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Functional studies of the
Arabidopsis thaliana
dormancy associated genes,
DRM1 and DRM2

Georgina Martha Rae

A thesis submitted in fulfilment of the requirements for
the degree of Doctor of Philosophy in Biological Sciences,
The University of Auckland, 2013
Abstract

Plants must carefully regulate their development in order to survive the prevailing conditions. One aspect of this of particular importance is dormancy release - meristematic tissues deciding when to grow and when not to, given varying conditions. In order to better understand the growth release mechanism of dormant tissue at the molecular and physiological levels molecular markers can be used. Members of the DRM1/ARP (DORMANCY ASSOCIATED GENE-1/AUXIN-REPRESSED PROTEIN) gene family are routinely used as markers for dormancy release. This plant-specific gene family has high sequence identity at the protein level throughout the plant kingdom, but its function in planta remains undetermined.

The aim of this PhD project is to gain insight into the function of the dormancy associated genes DRM1 and DRM2 in Arabidopsis thaliana. A multi-faceted approach, including bioinformatic, molecular and biochemical studies, was adopted.

The Arabidopsis DRM1/ARP gene family includes five members, the resulting proteins of which are predicted to be intrinsically disordered in nature. These family members are differentially regulated across development at the transcript level, with both constitutive and floral-specific, non-meristematic profiles evident. Both AtDRM1 and AtDRM2 produce splice variants which differ in their transcriptional response to various abiotic factors. Over-expression of AtDRM1 or AtDRM2 causes subtle developmental retardation. While null mutants do not exist for either gene, down-regulation amiRNA lines were analysed and exhibited subtle increases in bolt height compared with wild-type. Attempts to express His6-tagged AtDRM1 or AtDRM2 recombinant protein in E.coli yielded no expression, while expression attempts in a cell-free system produced an insoluble product. This protein is readily degraded in vitro, possibly as a result of its lack of intrinsic structure. Yeast-2-hybrid assays showed that neither AtDRM1 nor AtDRM2 bind with the branching pathway protein AtBRC1 and a lack of putative binding partners retrieved from a Yeast-2-hybrid library screen suggests that binding may be phosphorylation-dependent.

Overall these findings lead to the hypothesis that AtDRM1 and AtDRM2 may be hub proteins acting in a general stress response pathway, specifically involved in the early modulation of protective growth prevention signals.
Acknowledgements

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Rosemary Bellamy, thank-you for showing me that science was there for the taking.

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# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>3AT</td>
<td>3-Amino-1,2,4-Triazole</td>
</tr>
<tr>
<td>α-MoRF</td>
<td>α-molecular recognition features</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid(s)</td>
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<tr>
<td>ABA</td>
<td>abscisic acid</td>
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<tr>
<td>AGRIKOLA</td>
<td><em>Arabidopsis</em> Genomic RNAi Knock-Out Line Analysis</td>
</tr>
<tr>
<td>amiRNA</td>
<td>artificial microRNA</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ARP</td>
<td><em>AUXIN-REPRESSED PROTEIN</em></td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>BiFC</td>
<td>bimolecular fluorescent complementation</td>
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<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>bp</td>
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<td>C-terminus</td>
<td>carboxy-terminus</td>
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<tr>
<td>CATMA</td>
<td>Complete <em>Arabidopsis</em> Transcriptome Microarray</td>
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<tr>
<td>Cp</td>
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<tr>
<td>DISPHEROS</td>
<td>disorder enhanced phosphorylation sites predictor</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DNAsE</td>
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dNTP  deoxynucleotide 5′-triphosphate

**DRM1/2**  *DORMANCY ASSOCIATED GENE 1/2*

DTT  dithiothreitol
e.g.  for example

EST  expressed sequence tag

EtOH  ethanol

FRET  fluorescence resonance energy transfer

GA  gibberellic acid

GA-3  gibberellic acid
gDNA  genomic DNA

GFP  green fluorescent protein

GST  gene-specific sequence tags

GUS  β-Glucuronidase

h  hour(s)

H₂O  water

HC  hydrogen cyanamide; HiCane™

HIS  histidine

HSP  HEAT SHOCK PROTEIN

i.e.  that is

IAA  indole-3-acetic acid

IDP  intrinsically disordered protein

IDR  intrinsically disordered region

IPTG  isopropyl β-D-1-thiogalactopyranoside

Kb  kilobase pairs

kD  kilo-dalton

KLH  Keyhole Limpet Haemocyanin

LEU  leucine
<table>
<thead>
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<tr>
<td>MEME</td>
<td>Multiple Em for Motif Elicitation</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MJ</td>
<td>methyl jasmonate</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog</td>
</tr>
<tr>
<td>N$_2$</td>
<td>nitrogen</td>
</tr>
<tr>
<td>NAA</td>
<td>Naphthaleneacetic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride; salt</td>
</tr>
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<td>plant cis-acting regulatory DNA elements database</td>
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<tr>
<td>PONDR®</td>
<td>predictor of natural disordered regions</td>
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<tr>
<td>PTI</td>
<td>pathogen triggered immunity</td>
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<td>RNase</td>
<td>ribonuclease</td>
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ROS  reactive oxygen species
rpm  revolutions per minute
RQ   relative quantity
RT-qPCR  real time quantitative-polymerase chain reaction
SA  salicylic acid
SAM  shoot apical meristem
SAS  shade avoidance syndrome
SD  standard deviation
SDS  sodium dodecyl sulfate
s  second(s)
SEM  standard error of the mean
siRNA  short interfering RNA
SRE  sugar responsive elements
T₀  timepoint 0
T1  first generation
T2  second generation
T3  third generation
TAIR  The *Arabidopsis* Information Resource
TBLASTn  BLAST search of translated nucleotide databases using a protein query
T-DNA  transfer DNA (from *Agrobacterium tumefaciens*)
*Taq*  *Thermus aquaticus*
TBS  Tris-buffered saline
TEV  tobacco etch virus
TF  transcription factor
TRP  tryptophan
U  unit of enzyme activity
Up1  Up-Regulated-1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>UP2</td>
<td>Up-Regulated-2</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>WMD</td>
<td>Web MicroRNA Designer</td>
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<tr>
<td>Ws</td>
<td>Wassilewskija</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
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<tr>
<td>Y2H</td>
<td>Yeast-2-hybrid</td>
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1 Introduction

The development of a plant over its lifecycle is determined at both the genetic and the environmental levels, and as such is not random but rather a series of highly coordinated events. The plant’s survival is ensured via a number of complex and co-ordinated mechanisms. Timing and location of growth in response to the given environment, whether that growth be vegetative or floral, is critical and can be via cell division, cell elongation or a combination of the two.

Plants can be categorised by their type of life cycle. Plants which grow from seeds, transitioning from vegetative to floral growth irreversibly and dying after one flowering, are referred to as annuals. Examples of annuals include *Arabidopsis thaliana* and *Petunia hybrida*. By contrast, perennial plants such as kiwifruit (*Actinidia* sp.) and grape (*Vitis vinifera*), can cycle between vegetative and floral growth. Making decisions on when to grow and when not to grow is of particular importance for these perennial plants. Meristems are the key sites of growth in plants. Like stem cells in animals, these cells can undergo indeterminate growth via cell division to produce various tissue types. Meristems can be found in the shoot apex (shoot apical meristem; SAM), in the axil of the stem (axillary meristem), the root apex (root apical meristem) and secondary meristems. They allow both vertical and lateral growth (vascular- and cork-cambium) and are the only tissue in plants which, by strict biological definition, can become ‘dormant’.

A combination of a general introduction to concepts described in this thesis, including plant development, dormancy, stress and IDP is combined with a published review paper more specifically covering the DRM1/ARP family, in this introduction. For the published review, Georgina Rae generated the original manuscript, while Karine David and Marion Wood critically reviewed the manuscript, providing suggestions for concepts to be included.

1.1 Dormancy

1.1.1 What is dormancy?

Dormancy is crucial to a number of key physiological processes occurring in the plant’s lifecycle - such as defining plant architecture, plant growth and development and providing a long term survival strategy. Dormancy has been defined as the “transitory delay of visible growth that occurs in plant meristematic tissues” and is divided into a number of different types (Lang et al., 1987) defined by the primary effector controlling growth: endodormancy, ecodormancy, and paradormancy (reviewed in Horvath et al., 2003) (Figure 1.1).
Figure 1.1. Types of dormancy in relation to photoperiod, temperature, cell cycle and growth conditions.

Figure constructed as a compilation of the current literature. The seasons provide different photoperiod and temperature conditions which reach critical threshold levels for bud set and bud break. Cell division ceases with bud set, but resumes prior to visible bud break. Growth permissive conditions do not directly overlap with either the dormancy or cell division status of the bud. The combination of these factors, allows division of dormancy into the three types: paradormancy, endodormancy and ecodormancy.

In perennial species, the cycle in and out of dormancy is related to environmental conditions which affect growth and which are dictated by the season. This cycling described in perennials requires at least one indeterminate meristem to undergo dormancy when conditions are unsuitable for growth and then continue growing vegetatively in the season that follows (Battey, 2000; Thomas et al., 2000).

Plants will transition into dormancy from a growing state when temperature and/or the photoperiod reach a critical threshold, which varies between species. The affected structure perceives either an environmental or endogenous signal and becomes dormant. This type of dormancy is referred to as endodormancy (Lang et al., 1987). At dormancy
induction, there is generally a buffer of time between the time growth permissive conditions are sensed and the critical threshold being reached. These buffer periods at dormancy induction and dormancy release constitute ecodormancy (Figure 1.1).

Endodormancy is described as the dormant state which is maintained by internal signals in the meristem preventing bud out-growth irrespective of the presence of growth-permissive conditions (Lang et al., 1987). For example, in perennial species such as kiwifruit the bud needs to perceive sufficient chill units before it is released from endodormancy, even if the conditions are suitable for growth (Brundell, 1976; Lionakis and Schwabe, 1984; Warrington and Stanley, 1986).

Ecodormancy is described as the dormant state associated with unsuitable environmental conditions when the hard bud physically protects the delicate, but highly important indeterminate meristematic tissue (Lang et al., 1987). Environmental factors include nutrient deficiencies, water stresses, and temperature extremes.

The third defined state is paradormancy (also referred to as apical dominance or correlative inhibition) where the dormant state is regulated by a biochemical signal external to the target tissues. Upon bud break, non-apical (lower/basal) buds can become paradormant as a result of apical dominance (Thimann and Skoog, 1934). This form of dormancy is important for the control of plant architecture such that during times of favourable growth conditions the plant is able to optimise resource usage. The correct architecture of a plant will ensure that light perception and utilisation is maximised and that reproductive structures are maximally exposed for pollination and dispersal (Horvath et al., 2003). Apical dominance is released following damage or loss of the apical bud and in this way a lower axillary bud is released from dormancy and the overall architecture of the plant is maintained. Shoot architectural studies in the annual Arabidopsis exploits this phenomenon in decapitation studies, whereby removal of either a vegetative or floral phase apical bud can yield information on dormant versus growing buds in the rosette.
1.1.2 Cell division and bud dormancy

Visible growth (occurring mutually exclusively from dormancy by definition from Lang et al., (1987)) includes both cell division and cell elongation or expansion, with data on elongation and dormancy scarce (Rohde and Bhalerao, 2007).

As displayed in Figure 1.1, cell division ceases during dormancy. Using flow cytometry and cell cycle markers two independent research groups determined that cells present in dormant axillary buds of pea were predominantly arrested in the Gap-1 (G₁) phase of the cell cycle (Devitt and Stafstrom, 1995; Shimizu and Mori, 1998b). Devitt and Stafstrom, (1995) capitalised on the potential of Alaska pea plants (Pisum sativum) to undergo multiple growth-dormancy cycles over a period of days by decapitation, i.e. removing the apical bud of plants with dormant lower lateral buds, to consider the relationship between expression of cell cycle-related genes and axillary bud dormancy. Their data showed that there was a distinct correlation between dormancy release and cell cycle continuation, as indicated by increased expression of a number of recognised cell cycle markers (MITOGEN ACTIVATED PROTEIN (MAP) KINASE; CYCLIN-DEPENDENT PROTEIN KINASE-2 (CDK2); RIBOSOMAL PROTEIN L27 (RPL27) and RPL34). Furthermore, subsequent work confirmed that RPL27 expression was negatively correlated with expression of DORMANCY ASSOCIATED GENE-1 (PsDRM1) and a closely related gene, PsDRM2 (Stafstrom et al., 1998).

The primary control on the cell cycle is provided by the activity of CDKs. The enzymatic activity of CDKs can be positively regulated by the binding of a CYCLIN (CYC) and negatively regulated by the binding of a CDK inhibitor (CKI) (reviewed in Campbell, 2006). Many types of CYCLINs have been identified in plants. In Arabidopsis, A-type and D-type CYCLINs have been shown to be associated with G₁ to S-phase transition of the cell cycle (Dewitte and Murray, 2003) and are of direct interest to dormancy studies. Expression levels of the mRNA of one such gene, CYCLIND3, has been consistently shown to increase following decapitation and has been used as a marker for dormancy release (Shimizu and Mori, 1998a). Interestingly, data in pea has shown that the CYCLIND3 protein forms a complex with PCNA (PROLIFERATING CELL NUCLEAR ANTIGEN) only in dormant axillary buds when cells are arrested at the G₁ phase. It is proposed that decapitation promotes the dissociation of the PCNA/CYCLIND3 complex resulting in the activation of each protein (Shimizu and
Mori, 1998b). With this the dormant axillary bud enters the S-phase of the cell cycle and growth is resumed (Shimizu and Mori, 1998b).

### 1.1.3 Environmental signals implicated in dormancy and shoot outgrowth

Plants are sessile and as such have evolved complex mechanisms in order to perceive and respond to environmental signals which may be otherwise unfavourable to the plant’s survival (Horvath et al., 2003). This is particularly true when tissues transition between dormant and non-dormant status, as uncontrolled vegetative bud growth would have highly negative effects on the plant’s architecture, reproduction and survival (Horvath et al., 2003). These environmental signals include responses to light and temperature, although the contribution of each factor to dormancy is known to vary both between and within species (reviewed in Dogramaci et al., 2013). Recent work has attempted to clarify the intricate relationship between photoperiod and temperature signals in leafy spurge (Dogramaci et al., 2013).

On its own, light (or photoperiod) can have a significant impact on a plant’s architecture (Snowden and Napoli, 2003). By increasing irradiance levels, apical dominance is weakened (Andersen, 1976; Cline, 1996) meaning that with increased light levels plants increase their number of branches. Moreover, the converse is true in a phenomenon known as the shade avoidance syndrome (SAS) (reviewed in Casal, 2012). In the SAS, high density planting leads to a reduction in red:far-red light ratio (R:FR) in the microenvironment, which communicates to plants that neighbouring plants are in close proximity (Smith and Whitelam, 1997). SAS subsequently induces plants to grow taller, have reduced root growth and bud outgrowth, as well as causing the plants to flower early (Franklin and Whitelam, 2005). More recently it has been shown that this bud outgrowth repression in SAS also occurs in newly formed axillary buds of adult plants grown in standard (white light) conditions only transiently exposed to low R:FR (Gonzalez-Grandio et al., 2013), demonstrating a direct relationship between the dormancy status of buds and light conditions.

Integral to the SAS response is the photoreceptor PHYTOCHROME B (PHYB) (Childs et al., 1997; Devlin et al., 1992; Kerckhoffs et al., 1999; Lopez-Juez et al., 1992; Reed et al., 1993; Sheehan et al., 2007; van Tuinen et al., 1995). phyb mutant plants have
reduced bud outgrowth; tissue hyper-elongation; reduced chlorophyll levels and early flowering (Childs et al., 1992; Childs et al., 1997; Reed et al., 1993).

Mutants involved in perception and signal transduction of another phytochrome: PHYTOCHROME A (PHYA), such as far-red elongated hypocotyls-3 (fhy3), similarly exhibit reduced bud outgrowth (Stirnberg et al., 2012). FHY3 is a transcription factor involved in very early transduction of the PHYA signal (Hudson et al., 1999; Wang and Deng, 2002; Wang et al., 2002). This mutant provides further links between plant growth, development and light signalling.

