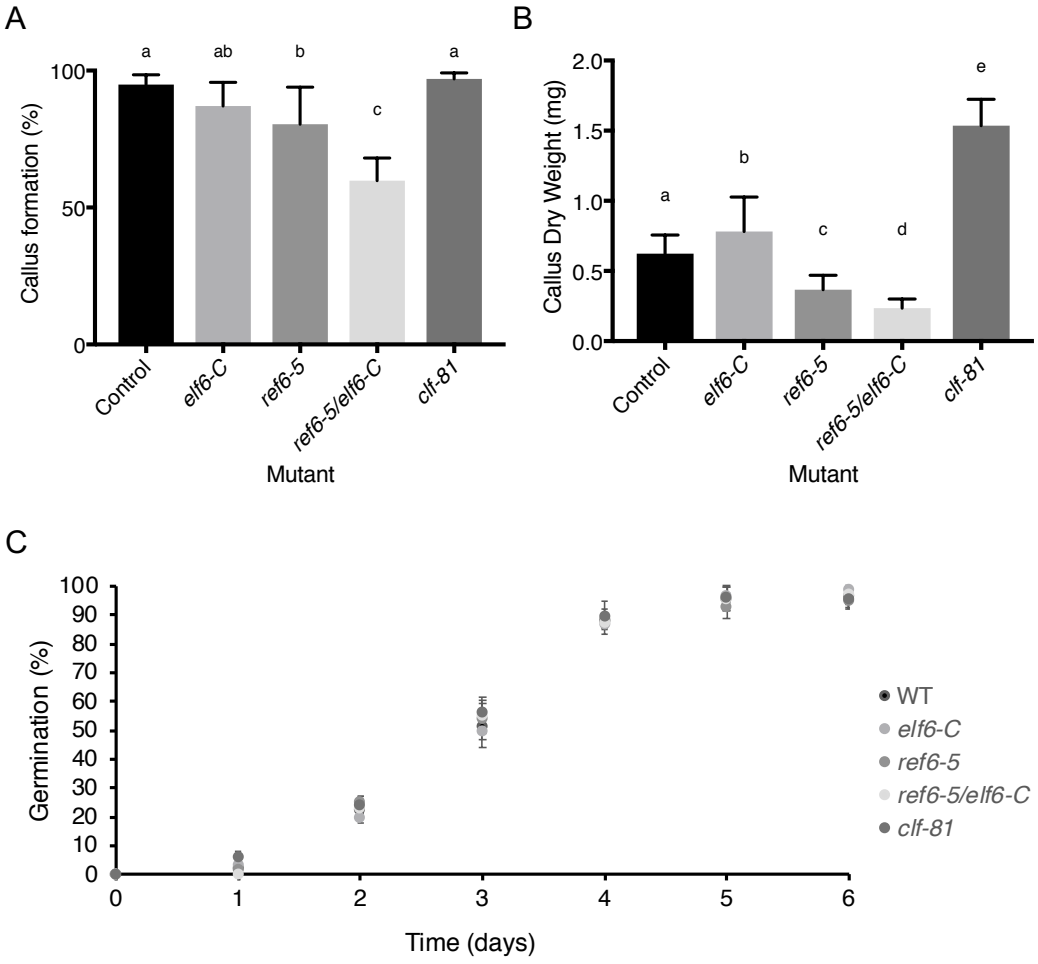


Figure 4.4: RKD4-mediated Callus Initiation in JmJ-C and PcG Mutants. Control, *elf6-C*, *ref6-5*, *ref6-5/elf6-C* and *clf-81* mutants were germinated on media inducing *RKD4* over expression with dexamethasone. Callus formation was reduced in *ref6-5/elf6-C* after 14 days with successful callus being the complete reprogramming of the root and shoot meristems and bleaching of cotyledons (A, n=350). Successfully induced calluses were collected, and dry weight measured after drying for 48 hours at 65 degrees (B, n=220-330). There was no difference between germination rate on MS media of age matched seeds between wild-type, *elf6-C*, *ref6-5*, *ref6-5/elf6-C* and *clf-81* mutants (C, n=300). Significant differences in the data were supported by ANOVA and indicated with letters ($n \geq 119$, $p \leq 0.025$).

regeneration system. As callus growth should develop exponentially (Henshaw et al., 1966), early differences in induction caused by germination could result in large differences in callus size after 14 days. However, there were no differences in germination between any of the mutant lines, suggesting the differences observed were due to the effects of the mutation on cellular growth or delay in transcriptional 'access' of RKD4 to its gene targets (Fig.4.4 C).

4.2.3 Reprogramming of Differentiated Tissue by Ectopic Expression of *RKD4*

A number of transcription factors have been observed to induce SE but this is often a tissue specific response: ectopic expression of *LEAFY COTYLEDON 2 (LEC2)* leads to the formation of somatic embryos in early hypocotyls (Stone et al., 2008; Wójcikowska et al., 2013) while *BABY BOOM (BBM)* was able to induce the formation of somatic embryos in mature embryos and cotyledons (Horstman et al., 2017b). In contrast, reprogramming by RKD4 over expression provides a longer window in which regeneration can be investigated, as cells within the shoot apical and root apical meristems remain susceptible 10 days after germination. While reprogramming in germinating tissue provides an interesting snapshot of inherent susceptibility to reprogramming it does not provide insight to the epigenetic barriers to reprogramming of terminally differentiated tissues. To probe the effect of PcG and JmJ chromatin remodelling on the ability of RKD4 to reprogram terminally differentiated cells, *RKD4* over expression was induced in five day old seedlings once terminal differentiation had been established in some tissues.

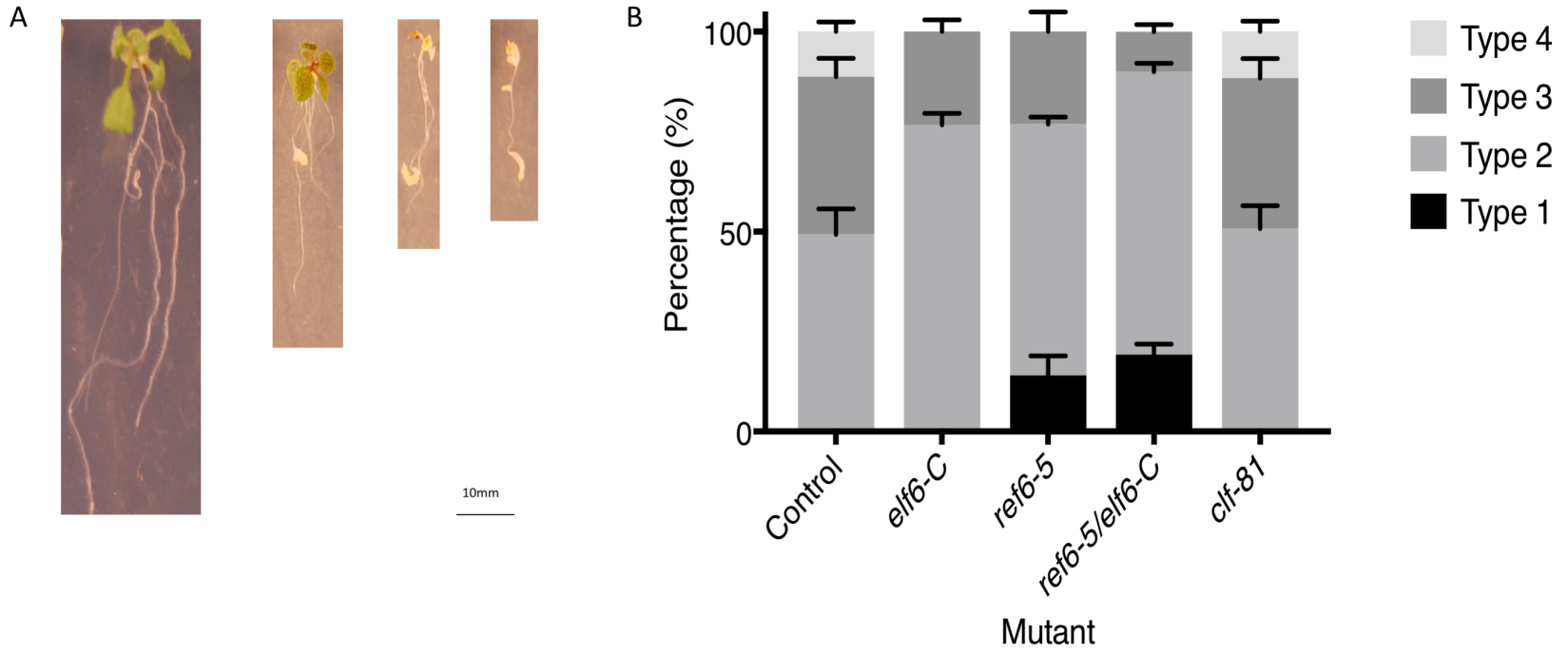


Figure 4.5: The role of JmJ-C and PcG in RKD4 Induced Reprogramming. *RKD4* over expression was induced in five day old seedlings in mutant lines. Initiation continued for 14 days before callus formation was grouped into four categories: Type 1 - 'Insensitive'; Type 2 - 'Root tip only'; Type 3 'root and shoot'; and Type 4 - 'complete induction' (A, left to right respectively), percentage grouping from 3 biological replicates (n≥37) (B).

Although RKD4 over expression is ubiquitous throughout the plant, induced reprogramming was found to be restricted to the actively growing regions, namely the root and shoot meristems (Chapter 3, Fig.3.1). This suggests that once terminal tissue development is established, RKD4 cannot initiate cell division directly. However, the severity of the reprogramming varied within the populations (Fig.4.5).

Similarly to the previous experiment, reprogramming profiles for *clf-81* was indistinguishable from control; while *ref6-5* and *ref6-5/elf6-C* mutants inhibited response to RKD4 induction as they were the only lines to display Type 1 responses. They developed normally, with no evidence of RKD4 induced reprogramming. However, within the JmJ-C group, plants failed to undergo complete induction (Type 4), suggesting a role for both REF6 and ELF6 in the deviation from normal developmental growth to a new developmental signal or the activation of RKD4 gene targets in older differentiated tissues.

To investigate whether native REF6 expression was limiting regeneration potential of differentiated tissues, REF6 over expression was induced using estradiol (Ref6ox, pMDC7 -OLE -REF6). However, reprogramming profiles of REF6ox plants in conjunction with RKD4ox were indistinguishable from control plants indicating that REF6 expression was not limiting response in established tissues.

4.2.4 RKD4 Induced Reprogramming in Different Cell Types

Recent regeneration studies have suggested the role of a dispersed network of cells that remain regeneratively competent, these regeneration-initial cell are thought to involve the pericyle and pericycle-like cells surrounding the vasculature (Sang et al., 2018). While the broad regions of the plant that are susceptible to RKD4 mediated reprogramming have already been reported (Waki et al., 2011), the cell types responsible for this regeneration are currently unknown. To generate a growing three-dimensional structure, the root meristem undergoes several rounds

of ordered cell divisions. Those divisions underlying the longitudinal growth of the root are called anticlinal divisions, whereas radial growth is controlled by periclinal divisions (De Rybel et al., 2016). Root tip development is a tightly regulated process forming concentric rings of defined cell layers (De Rybel et al., 2016). This defined cell structure provides an ideal system in which to study the cell type responses to ectopic *RKD4* expression (Chapter 1, Fig.) as perturbation from the established developmental norms are easy to identify morphologically. To investigate this response, confocal analysis and 3D z-stack images were produced to map the early cell divisions initiated by *RKD4* over expression, within the root meristem. Samples were collected in a timecourse at 0, 6, 12, 18, 24, 48 and 72 hours after *RKD4* induction, and samples were fixed in ethanol:acetic acid (3:1 ratio) to prevent further cell divisions occurring before imaging.

The initial adventitious cell divisions were observed within the meristematic region of the root tip 12 hours after *RKD4ox* induction (Fig.4.6). During the early stages (12-24 hours) anticlinal cell divisions were limited to the epidermal and cortex layers of the root leading to a decrease in cell volume across the sample window and an inhibition of cell elongation (Fig.4.6). Later in induction (24-48hrs) adventitious divisions begin to occur within the endodermal cell layer, and after 48 hours root developmental patterning within these layers begins to break down with a mix of periclinal and anticlinal divisions continuing within the epidermis, cortex and endodermis, distorting the root cell layers leading to the rough, amorphous cell texture associated with callus formation. Throughout the induction time-course pericycle and vascular cell layers of the root were indistinguishable from mock treated control plants. This indicated that there were no developmental effects of *RKD4* over expression in these cell layers, suggesting that these cells may be resistant to reprogramming by RKD4.

Most cell types divide down the shortest cell wall, unless affected by external pressures or internal signals (Smith, 2001; De Rybel et al., 2016). In root cells, outside of the areas immediately surrounding the quiesent center most cell division is limited to the anticlinal division plane (De Rybel et al., 2016). Interestingly,

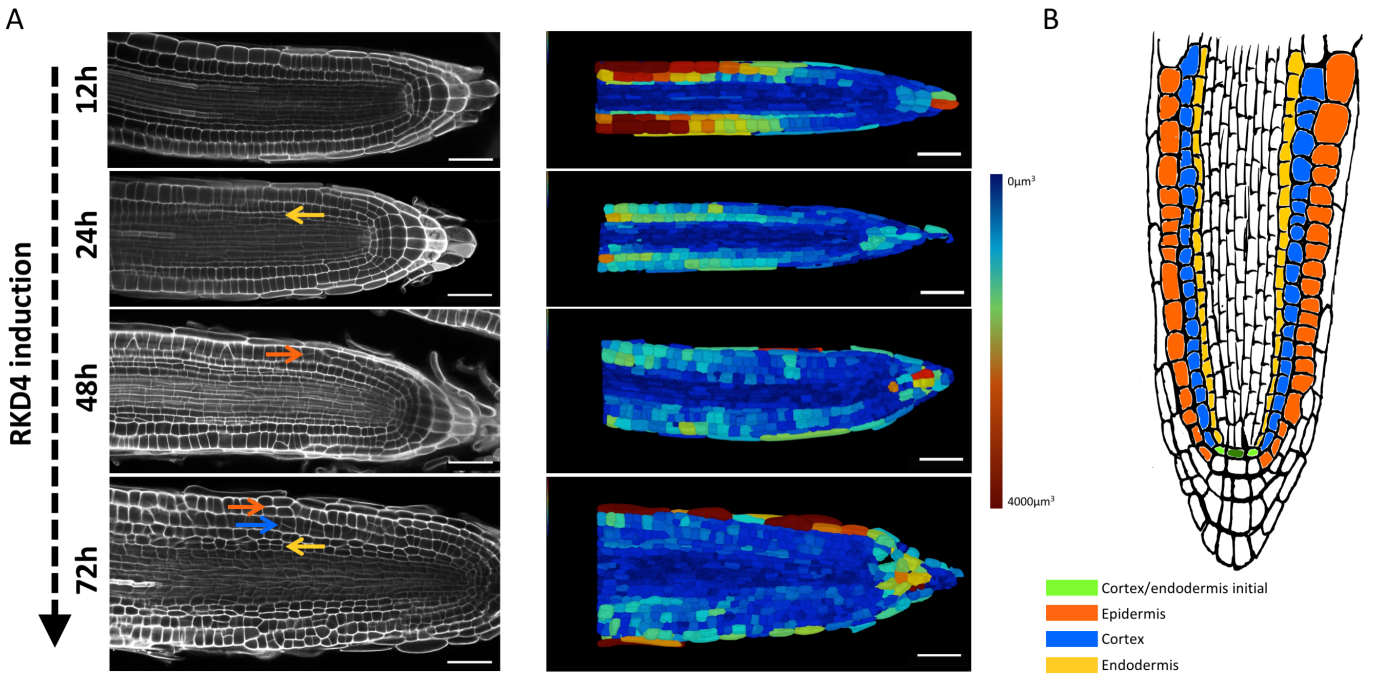


Figure 4.6: Root cell responses induced by RKD4. Left- Confocal cross sections collected after *RKD4* induction in roots fixed with ethanol:acetic acid, cell walls stained using propidium iodide and cleared using chloral hydrate. Right - Heat map of segmented cell volume (μm^3) (A). Schematic representation of the cell layers affected by RKD4 mediated reprogramming (B). Arrows: orange – Epidermis, blue – Cortex, Yellow – Endodermis, bars = 20μm.