Along with light conditions, temperature is another well characterised effector of dormancy and bud outgrowth. Without the required chilling through the dormancy period, many perennials will not go on to break bud and develop flowers efficiently. Examples of this phenomenon include Pinus taeda (Garber, 1983); kiwifruit (Linsley-Noakes and Allan, 1987); apple (Naor et al., 2003); peach (Wagner Jr et al., 2006); and grape (Reeder et al., 1998). DORMANCY-ASSOCIATED MADS-BOX (DAM) genes are one group of temperature-responsive genes which have been the focus of attention for a putative role in dormancy induction and maintenance. The MADS box transcription factor (TF) family, of which the DAM genes are members, are found in plants, fungi and animals (Alvarez-Buylla et al., 2000; Shore and Sharrocks, 1995; Theissen et al., 1996). DAM gene family members from peach are candidates for roles in dormancy induction, in particular for growth cessation and terminal bud formation (Bielenberg et al., 2008). Early studies exploited a naturally occurring peach mutant from Mexico called ‘evergrowing’ (evg), which fails to cease growing and enter dormancy despite conditions normally inducing dormancy (short day length and cold). (Rodriguez-A et al., 1994). It was not until more than a decade after the characterisation of the mutant that a group of MADS-box TFs, designated as DAM genes, were shown to be undetectable in the evg mutant (Bielenberg et al., 2008). Further characterisation of the six genes showed that transcriptional expression of DAM1, DAM2, and DAM4 are associated with growth cessation and bud set (Li et al., 2009), making them candidates for the regulation of transition into endodormancy. Conversely, transcriptional expression of DAM3, DAM5 and DAM6 was detected over the winter (i.e. when buds had reached chilling requirements and had been exposed to short day length), reaching a maximum immediately prior to bud break (Li et al., 2009). Similarly, increased
transcript expression of homologues from kiwifruit, *SHORT VEGETATIVE PHASE-1* (*SVP1*), *SVP2* and *SVP4*, were associated with winter dormancy (Wu et al., 2012). Empirical evidence for cold-regulation of *DAM* gene homologues has been seen in leafy spurge (Horvath et al., 2008), Raspberry (Mazzitelli et al., 2007) and peach (Jiménez et al., 2010).

**1.1.4 Dormancy, shoot outgrowth and stress**

While ‘standard’ growth conditions clearly regulate plant architecture, extreme conditions which create potentially damaging physiological changes (Shao et al., 2008), or stresses, have repercussions upon a plant’s growth. With perception of unfavourable conditions a plant will cease or minimise growth until more favourable conditions return (Rohde et al., 1999). This reduction in growth allows plants to divert resources to deal with a given stress (Xiong and Zhu, 2001).

As mentioned previously, low R:FR light will cause plants to grow taller, with reduced root growth and branching (Franklin and Whitelam, 2005). By contrast, exposure to wind leads to an increase in basal branching in *Arabidopsis* (Pigliucci, 2002) and mechanically stressed *Potentilla reptans* produced more stolons (Liu et al., 2007). Nutrient starvation also causes decreased shoot branching (Lafever, 1981) via a strigolactone-mediated pathway (Kohlen et al., 2011; López-Ráez et al., 2008; Umehara et al., 2010; Umehara et al., 2008; Yoneyama et al., 2007).

At the molecular level, the SNF2/Brahma-type *AtCHR12* chromatin-remodelling gene appears to have a key role in growth arrest in response to stress (Mlynárová et al., 2007). Only upon exposure to stress conditions does over-expression of the SNF2/Brahma-type *AtCHR12* chromatin-remodelling gene cause normally active buds to arrest in their growth, as well as causing cessation of inflorescence growth, compared with wild-type plants. Conversely, *Atchr12* mutant plants exhibit reduced growth arrest compared to wild-type plants, upon stress. Interestingly, in the absence of any stress there are no discernible morphological differences between either mutant and wild-type plants.

To release axillary buds from dormancy, plants will often endure sub-optimal conditions, whether they be wounding in the form of decapitation or very cold
temperatures. For this reason, dormancy release is increasingly being referred to as a sub-lethal stress response (Ophir et al., 2009; Walton et al., 2009). High temperature and treatment with the compound hydrogen cyanamide (H$_2$CN$_2$) are examples of treatments used to trigger dormancy release in grape (Ophir et al., 2009) and kiwifruit (Erez, 1995; Henzell et al., 1992; Linsley-Noakes, 1989). The mechanism of action of these treatments is hypothesised to be induction of respiratory stress, such as the generation of reactive oxygen species (ROS). Transcriptional studies show that upon application of either treatment, the expression profiles of CATALASE, ALCOHOL DEHYDROGENASE, PURUVATE DECARBOXYLASE, ASCORBATE PEROXIDASE, GLUTATHIONE REDUCTASE, THIOREDOXIN H, GLUTATHIONE S-TRANSFERASE and SUCROSE SYNTHASE genes were similarly altered (Halaly et al., 2008). In a more general sense, production of ROS is increased in response to abiotic stresses (Foyer and Noctor, 2009), such as those experienced during budbreak.

As well as ROS being inextricably linked to stress, evidence is emerging linking this intracellular process with plant physiology - specifically with shoot outgrowth. The highly branched more axillary growth (max)-2 mutants in Arabidopsis have increased tolerance to ROS (Woo et al., 2004). This phenotype can be rescued by the fhy3 mutant, which shows a greater susceptibility to an inducer of chloroplast-derived ROS – methyl viologen (Stirnberg et al., 2012). In addition, over-expression of the hydrogen peroxide-responsive UDP GLUCOSYLTRANSFERASE UGT74E2 caused increased survival during salt and drought stress as well as shorter plants with an increased number of branches. Outside of Arabidopsis, tomato plants down-regulated in PLANT RESPIRATORY BURST OXIDASE HOMOLOGS (RBOH) produced more branches (Sagi et al., 2004). The increasing associations between stress, shoot outgrowth and respiratory stress make this an area of importance for these fields of research going forward.

1.1.5 Hormones involved in dormancy and shoot outgrowth

Hormones are critical players throughout plant development, with several hormones implicated in dormancy including auxin, cytokinin, abscissic acid (ABA) and the more recently identified strigolactone (Gomez-Roldan et al., 2008; Umehara et al., 2008).
1.1.5.1 Auxin

Indole acetic acid (IAA) is the most abundant naturally occurring isomer of auxin in plants. Initial experiments revealed that decapitation of plants resulted in a perturbed auxin flow down the stem, i.e. no auxin flow, concomitant with bud break. This phenomenon was later described as apical dominance or paradormancy (reviewed in Domagalska and Leyser, 2011).

The auxin response mutant auxin resistant-1 (axr1), which regulates activity of the auxin receptor TRANSPORT INHIBITOR RESPONSE-1 (TIR1) (Ruegger et al., 1998), exhibits a characteristic increased lateral branching phenotype (Lincoln et al., 1990) similar to that seen in tir1 mutants (Gray et al., 2001) due to a strongly reduced auxin response (Stirnberg et al., 1999).

1.1.5.2 Cytokinin

Cytokinins are a family of root-derived branching signals (Cline, 1991) which promote the outgrowth of axillary buds, in contrast to the outgrowth inhibitors auxin and strigolactone. Cytokinins are synthesised via an ADENOSINE PHOSPHATE-ISOPENTYLTRANSFERASE (IPT). In Arabidopsis seven IPT members have been confirmed in the family (AtIPT1, AtIPT3-8) (Kakimoto, 2001; Miyawaki et al., 2004; Takei et al., 2001). Mutants with increased cytokinin, altered meristem program 1 (amp1), show an increased branching habit as well as altered embryonic patterning, faster vegetative growth, early flowering and constitutive photomorphogenesis (Chaudhury et al., 1993; Chin-Atkins et al., 1996; Saibo et al., 2007). The increased branching in particular highlights an important role for cytokinins in the regulation of bud outgrowth.

1.1.5.3 Strigolactone

The group of terpenoid lactones known as strigolactones are the most recently identified plant hormones (reviewed in Seto et al., 2012). Originally associated solely with mycorrhizae and parasitic plant responses, this carotenoid-derived signal is now identified as the previously undiscovered signal for inhibition of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008).
Prior to the discovery of the branching inhibition role of strigolactones, it was hypothesised that as strigolactones were carotenoid derivatives they could be downstream products of CAROTENOID CLEAVAGE DIOXYGENASES (CCDs) (Matusova et al., 2005). The decreased apical dominance (dad) mutants first identified in petunia, which showed an increased branching phenotype, have been used extensively to dissect the branching pathway (Napoli, 1996; Napoli and Ruehle, 1996; Simons et al., 2007). Homologues of these genes have now been identified in a number of species, including both monocotyledons and dicotyledons. For example homologues have been identified in pea (ramosus (rms) mutants) (Beveridge et al., 1996; Beveridge et al., 1997; Morris et al., 2001), in Arabidopsis (max mutants) (Booker et al., 2004; Booker et al., 2005; Sorefan et al., 2003; Stirnberg et al., 2002; Turnbull et al., 2002) and in rice (dwarf (d) or high tillering dwarf (htd) mutants) (Arite et al., 2007; Arite et al., 2009; Gao et al., 2009; Ishikawa et al., 2005; Lin et al., 2009; Liu et al., 2009; Zou et al., 2006). MAX1, MAX3, MAX4 and DWARF27 are all hypothesised to be involved in carotenoid metabolism to form strigolactone preventing axillary bud outgrowth, with mutants exhibiting reduced strigolactone levels (Gomez-Roldan et al., 2008; Umehara et al., 2008). In addition, exogenous application of the terpenoid inhibits shoot branching in these strigolactone synthesis mutants.

1.1.5.4 Auxin, cytokinin and strigolactone cross-talk

While each hormone is important in their own right, the homeostatic balance and interactions between the hormones is paramount to a plant’s development and to the decision to branch or remain dormant. Shoot-derived auxin works in co-ordination with root- and stem-derived cytokinin and root-derived strigolactone to regulate axillary bud outgrowth (reviewed in Domagalska and Leyser, 2011; Ferguson and Beveridge, 2009) (Figure 1.2).
A) Dormant buds must be triggered to a responsive state where they are receptive to outgrowth signals. B) Loss of the shoot tip (apical dominance) leads to a rapid trigger that is not IAA (auxin) mediated. C) Growing axillary buds/branches (correlative inhibition) affect whether buds respond to depleted strigolactone and also reduce nutrient availability for bud growth and elongation. D) Strigolactone inhibits bud outgrowth, whereas cytokinin (CK) promotes it. In intact plants, IAA negatively regulates bud outgrowth by maintaining high strigolactone and low CK contents. E) The IAA content of responsive buds increases and is exported into the stem, allowing the bud to develop a more effective and substantive vascular system and attract nutrients for growth. The extent of subsequent bud/branch growth is then strongly dependent on nutrient availability. This model illustrates the developmental stages of a growing bud/branch. The arrows shown between stages represent the progression toward outgrowth but do not preclude the fact that bidirectional development can occur, where inhibition can cause buds to revert to a previous stage. References are indicated as follows: a, Morris et al. (2005); b, Beveridge (2000); c, Foo et al. (2005); d, Tanaka et al. (2006); e, Miyawaki et al. (2004); Takei et al. (2001); f, Stafstrom (1995); g, Napoli et al. (1996); h, Bangerth (1989); i, Davies and Wareing (1965); j, Phillips (1968); k, Ongaro and Leyser (2008); l, Ferguson and Beveridge (2009). Figure and figure legend taken directly from Ferguson and Beveridge (2009).

Axillary bud-break is inhibited by a flow of auxin from the terminal or apical bud (Booker et al., 2003). Upon decapitation the flow of auxin ceases and cytokinin produced in the stem promotes the bud outgrowth (Tanaka et al., 2006). Strigolactones
act downstream of auxin to inhibit axillary bud outgrowth (Brewer et al., 2009). Auxin acts on cytokinin flux and inhibits cytokinin synthesis in the node (Bangerth, 1994; Tanaka et al., 2006). It has been proposed that auxin from the terminal bud represses expression of a key gene in the cytokinin biosynthesis pathway, *IPT*, in the stem (Kakimoto, 2001; Takei et al., 2001). Upon decapitation, stem-localised auxin decreases, releasing repression of *IPT*. This facilitates cytokinin biosynthesis allowing subsequent cytokinin-mediated bud outgrowth. After the new shoot has grown out auxin is produced in its apex which flows to the stem. Once again, *IPT* is repressed inducing *CYTOKININ OXIDASE (CKX)* which reduces the cytokinin levels and the apical dominance cycle can begin again (Nordström et al., 2004; Shimizu-Sato et al., 2009). In addition, basal cytokinin levels can be restored with exogenous application of auxin to the decapitated stump (Bangerth, 1994; Bangerth et al., 2000; Li et al., 1995).

Critical to the action of auxin is its location, acting outside of the young expanding leaves at the shoot apex. Therefore, transport and regulation of the hormone is important to its activity. The flow of auxin, which is critical to the apical dominance phenomenon, is not passive, but rather is highly regulated. Auxin is a highly mobile molecule that exhibits directional and responsive movement (Teale et al., 2006), commonly referred to as the polar auxin transport system (PATS). In *Arabidopsis* auxin is transported by two different transporters: AUXIN INFLUX CARRIER PROTEIN 1 (AUX1) / members LIKE-AUX1 (LAX) (Parry et al., 2001; Swarup et al., 2001) and the PIN-FORMED (PIN) proteins (Gälweiler et al., 1998; Paponov et al., 2005).

Auxin depletion causes significant reduction in transcript levels of the strigolactone biosynthesis genes, which return to wild-type levels with application of exogenous IAA (Arite et al., 2007; Bainbridge et al., 2005; Bennett et al., 2006; Foo et al., 2005; Hayward et al., 2009; Johnson et al., 2006; Sorefan et al., 2003; Zou et al., 2006). Furthermore, auxin depleted pea plants and *Arabidopsis* auxin-signalling mutants respond to strigolactone application (Brewer et al., 2009). Strigolactone has been implicated in auxin transport, regulating growth redistribution in the plant. Strigolactone enhances PIN1 and therefore PATs depletion (Crawford et al., 2010). More recently, these effects have been shown to be independent of protein synthesis and dependent on clathrin-mediated membrane trafficking, suggesting that strigolactone is creating these effects by increasing endocytosis of PIN1 from the plasma membrane, limiting auxin
transport through the PATs in xylem parenchyma cells (Shinohara et al., 2013). This finding supports the auxin canalisation hypothesis for auxin transport which requires systemic strigolactone to be involved in positive feedback between auxin flux and auxin flux capacity (reviewed in Domagalska and Leyser, 2011).

More recently, the involvement of cytokinin in this complex regulation of branch outgrowth by auxin and strigolactone has also been considered (Dun et al., 2012). Application of cytokinin to pea buds stimulated outgrowth with lower concentrations in strigolactone-deficient mutants compared with wild-type. Furthermore, cytokinin supplied via the vasculature was still effective in strigolactone-deficient mutants while wild-type plants were almost completely resistant. In combination, these results provide evidence for these hormones both acting directly on buds to antagonistically regulate bud outgrowth.

An example of this hormone co-ordination and homeostasis is found in the bushy and dwarf-2 (bud2) mutation in Arabidopsis, associated with increased branching and reduced overall plant height, in response to changes in auxin and cytokinin responses (Cui et al., 2010). BUD2 encodes a polyamine synthesis enzyme named S-ADENOSYL METHIONINE DECARBOXYLASE 4 (SAMDC4) (Ge et al., 2006). Polyamines are known to regulate axillary bud outgrowth in the bud2 mutant (Ge et al., 2006), with subsequent work suggesting the polyamines may have a role in cytokinin homeostasis as well as auxin and cytokinin sensitivity (Cui et al., 2010). Overall, it is hypothesised that polyamines increase auxin perception sensitivity, therefore repressing cytokinin biosynthesis (Cui et al., 2010).

1.1.5.5 ABA

Abscisic acid (ABA) is another hormone integral to plant development. In particular, ABA is associated with stress response and seed dormancy.

During seed dormancy a primary role for ABA is in the inhibition of precocious germination and the induction of primary dormancy (Nambara et al., 2010). ABA synthesis mutants have enhanced germination potential, sometimes even producing viviparous seeds (McCarty, 1995). Conversely, ABA response mutants show enhanced dormancy (Okamoto et al., 2006; Okamoto et al., 2010; Qin and Zeevaart, 2002; Thompson et al., 2000).
In addition to ABA’s role in seed dormancy, it has also been associated with dormancy induction and maintenance in buds. Work completed in poplar showed a correlation between increasing ABA levels and short day treatment in the apex (Rohde et al., 2002). Furthermore, a poplar mutant for \textit{ABSCISSION-ACID INSENSITIVE3} (\textit{ABI3}), a transcription factor involved in ABA signalling, exhibited altered bud development (Rohde et al., 2002).

Additional evidence for ABA’s role in dormancy is seen in transcript expression analysis from poplar which showed that components of the ABA receptor (reviewed by Sheard and Zheng, 2009) are induced in the shoot apex with short day treatment and therefore with induction of dormancy (Karlberg et al., 2010).

### 1.1.6 Integration at the bud level

Outside of the various hormone mutants already described, another mutant, \textit{tb1} (\textit{teosinte branched 1}), first discovered in maize exhibits a characteristic increased branching phenotype (Doebley et al., 1997). Variation at the \textit{TBI} locus explains much of the divergence in branching phenotype between maize (no axillary branches) and its presumed ancestor teosinte (highly branched) (Doebley et al., 1997). TB1 belongs to the plant-specific TCP domain group of proteins including TB1, CYCLOIDEA and PCF – a group involved in the control of cell division. However, the mechanism of action of \textit{TBI in planta} remains unknown, despite well characterised mutants being identified in many species including rice: \textit{Ostb1/Osfine-culm1} (\textit{Osfc1}) (Hu et al., 2003; Minakuchi et al., 2010; Takeda et al., 2003), sorghum: \textit{Sbtb1} (Kebrom et al., 2006), tomato: \textit{Slbranched1a} (\textit{Slbrcl1a}) and \textit{Slbrcl1b} (Martín-Trillo et al., 2011) and \textit{Arabidopsis}: \textit{Atbrcl} (\textit{Atbranched1})/\textit{Attbr1} (Aguilar-Martínez et al., 2007; Finlayson, 2007). Transcript expression of this gene is correlated with bud development arrest, with expression decreasing upon release of buds from dormancy (Aguilar-Martínez et al., 2007; Gonzalez-Grandio et al., 2013; Martín-Trillo et al., 2011).

The initial hypothesis suggested that this gene may be the branching signal integrator at the bud level, controlling whether or not axillary buds grew out, which is influenced by a number of different endogenous and exogenous signals, including hormonal signals and light conditions (Aguilar-Martínez et al., 2007). However, this hypothesis has evolved to specify a role for \textit{BRC1} as a key branching signal step in light perception via
the phyB pathway but not defoliation pathways (Kebrom et al., 2010). Interestingly, BRC1 transcript levels were shown to be decreased in max mutants 1-4 (Aguilar-Martínez et al., 2007). Conversely, MAX2 levels were unaffected in brc1 mutants (Finlayson, 2007), suggesting that BRC1 is acting downstream of the MAX genes in an axillary bud outgrowth pathway (Finlayson, 2007). The MAX genes have also been implicated in light signal transduction (Shen et al., 2007), providing support for the hypothesis that these genes are involved in the same branching pathway. Recent work considering the relationship between the antagonistic effects of strigolactone and cytokinin on bud outgrowth showed that PsBRC1 is regulated by both hormones and as such may still be involved in the convergence of these two critical signalling pathways in the bud (Dun et al., 2012).

Protein interaction studies have shown that AtBRC1 binds NUCLEAR FACTOR Y, SUBUNIT C9 (NF-YC9) and SNF1-RELATED PROTEIN KINASE CATALYTIC SUBUNIT ALPHA KIN10 (AKIN10), proteins associated with photoperiod (Kumimoto et al., 2010) and phosphate starvation (Fragoso et al., 2009) responses, respectively (Chevalier et al., 2012). These preliminary results are interesting in that both processes have been closely linked with branch outgrowth, providing additional evidence for a possible role as an integrator of branch outgrowth signals.

1.1.7 Importance of proteins in plant development

While an understanding of transcriptional profiles relating to various plant processes is important, an organism’s genetic make-up generally acts upon cellular processes at the level of proteins. Regulation of a trait can occur at multiple levels from transcriptional regulation through to post-translational modifications. Post-translational modifications can be particularly important for various processes including the protein’s ability to interact with binding partners as well as targeting proteins for degradation. Indeed, such post-translation modifications, including phosphorylation and oxidation, have been linked to the presence of reactive oxygen species (ROS), which are associated with both bud dormancy release and stress responses (Gill and Tuteja, 2010; Halaly et al., 2008; Piterková et al., 2013).

Historically, functional proteins have been described as maintaining a rigid tertiary structure and as a consequence of the loss of this order, lose their functionality. A new school of thought acknowledges that proteins can lack an ordered configuration without
compromising functionality. Such proteins are known as intrinsically disordered proteins (IDPs) or intrinsically unstructured proteins (IUPs), referring to the sequence (or region of sequence) being biologically active but not exhibiting a stable secondary and/or tertiary structure (reviewed in Uversky, 2013a). IDPs are increasingly being associated with roles in signal transduction, transcriptional regulation, stress responses and disease (Garay-Arroyo et al., 2000; Iakoucheva et al., 2002; Minezaki et al., 2006b; Mouillon et al., 2006). As dormancy and shoot outgrowth are showing increasing parallels with stress, it is likely that a role for IDPs in these processes will emerge.
1.2 The dormancy marker $DRM1/ARP$: associated with dormancy but a broader role \textit{in planta}

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Abstract

Plants must carefully regulate their development in order to survive a wide range of conditions. Of particular importance to this is dormancy release, deciding when to grow and when not to, given these varying conditions. In order to better understand the growth release mechanism of dormant tissue at the molecular and physiological levels, molecular markers can be used. One gene family that has a long association with dormancy, which is routinely used as a marker for dormancy release, is $DRM1/ARP$ ($DORMANCY\ ASSOCIATED\ GENE-1/AUXIN-REpressed\ PROTEIN$). This plant-specific gene family has high sequence identity at the protein level throughout several plant species, but its function \textit{in planta} remains undetermined. This review brings together and critically analyses findings on the $DRM1/ARP$ family from a number of species. We focus on the relevance of this gene as a molecular marker for dormancy, raising questions of what its role might actually be in the plant.
1.2.1 Introduction

Plants are sessile; therefore, a number of mechanisms are elegantly managed in the plant to ensure its survival and optimisation in its given setting. Integral to this is the temporal and spatial determination of growth in relation to the surroundings. This growth may be vegetative, such as axillary budbreak and branch development; or floral, including flower and fruit development. To achieve this, cell division, cell elongation, or a combination of the two have been shown to play a pivotal role in plant growth regulation.

Dormancy is a means of ensuring a plant’s long-term survival and is defined as the “transitory delay of visible growth that occurs in plant meristematic tissues” (Lang et al., 1987). An example of this occurs in perennials over winter. When temperatures are low and daylight hours are limited, plants protect themselves by ceasing growth, waiting for the more favourable conditions of spring in which to focus their resources into growing and reproducing. As such, the key decisions around when to release dormant tissue from dormancy (dormancy release/shoot outgrowth/shoot branching) is an important mechanism for plant biologists to understand.

Both perennial and annual plants (e.g. Arabidopsis) also undergo a type of dormancy known as apical dominance or paradormancy, where endogenous signals/hormones from the apical bud are received by lower axillary meristems, preventing their bud outgrowth. This phenomenon was first described in 1934 (Thimann and Skoog), when it was shown that axillary buds of decapitated plants were released from dormancy due to a lack of auxin flow down the stem. Since then, the interactions between auxin and other hormones including cytokinin (growth promoting) and strigolactone (branching inhibition) have been studied in-depth in order to better understand the complex cross-talk which is controlling this process (reviewed by Domagalska and Leyser, 2011).

Mutant lines exhibiting hyper-branching phenotypes have been integral to these dormancy release studies. These included characterisation of the MAX/CCD/RMS pathway involved in strigolactone biosynthesis and perception (reviewed by Seto et al., 2012); and more recently, additional hyper-branching mutants, the bud-specific dormancy release signal integrator brc1/tbl1, have also been studied in order to elucidate alternate pathways involved in this critical process. These studies relied
heavily on the use of a genetic marker for dormancy status — _DORMANCY ASSOCIATED GENE 1 (DRM1)/AUXIN REPRESSED PROTEIN (ARP)_). This review will present literature associated with _DRM1/ARP_ from a number of species (summarised in Table 1.1) and will discuss the relevance of using _DRM1/ARP_ as a molecular marker for dormant tissues.
Table 1.1. Summary of the characteristics and putative functions of known DRM1/ARP family members.

<table>
<thead>
<tr>
<th>Species</th>
<th>Protein Identifier</th>
<th>Gene name</th>
<th>Possible function</th>
<th>Predicted protein length (aa)</th>
<th>Predicted protein size (kD)</th>
<th>Predicted pI</th>
<th>Predicted extinction coefficient</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Actinidia deliciosa</strong></td>
<td>FG458205</td>
<td>Ade_DRM1_ID / AdDRM1</td>
<td>Axillary bud dormancy maintenance and growth repression in mature fruit</td>
<td>115</td>
<td>12.722</td>
<td>10.58</td>
<td>19,480</td>
<td>Wood et al., 2013</td>
</tr>
<tr>
<td><strong>Arabidopsis thaliana</strong></td>
<td>NP_001154378</td>
<td>AtDRM1</td>
<td>Axillary bud dormancy maintenance and leaf maturation</td>
<td>122</td>
<td>13.406</td>
<td>10.71</td>
<td>19,480</td>
<td>Tatematsu et al., 2005</td>
</tr>
<tr>
<td></td>
<td>NP_850220</td>
<td>AtDRM2</td>
<td>Axillary bud dormancy maintenance</td>
<td>108</td>
<td>11.500</td>
<td>10.32</td>
<td>19,480</td>
<td></td>
</tr>
<tr>
<td><strong>Arachis hypogaea</strong></td>
<td>AAZ20292</td>
<td>AbDRM1</td>
<td>Drought stress response</td>
<td>121</td>
<td>13.459</td>
<td>10.19</td>
<td>22,460</td>
<td>Govind et al., 2009</td>
</tr>
<tr>
<td><strong>Brassica oleracea</strong></td>
<td>AAL674436</td>
<td>BoDRM1</td>
<td>Germination in sub-optimal conditions</td>
<td>105</td>
<td>11.352</td>
<td>10.10</td>
<td>19,480</td>
<td>Soeda et al., 2005</td>
</tr>
<tr>
<td><strong>Brassica rapa</strong></td>
<td>ACQ0305</td>
<td>BrDRM1</td>
<td>Prevention of germination; prevention of hypocotyl elongation; temperature and</td>
<td>128</td>
<td>14.035</td>
<td>9.35</td>
<td>18.115</td>
<td>Lee et al., 2013</td>
</tr>
<tr>
<td></td>
<td>AAO32054</td>
<td>BrARP1</td>
<td>Cold and salt stress response</td>
<td>108</td>
<td>11.651</td>
<td>10.10</td>
<td>19,480</td>
<td></td>
</tr>
<tr>
<td><strong>Capsicum annuum</strong></td>
<td>AAR83888.1</td>
<td>CaARP1</td>
<td>Pathogen response in root nodules</td>
<td>120</td>
<td>12.985</td>
<td>10.26</td>
<td>19,480</td>
<td>Hwang et al., 2005</td>
</tr>
<tr>
<td><strong>Elaeagnus umbellata</strong></td>
<td>AAC62104</td>
<td>EuNOD-ARP1</td>
<td>Pathogen response in root nodules</td>
<td>111</td>
<td>12.416</td>
<td>9.69</td>
<td>19,480</td>
<td>Kim et al., 2007</td>
</tr>
<tr>
<td><strong>Fragaria x ananassa</strong></td>
<td>Q05349</td>
<td>FaSAR5</td>
<td>Growth repression in mature fruit</td>
<td>119</td>
<td>13.060</td>
<td>10.01</td>
<td>19,480</td>
<td>Reddy and Poovaiah, 1990</td>
</tr>
<tr>
<td><strong>Malus domestica</strong></td>
<td>AAA71994</td>
<td>MdAPI</td>
<td>Growth repression in mature fruit</td>
<td>127</td>
<td>13.623</td>
<td>10.36</td>
<td>16,960</td>
<td>Lee et al., 1993</td>
</tr>
<tr>
<td><strong>Nicotiana benthamiana</strong></td>
<td>GW691612</td>
<td>NbDRM3</td>
<td>PTI immunity</td>
<td>137</td>
<td>14.636</td>
<td>11.39</td>
<td>20,970</td>
<td>Chakravarthy et al., 2010</td>
</tr>
<tr>
<td><strong>Nicotiana tabacum</strong></td>
<td>AAO21304</td>
<td>NtARPL1;1</td>
<td>Growth repression in mature pollen and flowers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Steiner et al., 2003</td>
</tr>
<tr>
<td>Species</td>
<td>Accession</td>
<td>Gene</td>
<td>Function Description</td>
<td>E-value</td>
<td>I-value</td>
<td>P-value</td>
<td>q-value</td>
<td>References</td>
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</tr>
<tr>
<td><em>Paeonia suffruticosa</em></td>
<td>ABW74471</td>
<td><em>PsARP</em></td>
<td>Axillary bud dormancy maintenance</td>
<td>126</td>
<td>13.725</td>
<td>10.39</td>
<td>20.970</td>
<td>Huang et al., 2008</td>
</tr>
<tr>
<td><em>Pisum sativum</em></td>
<td>AAB84193</td>
<td><em>PsDRM1</em></td>
<td>Bud dormancy maintenance and growth repression in mature tissues</td>
<td>111</td>
<td>12.284</td>
<td>10.26</td>
<td>19.480</td>
<td>Stafstrom, 2000; Stafstrom et al., 1998</td>
</tr>
<tr>
<td></td>
<td>AGG19166</td>
<td><em>PpARP2</em></td>
<td></td>
<td>139</td>
<td>14.273</td>
<td>10.58</td>
<td>9.970</td>
<td></td>
</tr>
<tr>
<td><em>Solanum lycopersicum</em></td>
<td>ABH07900</td>
<td><em>SIDRM1</em></td>
<td>Growth repression in ‘dormant’ ovaries prior to fruit set</td>
<td>123</td>
<td>13.442</td>
<td>10.11</td>
<td>20.970</td>
<td>Vriezen et al., 2008</td>
</tr>
</tbody>
</table>
1.2.2 DRM1/ARP and dormancy

The first studied member of the DRM1/ARP gene family whose transcript expression was linked with dormancy physiology, \( \text{PsDRM (DORMANCY ASSOCIATED GENE)} - 1 \), was isolated from differential display performed with cDNA libraries from dormant and growing axillary buds of pea. To consider the relationship between expression of this gene and axillary bud dormancy, Stafstrom et al., (1998) decapitated Alaska pea plants. By removing the apical buds of the pea plants, the bud can be released from paradormancy (apical dominance), allowing outgrowth of the axillary buds (Figure 1.3). \( \text{PsDRM1} \) expression was analysed by northern blot, in the bud at various time points after decapitation and revealed that upon release of dormancy, \( \text{PsDRM1} \) levels decreased 20-fold with a concomitant increase in expression of the cell cycle marker gene, \( \text{PsRPL27} \) (Stafstrom et al., 1998).

![Figure 1.3. The 'decapitation' experiment used by Stafstrom et al., (1998) to induce release of dormancy.](image)

Two homologues to \( \text{PsDRM1} \) have been identified in Arabidopsis: \( \text{AtDRM1 (At1g28330)} \) and \( \text{AtDRM2 (At2g33830; formally known as AtDRM1-Homologue).} \) As in pea, a decapitation was conducted in Arabidopsis showing that \( \text{AtDRM1} \) and \( \text{AtDRM2} \) expression levels decreased with release of paradormancy (Tatematsu et al., 2005). Subsequent expression levels increased from 24 h after decapitation, due to a putative apical dominance from the primary axillary outgrowth, leading to paradormancy in the lower buds.
Dormancy release experiments performed with AdDRM1, the DRM1/ARP homologue in Actinidia deliciosa (kiwifruit), using hydrogen cyanamide (commercially available as HiCane™) as a dormancy release agent, show the same expression profiles (Wood et al., 2013). Treatment with hydrogen cyanamide was used to release the apical buds from endodormancy (compared with decapitation initiating release of apical buds from paradormancy in pea and Arabidopsis). Upon release of bud dormancy, the characteristic decrease in AdDRM1 expression was seen. In the same experiment, the expression of AdCDKB (CYCLIN-DEPENDENT PROTEIN KINASE B), a marker for growth from kiwifruit, was assessed. CYCLIN-DEPENDANT KINASES (CDKs) are known to be involved in the re-initiation of cell cycle progression through the G1-S phase transition (Horvath et al., 2003). As AdDRM1 levels decrease, AdCDKB expression increases (Wood et al., 2013).