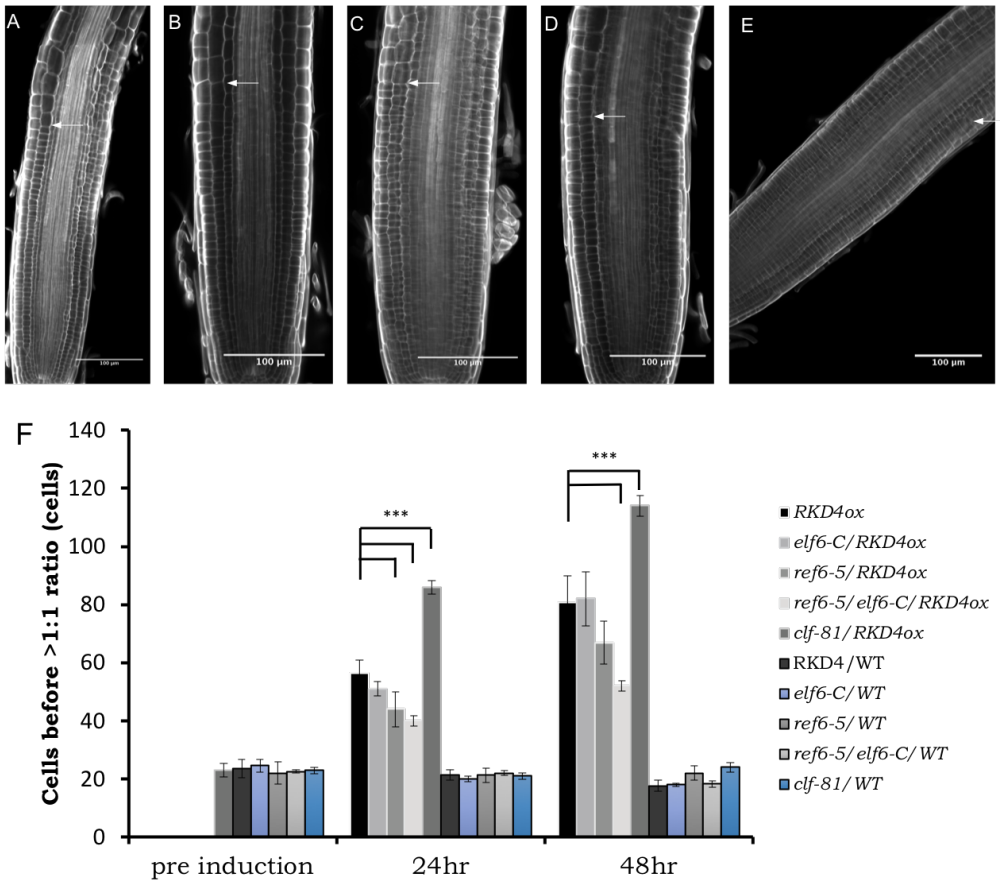


Figure 4.7: Characterization of root meristem during *RKD4* induced reprogramming. A to E, Confocal optical sections through the root meristem of the control (A), *elf6-C* (B), *ref6-5* (C), *ref6-5/elf6-C* (D) and *clf-81* (E) 24 hours after *RKD4* induction. F, meristem length measured by counting cortex cell number before exceeding 1:1 cell ratio wall ratio indicating the start of the elongation zone in induced (RKD4ox) and non-induced (WT) plants. White arrows indicate the transition zone between the meristem and the elongation-differentiation zone. Bars = 100 μm. Values shown represent means of at least 14 seedlings. Error bars indicate sd. '***' indicate significant differences supported by Student's t test ($p \leq 0.01$).

during *RKD4* induction, the endodermal cell layer, preferentially divide in a periclinal direction rather than the anticlinal direction of the epidermis and cortex cells. This division preference is observed in all endodermal cells, leading to the creation of an additional cell layer, on average 48 hours after induction (Fig.4.6). This division preference was similar to the middle cortex formation generally seen in older seedlings (around 10-14 days) and is thought to be genetically controlled (De Rybel et al., 2016), so this observation suggests that endodermal cells are functionally primed to divide along this axis once cell division is initiated.

When early cell division events were similarly tracked in the mutant lines, cell type responses were indistinguishable from control, indicating that REF6, ELF6 and CLF were playing no part in the cell type response (Fig.4.7). However, in this analysis the mutants did show similar 'cell division rate' effects to those observed previously in the seed induction experiment (Fig.4.4). As early as 24 hours after *RKD4ox*, callus induction (scored by cortex cell number before cell elongation zone of the root tip) was increased in *clf-81* and inhibited in *ref6-5* and *ref6-5/elf6-C* despite this cell number being similar in non-induced conditions (t-test, $p \leq 0.001$). These data in combination with the callus initiation rates suggest an increased 'sensitivity' of cells within *clf-81* and a decreased 'sensitivity' in *ref6-5/elf6-C* to RKD4, potentially by delaying transcriptional access to its gene targets.

4.2.5 Meristem Integrity During RKD4 Induced Reprogramming

The susceptibility of plants cells to new developmental stimuli must be carefully controlled to ensure responses to intermittent or low level signals are not instantly followed to the detriment to the plant as a whole (Bassel, 2016). *RKD4* over-expression results in an irreversible developmental fate switch into a embryonic program. The variation in response between the mutant lines suggests a differing susceptibility within the responsive cells to *RKD4* induction. This suggests that

while the ectopic expression of this gene induced callus formation in all genotypes, the resultant commitment to the embryonic program could vary between the mutants.

To ensure continual development throughout it's life cycle, plants maintain a pool of stem cells which are located in meristems, composing of a central stem cell niche and actively dividing stem cell progeny (Pierre-Jerome et al., 2018). The maintenance of these organizing cells is tightly regulated by layered feedback mechanisms (Pierre-Jerome et al., 2018). To investigate the role of JmJ-C and CLF on determining the susceptibility to cells to new reprogramming signals, transient *RKD4ox* was induced in the mutant lines from germination for either seven or 14 days before recovery on non-inducing media. After a recovery period of 10 days, the perturbation of the root and shoot meristem was scored based on the re-establishment of normal root and shoot development (Fig.4.8).

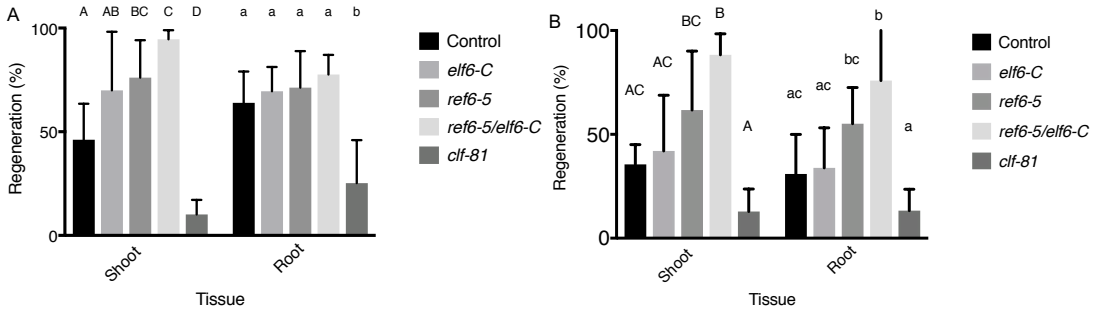


Figure 4.8: Re-establishment of Root and Shoot Meristematic Programs After *RKD4* Induction. *RKD4* over expression was induced for either 7 or 14 days (A and B respectively) calluses were then transferred to non inducing media. Re-establishment of root and shoot was scored based on the development of root and shoot structures after 10 days. Significant differences in the data were supported by ANOVA and indicated with letters ($p \leq 0.05$) data from biological 6 replicates (n=30).

Interestingly, the root meristem was more resistant to short term reprogramming by RKD4 but responded similarly to the shoot after longer induction in control plants. It is clear that the root and shoot meristems in the *clf-81* mutant line were significantly more susceptible to RKD4 induced reprogramming, as even after a 7 day induction treatment, 90% of the root and shoot meristems were unable to recover normal development (Fig. (Fig.4.8), ANOVA, $p \leq 0.0005$). Contrastingly, the double mutant *ref6-5/elf6-C* was more resistant to the *RKD4* pulse, re-establishing root and shoot developmental programs on an average of 75% of cases even after a longer 14 day treatment (Fig. 4.8, ANOVA, $p \leq 0.001$). These data suggests that the maintenance of the stem cell niche by PRC2, prevents the activation of alternate developmental programs, and that REF6 and ELF6 contribute to the integration of new developmental signals.

4.2.6 Somatic Embryo Production by Calluses

Plant regeneration is a two step process where cells must first lose the established cell fate, before re-developing new ones (Orłowska et al., 2017). However, successful de-differentiation to a callus state does not necessarily allow for re-differentiation to new cell fates, as the two systems require both the activation and repression of different developmental pathways (Orłowska et al., 2017; Sang et al., 2018; Kadokura et al., 2018). The development of somatic embryos within the *RKD4ox* system only occurred after the removal of the chemical inducer, indicating that this TF re-codes and primes somatic cells to be embryonically competent, but does not initiate embryo formation directly.

To investigate the role of JmJ-C and PRC2 on the acquisition of cell competency, *RKD4ox* was induced from germination in *ref6-5*, *elf6-C*, *ref6-5/elf6-C* and *clf-81* mutant lines. Induction was ceased by the transfer to fresh plates and somatic embryos allowed to develop over 10 days before being counted. Embryo counts were then normalised to the average callus fresh weight weight at the point of transfer (7 or 14 days), to estimate the proportion of cells within the callus that

become competent to initiate a somatic embryonic program (Fig. 4.9).

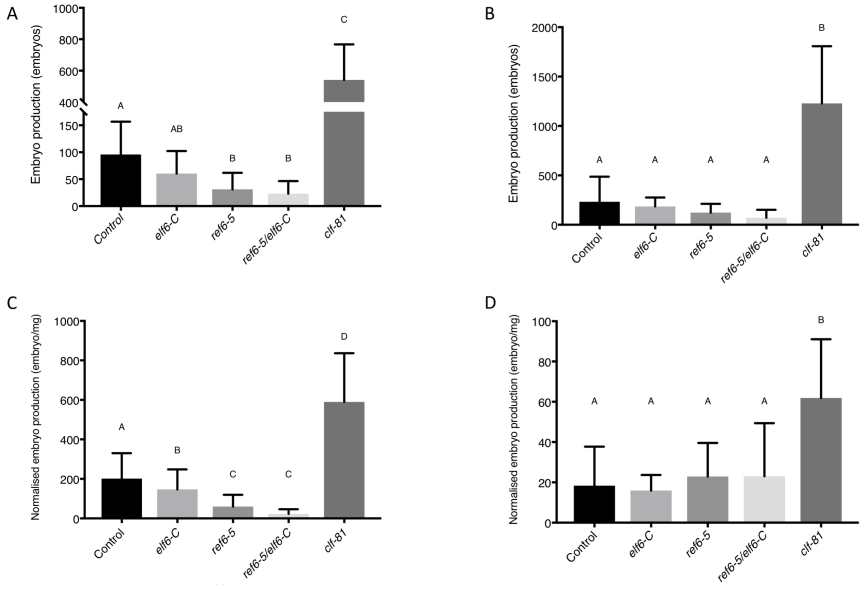


Figure 4.9: Somatic Embryo Production Induced by RKD4. Control, *elf6-C*, *ref6-5*, *ref6-5/elf6-C* and *clf-81* mutants were germinated on media inducing *RKD4* over expression. Calluses were induced for 7 or 14 days before being transferred to non inducing media. Embryo formation was progressed for 10 days before counting (A and B). Embryo counts were then normalised to average callus weight at 7 and 14 days (C and D). Significant differences in the data were supported by ANOVA and indicated with letters ($p \leq 0.05$), (n=104).

After 7 days of induction, embryo production was inhibited in *ref6-5* and *ref6-5/elf6-C* but enhanced in *clf-81* (Fig. 4.9). These data demonstrates that availability of the REF6 is important for rapid activation of the embryonic program, while suggesting that the reduction of H3K27me3 facilitates cells becoming embryonically competent. When these counts were normalised to callus mass, embryo production was still significantly enhanced in *clf-81* mutants demonstrating that proportionally more cells within the callus have committed to an embryonic program. Conversely, embryo production is reduced in all *ref6-5*, *elf6-C* and *ref6-5/elf6-C* lines indicating a smaller proportion of cells were embryonically competent at this timepoint (Fig. 4.9).

These data reveals that REF6 and ELF6 are necessary for the activation of new developmental transitions. However, after 14 days the effect of mutations in the histone demethylases was less marked, suggesting that the reprogramming to SE requires longer in these mutants to allow cell competency to be firmly established. This is further supported by the normalised embryo counts, which shows proportional equivalence between the 'control' and *JmJ-C* mutant calluses (Fig. 4.9). Enhanced embryo production is maintained within the *clf-81* line, producing on average 1000 embryos from the 14 day old callus, a four fold increase from control (ANOVA, $p \leq 0.0001$).

Interestingly, somatic embryo induction was not proportional to callus growth, with normalised embryo production dropping by a factor of 10 between 7 and 10 days (Fig. 4.9). This suggests that a subset of de-differentiated cells may be competent to produce somatic embryos; while the rest develop into callus and have either lost the ability to re-differentiate or are prevented from de-differentiating sufficiently to achieve competency.

4.2.7 Expression Analysis of RKD4 Targets

Our data supports the view that RKD4 developmental reprogramming is underpinned by dynamics in H3K27me3. To test this hypothesis, we asked if there was an overlap between genes misregulated in *rkd4-1* embryos (Waki et al., 2011), and genomic regions affected in H3K27me3 in PcG and JmJ-C mutants (Yan et al., 2018, Julia Engelhorn personal communication). From this list we identified *LEC1*, an embryonic TF that has previously been implicated in somatic embryo induction (Lotan et al., 1998; Horstman et al., 2017b).

In all genotypes expression of *LEC1* was significantly up-regulated after RKD4 induction (Welch's t-test $p \leq 0.024$). However, *LEC1* expression in *ref6-5/elf6-C* showed a ten-fold reduction (Fig. 4.10) while *clf-81* showed a 7.5-fold increase relative to control samples (Fig. 4.10). This demonstrates that *LEC1* is being transcriptionally activated upon *RKD4* induction, and suggests that the chromatin state of these genes is an important component of the developmental reprogramming mediated by RKD4.