Further evidence of an association of DRM1/ARP transcripts with periods of dormancy is seen in PsARP (Paeonia suffruticosa AUXIN REPRESSED PROTEIN), a gene identified as associated with the release of dormant buds of tree peony. PsARP showed a sharp increase prior to bud break, then dropped to low levels as the buds grew (Huang et al., 2008).

In most cases, increases in DRM1/ARP expression are associated with reduced branching phenotypes: phyb (Finlayson et al., 2010), tin (Kebrom et al., 2012) and CHR12 OX (Mlynárová et al., 2007); and decreases in DRM1/ARP expression are associated with increased branching phenotypes: axr1-12 (Finlaysen, 2007), max2 (Finlaysen, 2007) and chr12 (Mlynárová et al., 2007) (Table 1.2). However, DRM1/ARP has also been used as a marker for dormancy in Arabidopsis in studies of Teosinte Branched 1-Like or Branched 1 (TBL1 or BRC1) (Finlayson, 2007) with less dependability. Results showed that a reduction in mRNA expression of DRM1/ARP was not required for bud outgrowth leading to the conclusion at the time that DRM1/ARP was probably acting upstream of TBL1 (Finlayson, 2007). Alternatively, DRM1/ARP could be acting independently of TBL1. In the same study, there was no change in DRM1/ARP transcript levels in hyper-branching 35S::YUCCA where you may anticipate a reduction in expression, providing further evidence that DRM1/ARP may not be playing a role specifically in holding back bud outgrowth.
These findings weaken the argument for DRM1/ARP as a molecular marker for dormancy in axillary buds. Alternatively, these data suggest that DRM1/ARP is playing a role as an integrator only under a subset of specific dormancy-related conditions.

Table 1.2. Transcript expression profiles of DRM1/ARP family members in characterised mutants.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mutant</th>
<th>Mutant phenotype</th>
<th>Transcript expression profile in mutant cf. WT</th>
<th>Correlation between DRM1/ARP expression and reduced branching/enhanced dormancy?</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis thaliana</td>
<td>phyb</td>
<td>Reduced branching, hyper-elongation</td>
<td>↑</td>
<td>y</td>
<td>Finlayson et al., 2010</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>tbl1/brc1</td>
<td>Highly branched mutant</td>
<td>¬</td>
<td>n</td>
<td>Finlayson, 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>axr1-12</td>
<td>Highly branched mutant</td>
<td>↓ (buds)</td>
<td>y</td>
<td>Finlayson, 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>max2</td>
<td>Highly branched mutant</td>
<td>↓ (buds)</td>
<td>y</td>
<td>Finlayson, 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>35S:YUCCA</td>
<td>Reduced branching</td>
<td>¬</td>
<td>n</td>
<td>Finlayson, 2007</td>
</tr>
<tr>
<td>Wheat (Triticum aestivum)</td>
<td>tin</td>
<td>Reduced tillering/branching</td>
<td>↑</td>
<td>y</td>
<td>Kebrom et al., 2012</td>
</tr>
<tr>
<td>Sorghum (Sorghum bicolor)</td>
<td>phyb-1</td>
<td>Reduced branching</td>
<td>↑</td>
<td>y</td>
<td>Kebrom et al., 2006; Kebrom et al., 2010</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>chr12</td>
<td>Reduced growth arrest when exposed to moderate stress</td>
<td>↓</td>
<td>y</td>
<td>Mlynárová et al., 2007</td>
</tr>
<tr>
<td>Arabidopsis thaliana</td>
<td>CHR12 OX</td>
<td>Growth arrest of normally active primary buds, and reduced growth of primary shoot, only upon perception of stress conditions</td>
<td>↑</td>
<td>y</td>
<td>Mlynárová et al., 2007</td>
</tr>
</tbody>
</table>
1.2.3 The DRM1/ARP protein family

Database searches show that the DRM1/ARP family is unique to plants, with homologues found in both monocotyledons and dicotyledons (Park and Han, 2003). Multiple protein sequence alignments show high conservation of the DRM1/ARP protein product, with two domains clearly identifiable at the amino-terminus and at the carboxy-terminus (Park and Han, 2003; Wood et al., 2013). This high conservation across species, and indeed monocotyledons and dicotyledons, suggests a potential importance of the action of this protein in the normal development and physiology of plants. Moreover, the protein product is conserved in its size, with all eleven species analysed by Park and Han (2003) between 11 and 14 kDa (between 109 and 126 amino acid residues). GFP fusion constructs of EuNOD-ARP1 (EuNOD-ARP1; Elaeagnus umbellata nodule AUXIN-REPRESSSED PROTEIN GENE-1), a DRM1/ARP homologue in the ornamental shrub E. umbellata (Japanese silverberry, Autumn-Olive), as well as GFP fusion constructs of Brassica rapa DRM1 and ARP1, suggest that it is cytosol-localised (Kim et al., 2007; Lee et al., 2013). However, in silico prediction shows AtDRM1 in the nucleus and mitochondria, possibly suggesting differing roles for family members from different species. No characterised localisation signals or DNA-binding domains are found in the protein sequence. Additionally, the PFAM motif (PF05564), ‘dormancy/auxin associated protein’, represents almost the entire protein, leaving few hints on the role of this protein in planta.

Recently evidence has emerged for DRM1/ARP family members as intrinsically disordered proteins (IDPs) (Wood et al., 2013). IDPs, found in plants and animals, have a large proportion of their sequence yielding no fixed tertiary structure, yet still providing some biological function (Dunker et al., 2001). Signal transduction and transcriptional regulation are key processes within which structural disorder is prominent (Iakoucheva et al., 2002; Minezaki et al., 2006b). A well characterised example of IDPs in the plant kingdom is the late embryogenesis abundant (LEA) class of proteins, involved in abiotic stress tolerance (Garay-Arroyo et al., 2000; Mouilllon et al., 2006) as chaperones for other stress response genes (Chakrabortee et al., 2007; Kovacs et al., 2008a; Kovacs et al., 2008b). ERD10 and ERD14 (EARLY RESPONSE TO DEHYDRATION) are members of the dehydrin type of LEA proteins, shown to be both intrinsically disordered and responding to high salinity, drought and low
temperature stresses in *Arabidopsis* (Kovacs et al., 2008b). Similarly, a (LEA)-like phosphoprotein (CDeT11-24) whose expression is associated with periods of dessication, has been identified in the resurrection plant *Craterostigma plantagineum* (Petersen et al., 2012). Indeed with these findings in mind, DRM1/ARP family members may have roles in chaperoning proteins critical to the no-growth phase in tissues.

Despite the high degree of conservation across species, the biological significance of the DRM1/ARP protein family remains unknown.

### 1.2.4 Potential role of DRM1/ARP in the growth/no-growth decision outside of meristematic tissues

Despite being a commonly used marker for dormancy, DRM1/ARP is expressed in tissues other than dormant axillary buds with high expression often localised to non-growing tissues.

Fruit development is an example of the growth/no-growth homeostasis in floral tissues (cf. vegetative tissues) and as such provides further evidence of DRM1/ARP’s association with non-growing tissues. For example, one DRM1/ARP homologue, AP1, was first discovered in cortical tissue from ripe apple fruit (Lee et al., 1993). In addition, Reddy and Poovaiah (1990) showed a positive correlation between strawberry (*Fragaria ananassa*) fruit growth and repression of a homologue identified from a library of fruit starved for auxin, λSAR5, while being highly expressed in mature fruit which have stopped growing. More recently, *Pyrus pyrifolia* ARP1 (*PpARP1*) and *PpARP2* expression was detected in the mesocarp tissue of Pear (*Pyrus pyrifolia*) fruit (Shi et al., 2013).

Similar data has been seen in kiwifruit with *AdDRM1* levels reaching a peak in ripe, mature fruit (unpublished: M. Wood) and in tomato where high levels of the gene transcript were localised to the ovaries, with a decrease exhibited after fruit set (Vriezen et al., 2008). It is hypothesised that unpollinated ovaries are in a temporally dormant state, supported by expression of cell cycle genes induced after fruit set (Vriezen et al., 2008). As these tissues may have included seeds, and *in silico* expression profiles show seeds have high levels of *SlDRM1* expression, further analysis is required in these
species to clarify whether this increase in expression is a result of increases in the maturing fruit flesh, or rather in the associated maturing seeds.

Increased *DRM1/ARP* gene expression has also been described in tobacco flowers (Steiner et al., 2003); mature stems, leaves and roots of pea (Stafstrom et al., 1998); dehisced pollen of tobacco (Steiner et al., 2003); and root nodules of *E. umbellata* (Kim et al., 2007). While not at particular high levels, *DRM1/ARP* transcript has also been detected in petioles, seeds, various floral organs, and across trunk wood of mature trees (Lee et al., 1993; Park and Han, 2003; Ross et al., 1992; Stafstrom et al., 1998; Steiner et al., 2003).

A classical example of tissue aging and transitioning into growth suppression is provided in the leaf senescence process. *Arabidopsis max2* mutants, already described earlier for their hyper-branching phenotype, also exhibit increased longevity during leaf senescence (Woo et al., 2001). In *Arabidopsis*, *MAX2* transcript expression peaks during the early stages of senescence; while *AtDRM1* expression continually increases throughout senescence, reaching a maximum when yellowing begins, at which point expression remained reasonably constant (Breeze et al., 2011). These data suggest that *DRM1/ARP* is involved in leaf senescence, but later in the process than *MAX2*, raising the question; is *MAX2* controlling *DRM1/ARP*?

An interesting hypothesis given by Park and Han (2003) is for a putative *in planta* role for *DRM1/ARP* family members in repression of cell elongation. The authors introduced an inverse correlation between expression of the *DRM1/ARP* homologue in black locust, *RpARP* (*Robinia pseudoacacia auxin repressed protein*) and the elongation zone of 7-day old hypocotyls. Possible contradictory evidence is found in the hyper-elongated *phyb* mutant which has increased *DRM1* transcript expression in *Arabidopsis* axillary buds (Finlayson et al., 2010). To clarify this, *AtDRM1* transcript expression would need to be measured in the hyper-elongating hypocotyl tissue of the *phyb* mutant.

*PsAD2*, another gene identified as being associated with periods of dormancy, showed similar expression profiles outside of purely dormant meristematic tissues (accumulated in axillary buds, roots, mature leaflets and elongated stems) (Madoka and Mori, 2000).
1.2.5 **DRM1/ARP and seed dormancy**

While characteristics of seed dormancy are not generally linked with bud dormancy, some striking similarities do exist. Seed dormancy is defined as the failure of a seed to germinate despite favourable conditions (Bewley, 1997; Simpson, 1990). Some chilling is often required for either seed germination or bud break, in perennial species at least. However, dormancy requires long-term exposure to low temperatures, while stratification of seeds is relatively short-term (Rohde and Bhalerao, 2007). Microarray expression data show that in *Arabidopsis*, high levels of *AtDRM1* transcript significantly reduce with imbibition (Schmid et al., 2005); while transgenic *Arabidopsis* lines over-expressing *BrDRM1* or *BrARP1* exhibited delayed germination phenotypes, providing evidence that *DRM1/ARP* family members might be involved in seed dormancy maintenance. Assessment of relative *DRM1/ARP* levels in a seed germination microarray of barley showed that *DRM1/ARP* was unaffected (Barrero et al., 2009).

Osmopriming of seeds is a process which initiates germination related processes but does not allow radicle protrusion due the high osmolarity solutions used, limiting water availability. A study completed in *Brassica oleracea*, comparing the transcriptome of osmoprimed seeds with that of water germinated seeds, identified that *DRM1/ARP* was expressed in seeds germinated on water and not the osmoprimed seeds (Soeda et al., 2005). This finding conflicts with the reduction in transcript with imbibition seen in closely related *Arabidopsis* (Schmid et al., 2005), suggesting that *DRM1/ARP*’s expression levels throughout germination are not integral to the process, but might be indirectly linked. This profile in *B. oleracea* was shared with a number of stress-associated genes, including *CYCLOPHILLIN*, *SUPEROXIDE DISMUTASE*, *GRP2* and *GLUTATHIONE-S-TRANSFERASE* (Soeda et al., 2005), providing evidence that *DRM1/ARP* might be playing a more general role in stress response in the seed.

1.2.6 **Regulation of *DRM1/ARP* family gene expression**

Regulation of *DRM1/ARP* has been assessed in response to a number of treatments: hormonal, sugars, abiotic and biotic; across a number of plant species.
1.2.6.1 **Hormonal regulation of DRM1/ARP**

As with many plant genes, DRM1/ARP is regulated hormonally. In particular, a number of often conflicting lines of evidence exist suggesting that DRM1/ARP family members are regulated by auxin, hence many family members being named ARPs (*AUXIN REPRESSED PROTEINS*).

In 1990, a clone named λSAR5 was identified from a cDNA library of strawberry (*Fragaria ananassa*) fruit starved for auxin (Reddy and Poovaiah, 1990) and was later found to be a homologue of DRM1/ARP. Strawberry receptacles (the red-fleshed, seed/achene-coated organ) were used as a model for auxin action as the achenes are a source of auxin which controls the receptacle growth in a pollination-dependent manner. Receptacles can be de-achened then auxin applied exogenously mimicking normal fruit development.

Within 6 h of the start of auxin treatment, λSAR5 mRNA levels were significantly lower than those of receptacles with no auxin, suggesting that high levels of auxin may have a role in the down-regulation of the DRM1/ARP gene. Repression was not shown with a weak acid control suggesting that any regulatory effects were not simply a result of auxin’s acid effect. The auxin analogue selected for these experiments was the synthetic naphthalene acetic acid (NAA), as opposed to naturally occurring indole-3-acetic acid (IAA), for example. NAA can induce stronger effects than IAA due in part to it being metabolised more slowly in the plant (Beyer and Morgan, 1970), therefore any discrepancies observed between NAA and IAA may be a consequence of the nature of the two compounds, although this remains to be determined. Furthermore, NAA was applied at 1 mM, significantly greater than an accepted biologically relevant pmol gram⁻¹ fresh weight concentrations as determined in *Arabidopsis* and tobacco (Izumi et al., 2009). Pollinated fruit which have an endogenous source of auxin from the resulting achenes provide a more biologically relevant assessment of λSAR5 mRNA levels in response to auxin. As expected, λSAR5 mRNA levels are higher at 3- and 5-days after pollination (with auxin) than the unpollinated equivalents (no auxin); allowing the conclusion to be drawn that λSAR5 is repressed during normal development of strawberry fruit in response to exogenous auxin. It is noteworthy that the process of de-achening, which was integral to this approach, would likely have
induced a number of stress response pathways not specific to the exogenously applied auxin, which may have had separate effects on DRM1/ARP transcription.

A further auxin-repressed homologue from black locust, RpARP, was identified from an EST database of trunk wood from 10-year old tree, with gene expression seen across all sections of the trunk wood (Park and Han, 2003). RpARP levels decrease upon application of exogenous auxin in concentrations as low as 0.1 μM. Upon removal of the source of auxin, RpARP levels increased again, showing a negative relationship between DRM1/ARP family member expression and auxin treatment. Unfortunately, this study also used the synthetic auxin isomer NAA and most of the experiments were completed with 1 mM auxin, beyond the accepted biologically relevant concentration.

Analysis of the roles of non-coding sequences showed that regulatory sequences for auxin repression were located in the UTR sequence as opposed to the promoter sequence for RpARP in transgenic Arabidopsis plants, supporting a possible post-transcriptional regulatory mechanism (Park and Han, 2003). This regulation, however, is not mediated by the characterised mRNA instability sequences including cis-elements such as the small auxin up-regulated transcript (SAUR) sequences found in the 3’ UTR of several plant genes (Gil and Green, 1996); or the mammalian AU-rich elements (AREs) (Chen and Shyu, 1995; Park and Han, 2003). The data suggest a novel regulation mechanism that warrants further validation.