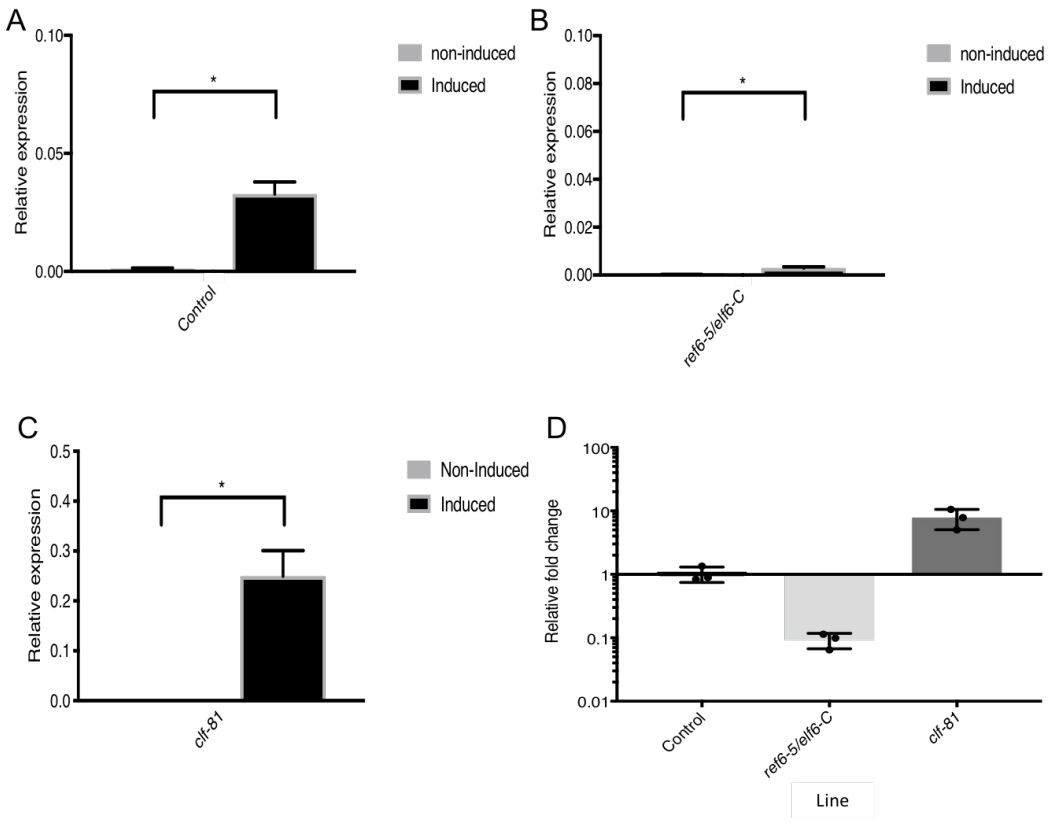


Figure 4.10: *LEC1* Expression During *RKD4* Induction in Control, *ref6-5/elf6-C* and *clf-81*. Up-regulation of *LEC1* in control and mutant lines in response to *RKD4ox* after 72 hours compared to non-induced controls (A, B, C) Relative expression of *LEC1* in induced samples compared to control (D). The expression level in whole seedlings was measured by qPCR and was normalized to that of *PPA2* gene (AT2G18230). Data shown here represents the mean value of three biological replicate pools of 12 whole seedlings. Significant differences supported by Welch's t-test indicted by '*' ($p \leq 0.05$), Bar=s.d.

4.2.8 Inheritance of the Chromatin State

If the chromatin state of genes targeted by RKD4 is critical for induction of SE, then the resistance to reprogramming of *ref6-5/elf6-C* plants could be variable and may lead to heritable responses. To investigate if this hypothesis is correct, we selected plants resistant to developmental reprogramming upon *RKD4* induction and allowed them to propagate by selfing. Progeny from these lines were tested for callus proliferation induced by *RKD4ox* (Fig. 4.11).

This analysis showed that developmental reprogramming resistance was not heritable, and that callus induction was highly variable between individual *ref6-5/elf6-C* lines, ranging from 86% regeneration to 16% (Fig.4.11). The original *ref6-5/elf6-C* stock used as a control was the result of the pooling of seed between multiple individuals all harvested together, meaning that the results within this population should represent an average for the genotype. However, the large variation observed between lines suggests that resistance to developmental reprogramming may not be solely due to loss of function in JmJ-C demethylases but instead to genetic or epigenetic variation generated in these mutants.

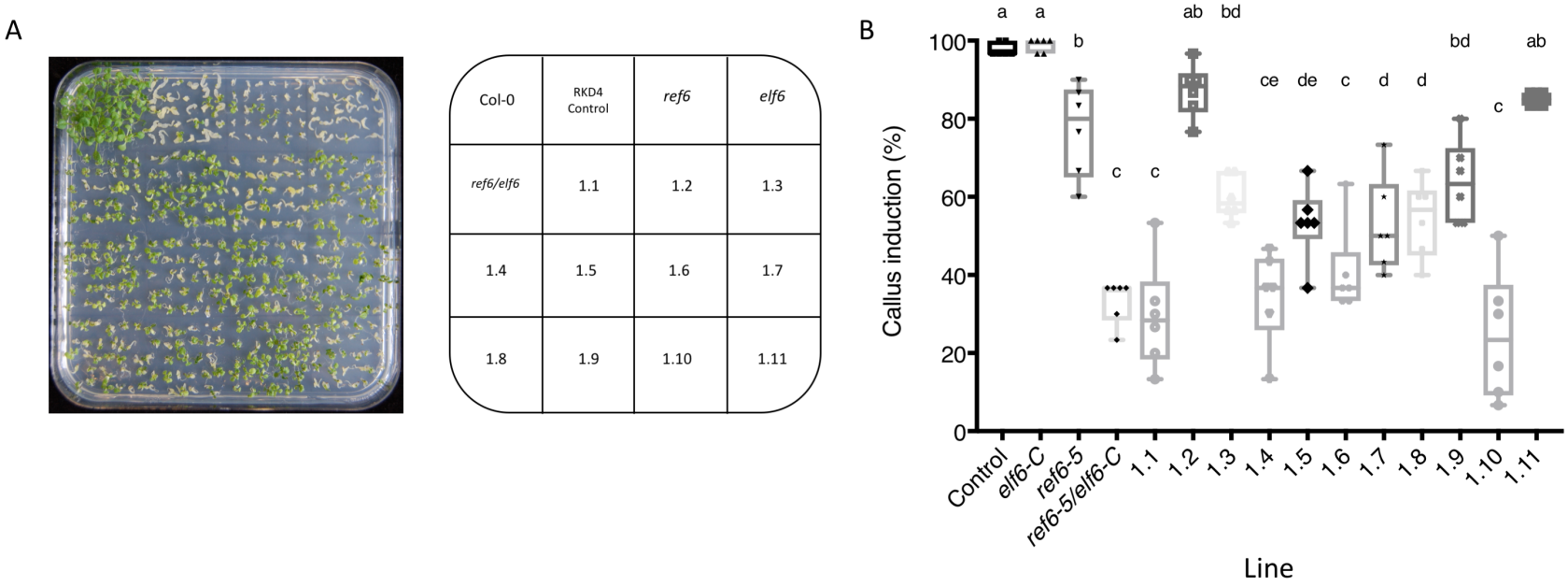


Figure 4.11: Callus initiation in hypo-sensitive *ref6-5/elf6-C* progeny. Seeds of 12 *RKD4ox* resistant*ref6-5/elf6-C* individuals (1.1-1.11) were germinated on media inducing RKD4 over expression with dexamethasone (A). Callus formation was reduced in *ref6-5/elf6-C* after 14 days on induction. However, resistance to regeneration was not inherited and responsiveness from individual lines was stochastic (B). Significant differences in the data were supported by ANOVA and indicated with letters ($p \leq 0.05$), data from biological 6 replicates (n=30).

Wound Induced Organogenesis

While the *RKD4* induction system provides a good model to study developmental reprogramming, it is an artificial system, thus limiting wider interpretations. Somatic cell re-differentiation in plants can be achieved in response to multiple environmental and developmental signals, such as wounding (Ikeuchi et al., 2016). In *Arabidopsis*, true leaves excised from seedlings undergo *de novo* organogenesis to regenerate adventitious roots from the cut site, without the exogenous application of hormones (Chen et al., 2014). Therefore, we utilised this leaf-to-root regeneration system within the *ref6-5*, *elf6-C* and *ref6-5/elf6-C* mutant lines, to investigate whether different reprogramming signals (such as wounding) require similar chromatin remodelling pathways to activate new developmental programs during *de novo* organogenesis. This assay was attempted using *clf-81*, however, all leaves from this line became necrotic after wounding and thus was excluded from the analysis.

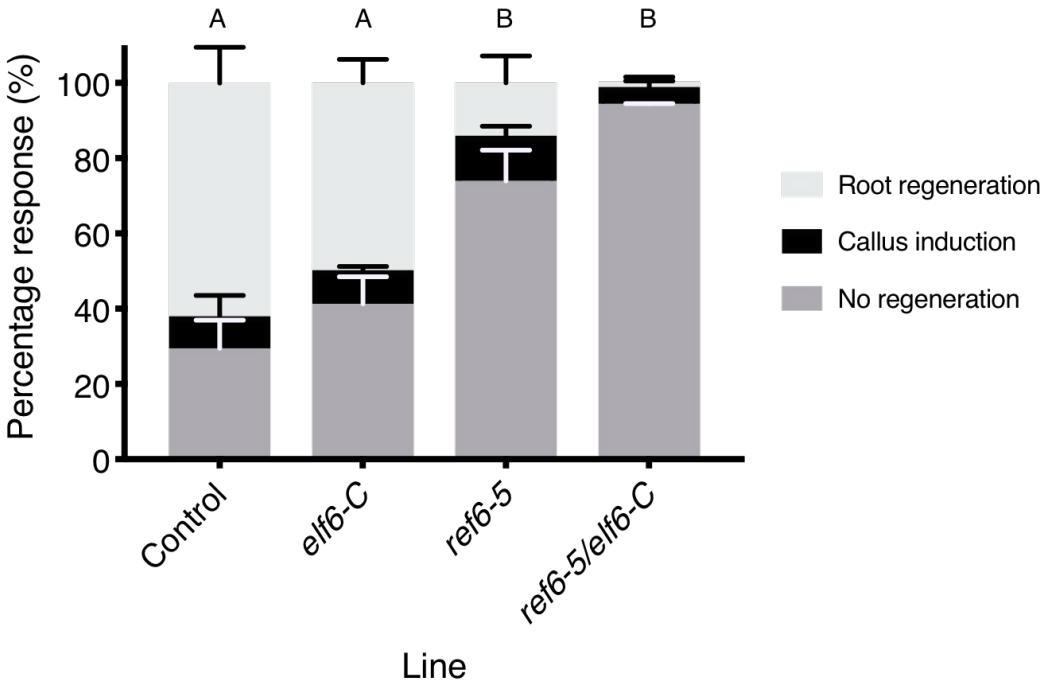


Figure 4.12: *De novo* Root Organogenesis in Leaf Explants. Percentage of leaves undergoing *de novo* root organogenesis, callus formation, or no regeneration in explants from 12 day old seedlings in control (*inRKD4ox*), *ref6-5*, *elf6-C* and *ref6-5/elf6-C* 20 days after cutting. Significant differences in root regeneration were supported by ANOVA and indicated with letters ($p \leq 0.0001$) Bars show s.d. with a minimum of two biological repeats. n=105, 244, 180, 162 in Control (inRKD4ox), *ref6-5*, *elf6-C* and *ref6-5/elf6-C* respectively.

We found that *de novo* root organogenesis in *elf6-C* was indistinguishable from wild type with adventitious root formation occurring in 50% of excised leaves (Fig.4.12, 2-way-ANOVA, p=ns). However, in *ref6-5* plants the initiation of adventitious root production was significantly impaired, initiating root development in only 14% of leaves (Fig. 4.12, 2-way-ANOVA, $p \leq 0.001$). Moreover, in *ref6-5/elf6-C* plants, root organogenesis was limited to just 1% of excised leaves (Fig. 4.12, 2-way-ANOVA, $p \leq 0.001$). This analysis also showed that although some excised leaves did not undergo *de novo* organogenesis, they still developed a callus mass at the wound site. This initiation was independent of the mutant background, developing in an average of 8% of leaves (Fig. 4.12, 2-way-ANOVA, p=ns), and did not establish root organogenesis even after prolonged culture (data not shown).

These data indicate that although wound induced cell proliferation was not dependent on histone demethylases, plants utilise similar chromatin remodelling pathways during *de novo* organogensis, to those in RKD4-induced developmental reprogramming, to initiate re-differentiation to a new cell fate.

4.3 Discussion

4.3.1 Dual Recruitment Mechanism of JmJ-C Proteins

Chromatin re-modellers such as REF6 have active roles in regulating gene expression at the chromatin level (Lu et al., 2011a), and genome-wide mapping of REF6 binding sites showed that it binds to thousands of putative targets directly via a Cys2His2 zinc finger and a CTCTGYTY motif within target genes (Li et al., 2016a). This direct binding mechanism raises the question of how REF6 and related H3K27me3 demethylases exert a dynamic antagonistic control of PRC2 repression (Mozgová et al., 2017). In this regard, the molecular components that ensure the properly timed and selective release of PRC2 repression are still poorly understood.

The increased delay in flowering by the *ref6-5* and the novel double mutant phenotype in *ref6-5/elf6-C*, suggests the production of a truncated or semi-functional REF6 protein in the previously reported mutants of *ref6-1* and *ref6-1/elf6-3* (Yu et al., 2008) that is capable of partially complementing the phenotype observed in *ref6-5/elf6-C*. The *ref6-1* mutant line contains the insertion at amino acid 1082 (Noh et al., 2004), thus, the catalytic JMJ-C domain region (amino acids 200-369 (Yu et al., 2008)) of REF6 could still be expressed in a truncated form of the protein. This is further supported by the subsequent re-emergence of the novel phenotype in double mutant produced with T-DNA insertion lines *ref6-5* and *elf6-3*, indicating the phenotype was not the result of an off target mutation during the CRISPR-Cas9 targeting during the generation of *elf6-C*.

Recently, a similar phenotype for the double mutant has been described (Yan et al., 2018), where the authors produced a double mutant of *ref6* and *elf6* using a CRISPR targeted mutagenesis of the *REF6* gene and the previously described T-DNA insertion mutant *elf6-3*. This mutant displayed an identical phenotype to the double mutant produced in this study, and the authors go on to demonstrate that the phenotype is complemented by the expression of a truncated version of

REF6, lacking the C-terminal C2H2-ZnF domain. This supportis the hypothesis a truncated form of REF6 is produced in *ref6-1*. This study goes on to present a role for REF6 in defining the spatial boundaries of H3K27me3; as many of the differentially methylated regions within the *ref6-C* background were not true targets of PRC2 (Yan et al., 2018).

The novel phenotype observed in Yan et al (2018) and complementation study indicates a dual targeting mechanism of REF6; whereby the recruitment of REF6 by other transcription factors could allow demethylation of gene targets, independently of the DNA binding activity of the C-terminal C2H2-ZnF domain. REF6 has already been shown to interact with other chromatin remodelers, such as BRAHMA (BRM) (Li et al., 2016a), and recent work in rice has found that a JmJ H3K27me3 demethylase (JMJ705) binds to targets interdependently with WOX11 (Cheng et al., 2018). Thus, the TF-guided activity could play an important role in the targeted removal of H3K27me3 (and resultant gene regulation) in specific plant tissues or cell types. This dual targeting of REF6 could be important for further research studies into regeneration, as the spatial and temporal co-expression of REF6 and guiding TFs, and not just the REF6 binding motif would be required to control the reactivation of some previously silenced genes by a REF6 mediated pathway.

4.3.2 Active Reprogramming of Histone Modifications Facilitates Regeneration

Developmental reprogramming of somatic tissues into cells that form embryos is seen as the ultimate demonstration of plant cell totipotency (Horstman et al., 2017a). Utilising, the process is often problematic due to the fact that the molecular mechanisms to this process remaining largely unknown (Lee et al., 2018). Cells in young tissue such as in meristems are able to respond quickly to new positional signals such as levels of auxin and cytokinin, that can alter cell fate, enabling new cellular divisions just hours after a signal is received (Xu et al.,

2006; Sena et al., 2009; Efroni et al., 2016); while in older tissues regeneration is a much slower process and generally less efficient (Birnbaum and Sánchez Alvarado, 2008). The lengthy competence step could reflect the time needed to alter chromatin and DNA modifications through either active mechanisms or a passive replication and dilution (Birnbaum and Roudier, 2017). The results of this study similarly show a preference for regeneration in 'young' tissues within the meristematic region of the root and shoot, with no reprogramming occurring in mature cells. However, the active removal of H3K27me by histone methyltransferases REF6 and ELF6 (Li et al., 2016a; Yan et al., 2018) seems to be a key step in the reprogramming response, even in these 'young' tissues.

It was recently shown that during regeneration the cytokinin-rich environment of shoot inducing media promotes the removal of H3K27me3 at the WUSCHEL (WUS) locus in a cell cycle-dependent manner but the authors were unable to determine if this was an active or passive process (Zhang et al., 2017). The results of this study suggest that the initial reprogramming of plant cells is an active process, indicated by the facilitatory role of the histone demethylases REF6 and ELF6, in both RKD4ox developmental reprogramming and *de novo* organogenesis; rather than a passive dilution of epigenetic marks by cell division occurring after the regeneration signal is received. The further reduction of regeneration potential in *ref6-5/elf6-C* compared to single *REF6* mutants indicates partial redundancy within the JmJ-C protein family in this demethylation activity required for regeneration in *Arabidopsis*.

Based on the phylogeny of JmJ-C proteins, JMJ13 could be a likely candidate for this function as it shares a similar sequence with REF6 and ELF6 but lacks the C2H2-ZnF DNA binding domain (Fig.1.2). A recent study has demonstrated that global levels of H3K27me3 were strongly elevated in the triple mutant of *ref6/elf6/jmj13* compared to wildtype. Single and double mutants supporting the semi-redundant function of REF6, ELF6 and JMJ13 (Yan et al., 2018). Further work investigating the regeneration response within *ref6/elf6/jmj13* by RKD4 could help determine the role of these proteins in regeneration within *Arabidopsis*

and the extent to which they are essential or required. Furthermore, it would be interesting to elucidate whether RKD4 actively recruits and binds to REF6, ELF6 or and other JMJ-C proteins to facilitate SE or whether the interaction is passive.

The role of division within cell reprogramming raised in Zhang et al (2017) is an interesting one. It is not clear whether cell division is a requirement or a consequence of regeneration. In most studies the initiation of division accompanies the reprogramming response, whereby the regeneration stimulus activates new cell division that can replace a damaged area or further divide and grow into new tissues. Live cell tracking within a callus is difficult experientially but would be informative. Therefore, a key question remains over whether regeneration is just a process by which cell division is reactivated, leading to a erasure of cellular memory by dilution, and leaving only the daughter cells that are capable of producing new tissues. If this is the case, observation of regeneration and totipotency could be more dependent on the cells ability to re-enter the cell cycle rather than reprogram chromatin and gene expression directly.

There are some instances of direct SE where embryos develop without the need for a callus step, but it is often reported that both active and passive (through callus) somatic embryogenesis being initiated within the same plant and tissue (Horstman et al., 2017a). In root tips it was recently demonstrated that after excision of the quiescent center, the surrounding cells began to divide to replace the quiescent center rather than re-differentiate or re-establish a new stem cell niche within the remaining meristamatic tissue (Efroni et al., 2016), suggesting that re-differentiation is occurring after the initiation of cell division. As the RKD4 reprogramming system affects the same cells consistently within the root meristem it provides an interesting model in order to test this hypothesis. If new cellular divisions division can be stopped, such as with the application of a chemical such as Roscovitine or Chem7 (Cicenas et al., 2015; Nambo et al., 2016), then a period of *RKD4ox* could be induced while cell division was arrested and then both treatments could be relieved simultaneously. The results from this

experiment should indicate whether these cells are actually being reprogrammed directly or if reprogramming is achieved later on after multiple cell division cycles.

4.3.3 Role of H3K27me3 as an Epigenetic Barrier to Developmental Reprogramming

Deposition of H3K27me3 and H2AK119ub by PcG proteins was initially shown to control developmentally-regulated processes and maintain cell identity (Papp and Müller, 2006; Schuettengruber et al., 2007). Many of the gene targets of hormone and TF-mediated developmental reprogramming have been shown to accumulate these marks (Horstman et al., 2017a). In addition, double loss-of-function mutants in the PRC2 genes *CLF* and *SWN* or *VRN2* and *EMF2* lose established structures and form callus as the plants develop (Chanvivattana et al., 2004). However, the mechanistic link relationship between H3K27me3 and somatic cell regeneration is currently unclear.

This study found that mutation in *CLF* enhanced the proportion of cells within a callus that became embryonically competent, and allowed RKD4 reprogramming to override the feedback mechanisms maintaining the root and shoot meristem environments. REF6 and ELF6 activity facilitated the initial induction response to *RKD4ox* and in root organogenesis. These data provide evidence that H3K27me3 is an epigenetic brake to cellular reprogramming, that it must be removed to both allow regeneration to occur and new developmental pathways to become active.

It has previously been shown that PRC2 activity might provide a differentiation memory to prevent tissues from "slipping back" into earlier developmental programs, once cells have left the signalling environment of the meristem (Signolet and Hendrich, 2015). In double mutants between *clf* and *swn* there was no major defects during initial development or meristem patterning. However, at a late stage, endoreplicated root hairs re-initiated cell division and reverted back to an embryo-like development program, and up-regulation of genes involved in wound response and embryonic regulators, such as *LEC2* was found (Ikeuchi et al., 2015).

These data indicate that initial root hair differentiation does not depend on the activity of PRC2, but the stability of this differentiation is regulated (in part) by the activities of the PRC2 complex (Ikeuchi et al., 2015). A similar regression phenotype is seen in stomata development (Matos et al., 2014). During normal development, meristemoid mother cells self-renew in a stem cell like fashion and generate the precursors to the stomatal lineage (Bergmann and Sack, 2007; Pillitteri and Dong, 2013). These guard 'mother' cells undergo further differentiation into guard cells and this process has been shown to be mediated by the basic helix-loop-helix transcription factor FAMA (Ohashi-Ito and Bergmann, 2006). Recently it was shown FAMA interacts with RETINOBLASTOMA-RELATED (RBR) and the PRC2 complex, to aid in stabilizing guard cell identity. If this interaction was impaired, guard mother cells underwent a regression reverting back from a guard 'mother' cell precursor to the meristemoid stem cell state (Matos et al., 2014). A separate study demonstrated that H3K27 methylation of stomatal stem cell genes was mediated via FAMA (Lee et al., 2014). One potential model for this is through the interaction between FAMA/RBR as RBR proteins have been previously been shown to recruit chromatin modifying complexes to their binding regions (Burkhart and Sage, 2008; Gutzat et al., 2012). However, it has not been shown directly that that FAMA/RBR interaction plays a role specifically on H3K27 modification (Matos et al., 2014).

These studies provide strong evidence that PRC2 is able to stabilize cellular differentiation, independent of the early patterning mechanisms that establish cell fates. Therefore, in the context of this study H3K27me3 could be preventing developmental reprogramming by preventing the activation of RKD4 gene targets. Histone demethylases are required to reactivate these target genes once the *RKD4* signal is expressed. This mechanism is supported by the evidence that *LEC1* up regulation by *RKD4ox* is reduced in *ref6-5/elf6-C* and enhanced in *clf-81*. Furthermore, it was recently demonstrated that *REF6* expression becomes up-regulated in response to explants being moved onto callus induction media but *ELF6* expression was not changed during this experiment (Nakamura and

Hennig, 2017). These data fit with the observations in this study, where there may be some functional redundancy between REF6 and ELF6, however, repression of reprogramming is primarily observed or more severe in *ref6-5* compared to *elf6-C*. In recent years our understanding of the regulation of SE has increased dramatically and common elements within the gene networks identified are beginning to emerge (Braybrook et al., 2006; Stone et al., 2008; Wójcikowska et al., 2013; Horstman et al., 2017b). These findings indicate a dynamic network, in which chromatin, hormone and transcript factor pathways are all inter-regulated in order to achieve regeneration. In this study we present evidence for the role JMJ-C proteins play within this pathway, and thus add to a model for how these findings add to the literature for TF mediated SE (Fig.4.13).

However, mechanistically this relationship between H3K27me3 and regeneration may be more complicated. It has previously been shown that double mutants *clf-50/swn-1* were incapable of forming callus from leaf explants, while root explant regeneration was unaffected (He et al., 2012; Mozgová et al., 2017). The authors hypothesise that this is due to the failure of PRC2 to suppress the necessary leaf specific genes to allow re-differentiation to callus. Illustrating that some gene or gene networks may have a role positively reinforcing cell fate identity and these mechanisms are sufficient to repress cell fate changes even in a H3K27me3 depleted environment (Mozgová et al., 2017). Additionally, recent work has demonstrated that up-regulation of *CLF* occurs in the initial response to hormones in an embryonically competent genotype while non embryonic lines do not show this up-regulation (Orłowska et al., 2017). Furthermore, *REF6* over-expression does not cause loss of cell fate (Lu et al., 2011a) suggesting that the targeting of this activity determines the regeneration response. These examples and the failure of *clf-81* to affect the regeneration of other cell types and non-meristematic tissue within the *inRKD4ox* regeneration system show that chromatin remodelling in itself is not sufficient in isolation to enable cell fate transitions, even when TF drivers are expressed.

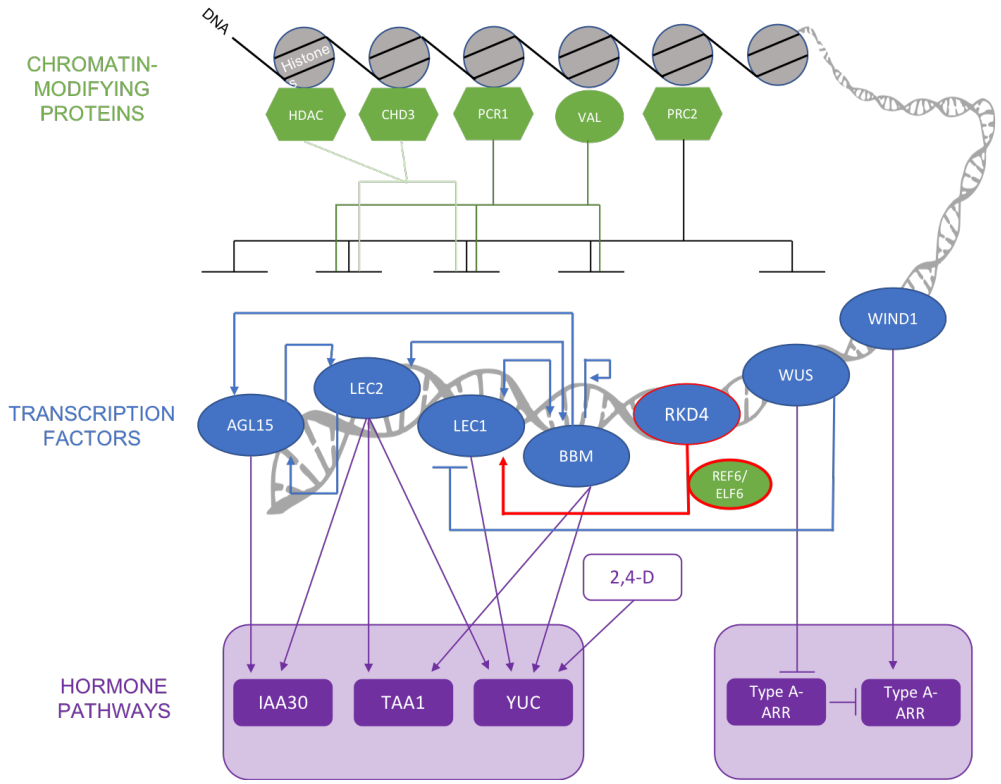


Figure 4.13: Proposed Model of RKD4 Activity Within TF Mediated Induction of Somatic Embryogenesis in *Arabidopsis*. Schematic overview summarising the molecular regulation of somatic embryogenesis in *Arabidopsis*. Chromatin modifying proteins (green) repress or facilitate activation of transcription factor (TF) expression (blue). TFs regulate expression of each other, as well as downstream targets of genes involved in auxin and cytokinin (purple). Schematic adapted from Horstman et al., 2017a. Novel contribution from the results of this study highlighted in red.

4.3.4 Cell Type Effects in Developmental Reprogramming

Recent somatic cell regeneration studies question whether the array of cells shown to demonstrate totipotency using hormone treatments truly represents the type of cells that participate in regeneration under endogenous conditions (Birnbaum and Roudier, 2017). It has been demonstrated that in callus culture some tissues were able to form callus after hormone treatment but were unable to establish new developmental fates even after lengthy culture, suggesting there is a disconnect between the actual proliferation of new cells and the ability to form new cell types (Orłowska et al., 2017; Kadokura et al., 2018). This study shows a similar disconnect, indicated by the 10 fold drop in the normalised embryo production between the two treatments. This suggests that only a subset of the callus cells are embryonically competent, while the rest actively divide and are unable to re-differentiate.

In *Arabidopsis*, hormone-induced callus from root explants were shown to originate specifically from pericycle tissue, while specific vascular cells within the leaf cambium were also competent to regenerate (Sugimoto et al., 2010; Feldman, 1976; Liu et al., 2014; Reinhardt et al., 2003). Further investigation illustrated that cells surrounding the vasculature express pericyle marker J0121, indicating that pericycle and pericyle-like cells act as the origin of callus upon application of exogenous hormones (Sugimoto et al., 2011). Although somewhat contradictory to finding of which cell types are responsible for RKD4 mediated regeneration, these results indicate that there may be a subset of cells dispersed within plant tissues that are able to fully regenerate once a regeneration signal is applied, and that it is these cells which enable plant pluipotentcy (Sang et al., 2018).

Investigation of this cell type specific hypothesis within the inRKD4ox system would with isolation of responsive cells from the epidermis, cortex and endodermial cell layers either by micro-dissection or cell sorting and investigation of the *RKD4ox* response in these separated cells. This could identify whether one of these cell types in isolation is responsible for somatic embryo production.

An alternative hypothesis is needed to explain that, Efroni et al, (2016) observed that after excision of the root tip, all cell types within the ground tissue and stele underwent cell division, and contributed to the regeneration of the root meristem. This indicates that to some degree, all cells within the root meristem do have the ability to regenerate. RKD4 reprogramming occurs in the same region of the root tip as Efroni et al, (2016) found, but cell responses were limited to the epidermis, cortex, and endodermis. These data imply that other factors are necessary to unlock plant cell regenerative capacity in a cell type, other than H3K27me3 removal, or there is a possible mechanistic distinction between pluripotency vs totipotency potential of cell types.

Ectopic *RKD4* expression causes no mechanical stress or damage to plant tissue and the application of dexamethasone does not elicit any developmental changes in the plants (Schena et al., 1991), suggesting that as regeneration systems go it is a 'light touch' approach. Regeneration is likely to be only effective in naive cell types that lack imprints that otherwise block developmental reprogramming.

In natural systems, a cell-fate switch is generally associated with biotic and abiotic stresses, and there are numerous examples demonstrating the dramatic effect that nutrient availability, pathogenic organisms, insects, or symbionts including bacteria, fungi and viruses can have on plant biology. Effects of these organisms can manifest as abnormal growths often referred to as nodules, galls or tumors each requiring the switching from one cell fate to another (Mani, 1964; Arnholdt-Schmitt, 2004; Malamy, 2005; Niu et al., 2013; Grafi and Barak, 2015). The impact of stresses, the limited cellular response to *RKD4ox*, together with the effect of Efroni et al, suggests that the control of regeneration of plant tissues is likely to require the integration of a number of stress signals such as, defence responses, tissue damage or cell death, in addition to a reprogramming signal such as *RKD4ox* or activation of *WOX* genes (Osipova et al., 2012), in order to allow regeneration pathways to become fully active in all differentiated cell types.

One factor not addressed in this study is the role that hormones play in both the

formation of calluses and regeneration to new cell fates. One advantage of the *inRKD4ox* system used in this study was the precision of the reprogramming. Regeneration systems using exongenous treatment of hormones cause a wide variety of unrelated responses, making the precise control and study of early regeneration difficult, while ectopic expression of *RKD4* allows for a targeted response. However, the role that hormones play cannot be completely excluded from the context of RKD4 mediated regeneration. Many TF-mediated regeneration systems have been shown to activate a number of auxin and cytokinin related genes, but in these cases the hormone response is downstream of TF activation (Horstman et al., 2017b). One factor that may have an important role in the activation of RKD4 mediated reprogramming could the the role of AUXIN RESPONSE FACTORS (ARFs). It has been shown that ARFs modulate cell sensitivity to auxin signals and are specifically involved in the de-compaction and chromatin acetylation required for plant cell differentiation (Li et al., 2016b). ARF7 and 19 have previously been reported to be involved in callus generation by regulating key genes *LATERAL ORGAN BOUNDARIES DOMAIN 16-18* (*LBD 16-18*) and *LBD29* (Fan et al., 2012; Tian et al., 2018). Over expression of each of these LBD genes is sufficient to induce callus without exogenous hormones, while silencing of these genes blocks callus formation (Sang et al., 2018).