While RpARP, and indeed λSAR5, are showing some evidence for down-regulation in their transcript levels in response to auxin, the timeframe in which this happens can provide insight into whether or not they are likely to be responding directly or indirectly to auxin. Gene expression of primary auxin response genes occurs within a matter of minutes (Abel and Theologis, 1996; Key et al., 1967; Theologis et al., 1985; Theologis and Ray, 1982). Those genes whose regulation by auxin takes longer than this are generally referred to as secondary auxin responders, and are likely to be further downstream in the signalling cascade.

In contrast, analysis carried out in E. umbellata showed that transcript levels in field-grown leaves of a clone identified from a root nodule cDNA library, EuNOD-ARP1, were induced with exogenous auxin treatment after 6 h (Kim et al., 2007). Furthermore, pear mesocarp discs exhibited an increase in transcript expression of PpARP1 and
*PpARP2* with IAA treatment (Shi et al., 2013). These pieces of data contradict previous findings in strawberry (Reddy and Poovaiah, 1990), black locust (Park and Han, 2003), and in pea (Stafstrom, 2000). The type and age of tissue assessed is a major difference between these studies which might explain these variations in auxin response.

A further conflicting finding has been described in *Arabidopsis*, where auxin over-producing *35S:YUCCA Arabidopsis* lines displayed no change in *AtDRM1* expression compared to wild-type plants (Table 1.2) (Finlayson, 2007). These mutants showed a reduced branching phenotype and approximately twice the amount of free auxin compared with wild-type plants (Zhao et al., 2001). As such Finlayson (2007) argued that *DRM1/ARP* is unlikely to be affected directly by auxin, if at all.

More recently, transgenic *Arabidopsis* lines over-expressing the coding region of *BrDRM1* and *BrARP1*, which exhibit reduced hypocotyl growth in dark-grown seedlings, showed a compounded growth suppression with NAA application (Lee et al., 2013). That these constructs used the coding region only and therefore do not contain any UTR sequence which would be required for an auxin response based on the *Robinia pseudoacacia* data (Park and Han, 2003), suggests that any auxin responses in this gene family might be regulated by different mechanisms in different species.

Overall, these data question whether or not *DRM1/ARP* family members are regulated by auxin with conflicting data emerging from various experimental set-ups. Thorough analyses of auxin regulation *in planta*, with a focus on biological relevance are therefore required.

Only scarce data are available pertaining to the effects of hormones other than auxin on expression of *DRM1/ARP* family members. In pea it was shown that *PsDRM1* is induced when samples were treated with ABA (Stafstrom, 2000), while GA had no effect on transcript levels. Despite being the sole piece of published data on ABA regulation of *DRM1/ARP*, the gene has been used as a genetic marker for ABA response in studies of fruit set in tomato (Vriezen et al., 2008). Vriezen et al. (2008) argue that the decrease in *DRM1/ARP* after fruit set might be due to lower levels of ABA in the ovary after induction of fruit growth, although this remains to be assessed further.

Few data are available on any response of *DRM1/ARP* family members to cytokinin treatment; however, it would be expected that cytokinin treatment, which is generally
associated with an increase in cell division, would lead to a decrease in transcript levels of DRM1/ARP. The only data available are from a transcriptomic approach comparing Arabidopsis seedlings treated with BAP with non-treated plants. A decrease in both AtDRM1 and AtDRM2 levels was observed but further validation of this finding remains to be completed (Lindsay, 2006).

1.2.6.2 Regulation of DRM1/ARP family members by sugars

A plant needs carbohydrates in the form of sugars to grow. In particular, sugar is required for expression of the cell cycle gene CYCLIND3 (Healy et al., 2001; Oakenfull et al., 2002). Similarly, carbohydrate metabolism genes have been shown to change over the three distinct phases of dormancy in the buds of the perennial weed, leafy spurge (Chao and Serpe, 2010), making a clear link between sugar levels and ratios to dormancy.

An association between sugar and DRM1/ARP was first described in black locust in 2003 (Park and Han). In an attempt to further understand auxin regulation of RpARP, sucrose was analysed as a factor associated with auxin-mediated growth. Sugar-deprivation led to an increase in RpARP gene expression. As discussed above in this paper’s findings on auxin regulation, the age of this tissue, and therefore whether or not it was growing, was not mentioned in the text and age of the tissue may have an impact on the findings. Upon discovery of the sugar response, the upstream promoter sequence was screened and four sugar responsive elements (SREs), known to be responsible for sugar repression of the α-amylase gene in rice (Lu et al., 1998), were identified (Nambara et al., 2010). Furthermore, it was shown that the promoter sequence was required to mediate the sugar response (Park and Han, 2003).

A subsequent, in-depth study of sugar regulation of DRM1/ARP, amongst other genes, was carried out by Gonzali et al., (2006). In support of the findings of Park and Han (2003), they described almost a complete repression of AtDRM1 by sucrose, fructose and glucose, but not by other sugars including turanose and mannitol. As modulation of AtDRM1 was seen as a result of more than one sugar type, it was concluded that AtDRM1 could be a low-specificity sensor of sugars (Gonzali et al., 2006).

More recently, studies of the reduced branching tin (tiller inhibition) mutant in wheat (Triticum aestivum), have drawn a link between sugar levels, dormancy status and
DRM1/ARP (Kebrom et al., 2012). The dormant buds of tin show reduced sucrose content, transcriptional responses in both sucrose-inducible and sucrose starvation–inducible markers (down-regulation and up-regulation, respectively) and reduction in transcript expression of cell cycle markers leading to the hypothesis that the extensive bud dormancy seen in the mutant is due to cessation of cell division due to diversion of sucrose away from the buds. The dormancy status of the buds of tin plants was confirmed by DRM1/ARP and TBL1 transcript expression assessment leading to an additional hypothesis that DRM1/ARP (as well as TBL1) are involved in dormancy responses specifically related to sucrose starvation (Kebrom et al., 2012). Data from kiwifruit have shown that sucrose levels accumulate in dormant buds over winter and reduce again prior to bud break in spring (Richardson et al., 2007). Indeed, application of hydrogen cyanamide is associated with a rapid decrease in both detectable sucrose levels (Richardson et al., 2007) and DRM1/ARP transcript levels (Wood et al., 2013).

Work in Arabidopsis showed a somewhat contradictory result (Tatematsu et al., 2005). A microarray was carried out allowing categorisation of genes categorised as either up- or down-regulated in response to inflorescence decapitation. Both AtDRM1 and AtDRM2 were down-regulated in buds upon decapitation (Tatematsu et al., 2005). A search for regulatory elements enriched in promoter sequences of either group identified SREs as being highly conserved in genes down-regulated with decapitation. It must be noted, however, that this work only analysed the initial 500 bp upstream of the start codon, and as such possibly neglected the majority of the promoter sequences. Furthermore, these motifs remain to be assessed biologically, which will inevitably provide a more robust basis for any hypotheses. These conflicting pieces of data suggest that DRM1/ARP family members are unlikely to be directly regulated by sugar levels.

1.2.6.3 Regulation of DRM1/ARP family members by abiotic and biotic stresses

Across the plant kingdom, DRM1/ARP is increasingly being linked to stress responses, whether they are a result of abiotic or biotic factors.

Increases in DRM1/ARP transcript levels have been associated with the light signalling pathway mutant, phyb, in both Arabidopsis and Sorghum (Sorghum bicolor) (Finlayson et al., 2010; Kebrom et al., 2010; Kebrom et al., 2006) (Table 1.2). phyb mutants have reduced bud outgrowth, with a concomitant increase in DRM1 expression.
(Finlayson et al., 2010; Kebrom et al., 2010; Kebrom et al., 2006), providing support for the hypothesis that DRM1/ARP is involved in growth suppression in bud and hypocotyl tissues.

Other abiotic stress factors have also been considered. An increase in DRM1/ARP expression with cold treatment or salt stress has been documented in pea (Stafstrom, 2000), Capsicum annuum (Hwang et al., 2005) and the close relative of Arabidopsis, B. rapa (Lee et al., 2013). During times of eco-dormancy due to cold, it might be anticipated that DRM1/ARP expression is high, suggesting a role for DRM1/ARP in temperature signalling. An interesting set of experiments considered the effect of various growth regulators and abiotic stresses in conjunction with auxin application on RpARP gene expression (Park and Han, 2003). Data showed that RpARP expression was induced either with or without auxin by cold stress providing evidence for cold stress overriding any auxin response. Other treatments showed no significant effect on transcript profiles. These data provide further evidence for DRM1/ARP family members not being directly regulated by auxin.

DRM1/ARP transcript expression has been assessed in response to further abiotic factors including drought conditions in peanut (Arachis hypogaea) (Govind et al., 2009) and heat shock in B. rapa (Lee et al., 2013); with both conditions leading to an increase in expression.

AtDRM1 and AtDRM2 levels are increased in the SNF2/Brahma-type AtCHR12 chromatin-remodelling gene over-expression lines, introduced earlier, which undergo arrest of axillary bud outgrowth and a reduction in inflorescence bolt growth, only in response to stress conditions (Mlynárová et al., 2007). Conversely, transcript levels of AtDRM1 and AtDRM2 are down-regulated in Atchr12 knock-out mutants (Mlynárová et al., 2007). These findings suggest that AtDRM1/ARP genes might be acting downstream of AtCHR12 in a stress-modulated pathway.

As well as being involved in responses to abiotic factors, DRM1/ARP family members have also been linked to biotic stress responses. A cell death-based assay was undertaken using transient virus-induced gene silencing of DRM3 (homologue of a closely related DRM family member At1g56220) in Nicotiana benthamiana. Plants silenced for NbDRM3 and challenged with a number of bacterial effectors were
compromised in their pathogen-associated molecular pattern-triggered immunity (PTI) (Chakravarthy et al., 2010).

These data combined with the fact that DRM1/ARP proteins in kiwifruit have been shown to be IDPs (Wood et al., 2013), a class of proteins often associated with stress response pathways in plants, as well as multiple lines of evidence showing transcript expression outside of purely dormant tissue provide increasing evidence that DRM1/ARPs are involved in responding to sub-optimal conditions, whether they be abiotic or biotic, and whether this be in meristematic tissue with (by definition) the potential to go dormant, or not.

1.2.7 DRM1/ARP mutants

Analysis of plant lines either over-expressing a gene and its resulting protein, or down-regulating expression, is a powerful tool for characterising a gene’s role in planta.

Studies conducted in black locust (Park and Han, 2003) stated that over-expression of full length RpARP cDNA either with or without the UTRs resulted in Arabidopsis plants with no change in phenotype compared with wild-type plants. Similarly, over-expression of EuNOD-ARP1 cDNA in Arabidopsis had no noticeable phenotype (Kim et al., 2007).

More recently, data have emerged claiming a number of alterations to the visible phenotype of Arabidopsis plants over-expressing cDNA of either of two homologues of DRM1 and ARP1 from B. rapa. These included a reduction in vegetative growth (petiole length), as well as a reduction in seed productivity (siliqule length). A general delay in growth with no significant impact on bolting times was also reported, resulting in a reduction in the number of leaves associated with the vegetative-floral phase transition. In addition, transgenic plants exhibited a reduction in bolt length and a delay in seed germination. Double knock-outs to AtDRM1 and AtDRM2 exhibited no change in phenotype (Lee et al., 2013). These findings are of particular interest as they conflict with other species assessed thus far, providing further evidence of differing roles of DRM1/ARP family members across different plant species.
1.2.8 Conclusions and future directions

As highlighted in this review, DRM1/ARP transcript expression has generally been associated with dormant buds. Exceptions to this association are seen in tbl1/brc1 and 35S:YUCCA mutants, suggesting that DRM1/ARP is not playing a specific role in dormancy maintenance and as such may not be a reliable maker for dormancy release in future studies. Furthermore, the detection of transcripts outside of meristematic tissues, by definition the site of dormancy, often in non-growing tissue means that the correlation between DRM1/ARP and dormant tissues is more likely representative of a link between the non-growth status of these tissues, as opposed to dormancy per se (Figure 1.4).
Mitogen activated protein (MAP) kinase, kinase, kinase (M KK K); MAP kinase, kinase (M KK); and MAP kinase (M K) are representative of a generalised signalling cascade resulting in expression of DRM1/ ARP’s, in response to a range of abiotic, biotic and endogenous factors. DRM1/ ARP proteins are hypothesised to play a role in growth inhibition via a currently unknown pathway.

This plant-specific gene and its resulting low molecular weight protein are highly conserved, and it may be negatively regulated by auxin. Ever since the identification of the dormancy-associated gene DRM1/ ARP in pea more than a decade ago, and more recently in other species, its mode of action has still to be elucidated. Future work will require a multi-faceted approach in order to draw conclusions on the function of DRM1/ ARP in planta. Due to the conflicting findings around DRM1/ ARP mutants, further analysis in a number of species is required to clarify the effect that either over-
expression or down-regulation of DRM1/ARP genes has on the plant. Transcriptome analysis of these various mutants will provide further information on the function of DRM1/ARP. Further physiological and transcriptional studies considering additional abiotic and biotic stress factors, as well as those already described in more depth, will be of interest. Antibodies would be helpful to use for protein localisation studies which could complement promoter:GUS analysis looking into the means of transcriptional regulation of this gene family. Finally, protein-protein interaction experiments, such as yeast-2-hybrid assays, could allow elucidation of binding partners.

Through identification of genetic pathways the genes are involved in; identification of the regulatory mechanisms of the genes; localisation studies at the protein level; and identification of binding partners, the function of DRM1/ARP will be clarified.
1.3 Aims of this thesis

As highlighted in the introduction, DRM1/ARP expression is associated with dormancy. However, increasingly lines of evidence are demonstrating a broader role for this gene family in planta.

This project aims to gain insight into the biological function of Arabidopsis DRM1 and DRM2.

Within this overall objective, an initial aim was to undertake a thorough bioinformatic analysis of all Arabidopsis DRM1/ARP family members, particularly leveraging available in silico predictive packages for analysis of putative protein sequence (Chapter 2 and 3). RT-qPCR was subsequently used to analyse each family member across development and in response to dormancy release via decapitation in order to evaluate the possible similarity of function within the family (Chapter 2).

A second aim was to assess putative regulators of AtDRM1 and AtDRM2 by treating plants and assessing transcripts using RT-qPCR (Chapter 3).

For both the aforementioned objectives, available over-expression lines were used and characterised to provide further information on AtDRM1 and AtDRM2 across plant development and in response to stress conditions (Chapter 2 and 3). As null mutants to these genes were not available, down-regulation lines developed using amiRNA technology were assessed to complement over-expression data (Chapter 4).

The final objective of this work was to undertake biochemical analyses of both AtDRM1 and AtDRM2 (Chapter 5). This work involved expressing the protein, developing antibodies targeting either protein and protein:protein interaction studies using yeast-2-hybrid technology.

The model plant Arabidopsis is being used due to the plethora of resources available for the scientist, including a sequenced genome, various mutants and online databases of transcriptional and biochemical data. Moreover, quick generation time and ease of growing makes Arabidopsis a suitable organism in which to undertake plant research.
The thesis is presented as a ‘thesis with publication’ with Chapters 2 and 3 being two independently submitted papers, while the remainder of the results are presented in chapter format (Chapter 4 and Chapter 5).


2 AtDRM1/ARP family members

Investigations into *Arabidopsis* family members were undertaken during the course of this PhD project and the findings presented as a paper entitled: ‘The DRM1/ARP gene family in *Arabidopsis thaliana* and its proteins’, submitted to the journal PLoS ONE.

Unless otherwise stated, all experiments and analysis were undertaken by the candidate, Georgina Rae. IDP predictions and analyses were undertaken in collaboration with Bin Xue and Vladimir Uversky. GUS histochemical analyses were undertaken by Georgina Rae during her Masters project (Rae, 2009).

Georgina Rae wrote the entire manuscript and critical reading of the manuscript was provided by Vladimir Uversky, Karine David and Marion Wood.
The **DRM1/ARP** gene family in *Arabidopsis thaliana* and its proteins

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**Abstract**

Plant development is highly regulated at both the genetic and environmental levels, ensuring the plant’s survival in its given environment. The ability for a plant’s meristematic tissue to become dormant is one aspect critical to this. *DORMANCY ASSOCIATED GENE-1/AUXIN-REPPRESSED PROTEIN (DRM1/ARP)* is routinely used as a genetic marker for dormancy status in a range of species. However, its biological function remains unknown.