ARFs are expressed widely in the root and are nuclear localised within the root tip (Okushima et al., 2007). However, these factors are exported out of the nucleus and have been shown to aggregate within the cytoplasm as root cells mature, preventing re-migration to the nucleus and limiting auxin responsiveness of the plant cells (Lucie Strader, personal communication). The presence of these aggregation bodies occurs at the transition from the meristematic to the elongation region of the root meristem, which is similar to the point at which cells become developmentally resistant to the RKD4 signal. Disruption of the ARFs by cytosolic compartmentalization could be preventing the RKD4 signal being translated into cell division and restricting subsequent reprogramming. Whether these factors are involved in the downstream responses to *RKD4ox* and compart-

mentalization acts as a second layer of control over plant cell regeneration would be an interesting avenue to explore to take this research forward. One potential way to investigate this hypothesis would be to monitor the expression of initial RKD4 gene targets within the unresponsive mature tissue, such as *LEC1*. If the blockage of downstream signalling is causing the lack of regeneration in these tissues then these genes should still be up-regulated. Assuming these genes are being activated, then identifying factors that are able to resolubilize the ARFs from the aggregation bodies, could then be used to demonstrate whether these ARFs are indeed the blocks to RKD4 mediated reprogramming in all tissues.

4.4 Summary

In summary, the findings of this study reveal that proteins involved in the regulation of H3K27me3 dynamics play a key role in facilitating plant cell redefferentiation and cell fate transition potential. These findings indicate that H3K27me3 acts as an epigenetic barrier to cell fate reprogramming on a genetic level, potentially by regulating accessibility of zygotic transcription factors such as RKD4 and LEC1. The results presented also indicate that H3K27me3 disruption is not sufficient to effect the spatial responsiveness of plant cells to a reprogramming signal, indicating that additional mechanisms are active within plant cells to suppress cell fate transitions, despite activation of a reprogramming signal.

Chapter 5

The Role of REF6 and ELF6 in Maintaining the Epigenetic Stability of the *Arabidopsis* Genome

5.1 Introduction

Inter-individual variation in chromatin states at a locus (epialleles) can result in changes in gene expression that can be transmitted across generations (Heard and Martienssen, 2014).This variation can contribute to heritable phenotypic variation in natural and experimental populations, independently of DNA sequence (Johannes and Colomé-Tatché, 2011). Recent molecular evidence shows that epialleles gives rise to a dynamic dimension in phenotypic inheritance, due to elevated levels of trans-generational instability (Rakyan et al., 2002; Mathieu et al., 2007; Johannes et al., 2009; Reinders et al., 2009). Stochastic or systematic changes in chromatin states, such as gain or loss of DNA and/or histone methylation have been previously shown to be transmitted across generations with significant phenotypic effects (Richards 2006). However, for epigenetic vari-

ation in these chromatin states to affect inheritance, mitotic propagation is not sufficient - transmission through meiosis is essential. As with genetic mutations, most epialleles are either neutral or deleterious to plant development, frequently involving the unleashing of transposable elements (Heard and Martienssen, 2014). However, trans-generational inheritance is relatively common in plants, and has the potential to be beneficial. In some cases it might enable novel responses to environmental challenges with major positive implications for heredity, breeding, and evolution (Weigel and Colot, 2012).

In principle, epigenetic inheritance and germline reprogramming are two sides of the same coin. In plants, germline cells are not specified in the early embryo, as they are in mammals, but are formed later in development from somatic cells. The projenitors of these cell, or the cells themselves have been exposed to developmental and environmental cues, meaning epigenetic reprogramming within the germline generation aids the removal of accumulated epigenetic signatures, and facilitating the totipotency of the zygote (Heard and Martienssen, 2014).

Gametogenesis in *Arabidopsis*

In *Arabidopsis*, the male and female gametophyptes are produced in the flowers, formed from the shoot apical meristem after a developmental phase transition from vegetative to inflorescence development.

The development of the male pollen occurs in the stamen; these structures are formed of four anther locules, each with a microsporagium in which the pollen grains are formed and mature (Twell, 2011). The process starts with the specification and differentiation of a pollen mother cell (Chen et al., 2010). This is perceived by the enlargement of the cell volume, along with the nucleus which is associated with a de-condensation of the chromatin (Fig. 5.1). The pollen mother cell then undergoes two rounds of meiosis to produce four haploid microspores. Each microspore then asymmetrically divides, generating a vegetative cell and a sperm cell. The sperm cell then undergoes a further round of division, resulting

in a tricellurlar structure containing a vegetative nucleus and two sperm cells (Brownfield and Köhler, 2011). Once the pollen lands on specialized cells at the top of the female reproductive structure (stigma), these pollen grains rehydrate, germinating to produce a pollen tube that enables delivery of the two sperm cells to the female gametophypte; allowing fertilization of the egg and central cell by the two sperm cells, forming the embryo and endosperm respectively (Palovaara et al., 2013).

The female gametophyte is formed within a subepidermal layer of the pistil (Fig. 5.1). Megaspore mother cells undergo meiosis to form a tetrad of haploid megaspores. In most flowering species only one of these megaspores survives to become a functional megaspore and the rest degenerate as the ovule develops (Drews and Koltunow, 2011). The surviving haploid megaspore undergoes mitosis to generate two syncytium nuclei that migrate to the polar ends of cell. These nuclei undergo a second and third round of mitosis to result in a eight-nucleate embryo sac (Yadegari and Drews, 2004). The mechanism underlying the arrangement of these nuclei is currently debated controlled either by a local gradient of auxin (Crismani et al., 2013), or by signalling peptides and auxin independent ARFs (Lituiev et al., 2013). This results in distinct cell fates established during a cellularization step, which form a mature ovule consisting of a seven-celled, 8-nuclei embryo sac containing 2 synergids, 1 egg, 1 central, and 3 antipodal cells (Crismani et al., 2013).

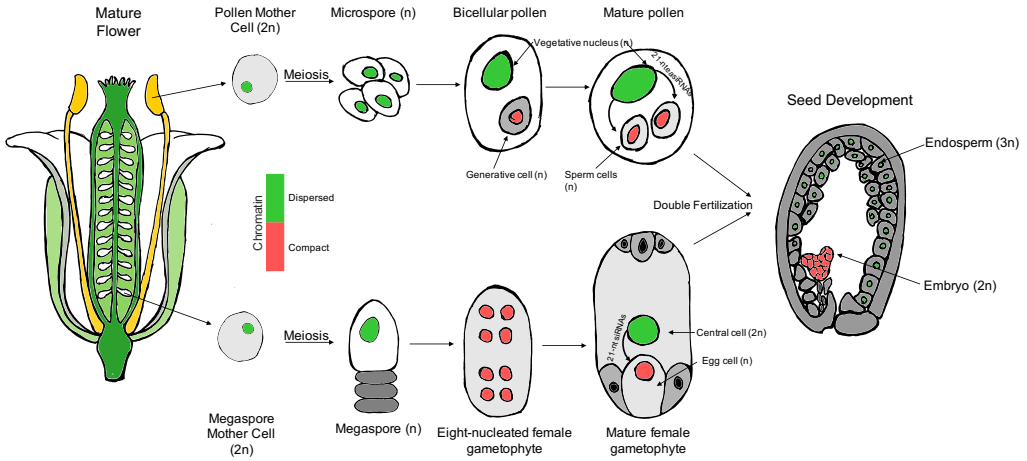


Figure 5.1: Sexual Reproduction in *Arabidopsis thaliana*. In the flower of *Arabidopsis thaliana*, pollen mother cells and megaspore mother cells are generated from somatic cells in the male and female reproductive tissues, respectively. Meiosis takes place in the pistil and stamens generating microspores from pollen mother cells and megaspores from megaspore mother cells. The microspore undergoes asymmetrical division to give rise to the vegetative cell and the generative cell. The generative cell divides further to create two sperm cells within the vegetative cell. The megaspore is subjected to three rounds of nuclear division to generate a syncytial female gametophyte with eight nuclei. Cellularization then takes place to establish the mature female gametophyte, which consists of the egg cell, the central cell and accessory cells (antipodals and synergids). The egg cell and the central cell are each fertilized by one sperm cell to produce the zygote and the endosperm, respectively. Dynamic chromatin remodelling occurs at each stage of both pollen and ovule development. Red and green nuclei indicate compact and dispersed chromatin respectively.

Chromatin Reorganization During Gametogenesis

In *Arabidopsis*, during the differentiation of the pollen mother cell, an enlargement of the nucleus is one result of large scale chromatin reorganisation. She and Baroux (2015) reported that during this differentiation there was drastic remodelling of the histone variants, particularly the linker histone H1 and the histone varient H2A.Z (She and Baroux, 2015). However, this study was unable to verify whether this histone depletion was due to the loss of H1 or the replacement with a sperm specific histone varient such as H3.10 (Ingouff et al., 2007; Okada et al., 2005). This chromatin de-condensation is associated with the transition to a more transcriptionally active state, which is supported by the reduction of repressive histone marks H3K27me1 and me3, while the permissive mark H3K4me3 is increased. This data suggests that drematic shifts in chromatin state are required to activate meiosis specific genes and transposons needed for meiotic progression (Ibarra et al., 2012; Kawashima and Berger, 2014).

After meisosis, the four microspores undergo an asymetic cell division to form the larger vegetative cell and the smaller sperm cell. These two cells differ in chromatin composition and there is evidence of cross-talk between cell types. After the initial asymmetric division, but during the binucleate stage of pollen development, the vegetative nucleus loses the centromere-specific histone varient (CenH3) resulting in the loss of centromere identity and de-condensation of the centromeric heterochromatin (Ingouff et al., 2007; Schoft et al., 2009). This de-condensation also coincides with the loss of CG DNA methylation and the downregulation of DDM1. This de-condensation of chromatin and loss of DDM1 activity within the vegetative nucleus results in the activation of transposable elements within the genome (Hirochika et al., 2000; Lippman et al., 2004; Feng and Jacobsen, 2011). Transposon activation results in the production of a class of 21-nucleotide siRNAs called epigenetically activated small RNAs (easiRNAs) that are transported to and accumulate at high levels within the sperm cells (Martinez et al., 2018).

The sperm cell nucleus does not de-condense in a similar manner to the vegetative nucleus (Kawashima and Berger, 2014). Whole-genome bisulphite sequencing profiles revealed that more than 80% of mC residues are retained in the symmetric CG and CHG methylation (mCG. mCHG) sequence contexts but asymmetric CHH methylation (mCHH) is specifically reduced (Heard and Martienssen, 2014). Studies have demonstrated that maintance of CG and CHG methylation throughout sperm cell development is reliant on two genes *METHYLTRANSFERASE 1* (*MET1*) and a SNF2 family nucleosome remodeller DECREASE IN DNA METHYLATION 1 (*DDM1*) (Johannes et al., 2009; Reinders et al., 2009). As mCHH is guided by small RNA via the RNA directed DNA methylation (RdDM) pathway, this allows for re-establishment of this epigenetic mark after fertilization (Jullien et al., 2012), when the majority of 24-nt heterochromatic siRNA are provided by the maternal genome, allowing for a mechanism of maternal and paternal inheritance (Mosher et al., 2009).

During female gametogenesis, similar to pollen mother cell formation, the formation of the megaspore mother cell is associated with visible changes in nuclear morphology, including nuclear enlargement and chromatin de-condensat-ion (Wollmann and Berger, 2012). It has been shown that these changes occur with the reduction of linker histone H1 and a possible depletion of CenH3, suggesting that both pollen and egg cells follow similar chromatin remodelling pathways required to activate meiosis specific genes (Kawashima and Berger, 2014). Consistent with the view that these cells are becoming more transcriptionally active, increases in H3K4me3 histone modification suggest a dynamic change toward a more active chromatin landscape within the megaspore mother cell (She et al. 2013). As the female gametophyte develops and becomes cellular, the central cell becomes hypo-methylated in comparison to the egg cell in a number of plant species tested so far including *Arabidopsis*, rice, maize, and castro bean (Xu et al. 2016; Gehring et al. 2009; Hsiehet al. 2009; Lauria et al. 2004; Zemach et al. 2010). This is thought to be principally caused through the transcriptional repression of DNA methyl-transferases and activity of DME in the central cell

(Gutierrez-Marcos and Dickinson, 2012) and displays a de-repression of transposable elements, resulting in the production of siRNAs that is believed to affect methylation within the egg cell (Ibarra et al., 2012; Kawashima and Berger, 2014).

In contrast to the mechanism of DNA methylation inheritance, the erasure or inheritance mechanisms of histone modifications during gametogenesis are not well understood. While histone modifications such as H3K27me3 have been shown to be mitotically heritable (Jiang and Berger, 2017; Carter et al., 2018), inheritance through meiosis has not been directly demonstrated in plants. The repression of *FLC* expression provides evidence that histone modification is active within gametogenesis (Crevillén et al., 2014). The vernalized state occurs during exposure to cold, caused by accumulation of H3K27me3 at the *FLC* gene locus. This causes a repressive gene state that is maintained mitotically after returning to warmth in the spring (De Lucia et al., 2008; Song et al., 2012; Crevillén et al., 2014; Tao et al., 2017, and many others). However, the vernalized state is consistently reset through gametogenesis, requiring another cold period to again repress *FLC* and induce flowering in progeny (Song et al., 2012). It has been shown that re-activation of *FLC* occurs within one day after pollination, indicating the inheritance of a *FLC* gene lacking the repressive H3K27me3 state (Tao et al., 2017). In addition, it has been shown that mutation in the histone demethylase *ELF6* leads to the partial inheritance of H3K27me3 at the *FLC* locus in the embryos (Crevillén et al., 2014). However, recent studies have indicated that ELF6 is not wholly responsible for resetting of the parental vernalized state at *FLC* in offspring (Tao et al., 2017). It was shown that ELF6 has partial redundancy with two other proteins, REF6, and JMJ13, in removing H3K27me3 in *Arabidopsis* flowers (Yan et al., 2018). These data place JmJ-C proteins as good candidates for the active erasure of histone methylation during gametogenesis.

5.1.1 Experimental Rationale

In summary, epialleles can result in changes in gene expression that can be transmitted across generations. Therefore, failure to correctly 'reset' or maintain epigenetic patterns can lead to a wide variety of developmental impacts. Recent data indicates that JmJ-C proteins may have a role in the reprogramming of histone modifications during gametogenesis. However, the consequences of failing to correctly reset repressive histone modification H3K27me3 during gametogenesis remains largely unknown. This study aims to test the role of REF6 and ELF6 in maintaining the correct epigenetic pattern during sexual reproduction using *Arabidopsis thaliana* as a model system.

5.2 Results

5.2.1 Aberrant phenotypes Arising from *ref6-5/elf6-C*

Trans-generation accumulation of histone modifications negatively affected the growth of *Caenorhabditis elegans* (Greer et al., 2014), suggesting that histone remodelling plays an important role during gametogenesis. However, the molecular components that ensure the correct inheritance of these modifications are still poorly understood. Reciprocal genetic crosses between *ref6-5/elf6-C* and wild type plants, produced plants with unexpected phenotypes, which were not found in either of the single mutants*ref6-5*, *elf6-C* or the double mutant *ref6-5/elf6-C* in the F2 generation.

To assess the generation frequency of aberrant phenotypes, ten independent reciprocal crosses (five with male and five with female contribution from *ref6-5/elf6-C*) were performed between *ref6-5/elf6-C* and WT plants. In all cases, F1 hybrid plants were indistinguishable from wild type. Approximately 1500 F2 progeny from the F1 hybrids were grown (approximately 300 seed contribution from each independent cross). It was found that 4.5% of the progeny displayed aberrant developmental growth defects, which were not found in either of the single mutants*ref6-5*, *elf6-C* or the double mutant *ref6-5/elf6-C* (Fig.5.2). Development of these phenotypes occurred independent of their parental origin (4.65 and 4.42% Table:5.1), suggesting that inheritance occurred through male and female gametogenesis.