In this study, the five *Arabidopsis DRM1/ARP* family members were investigated. Of the protein family’s two identified clades, *Arabidopsis* family members span both clades. AtDRM1 and AtDRM2 are part of a *Brassicaceae*-specific sub-clade, possibly indicative of an ancient gene duplication event. All family members are predicted to be intrinsically disordered proteins and are differentially regulated across development, as well as in response to decapitation (paradormancy release). Over-expression of either *AtDRM1* or *AtDRM2* is associated with a subtle retardation in development, with no detectable alteration in the plant’s final morphology.

Overall, the *DRM1/ARP* family is not involved exclusively in the maintenance of dormancy; rather they are more likely to be concomitantly present due to the inherent growth cessation associated with dormant tissues.
2.1 Introduction

Due to their sessile nature, plants elegantly manage their development in response to their given environment through a combination of complex mechanisms. One example of which is dormancy, defined as “the transitory delay of visible growth that occurs in plant meristematic tissues” (Lang et al., 1987) and in particular dormancy release.

Understanding the molecular changes that initiate the release from dormancy and promotion of growth is a goal for many crop species. One tool to facilitate a better understanding of the molecular mechanisms underlying bud dormancy is to consider genetic markers associated with it. Molecular studies of bud dormancy and bud outgrowth in pea, Arabidopsis, kiwifruit and maize, commonly refer to DRM1/ARP (DORMANCY ASSOCIATED GENE-1/AUXIN-REPRESSED PROTEIN) as a marker for dormant buds (Finlayson, 2007; Gonzalez-Grandio et al., 2013; Stafstrom et al., 1998; Tatematsu et al., 2005; Wood et al., 2013). However, to date the biological function of this gene remains undetermined.

The first member of the DRM1/ARP gene family associated with dormancy was identified in a cDNA library from dormant pea axillary buds, where upon release of paradormancy by decapitation of the apical bud, PsDRM1 levels decreased 20-fold in the remaining uppermost bud (Stafstrom et al., 1998).

Two homologues of PsDRM1 have been identified in Arabidopsis, namely AtDRM1 and AtDRM2 (also referred to as AtDRM1 Homologue). Analogous to the situation in pea, decapitation of Arabidopsis inflorescence bolts is associated with a decrease in AtDRM1 and AtDRM2 expression levels in axillary buds concomitant with the release of dormancy and the onset of growth (Tatematsu et al., 2005).

However, despite its usefulness as a dormancy marker, DRM1/ARP has also been shown in several species to be expressed in non-dormant tissues (reviewed in Chapter 1). Stafstrom et al. (1998) demonstrated that PsDRM1 transcripts accumulated preferentially in non-meristematic, non-growing stems and roots, with moderate levels in sepals, stamens and carpels, but not in leaflets or petals. This observation is further supported in black locust (Robinia pseudoacacia), in which RpARP was detected in non-meristematic tissues, namely the non-growing section of hypocotyls (Park and Han, 2003). Additional data in Brassica rapa indicates that this gene was also detected in
mature tissues such as roots, mature leaves, petals and sepals (Lee et al., 2013). A
DRM1/ARP homologue was also identified in apple fruit tissue (*Malus x domestica*
Borkh. ‘Golden Delicious’) (Lee et al., 1993). Northern blot analysis showed highest
expression in non-meristematic fruit tissue, but also had detectable expression in fruit,
leaf, stem, petiole, seed and root tissue; while sorghum DRM1/ARP (*SbDRM1*)
expression has been shown to be in leaves, roots of seedlings and only at low levels in
outgrowing buds (Kebrom et al., 2006). An additional tomato DRM1/ARP homologue
also displayed high levels of the gene transcript localised to the ovaries, with a decrease
exhibited after fruit set (Vriezen et al., 2008). Vriezen *et al.* (2008) hypothesised that
unpollinated ovaries are in a temporally dormant state, supported by expression of cell
cycle genes induced after fruit set.

What is clearly emerging is the possible association between periods of growth
suppression and DRM1/ARP expression that is not associated with meristematic tissue.
This is an important observation in that, by definition, dormancy is associated with
meristematic tissue only (Lang et al., 1987). Analyses of this putative DRM1/ARP role
outside dormancy are only just beginning to emerge.

While a great deal is known about DRM1/ARP at the transcript level, less is known
about the DRM1/ARP protein. No domains of known function are associated with the
primary sequence of the protein; including any putative signal sequences and DNA-
/protein-binding domains. Of note, the PFAM motif (PF05564), ‘dormancy/auxin
associated protein’ domain, is represented by >90% of the predicted protein sequence.
GFP fusion constructs using *Elaegnus umbellata* Nodule AUXIN-REPRESSED
PROTEIN GENE 1 (*EuNOD-ARP1*), a DRM1/ARP homologue in *E. umbellata*
suggest that it is cytosol-localised (Kim et al., 2007). A trend was similarly observed
with *Brassica rapa* DRM1 and ARP1 proteins, exhibiting a cytosolic localisation in
*Arabidopsis* protoplasts (Lee et al., 2013).

In a recent publication, it has been proposed that the DRM1/ARP family in kiwifruit are
intrinsically disordered proteins (IDPs) (Wood et al., 2013). IDPs are found in plants
and animals and have a large proportion of their sequence yielding no fixed tertiary
structure, yet still providing some biological function (Dunker et al., 2005; Dunker et
al., 2001; Dunker et al., 2008; Dyson and Wright, 2005; Tompa, 2002; Uversky, 2002;
Uversky and Dunker, 2010; Uversky et al., 2000; Uversky et al., 2005; Wright and
Dyson, 1999). Signal transduction and transcriptional regulation are among the key processes within which structural disorder is prominent (Iakoucheva et al., 2002; Minezaki et al., 2006b). Few examples of IDPs in plants have been described to date. One notable example of an IDP plant gene family is the late embryogenesis abundant (LEA) class of proteins, involved in abiotic stress tolerance (Garay-Arroyo et al., 2000; Mouillon et al., 2006) and as chaperones for other stress response genes (Chakrabortee et al., 2007; Kovacs et al., 2008a; Kovacs et al., 2008b). It is predicted that 23% of all the proteins coded for in the Arabidopsis genome are mostly disordered in nature (Oldfield et al., 2005).

Here we report that the DRM1/ARP family in Arabidopsis is a conserved family comprising of five members. In silico predictions indicate that proteins from the Arabidopsis DRM1/ARP family are intrinsically disordered. Temporal and spatial transcript expression analyses of family members indicate that the DRM1/ARP members are not associated exclusively with axillary buds and meristematic tissue. Over-expression of the AtDRM1 and AtDRM2 genes in Arabidopsis resulted in a subtle growth retardation, suggesting that these genes AtDRM1 and AtDRM2 may play a role in plant growth modulation.
2.2 Methods

2.2.1 Sequence alignments and construction of phylogenetic trees

All multiple alignment analyses were performed with the Geneious Alignment programme as part of the Geneious Pro 5.3.4 software, using an opening penalty of 12 and an extension penalty of 3 (http://www.geneious.com). All sequences used in the protein alignments represent the putative full-length protein sequences from Arabidopsis thaliana: AtDRM1 (At1g28330); AtDRM2 (At2g33830); AtDRM3 (At1g54070); AtDRM4 (At1g56220); and AtDRM5 (At5g44300). Phylogenetic and molecular evolutionary analyses were performed with Tree Builder programme as part of the Geneious Pro 5.3.4 software using the Jukes-Cantor genetic distance model, inferred by the neighbour-joining method (http://www.geneious.com). The resulting tree topology was evaluated and bootstrap analysis based upon 100 replicates. Numbers on nodes represent bootstrap values and branches are supported by ≥50% bootstrap values, which are statistically supported. All sequences used in the analyses represent the non-redundant, deduced full-length amino acid sequences. The DRM1/ARP conceptual protein translation sequences used in these alignments are listed in supplemental data (Appendix 7.1).

2.2.2 Bioinformatic analysis of Arabidopsis DRM1/ARP family: primary sequence analyses, disorder prediction and promoter analysis

Prediction of order/disorder

The order/disorder of DRM1/ARP proteins was predicted using PONDR® (Predictor Of Natural Disordered Regions) FIT (Xue et al., 2010). Output is represented by real numbers between 1 (ideal predictor of disorder) and 0 (ideal prediction of order). An arbitrary threshold of ≥0.5 was used to identify disorder.

Charge-hydropathy and cumulative distribution function plot

The CH-CDF plot analyses were performed as previously described (Huang et al., 2012; Wood et al., 2013).
Identification of putative α-MoRFs

An α-helix-forming molecular recognition feature predictor (α-MoRFs-II) based on PONDR® prediction and a large positive data set (Cheng et al., 2007; Oldfield et al., 2005) was used to identify putative α-MoRFs.

Prediction of putative PEST proteolytic cleavage sites

Putative proteolytic cleavage sites identified by the PEST motif were identified using epestfind (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::epestfind). PEST motifs are defined as hydrophilic stretches of at least 12 amino acids length with a high local concentration of critical amino acids.

Prediction of potential phosphorylation sites

The web-based phosphorylation predictor DISPHOS 1.3 (http://www.dabi.temple.edu/disphos/pref.html) was used to predict phosphorylation of serine, threonine and tyrosine residues in a query sequence as part of the PONDR® website (http://www.pondr.com). The Arabidopsis thaliana group predictor was used to minimise the misclassification of residues.

Identification of phosphorylated and oxidated residues from databases

Arabidopsis sequence identifier codes were used to interrogate the PhosPhAT4.0 database (http://phosphat.mpimp-golm.mpg.de/index.html) (Durek et al.; Heazlewood et al.).

Identification of putative regulatory motifs in the upstream sequence

Sequence upstream of the 5’ UTR of AtDRM1 and AtDRM2, but downstream of the neighbouring gene’s annotated UTR, was retrieved from TAIR (The Arabidopsis Information Resource: http://www.arabidopsis.org/) and designated ‘entire promoter sequence’. Sequences were analysed using PLACE, a database of plant cis-acting regulatory DNA elements (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999) and motifs of interest annotated on to the sequence.

Sequences were also submitted to the MEME (Multiple Em for Motif Elicitation) tool as part of the Meme Suite of motif-based sequence analysis tools (http://meme.nbcr.net/meme/). The ten most conserved motifs between AtDRM1 and AtDRM2 entire promoter fragments were then individually assessed by TOMTOM (also part of the Meme suite), a tool for comparing the identified motifs with biologically
characterised motifs, using the JASPAR core plant database as a reference. Motifs with a p-value of <0.05 were used to annotate the AtDRM1 and AtDRM2 promoter sequences.

### 2.2.3 Plant growth conditions

All plants used in this work were *Arabidopsis thaliana* (‘Columbia’ ecotype background).

Seeds were sterilised by soaking for 30 min in sterile water, followed by 5 min sterilisation in 95% ethanol and a subsequent 5 min incubation in 4% sodium hypochlorite, 0.0001% triton X-100 solution. Sterilised seeds were then washed ≥3x in sterile water.

For soil-grown plants, imbibed seeds were placed on moist filter paper and incubated in darkness at 4°C for 2-4 days. Seeds were then sown on to soil irrigated with 0.16% Dimilin® 25W (Veg Gro Supplies).

For plants grown on plates, seeds were sterilised and then transferred to ½ MS media (0.5 x Murashige & Skoog medium, including vitamins (Duchefa), adjusted to pH 5.6-6.0) plates (½ MS media, 0.75% select agar). Plates were grown in a light and temperature controlled growth room (12 h light (50 µE m⁻² s⁻¹), 12 h dark, at 22°C).

For plants grown on rockwool, seeds were placed on moist filter paper and incubated in darkness at 4°C for 2-4 days. Seeds were then sown either directly onto rockwool for transcript analysis, or onto an agar plug (½ MS) for phenotypic analysis, in the rockwool (Veg Grow Supplies) saturated with hydroponics media (KNO₃ 1.25 mM, Ca(NO₃)₂·4H₂O 1.5 mM, KCl 5 mM, C₁₀H₁₂FeN₂NaO₈ 72 mM, KNO₃ 1.25 mM, KH₂PO₄ 0.5 mM, MgSO₄·7H₂O 0.75 mM, H₃BO₃ 50 mM, MnSO₄·H₂O 10 mM, ZnSO₄·7H₂O 2.0 mM, CuSO₄·5H₂O 1.5 mM, (NH₄)₆Mo₇O₂₄·4H₂O 0.075 mM, KCl 50 mM, Ca₆H₁₃NO₄·SH₂O 0.5 mM; adjusted to pH 5.7 with orthophosphoric acid). Rockwool was saturated with hydroponics media every week to maintain nutrient status required for plant growth. Plants were grown in long day conditions (18 h light/6 h dark).

### 2.2.4 RNA Isolation and Quantitative RT-qPCR

RNA was isolated using TRIzol® Reagent (Invitrogen), according to the manufacturer’s instructions. DNA was digested using the RNAeasy® mini kit (Qiagen) with RNase-free DNAs (Qiagen) according to the manufacturer’s instructions. The quality of the
DNAse-free RNA preparation was visualised using standard agarose gel electrophoresis.

Superscript™ III Reverse Transcriptase (Invitrogen) was used for cDNA synthesis according to manufacturer’s instructions.

The LightCycler® 480 System (Roche) in the 384-well plate set-up was used for amplification and analysis by RT-qPCR. All reactions used LightCycler® 480 SYBR Green I Master Mix (Roche) and raw data analysed using the LightCycler® 480 software (version 1.5). Expression was normalised to reference genes.

Gene specific primers were designed using Primer3 (Rozen and Skaletsky, 2000) so that the resultant amplicon, preferably, spanned an intron or the stop codon and include a portion of the 3' UTR (Appendix 7.2).

### 2.2.5 Plant material for expression analyses

For the developmental series, tissue of wild-type plants grown on rockwool, was harvested throughout plant development (day 6; day 16; day 22 vegetative phase: mature leaves = 4 oldest, basal leaves, young leaves: 4 youngest, apical leaves, axil region: all leaves, petioles and root tissue removed and day 36 mature/floral phase: low bolt, basal 2 cm of primary inflorescence bolt; top bolt, apical 2 cm of primary inflorescence bolt; flowers: entire floral region of primary bolt) for total RNA extraction. Time-points were selected as they covered *Arabidopsis* development from young seedlings to mature plants. For day 6 and day 16 samples, approximately 100 and 20 plants, respectively, were harvested. For all other samples, tissue was harvested from 6 plants and pooled.

For expression in buds in response to decapitation, soil grown plants were grown as for the developmental series, until inflorescences were visible by eye; up to ~5 cm. Plants were either decapitated, by removing the inflorescence bolt with a scalpel, or not decapitated, for comparison (18 plants of each). After 12 h, axillary bud tissue was excised, removing all surrounding young leaf tissue as assessed macroscopically. The apical two buds were excised from each plant (not including cauline buds) for total RNA extraction.
2.2.6 Cloning and Arabidopsis transformation

For both promoter:GUS constructs and over-expression constructs, Arabidopsis gDNA was used as a template for PCR amplification of promoter and gDNA fragments, respectively, using the high fidelity polymerase Advantage® 2 PCR Enzyme System and primers (Appendix 7.3) with appropriate restriction digest sites. Upon ligation into pGEM®-T easy vector (Promega), sequence verified clones were subcloned upstream of the GUS gene in the binary vector pHEX14 (same as pHEX2, but without the 35SCaMV promoter (Hellens et al., 2005)) for promoter:GUS constructs, and downstream of 35SCaMV constitutive promoter in the binary vector pART277 for over-expression constructs.

Sequence verified constructs were transformed into Agrobacterium tumefaciens strain GV3101 by electroporation for transformation into wild-type Arabidopsis plants (ecotype ‘Columbia’) via floral dip method (Clough and Bent, 1998).

2.2.7 GUS histochemical analysis

T2 lines of interest expressing AtDRM1_{entprom}:GUS; AtDRM2_{entprom}:GUS; AtDRM1_{500bpprom}:GUS; AtDRM2_{500bpprom}:GUS; 35S:GUS (positive control); empty vector and wild-type negative controls were assayed. AtDRM1_{entprom}:GUS and AtDRM2_{entprom}:GUS both include fragments of the upstream neighbouring gene’s 5’ UTR (35 bp and 4 bp, respectively) as determined using an up-to-date TAIR annotation, which at the time did not identify this sequence as overlapping with neighbouring gene UTR sequence. Intact plants (N≥3) were sampled at day 6, or day 16 directly from plates. Where later developmental stages were required (vegetative phase and mature (reproductive) phase), individual plants were transferred to soil at day 21 (4 - 5 leaf stage) for further growth, corresponding to equivalent time points in the RT-qPCR developmental series analysis.

Harvested tissue was immediately immersed in ice-cold 90% acetone, and incubated on ice for 10 min to fix and facilitate the permeability of the tissue. Samples were washed 3x with non-stain solution (1 mM K_2Fe(CN)_6, 1mM K_4(CN)_6, 0.1% Triton X-100, 10% 1 M NaPO_4 buffer pH 7.0) and incubated in stain solution (1 mM cyano salt, 0.1% Triton X-100, 10% 1 M NaPO_4 buffer pH 7.0, 1 mg/mL X-Gluc (GoldBio), 0.5%
dimethylformamide) for 18 h at 37°C, in darkness. To remove chlorophyll, samples were incubated at room temperature on a slow shaking platform for 30 min with sequential washes in 20%, 30%, 50%, 70%, 80%, and 90% ethanol. Stained samples were stored in 95% ethanol in a sealed container prior to analysis.

Analyses were performed using at least three independent transgenic lines.

### 2.2.8 Statistical analysis

All statistical analyses were undertaken using the GenStat® 14th edition software (www.genstat.co.uk).
Chapter 2

AtDRM1/ARP family members

2.3 Results

2.3.1 Arabidopsis DRM1/ARP has five family members

DRM1/ARP is a useful marker for dormancy and to date family members have been studied extensively, namely AtDRM1 and AtDRM2 (previously referred to as AtDRM1 and AtDRM1-Homologue (At1g28330 and At2g33830, respectively)) (Tatematsu et al., 2005). Three additional members were identified following reiterative BLAST analysis and named AtDRM3 (At1g54070), AtDRM4 (At1g56220) and AtDRM5 (At5g44300) (Figure 2.1A). Sequence alignment highlights the presence of a conserved N-terminal domain in all family members (Domain I; 1-31 aa). AtDRM1, AtDRM2 and AtDRM5 are conserved at the C-terminal region (Domain II; 63-129 aa), while both AtDRM3 and AtDRM4 showed divergence in the C-terminal region (Figure 2.1A). Extended phylogenetic analysis of the DRM1/ARP family members using a range of other plant species was undertaken, indicating that the Arabidopsis DRM1/ARP proteins are distributed into two distinct clades (Figure 2.1B). Both AtDRM1 and AtDRM2 fall into Clade 1, together with other dormancy-associated proteins described in the literature, as well as AtDRM5, because of the presence of Domain II in their sequence (Figure 2.1B; Clade 1). By contrast, the other family members AtDRM4 and AtDRM3 fall into a separate clade (Clade 2) lacking Domain II in its entirety and only exhibiting the conserved Domain I. AtDRM3 possesses a partially conserved Domain II, making it a member of a Clade 2 extension (Clade 2b) (Wood et al., 2013).

AtDRM1 and AtDRM2 are designated to a Clade 1 sub-clade that contains only members of the Brassicaceae family, including Arabidopsis lyrata, Brassica oleracea, B. rapa and Thallungiella halophila (Figure 2.1B). Clades 1 and 2 both contain monocotyledons (e.g. Oryza sativum) and dicotyledons (e.g. Arabidopsis) (Figure 2.1B).
2.3.2 AtDRM1/ARP family is predicted to be intrinsically disordered

DRM1/ARP is routinely used as a marker for dormancy status, but no precise biological function has been determined. To gain insight into the potential mode of action of the Arabidopsis DRM1/ARP family members, a suite of predictive algorithms were employed using AtDRM1 as a representative example (Figure 2.2).
Chapter 2
AtDRM1/ARP family members

Figure 2.2. Predicted properties and secondary structure of AtDRM1.

Composite diagram illustrating predicted secondary structures and properties of Arabidopsis DRM1/ARP family member proteins, using AtDRM1 as a representative example. Potential nuclear localization signal (NLS), disulfide bonds, protein:protein interaction sites and secondary structure prediction (JNET; H = helix; E = β-strand) generated via PredictProtein (http://www.predictprotein.org). Phosphorylation prediction was generated via PhosPhAT4.0. Predicted putative PEST proteolytic cleavage sites were determined using EPESTFIND (* presence; - absence of predicted PEST motif). JNETCONF numbers represent the JNET prediction confidence on a scale of 0-9, where 0 indicates no confidence and 9 indicates high confidence. Solvent accessibility (JNETSOL25) is annotated with a B for solvent accessibility (buried) or a hyphen for solvent accessibility at a 25% cut-off. Putative α-MoRF motifs were determined using α-MoRFs-II based on PONDR® FIT prediction and a large positive data set.

A putative α-helix was predicted (20-29 aa) in AtDRM1, otherwise no secondary structures, such as α-helices or β-sheets, were predicted with any confidence in any of the other family members, suggesting the protein may consist of an intrinsically disordered region (IDR), or indeed be an intrinsically disordered protein (IDP). No transmembrane or nuclear localisation signal motifs, or predicted sites of disulfide bonding were identified (Figure 2.2). Protein:protein interactions sites are predicted across the length of AtDRM1 (Figure 2.2). Regions of hydrophobicity, as represented by ‘buried’ residues in JNETSOL25 analysis, are spread across the length of the AtDRM1 protein sequence. A number of predicted protein phosphorylation sites have been detected across all family members by PhosPhaAT4.0 and DISPHOS, although the localisation of these is not conserved within the family (Appendix 7.5). Biologically confirmed phosphorylation sites were identified in the PhosPhAT4.0 databases for AtDRM2 and AtDRM4 only (Appendix 7.5). PhosPhAT 4.0 was also used to identify oxidated methionine residues, one of which was detected in the phosphorylated C-terminal regions of AtDM4 and AtDM5 (Appendix 7.5). Cysteine residues were only detected in AtDM3 (Appendix 7.5).

Furthermore, prediction of structural disorder was undertaken using the PONDR® -FIT (Predictor Of Natural Disordered Regions) intrinsic disorder predictor, where scores...
>0.5 are indicative of predicted disordered residues and scores <0.5 are indicative of predicted ordered residues (Xue et al., 2010) (Figure 2.3).

The PONDR®-FIT score profiles were particularly highly conserved in the N-terminal regions of AtDRM1/ARP family members, with a conserved minima in the ~10-35 aa region (Figure 2.3). All sequences have a second conserved minima which is earlier in the sequence for both AtDRM2 and AtDRM5 (~60-95 aa) than for AtDRM1, AtDRM3 or AtDRM4 (~95-110 aa). AtDRM3 is the only family member with some order predicted at the C-terminus. All AtDRM1/ARP family members were predicted to be disordered to some degree. Based upon the six previously described distinct profiles of DRM1/ARP family proteins (Wood et al., 2013), AtDRM4 aligns with profile D with a long IDR from around residue 30 to residue 100 (Appendix 7.6). Within this work, Profile A is divided into two sub-profiles (A.i and A.ii). Profile A.i contains AtDRM1 and AtDRM3 and is similar to profile A.ii, but with the N-terminal conserved IDR being slightly longer, and some profile members not having the C-terminal IDRs. AtDRM2 and AtDRM5 exhibit profile A.ii, which has a shorter conserved IDR around residue 50, followed by conserved minima around residue 95.

Further predictive Charge Hydropathy - Cumulative Distribution Function analysis (CH-CDF) plots showed that AtDRM1, AtDRM3, AtDRM4 and AtDRM5 occupy
quadrant 3, indicating that they are predicted to be disordered by CDFs, but not by CHs, suggesting that they adopt a ‘molten globule’ structure that may undergo hydrophobic collapse but maintain their lack of stable structure (Appendix 7.7). Alternatively, proteins occupying quadrant 3 may represent a mixture of structured regions and IDRs. AtDRM2 occupies quadrant 4 and is thereby predicted to be disordered by both the CH and CDF methods (Appendix 7.7). Presence in either quadrant 3 or 4 is indicative of an intrinsically disordered protein. While all DRM1/ARPs fall into quadrants 3 or 4, the majority of family members occupy quadrant 3.

Despite all AtDRM1/ARP family members being predicted by both PONDR®-FIT and CH-CDF plot analyses to be IDPs, AtDRM5 does not have any predicted α-MoRFs (molecular recognition features) (Appendix 7.5). Found in intrinsically disordered regions of proteins, α-MoRFs are short binding regions, which facilitate interactions via a disorder-order conformational change forming an α-helix in their bound state. One to three α-MoRFs were predicted in the four other family members. AtDRM1 has one α-MoRF (83-100 aa) (Figure 2.2), which is conserved with AtDRM2 (67-84 aa) at the C-terminus. AtDRM2 has two additional predicted α-MoRFs in the N-terminal region (1-18 aa and 22-39 aa). AtDRM3 has only one α-MoRF, which is also predicted in the N-terminal region (3-20 aa). AtDRM4 has two α-MoRFs predicted, which are not conserved with those predicted in the other family members (16-33 aa and 99-116 aa) (Appendix 7.5).

AtDRM1 and AtDRM2 both display one predicted PEST domain (102-118 aa and 86-102 aa, respectively) (Figure 2.2; Appendix 7.5), which is also conserved with AtDRM3 and AtDRM5 in the C-terminal region (98-123 aa and 94-110 aa, respectively). AtDRM3 has three additional predicted PEST domains distributed across the entire protein sequence (6-23 aa, 34-47 aa and 65-98 aa). Only two PEST domains were predicted in the AtDRM4 sequence, located in the central region of the protein (33-47 aa and 47-60 aa). In addition to the conserved C-terminal PEST sequence, AtDRM5 has a large domain predicted through the central sequence (27-64 aa). Overall, all AtDRM1/ARPs have at least one predicted PEST domain downstream of α-MoRF sequences.
2.3.3 *AtDRM1/ARP* transcript expression is not limited to dormant meristematic tissues

The transcript expression profiles of all five family members in wild-type *Arabidopsis* were assessed using RT-qPCR across development from germination (6 days post-sowing) to flowering (36 days post-sowing) (Figure 2.4).
Figure 2.4. AtDRM1/ARP family member wild-type developmental expression profiles.

Graphs show the target/reference ratio for AtDRM1, AtDRM2, AtDRM3, AtDRM4 and AtDRM5 over (A) a developmental series in wild-type plants and B) decapitation series in wild-type plants. AtDRM5 decapitation data are not presented in this figure as no transcript was detected in either sample. SEM are represented in the error bars. Samples were normalised to the reference genes (SAND, TIP41-L and UBC9). For the developmental series, values were calibrated to the day 16 sample prior to calculating the mean over three biological replicates, with the exception of AtDRM5, which could not be calibrated to day 16 as expression was only detected in floral tissue. For the decapitation series, the mean of two biological replicates was calculated.
Two distinct transcription expression profiles (I and II) emerged within AtDRM1/ARP family members across development, neither of which is limited to meristematic tissue capable of undergoing periods of dormancy (Figure 2.4A). Messenger RNAs for AtDRM1, AtDRM2, AtDRM3 and AtDRM4 were detected in all tissues tested, while AtDRM5 was detected only in floral tissue. 

AtDRM1, AtDRM2 and AtDRM4 are representative of Profile I, with transcripts expressed constitutively across the time-points and tissues assessed within this development series. While Profile I genes appear to be constitutively expressed, the relative levels were shown to vary. High levels of expression were correlated with low bolt tissue, mature tissue that has ceased growth, compared with other tissues sampled. 

In contrast, Profile II members (AtDRM3 and AtDRM5) are expressed primarily in floral tissue, with little or no expression detectable in all other tissues sampled.

### 2.3.4 DRM1/ARP family members in response to decapitation

AtDRM1/ARP transcripts were detected for all genes assessed, except AtDRM5 (Figure 2.4B). Both AtDRM1 and AtDRM4 exhibited a decrease in expression, of approximately two-fold, upon decapitation compared with control plants (Figure 2.4B). AtDRM2 showed no significant change in expression levels between treatments, whereas AtDRM3 showed a contrasting profile, with transcript levels in axillary buds increasing upon decapitation. 

In order to identify putative upstream regulatory elements, bioinformatic analyses of promoter sequence upstream of the 5’ UTR (AtDRM1_{entprom}: 1771bp; AtDRM2_{entprom}: 1451bp; truncated AtDRM1_{500bprom} and AtDRM2_{500bprom}) were undertaken, with particular emphasis on the previously described Sugar-Repressive (SRE)-like elements, Up-Regulated (Up)-1 and Up2 elements (Lu et al., 1998; Tatematsu et al., 2005) (Table 2.1 and Appendix 7.8).
Table 2.1. Frequency of predicted upstream decapitation response elements in AtDRM1/ARP family member upstream sequence.

Frequency in ‘entire’ promoter sequence (frequency in 500 bp fragment) as spatially annotated in Appendix 7.8.

<table>
<thead>
<tr>
<th></th>
<th>SRE-like</th>
<th>Up1</th>
<th>Up2</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtDRM1</td>
<td>9 (4)</td>
<td>7 (0)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>AtDRM2</td>
<td>12 (3)</td>
<td>0 (0)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>AtDRM3</td>
<td>2 (2)</td>
<td>0 (0)</td>
<td>3 (3)</td>
</tr>
<tr>
<td>AtDRM4</td>
<td>19 (4)</td>
<td>6 (0)</td>
<td>12 (2)</td>
</tr>
<tr>
<td>AtDRM5</td>
<td>2 (1)</td>
<td>0 (0)</td>
<td>8 (5)</td>
</tr>
</tbody>
</table>

SRE-like and Up2 elements were identified in all upstream promoter sequences, while Up1 was detected only in AtDRM1 and AtDRM4. Similarly, SRE-like and Up2 elements were identified in all 500 bp upstream sequences. However, no Up1 motifs were identified in any of the 500 bp fragments. High frequencies of SRE-like motifs were identified in AtDRM1, AtDRM2 and AtDRM4.

### 2.3.5 Expression analysis of the DRM1/ARP using promoter:GUS fusion genes

Because of the high sequence similarity and transcript expression profile across development described above, the temporal and spatial expression profiles of the β-GLUCORONIDASE (GUS) reporter gene in transgenic plants driven by either the AtDRM1 or AtDRM2 promoter were studied. The promoter fragments of AtDRM3, AtDRM4 or AtDRM5 were not included in this analysis due to differences in either coding sequence of transcript expression profile. Moreover, studies considered both the entire promoter, not including 5' UTR (AtDRM1entprom:GUS 1771bp and AtDRM2entprom:GUS 1451bp, respectively) and a truncated 500 bp fragment of the aforementioned entire promoter immediately upstream of the 5' UTR sequence (AtDRM1500bpprom:GUS and AtDRM2500bpprom:GUS).
Figure 2.5. GUS histochemical analysis of *AtDRM1* and *AtDRM2*.

A) *AtDRM1* promoted *GUS* line day 6 samples. Scale = 2 mm. B) *AtDRM1* promoted *GUS* line day 16 samples. Scale = 2 mm. C) *AtDRM1* promoted *GUS* line day 6 samples. Scale = 2 mm. D) *AtDRM1* promoted *GUS* line day 16. Scale = 1 cm. E) *AtDRM1* promoted *GUS* line mature plant sample. Scale bar = 10 cm. F) *AtDRM2* promoted *GUS* line, global image of day 16 samples. Scale = 1 cm. G) *AtDRM2* promoted *GUS* line cotyledon. Scale = 1 mm. H) *AtDRM2* promoted *GUS* line cotyledon tip. I) *AtDRM2* promoted *GUS* line day 6 samples. Scale = 1 mm. J) *AtDRM2* promoted *GUS* young flowers. Scale = 1 cm. K) Empty vector negative control line at day 16. Scale = 1 cm. L) *35S:GUS* positive control line. Day 16 samples. Scale = 1 cm. M) *35S:GUS* positive control line mature plant sample. Scale = 10 cm.
Overall staining patterns were observed and are summarised in Table 2.2. All constructs except \textit{AtDRM2}_{500bpprom}\textcdot GUS lines exhibited \textit{GUS} expression in the tips of cotyledons and the axillary buds of 6-day-old seedlings (Figure 2.5A, C, G and I). No staining was associated with the \textit{AtDRM2}_{500bpprom}\textcdot GUS lines at any developmental stage (Figure 2.5F). For \textit{AtDRM1}_{500bpprom}\textcdot GUS, lateral root junction staining was seen from day 16 onwards (Figure 2.5B), while no staining was visible in mature plants (data not shown).