Table 5.1: Frequency of Aberrant Phenotype Occurrence After *ref6-5/elf6-C* Crosses

Cross Contribution	Aberrant Phenotypes isolated (%)	Total Number Screened (plants)
WT ♂ x *ref6-5/elf6-C* ♀	69 (4.42)	1562
ref6-5/elf6-C ♂ x WT ♀	73 (4.65)	1569

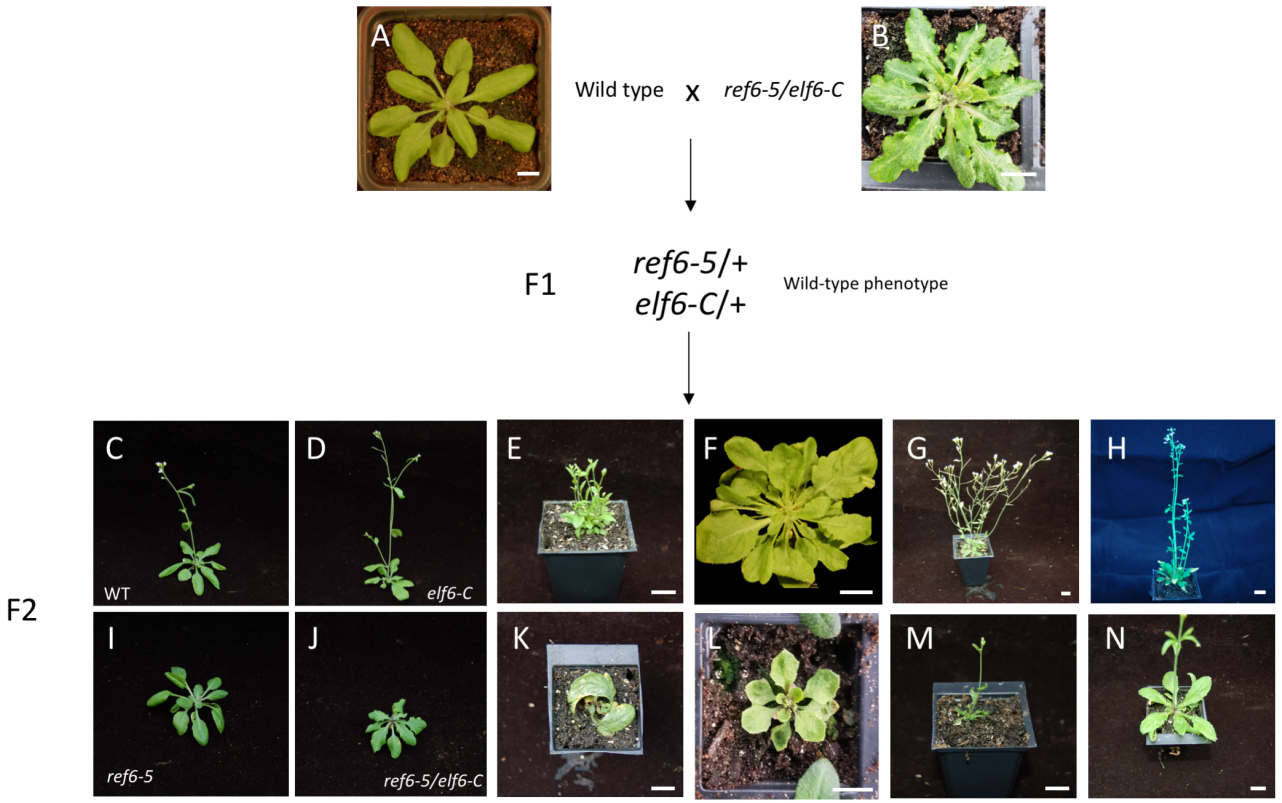


Figure 5.2: Aberrant phenotypes introduced during crosses between WT and *ref6-5/elf6-C*. Schematic of reciprocal crosses undertaken, representative pictures of single and double mutations (C,D,I,J), and aberrant phenotypes occurring in F2 (E,F,G,H,K,L,M,N). White bars =1cm

Aberrant plants isolated in the F2 population from this screen included a range of phenotypes. These included loss of apical dominance where plants had abnormal phyllotaxy and produced multiple inflorescences simultaneously (Fig.5.2, E and G). Production of enlarged rounded leaves, delayed flowering, and much reduced fertility (Fig.5.2 F). Inflorescence fasciation leading to increased flower production (Fig.5.2 H). Distinct leaf morphology variants (Fig.5.2 K and L), plant dwarfing associating with early flowering (Fig.5.2 M), and abnormal inflorescence phyllotaxy with several cauline leaves associated together (Fig.5.2 N).

These aberrant phenotypes varied within progeny from a single crossing event, or from the same parental F1 plant. This data suggests the potential for multi-gene involvement, leading to disruption of a number of regulatory pathways.

To screen out aberrant phenotypes induced by environmental factors, seed was collected from all aberrant phenotypes (n=142). 24 plants from each line are being grown on soil to investigate inheritance of the parental phenotype, and samples will be taken to determine genetic background of the isolated lines. Preliminary data from this screen indicates that these phenotypes are being introduced in wild-type and mutant background lines.

5.2.2 Characterisation of an Aberrant Phenotype

We selected a plant that displayed enlarged rounded leaves, delayed flowering, and much reduced fertility (Fig.5.3 B), despite carrying wild type alleles for *REF6* and *ELF6* (referred to as A5). This lines was used to explore the reason for this novel growth phenotype arising in JmJ-C crossed F2 populations.

A5 produced 23 viable seeds and single seed progeny was propagated for two generations by self-fertilisation. Segregating phenotypes were observed in the progeny, but following non-Mendelian inheritance and being comprised of a wide range of developmental abnormalities (Fig.5.3). Specifically, the 23 viable progeny presented with a spectrum of defects ranging from more wild-type like features

(frequency 5/23, Fig.5.3, C), to A5-like leaf development but lacking the infertility of the previous generation (frequency 3/23, Fig.5.3, D), elongated leaves with late flowering phenotypes (frequency 4/23), bushy leaves phenotype producing numerous small rosette leaves with highly infertile inflorescences producing leaves rather than flowers (frequency 1/23, Fig.5.3, E), and a curled leaf phenotype which was highly infertile also producing leaves rather than inflorescences (frequency 10/23, Fig.5.3, F-H).

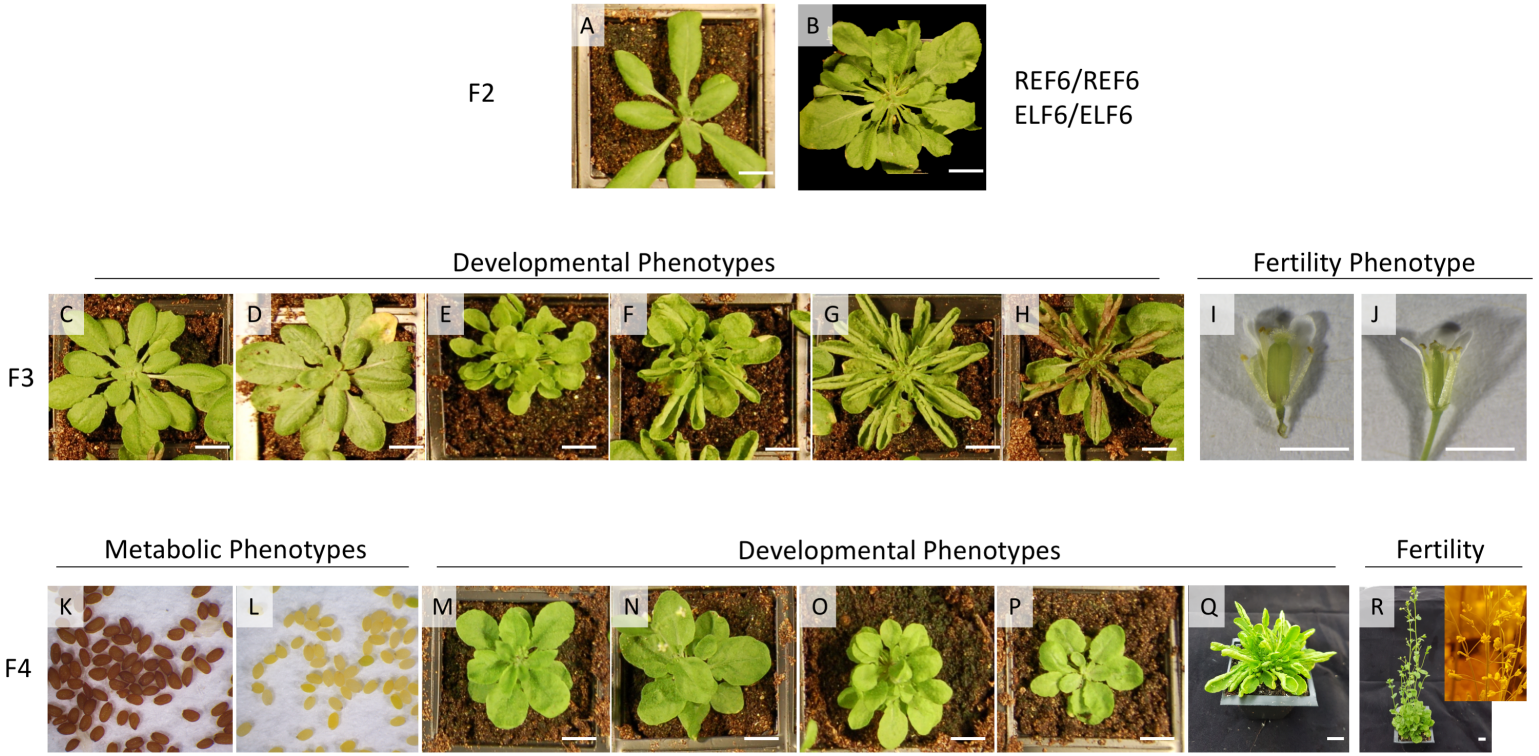
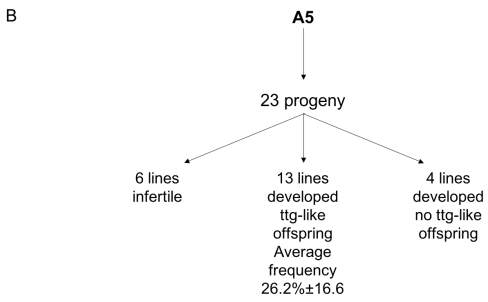


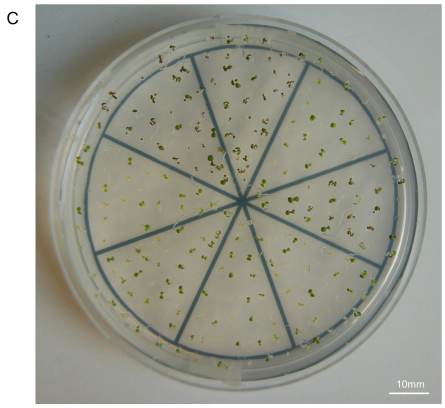
Figure 5.3: Phenotypic variation of aberrant line A5. Wild type (A), A5 phenotype occurring in F2 population after cross between *ref6-5/elf6-C* and wild type (B), Developmental phenotypes segregating within F3 population of A5 progeny: wild type-like (C, D), bushy (E) curled leaf phenotypes (F,G,H) flower phenotype of infertile lines (I) wild type flower (J), Wild-type seed (K), *ttg*-like seed phenotype (L), rounded leaf (M), A5-like (N,P), bushy (O), curled leaf (Q,R).

This segregation within and between generations meant that the phenotypes were very difficult to predict, and it was not possible to group plants from one generation to the next. Therefore, third generation plants were grown from all available lines, to determine if any of the observed phenotypes became more stably established.

In the third generation one phenotype emerged in 13 of the 23 progeny lines, independently of parental phenotype. These plants were unable to produce seed pigmentation giving rise to yellow seeds (Fig.5.3, L), similar to those described in the *TRANSPARENT TESTA* (*TT*) and *TRANSPARENT TESTA GLABRA* (*TTG*) gene mutants, which are impaired in flavonoid accumulation (Bowerman et al., 2012). This phenotype (referred to as *ttg*-like) in each line presented with an average segregation of 26.2%±16.6 S.D (n=477) indicating a single gene was responsible acting as a recessive allele somewhere within this pathway (Fig.5.4, B).

The testa (seed colour) is maternally inherited, therefore all seed produced by a *tt* or *ttg* mutant is deficient in pigmentation. One assay for the accumulation of flavonoid components in young seedling is the germination of seed on 3% sucrose media, resulting in germinating plants that present with anthocyanin accumulating in the hypercotyl and cotyledons (Fig.5.4, A) (Solfanelli, 2006). This allows for the *ttg*-like phenotype of progeny to be rapidly screened before seed production. Progeny from five *ttg*-like lines were tested using this assay, and were found to be unable to generate pigment in all cases (Fig.5.4, B - four biological replicates, n=30), indicating a stably inherited homozygous allele is responsible for this phenotype early in the pathway.





D

***ttg*-like Parental Line and Cross direction**	**F2 *ttg*-like frequency (%)**	**Total number screened (plants)**
A5.B1.4.D4 ♂ x WT ♀	14.3	112
A5.B1.4.B4 ♀ x WT ♂	25.7	113
A5.B1.4.B3 ♂ x WT ♀	18	111
A5.B1.3.B1 ♀ x WT ♂	24	129
A5.B1.3.B1 ♂ x WT ♀	25.1	430
A5.A4.3.B4 ♀ x WT ♂	19	142

Figure 5.4: Analysis of a *ttg*-like Phenotype Occurring Within Line A5. Anthocyanin accumulation after germination on 3% sucrose in wild type (A left) and *ttg*-like phenotpe plants (A right) inserts show seed colour of imaged plants (A). Schematic of *ttg*-like phenotype emergence within the A5 progeny (B), Consistent presentation of *ttg*-like phenotype in progeney after germination on 3% sucrose (C), F2 inheritance of *ttg*-like phenotype after back crosses between wild type and *ttg*-like parental plants (contribution through male and female gametes indicated) (D).

To determine whether the *ttg*-like allele could be stably inherited though back-crossing, five *ttg*-like plants from the third generation were reciprocally crossed to control (*inRKD4ox*) plants. F1 hybrids from these crosses were indistinguishable from wild-type including in seed pigmentation. Screening of F2 population using the anthocyanin assay showed the *ttg*-like phenotype segregated on average 22% of the offspring, independently of parental origin (Fig.5.4, D). These data demonstrate that the *ttg*-like phenotype was able to be stably inherited through back crossing acting as a recessive allele.

5.2.3 Aberrant Phenotype Methylation Analysis

As the developmental phenotypes within the A5 population were varied, and segregating from generation to generation, determining the nature of these phenotypes was difficult. The non-Mendelian inheritance of phenotypes between parent and offspring suggested that either multiple genomic mutations were introduced, which is unlikely, or non genetic variation in chromatin was the cause. The nature of the causal mutations in *ref6-5/elf6-C* would suggest that the histone profile of H3K27me3 would be a plausible candidate. However, these phenotypes were inherited from generation to generation, even after REF6 and ELF6 functionality was restored. This indicated that, if the phenotypes were caused by histone modifications directly, they should have been erased once the plants underwent a normal reproductive cycle in F1 (Tao et al., 2017). Therefore, another epigenetic mark is likely to be responsible for the phenotypes observed. One of the most well described heritable epigenetic modifications in plants is DNA methylation and it has been shown that methylation patterns are stable from one generation to the next (Johannes et al., 2009; Reinders et al., 2009). Therefore, bisulphite sequencing was used to identify potential causes of the phenotypic variation.

Four individuals were selected from the A5 line in the F4 generation, from differing parental plants. Three of the individuals selected developed the curled leaf phenotype (Fig. 5.3 Q, labelled Epi 1, 2 and 3), while the fourth was late flowering

and had bushy leaves (Fig 5.3 O, labelled Epi 4); in addition a WT (inRKD4ox) control was used. DNA was extracted from rosette leaves and bisulfite sequencing libraries prepared. Libraries were sequenced and the reads were aligned to the reference genome (TAIR10) providing a tenfold coverage of the genome in all samples. Methylated cytosines were identified and differentially methylated regions (MRs) were mapped using 'methyScore' (Unpublished, Computomics Tubingen).

Analysis found 30,316 differentially methylated regions (DMRs) between all samples, occurring in a range of contexts, but with a preference for CG sites (CG: 12,090, CG/CHG: 1,951, CG/CHG/CHH: 6,853, CG/CHH: 2,586, CHG: 1,112, CHG/CHH: 961, CHH: 4,763). Principle Component Analysis (PCA) of the DMRs showed clustering of the samples dependent on phenotype with Epi 1, 2 and 3 clustering together, with Epi 4 grouping separately in CG, CHG and CHH contexts (Fig.5.5). The observed variance in the CHH context was reduced (Fig.5.5 D) potentially indicating a replacement of hypo-methylation in this context. These data suggests that the phenotypes observed may be correlated to the DMRs in these lines.

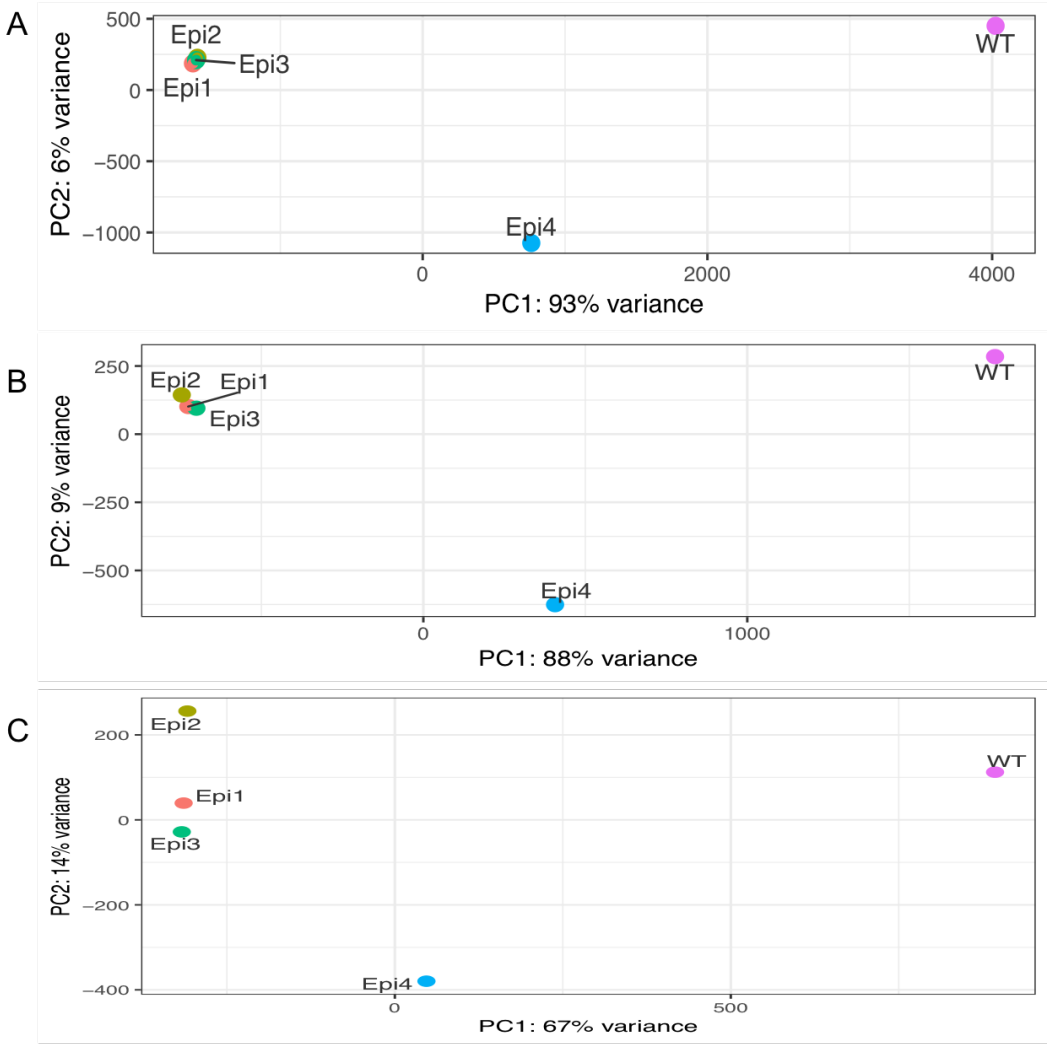


Figure 5.5: Principle Component Analysis of Differentially Methylated Regions. PCA of differentially methylated regions in CG (A) CHG (B) and CHH (C) sequence contexts between WT, Epi1, Epi2, Epi3 and Epi4 samples.

The DMRs observed occurred mainly in a hypo-methylated context in all Epi lines, with hierarchal clustering of the samples grouping with the observed phenotype (Fig.5.6). Analysis of Epi 1, 2, 3 and 4 samples showed reduction of methylated cytosines of 77%, 75%, 72% and 30% respectively compared to the WT sample (Fig.5.6, Fig.5.7 B). Interestingly, despite the reduction in global levels of DNA methylation, Epi 4 had an increased number of methylated regions compared to wild type (51,431 compared to 47,996 respectively) indicating a fragmentation of methylated regions across the genome in this sample.

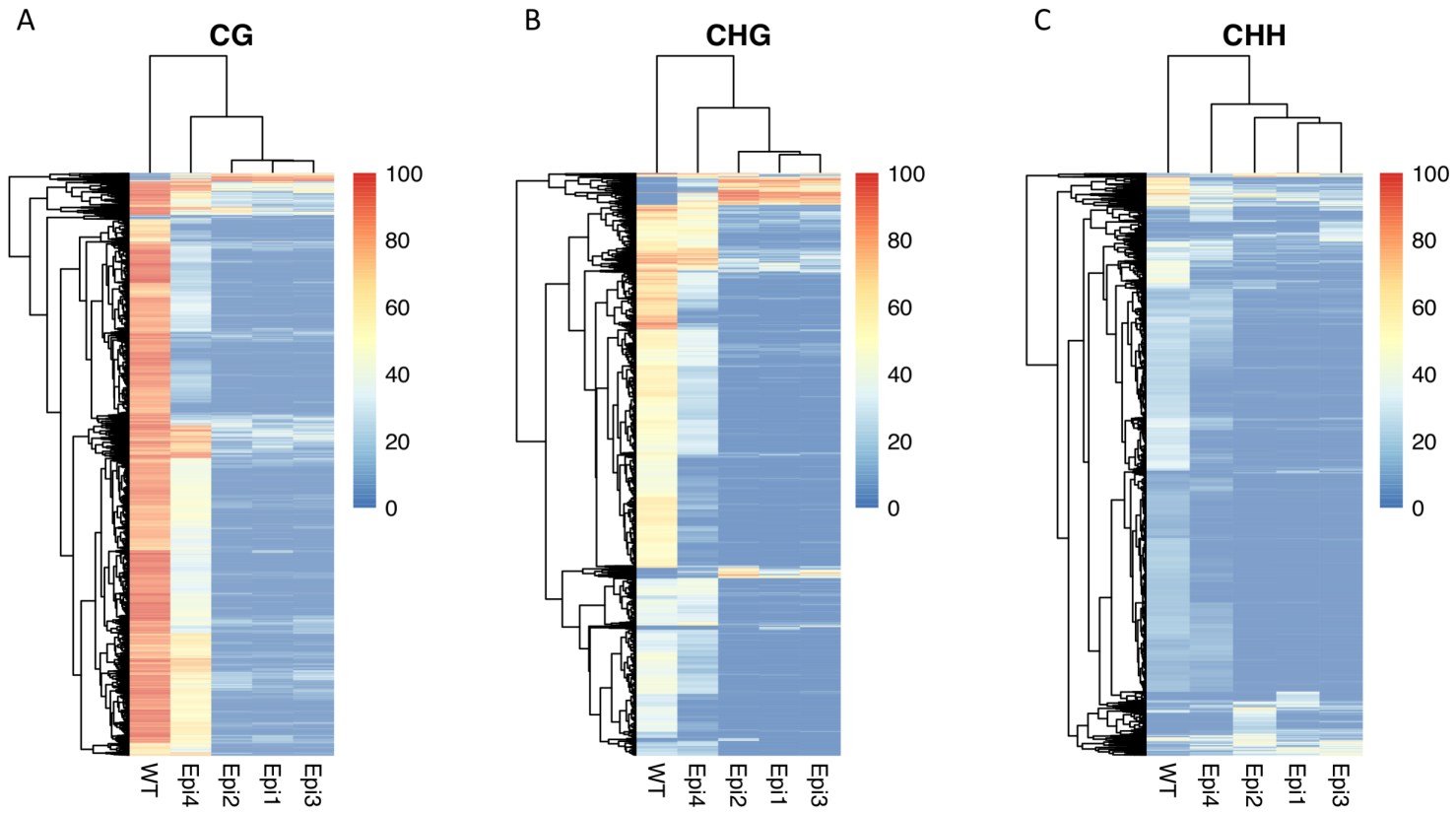


Figure 5.6: Hierarchal Heatmap analysis of differentially methylated regions (DMRs). Hierarchal Heatmap of all DMRs in CG (A) CHG (B) and CHH (C) sequence contexts between WT, Epi1, Epi2, Epi3 and Epi4 samples. Hierarchal clustering represents euclidean distance. Colours represent methylation percentage at each DMR.

Remaining MRs in the Epi samples were similarly biased for all genomic features compared to wild type (Fig.5.7 A) indicating that methylation at particular genomic features such as genes are not being specifically targeted. Similarly, DMRs between samples were not biased for any particular genomic features such as transposons (Fig.5.7 A). This indicated that the hypo-methylation being introduced was unbiased and genome wide, contrasting with hypo-methylation induced by biotic or abiotic stresses which often show enrichment for gene rich regions within the genome in the observed DMRs (Dowen et al., 2012; Wibowo et al., 2016; Hewezi et al., 2017).

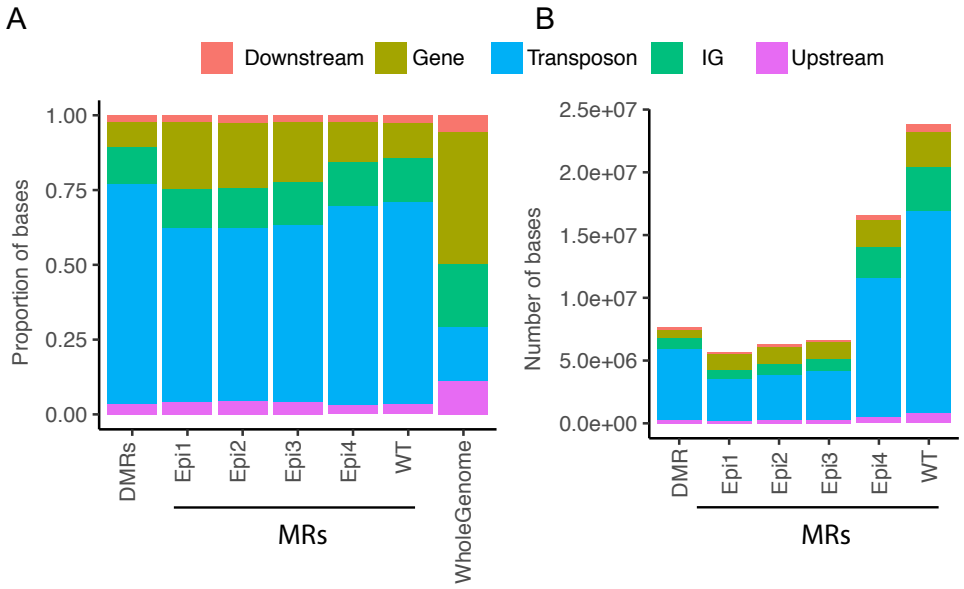


Figure 5.7: Distribution of Methylated Regions within Genomic Features. Proportion of methylated regions (MRs) within genomic features were equally biased in WT and Epi samples, with no feature enrichment in DMRs between samples (A) Number of methylated bases across the genome in samples (B) in WT, Epi1, Epi2, Epi3 and Epi4 leaf samples showing a reduction in DNA methylation of 77, 75, 72 and 30%, respectively compared to WT. IG= Intergenic

Although preliminary, these data shows genome wide hypo-methylation is likely introduced by crosses with *ref6-5/elf6-C*, Thus, these data indicates that removal of the histone modification H3K27me3, by REF6 and ELF6, is likely required for the correct maintenance of the genome wide methylation patterns through sexual reproduction in *Arabidopsis thaliana*.

5.3 Discussion

5.3.1 JmJ-C Histone Demethylases Protect the Genome from Epimutations

Previous studies have demonstrated that H3K9 methylation is required for CHG methylation (Law and Jacobsen, 2010), and reduction of DNA methylation is associated with gains in H3K27me3 and H3K4me3 in both plants and animals (Hon et al., 2012; Weinhofer et al., 2010; Zhang et al., 2009). Specific modification of the histone profile by mutants in *sdg8* (reduced H3K4me3 (Xu et al., 2008)), *atxr5/6* (reduced H3K27me1 (Jacob et al., 2009)), *sdg2* (reduced H3K4me3 (Berr et al., 2010)) and *ref6* were shown to have no significant impact on DNA methylation (Stroud et al., 2013b). This suggests that either REF6 and ELF6's role in DNA methylation was being masked by the less severe allele of *ref6*, or by redundancy between REF6 and ELF6. Data presented in this study provides evidence that double mutants in *ref6-5/elf6-C* can introduce epimutations. These epimutations can (in some cases) involve DNA methylation, and are heritable once *REF6* and *ELF6* are restored.

While it is clear epialleles are being introduced by these crosses, the mechanism behind how the histone environment introduces these changes to the methylation profile is not. *REF6* and *ELF6* are commonly expressed through most plant tissues including the leaf, root, inflorescence, and flowers (Noh et al., 2004; Yan et al., 2018) and ELF6 is highly expressed from the torpedo to green cotyledon stage during embryo development (Winter et al., 2007). Thus, the hypo-methylation observed in the A5 epimutant could be being introduced throughout the plants life cycle rather than at a specific point.

There are two main mechanisms that could explain the epigenetic variation observed. Firstly, epialleles could be introduced in *ref6-5/elf6-C* throughout development, and that these epi-imprints are stably inherited, through mitosis and meiosis, similarly to epigenetic imprints induced by environmental stresses

(Hauser et al., 2011; Paszkowski and Grossniklaus, 2011). Alternatively, REF6 and ELF6 may be involved in the epigenetic reprogramming of H3K27me3 during gametogenesis, leading to epigenetic changes in gametes that are transmitted to offspring.

In *Arabidopsis*, the hypo-methylation of the genome, such as observed in *met1* line leads to severe developmental defects and delayed flowering (Saze et al., 2003; Johannes et al., 2009; Zhang et al., 2018). However, in *ref6-5/elf6-C* lines grown from the same generation, leaf morphology and flowering time was consistent between individuals, varying only by 2 days across the population (Chapter 4). This suggests that the inter-individual variation is either established early on in development, so would be present in all soma cells (which may also be partially responsible for the novel phenotype of the *ref6-5/elf6-C* mutant), or are asymptomatic in most cases. In addition, it is not clear whether all epialleles introduced by *ref6-5/elf6-C* are of hypo-methethylation types. However, the increased susceptibility to somatic regeneration by *RKD4ox*, observed in the second generation of *ref6-5/elf6-C* lines, would be consistent with the genome becoming hypo-methylated (Chapter 4), as this has been previously shown to increase the susceptibility of plant tissue to regeneration using hormones (De-la Peña et al., 2015; Shemer et al., 2015).

If these epialleles were solely being introduced in the soma then you could expect the inheritance within a selfed line to be relatively consistent, leading to a gradual decrease of DNA methylation from one generation to the next. However, the effect on regeneration observed in was variable, suggesting that methylation changes are being introduced to a greater extent within some progeny than others. Additionally, the crosses between wild type and *ref6-5/elf6-C* took place within the same generation of the plants becoming homozygous for both mutations. Thus, the hypo-methyation observed in epimutants analysed, would need to be present in the *ref6-5/elf6-C* parent, without additional developmental defects, to have been inherited by epimutant A5. These data suggests that reprogramming of the DNA methylation within the gametes, or after the developmental phase

transition to the inflorescence meristem is the most likely source of this variation.

In order to separate these two potential sources of epigenetic variation, crosses should be carried out between plants heterozygous for both REF6 and ELF6 mutations. These plants would generate 1/4 pollen that was homozygous for both *ref6* and *elf6* while the soma tissue have functional copies of both proteins. If the resultant plants developed hypo-methylated regions then it would demonstrate that these changes were introduced directly in the gamete lineages and not in the somatic cells.

The near 25% inheritance of the *ttg*-like phenotype between the progeny of the A5 epimutant, and after the backcross suggests the epiallele responsible is behaving similarly to a genetic mutation. While this phenotype could have been introduced by a transposable element (TE) insertion, molecular analyses of epigenetic recombinant inbred lines (epiRILs) (Johannes et al., 2009; Reinders et al., 2009), show that a substantial fraction of the DNA methylation variation created through recombination was inherited in a Mendelian manner, and could persist through 8 generations (Johannes and Colomé-Tatché, 2011; Colome-Tatche et al., 2012; Cortijo et al., 2014). Additionally, within the F2 populations screened the frequency of *ttg*-like phenotye inheritance was usually under 25%, suggesting that some reversion to WT phenotype may occurred, but further generational observation in these *ttg*-like lines are needed.

5.3.2 Origin of DNA Hypomethylation Arising in JmJ-C mutants

As REF6 and ELF6 are not reported to interact with methylation directly (Stroud et al., 2013b), the mechanisms behind the introduction of hypo-methylated regions remains unclear. However, these changes could be hypothesised to be introduced by three mechanisms.

The first uses an observation that H3K27me3 is thought to be anti-correlated with

DNA methylation (Mathieu et al., 2005; Aichinger et al., 2009; Dong et al., 2012; Roudier et al., 2011). REF6 and ELF6 have been implicated in the setting of spacial boundaries of H3K27me3 deposition within the genome. Yan et al (2018) found that many of the genes accumulating H3K27me3 within a JmJ-C triple mutant of *REF6*, *ELF6* and *JMJ13* were not true targets of PRC2 in embryos, seedlings or inflorescences; meaning that, the effects of the spreading H3K27me3 across the genome would not be limited to PcG targeted genes and thus could affect a wide variety of genome regions. However, explicit anti-correlation of this interaction has not been demonstrated and in some systems this relationship is not upheld, for example in rice it has been shown that non-CG methylation and H3K27me3 at target loci are both required to suppress developmental genes (Zhou et al., 2016) and recently, in Arabidopsis it was shown that H3K27me3 is present at densely DNA methylated pericentromeric regions of the endosperm, indicating that H3K27me3 and DNA methylation are not necessarily exclusive marks in some contexts (Moreno Romero et al., 2016).

Therefore, the accumulation and spreading of H3K27me3 within *ref6/elf6* could potentially displace DNA methylation directly. Or, it could displace other epigenetic marks such as H3K9me2 or H3K4me3, which are closely correlated with DNA methylation and are involved in the maintenance during replication (Du et al., 2015). However, this mechanism would be a gradual dilution of DNA methylation from generation to generation and therefore does not fit with the high levels of hypo-methylation observed in the epimutants analysed. In addition, if these levels of hypo-methylation were possible within a generation you would expect a greater incidence of epimutations within the progeny derived from these parental lines rather than the consistent 4% frequency observed in the F2.

The second mechanism by which *ref6-5/elf6-C* could introduce the observed methylation changes, would be suppression of genes responsible for establishment or maintenance of DNA methylation by the accumulation or spreading of H3K27me3.

Hypo-methylation similar to that observed in this study, can be induced within *Arabidopsis* (Kakutani et al., 1999; Saze et al., 2003). Epigenetic recombinant inbred lines (epiRILs) are plants which have almost identical DNA sequences, but segregate many differences in DNA methylation and thus gene expression (Johannes et al., 2009; Reinders et al., 2009). Progeny of these these lines are capable of inheriting these differences independently of the causal mutation for many generations and have been used to study the effects of the methylome on phenotype (Cortijo et al., 2014). These epimutants can be created by single mutations in key genes involved in DNA methylation maintenance such as MET1 and DECREASE IN DNA METHYLATION 1 (*DDM1*). These mutations result in genome wide hypo-methylation (Johannes et al., 2009; Reinders et al., 2009), particularly at CG sites, similar to those observed in this study, leading to the release of transcriptional gene silencing and silencing of transposable elements (TEs). This would result in the mobilisation of TEs which could insert within genes, preventing correct transcription. These data suggests that this model is a good fit for the observed phenotypes produced and that the mechanism could be achieved with single gene suppression by H3K27me3 spreading.

However, there was no significant accumulation of H3K27me3 at genes previously linked to maintenance of DNA methylation: *MET1*, *DDM1*, *CMT2*, *CMT3*, *VIM2* and further candidates *HISTONE DEACETYLASE 6 (HDA6)* of which mutation leads to high acetylation of histone H4, increased methylation of histone H3 Lys-4 and hypo-methylation of DNA (Aufsatz et al., 2002); and *DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2)* which facilitates *de novo* methylation through the RdDM pathway (Data from Yan et al, 2018). Although, a slight increase is seen in VIM1 gene meaning that if this is the source of the hypo-methylation, repression may be occurring stochastically, within a specific tissue or stage of gamete development, but may be an interesting target for future work.

Another member of the JmJ-C protein family (JMJ14) has been found to be a H3K4me2/me3 demethylase, and is specifically involved in the maintenance phase

of DRM2-mediated RNA directed DNA methylation (RdDM) in non-CG contexts (Deleris et al., 2010; Searle et al., 2010; Greenberg et al., 2013). Other histone demethylases lysine-specific demethylase 1-like 1 and lysine-specific demethylase 1-like 2 (LDL1 and LDL2) were also shown to function in the RdDM pathway (Du et al., 2015). Although not closely releated to REF6 and ELF6 (Yan et al., 2018), this could potentially indicate wider functionality of REF6 and ELF6 in the contexts of other chromatin remodelling, but would not explain the preference for CG hypo-methylation within the A5 epimutant line.

The third mechanism by which epialleles could be introduced is through disruption of the histone remodelling occurring during gametogenesis. While the incorporation of germ cell specific histone variants is known (Ueda and Tanaka, 1995; Ueda et al., 2000), the specific role of histone modifications throughout both male and female gametogenesis is not well understood. In *Arabidopsis* a H3 variant, HTR10 (H3.10) has been shown to be specifically expressed in the germline and mature sperm cells (Ingouff et al., 2007; Okada et al., 2005), and is thought to be required to establish a sperm cell–specific transcriptome, initiated in the generative cell (Ingouff and Berger, 2010). However, the sperm-specific histones have been shown to be actively removed at fertilization, which leads to reprogramming of the composition of the zygotic chromatin (Kawashima and Berger, 2014).

The functional role of incorporation of histone variants during gametogenesis is currently an active area of research. It was recently shown that during pollen development the levels of H3K27me3 decreased to almost undetectable levels in mature pollen, which is hypothesised to allow the activation of pollen specific genes (Unpublished, Frederic Berger personal communication). This H3K27me3 reduction is thought to be be achieved by a multifaceted mechanism including the incorporation of the histone variant H3.10 which is biochemically incapable of being methylated at this site (Unpublished, Robert Martienssen personal communication), down regulation of the PRC2 complex which is not expressed during gametogenesis and the active removal of H3K27me3 by the proteins REF6, ELF6

and JMJ13 (Unpublished, Frederic Berger personal communication).

Exactly, why H3K27me3 is depleted through pollen development is unknown as PRC2 is dramatically up-regulated during embryogenesis, re-establishing the mark quickly after fertilisation (Kawashima and Berger, 2014). Failure to sufficiently erase this mark has dramatic effects on development, with double mutants of *HTR10* and *ELF6* producing an embryo lethal phenotype when inherited (Unpublished, Robert Martienssen personal communication). One current theory is that preservation of H3K27me3 could impact the nuclear de-condensation occurring within the vegetative nucleus, disrupting the activation of TEs and the sperm cell–specific transcriptome. This could disrupt the chromatin reprogramming, such as, production of easiRNAs which accumulate within the sperm cell and are thought to have a protective role, suppressing transposons within the CHH depleted sperm (Slotkin et al., 2009; Martinez et al., 2018). *ref6-5/elf6-C* could be allowing a proportion of H3K27me3 to be retained in the germ cells, providing a weaker phenotype to that of *elf6/hrt10*, causing missexpression of key developmental genes or TE mobilisation resulting in epiallele introduction in the embryo.

On the weight of the evidence and the genome wide, multi-context, nature of the hypo-methylation observed, the third mechanism discussed is the most likely source of this variation. Determining the exact mechanism behind the generation of these phenotypes would not be an easy task. Further investigation of the cause of some of the resultant phenotypes for example the ttg-like phenotype could provide a potential insights into the nature of the epialleles; whether they were caused by TE insertions or methylated alleles. Determining the H3K27me3 accumulation/distribution, as well as the methylation profile within the double mutant pollen would be important evidence to support the third mechanism as a viable option for the source of the variation.

5.4 Summary

In summary, this study demonstrated that double mutants in *REF6* and *ELF6* induce epiallelic variation, including genome-wide DNA hypo-methylation. These epialleles are heritable, independent of the *REF6* and *ELF6* mutations, and stably inherited after back crossing. These data postulate a protective role of histone demethylases in maintaining the epigenetic stability of the genome through sexual reproduction in *Arabidopsis thaliana*.

Chapter 6

General Discussion

Regeneration requires the ectopic activation of intrinsic developmental programs, in response to external stimuli. These responses require context-dependent integration of both environmental and developmental signals, leading to diverse strategies and efficiencies of regeneration (Ikeuchi et al., 2016). Cellular regeneration can range from the repair of a small wound to the formation of new organs or individuals, and the mode of regeneration varies markedly among taxa (Birnbaum and Sánchez Alvarado, 2008). As muticellularity evolved, so did the specialisation of cell types able to carry out distinct functions to the benefit of the organism. As increasing specialisms occurs, organisms require mechanisms for the stabilization of gene networks, often occurring at the expense of transcriptional plasticity. This phenomena occurs throughout the plant and animal kingdom, and can also be found in prokaryotes. A α-proteobacterium (*Caulobacter crescentus*) divide asymmetrically to produce a motile swarmer cell, and a sessile stalk cell. Replication of the motile cell is restricted, under the control of CtrA which binds to the origin of replication (Domian et al., 1997; Quon et al., 1998), under favourable environmental conditions CtrA is dephosphorylated and degraded mediating a cell fate change to a reproductively competent stalk cell, allowing colonisation of the new environment (Domian et al., 1997; Bastedo and Marczynski, 2009).

As organisms evolved in complexity a gradual shift in regenerative capacity can be observed, from organisms that can readily regenerate to those that can not. For example, multicellular algae, sponges, bryophytes and mosses, which contain limited number of cell lineages but can regenerate whole tissues or organisms without external hormone applications (Hoppe, 1988; Mandoli, 1998; Duckworth et al., 2003; La Farge et al., 2013); to a point where, in ferns, explants of the gametophyte retain the ability to regenerate in hormone free medium (Banks, 1999; Kaźmierczak, 2003; Menéndez et al., 2009; Abul et al., 2010; Somer et al., 2010), while tissue from the sporophyte are recalcitrant to regenerate an entire organism when no external hormones were supplied (Fernandez et al., 1993; Fernández et al., 1997). A similar reduction in regeneration capacity is seen in animals where metazoans, including earthworms, snails, and salamanders can regenerate but this is limited to specialised stem cells in higher animals (Birnbaum and Sánchez Alvarado, 2008), indicating the increasing layering of control mechanisms for induction of cell regeneration.

Fundamentally plants must retain mechanistic pathways to fully erase somatic cell identity in order to generate gametes (Kawashima and Berger, 2014). This contrasts with the situation in the animal kingdom where embryonic germ line specification and cell motility has allowed regenerative capacity to become limited to a number of embryonic stem cells, being traded off for increased complexity in the differentiated cell lineages. This has resulted in the increased plasticity of plant cells compared to animal cells, despite the two having similar chromatin features. For example, JmJ-C proteins in animals have a similar role demethylating histones particularly H3K4, recently JMJD6 and UTX were shown to be a histone demethylase targeting H3K27me3, activating gene expression in early cell-fate decisions, differentiation and organogenesis (Hong et al., 2007; Xiang et al., 2007). However, JMJD6 does not affect stem cell maintenance and self-renewal capacity (Mansour et al., 2012; Ohtani et al., 2013) and in fact is vital for causing senescence in mature cells, preventing further proliferation, acting as a tumour suppressor. Loss of function has been linked to cell over-proliferation

in at least 12 cancer types (Kandoth et al., 2013; Burchfield et al., 2015).

In this study, we demonstrate the role of a group of histone demethylases in plant regeneration pathways. Moreover, we have found that these proteins facilitate complete epigenetic reprogramming during gametogenesis, thus protecting against epiallele introduction, and initiating reprogramming responses to developmental signals or wound induced *de novo* organogenesis. While, developments in RNA sequencing and computational approaches have dramatically increased our understanding of the gene networks responsible for plant cell regeneration in recent years, revealing many key regulators such as *WUS* and *WIND1* (Iwase et al., 2011; Horstman et al., 2017a; Sang et al., 2018; Ikeuchi et al., 2018); current regeneration protocols remain ineffective on some closely related genotypes (Luo and Koop, 1997; Salvo et al., 2018), or introduce genetic elements such as TE *karma* in mantled palm (Ong-Abdullah et al., 2015), and residual epigenetic patterning remains from source tissues (Stroud et al., 2013a; Wibowo et al., 2018). This indicates that within these systems, there is a lack of understanding of reprogramming on an epigenetic level which is preventing the wider adoption of these regeneration techniques.

What is becoming increasingly clear is the blurring lines between TFs and chromatin regulators (Xiao et al., 2017). Some TFs in plants are more promiscuous in their genome occupancy than chromatin regulators, such as SPEECHLESS (Lau et al., 2014), while many developmental or environmental cues are directly integrated by chromatin regulators, modulating spatial, temporal, and condition-dependent accumulation or activity of proteins (Brady et al., 2007; Jeong et al., 2011; de Lucas et al., 2016). In plants, developmental transitions and regeneration responses are orchestrated by the combined activities of transcription factors, hormone response pathways, and regulators of chromatin state, with considerable crosstalk between these layers of regulation (Birnbaum and Roudier, 2017; Horstman et al., 2017a; Sang et al., 2018). For example, transcription factors can recruit chromatin remodelling proteins, but are also dependent on chromatin remodelling for the ability to bind target genes (Luo et al., 2012; Lodha et al., 2013;

Chhun et al., 2016). Hormonal pathways trigger chromatin state changes, and chromatin modification and remodelling that can alter hormone accumulation (Xiao et al., 2017; Horstman et al., 2017b). Finally, hormone environments alter transcription factor activity and transcription factors modulate hormone levels and response (Che et al., 2002; Wójcikowska et al., 2013; Perales and Reddy, 2012).

To better understand developmental reprogramming and regeneration, in the context of chromatin there are a number of challenges. Firstly, this study indicated the spatial sensitivity was not only due to a specific repressive modification H3K27me3. Determining the contributions of other epigenetic modifications could provide further insights into developmental reprogramming, such as other repressive histone modifications (e.g. H3K27me3, H2AK119ub and H3K9me3 (Bannister and Kouzarides, 2011)), microRNAs such as miR156 (Zhang et al., 2015) or active histone modifications (e.g. H3K4me3, H3K36me3, H3K27ac, H3S28ph, H1K26me3 (Bannister and Kouzarides, 2011)).

Secondly, the cell, tissue and condition dependent role of chromatin regulators has yet to be established, it is clear that the targeting of chromatin modifications can have context specific effects, and important roles in induction of new developmental programs, such as in the role of PRC2 in repressing tissue established gene networks at the onset of de-differentiation (He et al., 2012; Orłowska et al., 2017). Recent studies are beginning to identify factors that can recruit complexes like PRC2 to targets, such as the telomere-repeat-binding factors (TRBs) (Zhou et al., 2018), but much of this network remains to be uncovered. These mechanisms could be explored using spatial loss and gain of function mutants and a cell or tissue specific analysis. Recent developments in proteomics could provide an interesting avenue for the protein/protein interactions occurring during the onset and re-differentiation phases of regeneration, and how they may be controlled by extrinsic or intrinsic cues (Chin and Tan, 2018).

The results of this study provide a better understanding of how plant develop-

ment and regenerative capacity is affected by dynamic H3K27me3 regulation, facilitated by members of the Polycomb and Jumonji-C protein families. These findings could lead to the discovery of more robust regeneration protocols in economically important crops. Incorporation of these systems into breeding programs would allow quick establishment of phenotypic traits, and allow plant breeders to quickly produce lines able to adapt to the variable climate conditions the world is likely to face in the future.

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