Specific to those constructs containing the entire putative promoter fragment was \textit{GUS} expression in tips and hydathodes of vegetative phase leaves (Figure 2.5D). Staining of the vasculature of young leaves and cotyledons was seen when the entire putative \textit{AtDRM1} promoter was driving expression (Figure 2.5C and D), while stomatal guard cell staining in young, developing tissues was specific to \textit{AtDRM2}_{entprom}\textcdot GUS (Figure 2.5G, H and I).

In \textit{AtDRM1}_{entprom}\textcdot GUS lines, \textit{GUS} expression was visible in floral tissue, in particular the pedicle and the style, forming a gradient down the stem (Figure 2.5E). Furthermore, staining was visible in floral buds emerging from the axillary region of the rosette and inflorescence bolt (Figure 2.5I). A similar profile was exhibited by \textit{AtDRM2}_{entprom}\textcdot GUS,
but to a lesser extent than \textit{AtDRM1} \textsubscript{entprom-GUS} (Figure 2.5J). No staining was seen in mature rosette leaves of any constructs (Figure 2.5E).

Conserved regulatory motifs between the upstream sequences of \textit{AtDRM1} and \textit{AtDRM2} with possible roles in regulation of gene transcription were identified using Meme/TOMTOM analysis and PLACE analysis (Appendix 7.9). These included motifs associated with flowering time, DOF-binding, BZIP-binding, and ABA-response.

\textbf{2.3.6 Over-expression produces a subtle developmental retardation phenotype}

In order to gain additional insight into the role of \textit{AtDRM1} and \textit{AtDRM2} during plant development, full-length gDNA over-expressing lines were generated in \textit{Arabidopsis}, with morphological traits scored throughout the plant’s lifecycle from germination to maturity (Figure 2.6). Plants from all lines were confirmed as over-expressing the transgene at the transcript level, with representative lines (\textit{AtDRM1} OX2 and \textit{AtDRM2} OX7) confirmed at the protein level also (Appendix 7.10).
Figure 2.6. Phenotypic analyses of AtDRM1 and AtDRM2 over-expression lines.

(A) Mean leaf number of individual AtDRM1 and AtDRM2 over-expressing lines from day 12 until day 18. (B) Mean number of leaves at day 12. (C) Mean number of days to anthesis of DRM1/ARP over-expressing lines, as scored by visible presence of petals in floral buds. (D) Mean number of inflorescence bolts at 29 days post-sowing. (E) Mean height of plant (cm) at 29 days post sowing. (F) Mean number of inflorescence bolts at 1 week post-anthesis. (G) Mean height of plant (cm) at 1 week post-anthesis. All data sets were subjected to a one-way ANOVA test followed by Tukey’s test for multiple comparisons (95% confidence), with SEM represented in error bars (n ≥10).

Overall, over-expressing lines did not show any major phenotypic alterations, especially in branch number (Figure 2.6D & F), even after decapitation (data not shown). However, a subtle growth retardation phenotype was observed.

AtDRM1 over-expression lines had fewer leaves than wild-type plants 12 days after sowing (Figure 2.6B). AtDRM2 over-expression lines showed intermediate growth
retardation. Any differences in number of rosette leaves were maintained across vegetative development (Figure 2.6A).

The trend of a delay in reaching anthesis was evident, with both statistically significant and intermediate delays evident across the lines (Figure 2.6C). By 29 days after sowing there was no statistically significant difference in the number of inflorescence bolts (Figure 2.6D). However, there was a decrease in the height of the over-expression lines compared with those of wild-type (Figure 2.6E). By contrast, when these measurements were assessed at a standardised developmental timepoint (1 week post-anthesis), transgenic lines showed no difference in either plant height or the number of inflorescence bolts (Figure 2.6F & G).

This experiment was repeated, with similar trends observed (Appendix 7.11). Variation was evident in terms of which lines were demonstrating a statistically significant difference from wild-type plants.
Chapter 2

AtDRM1/ARP family members

2.4 Discussion

2.4.1 Divergence of sequence and function in the AtDRM1/ARP family

The AtDRM1/ARP family has been shown to consist of five family members. AtDRM1 and AtDRM2 have previously been described as robust genetic markers for dormancy status in a number of plant species (Aguilar-Martínez et al., 2007; Finlayson, 2007; Gonzalez-Grandio et al., 2013; Kebrom et al., 2010; Stafstrom et al., 1998; Tatematsu et al., 2005; Wood et al., 2013). However, the three additional family members, AtDRM3, AtDRM4 and AtDRM5, have not been previously documented in the literature. Arabidopsis is therefore represented in all clades and sub-clades of the DRM1/ARP family (AtDRM1, AtDRM2 and AtDRM5 in Clade 1; AtDRM3 in Clade 2b and AtDRM4 in Clade 2a) (Wood et al., 2013).

Alignment of all Arabidopsis family members in conjunction with family members from additional plant species identified a Brassicaceae-specific clade that includes AtDRM1 and AtDRM2, as well as members from Arabidopsis lyrata, Brassica oleracea and Thellungiella halophila. Reiterative BLAST searches of available whole genome sequence databases, including kiwifruit (genome unpublished) (Actinidia deliciosa), apple (Malus x domestica ‘Golden Delicious’) and avocado (Persea americana), yielded no potential homologues of AtDRM2 (Wood et al., 2013). This sub-clade may be a reflection of the very ancient duplication events that resulted in the common eudicot genome, followed by deletion of AtDRM2 in the aforementioned species (Schranz and Mitchell-Olds, 2006; Wood et al., 2013). Perhaps more likely is that duplicate genes arose as a result of an ancient paleoploidy event specific to the Brassicaceae family, hence since the radiation of the entire Brassicaceae family from eudicots 24-40 million years ago (Blanc et al., 2003).

Interestingly, despite all three Arabidopsis Clade 1 members having very similar sequence profiles, their transcript expression profiles are markedly different, possibly alluding to differences in functionality of these genes. Clade 1 members, AtDRM1 and AtDRM2, have a conserved constitutive profile of expression with a concomitant increase of expression in low bolt tissue, while the other Clade 1 member, AtDRM5, has a floral-specific transcription profile conserved with Clade 2 member AtDRM3. Furthermore, AtDRM4, which is a member of Clade 2, shows a constitutive transcription profile closer to those of AtDRM1 and AtDRM2. The disparity in sequence
homology and conservation of transcript expression profiles suggests that the function of these genes may be mediated by sequence outside of Domain II.

2.4.2 AtDRM1/ARP family members are predicted to be IDPs

Arabidopsis DRM1/ARP family members were subjected to a range of predictive in silico protein sequence analyses in order to gain insight into the potential nature of these proteins. Secondary structure predictions suggest no obvious ordered structure, further compounded by an absence of cysteine residues required for disulfide bonds in all Arabidopsis family members except AtDRM3 (Appendix 7.5). This is in agreement with findings in kiwifruit (Wood et al., 2013) and suggests that Arabidopsis DRM1/ARP family members are probably intrinsically disordered.

PONDR®-FIT predictions for Arabidopsis DRM1/ARP provided profiles of intrinsic disorder across the amino acid sequence for all family members. Family members were representative of three distinct disorder profiles (A.i, A.ii and D), including both profile A.i and A.ii, further sub-divisions of profile A (Wood et al., 2013). This further division highlights differences in the central IDR, with profile A.ii’s IDR being shorter in number of residues than that of A.i. The absence of any Arabidopsis family members and indeed, any dicotyledons from profile B suggests this highly disordered profile is monocotyledon-specific (Appendix 7.6). All family members except AtDRM5 are predicted to have α-MoRF motifs - short regions of sequence that undergo disorder-order transitions upon protein binding (Cheng et al., 2007; Mohan et al., 2006). An example of these motifs in plants is found in the N-terminal domain of the DELLA proteins. Upon perception of GA, two α-MoRF motifs facilitate binding to the GA receptor, GID1 (Sun et al., 2010). With three motifs predicted, AtDRM2 has a high number of α-MoRFs given its relatively small length (106 amino acids). This is a particularly high frequency of α-MoRFs, as most intrinsically disordered proteins contain a fewer number of MoRFs, with a rate of $3.69 \times 10^3$ MoRFs per residue (i.e., on average three α-MoRFs in a sequence of 1000 amino acid residues) (Oldfield et al., 2005). Biological evidence in plants for a disorder-order transition upon binding is seen in the DELLA proteins (Sun et al., 2010), which are known IDPs involved in gibberellic acid-mediated growth regulation (Sun et al., 2013). Interestingly, AtDRM1 and AtDRM5 lack an N-terminal α-MoRF, which is present in all other family members as well as DRM1/ARPs in kiwifruit (Wood et al., 2013). This lack of a potential binding
site may represent a divergence in functionality between AtDRM1 and AtDRM2, which remains to be determined experimentally. It may also be a limitation of the predictive algorithm, particularly considering that it is at the terminus of sequence and the algorithms utilise surrounding sequence in order to make the predictions (Cheng et al., 2007).

The distribution of Arabidopsis family members in CH-CDF plots was consistent with those described in kiwifruit family members (Wood et al., 2013). Arabidopsis family members are categorised into Q3 and Q4. Proteins falling into Q3 have associations with developmental or regulation pathways, while Q4 proteins are mainly associated with mitotic processes (Huang et al., 2012), consistent with evidence that AtDRM1/ARPs are developmentally regulated and differentially regulated in non-growing, compared with growing tissue.

Because of the predicted highly disordered nature of AtDRM1/ARP family members, combined with IDPs’ general prevalence in stress response pathways (Garay-Arroyo et al., 2000; Mouillon et al., 2006), it is possible that this family may bind multiple partners, as is common in IDPs (Dosztányi et al., 2006; Dunker et al., 2005; Ekman et al., 2006; Haynes et al., 2006; Patil and Nakamura, 2006; Singh and Dash, 2007; Singh et al., 2007; Uversky et al., 2005) facilitating a role of AtDRM1/ARP family members as hub proteins or chaperones in signalling cascades or transcriptional regulation.

Members of the DRM1/ARP family are conspicuously absent from many plant protein databases, which may be a reflection of their nature as IDPs, which are known to be susceptible to proteolysis (Suskiewicz et al.; Wright and Dyson, 1999) and attempts to express the AtDRM1 protein in vitro, or in heterologous systems, have proved unsuccessful (Chapter 5). AtDRM2 and AtDRM4 have been identified in the PhosPhAT 4.0 phosphoproteome database, providing biological evidence of phosphorylation. Those identified in AtDRM2 (Appendix 7.5) are spatially conserved with known phosphorylated peptides of DRM1/ARP from dormant buds of poplar (Liu et al., 2011). Furthermore, these sites are conserved with predicted phosphorylation in kiwifruit (Wood et al., 2013). Additional predicted sites of phosphorylation were identified in all AtDRM1/ARP family members, suggesting that current phosphoproteome databases may not be comprehensive. Furthermore, a lack of predicted phosphorylation sites in AtDRM1 compared with other family members (Appendix 7.5) may indicate greater
stability in terms of proteolytic cleavage in AtDRM1, or conversely represent a limitation in the predictive power of the DISPHOS and PhosPhAT 4.0 algorithms.

A means of predicting a protein’s propensity to degradation is via the identification of PEST motifs. PEST motifs are regions predicted to undergo proteolytic cleavage specific to the proteasome (Rechsteiner and Rogers, 1996) and were identified in all Arabidopsis DRM1/ARP family members. In addition, AtDRM3 and AtDRM5 have relatively large regions predicted to be PEST motifs. Despite the disparity in sequence of these genes at the C-terminus, the conservation of flower-specific transcript expression profile, this potential protein degradation may represent an additional degree of conserved regulation for these two proteins.

PhosPhAT 4.0 can also be used to identify oxidated methionine residues. A potential site was detected in each of the phosphorylated C-terminal regions of AtDRM4 and AtDRM5, possibly representing a means of protein regulation for these proteins via methionine oxidation. Both phosphorylation and methionine oxidation have been implicated in ROS-mediated signalling in plants (Emes, 2009) and may provide insight into the proteins’ biological role.

2.4.3 Transcript expression of AtDRM1 and AtDRM2 is not specific to meristematic dormant tissues

It was hypothesised that the Arabidopsis family members, AtDRM1 and AtDRM2, would display high levels of expression in dormant meristematic tissue, such as dormant axillary buds and roots. This was because DRM1/ARP is routinely used as a marker for dormancy in multiple species including pea (Stafstrom et al., 1998), Arabidopsis (Aguilar-Martínez et al., 2007; Finlayson, 2007; Gonzalez-Grandio et al., 2013; Tatematsu et al., 2005), maize (Kebrom et al., 2010) and kiwifruit (Wood et al., 2013). Transcripts detected outside these spatial and temporal designations (i.e. non-meristematic tissue) would suggest a role broader than dormancy per se.

Initial transcript expression studies of a general axil section (where samples consist of a vegetative phase plant with all roots removed and all leaves removed at the base of the petiole) showed that AtDRM1 and AtDRM2 mRNA levels were particularly low relative to those in other developmental samples, despite literature suggesting the contrary in pea (Stafstrom et al., 1998) and Arabidopsis (Tatematsu et al., 2005). As the sample taken was of the general axil region, as opposed to individual axillary buds, any high
transcript expression in distinct locations (i.e. the buds) may have been diluted by the inclusion of the surrounding tissue.

Upon decapitation, down-regulation of both AtDRM1 and AtDRM4 transcripts was shown in axillary bud samples. This complemented their high frequency of SRE (sugar-repressive elements)-like elements which were previously described as being common in the promoter sequence of genes that were down-regulated upon decapitation (Tatematsu et al., 2005). In the same study, AtDRM1 (Aguilar-Martínez et al., 2007; Tatematsu et al., 2005) and AtDRM2 (Tatematsu et al., 2005) were shown to be down-regulated upon decapitation. However, only the AtDRM1 down-regulation was reproduced in this work. Conversely, the floral-specific AtDRM1/ARP family member, AtDRM3, exhibited an increase in transcription with decapitation and high numbers of Up2 motifs, commonly associated with genes that are up-regulated following decapitation (Tatematsu et al., 2005), providing additional evidence for the divergence of biological function of the different family members.

Further evidence for a role solely in dormancy and dormancy release would be supported by a reduced branching phenotype with over-expression of either AtDRM1 or AtDRM2 in Arabidopsis. However, this observation was not reproduced in this study.

Results obtained from both RT-qPCR analyses and the GUS histochemical analyses showed that AtDRM1 and AtDRM2 had nearly identical general expression profiles over the developmental series, as has been described in the closely related species B. rapa (Lee et al., 2013). In combination with their high sequence similarity, these data suggest that there may be functional redundancy between these genes in both species.

2.4.4 Are regulatory motifs of AtDRM1 and AtDRM2 isolated to the upstream promoter?

Promoter:reporter analysis using a 500 bp truncated upstream promoter fragment, and a putative full length promoter for AtDRM1 and AtDRM2, combined with expression profiles generated by RT-qPCR, provided insights into the localisation of regulatory motifs potentially found in upstream promoter sequences.

Both approaches identified expression of these genes in vegetative phase tissue and flowers. However, differences also existed. In particular, transcripts were detected by RT-qPCR in mature leaves and at the base of the inflorescence bolt, yet no expression
of the GUS reporter gene was detected in these tissues when regulated by the upstream promoter sequences of AtDRM1 or AtDRM2. These differences may be indicative of required regulatory elements controlling mature tissue expression existing somewhere other than upstream of the 5′ UTR sequence, including the 5′ UTR, 3′ UTR or intron sequences. Previous work hypothesised that regulatory elements involved in DRM1/ARP-associated cell elongation were localised to the 3′ UTR (Park and Han, 2003).

While the entire promoter:GUS lines exhibited staining in floral tissue (particularly AtDRM1), lack of similar staining in the truncated promoter fragment constructs suggests that motifs of importance for this expression probably lie outside the minimal 500 bp fragment upstream of the 5′ UTR. An example of this could be the CaRG consensus sequence, the binding site of MADS transcription factors, known to have roles in floral organ identity (reviewed in Melzer and Theissen, 2011), which was found only in the entire AtDRM1 promoter fragment, but not that of AtDRM2.

Only the AtDRM1 500 bp promoter fragment yielded GUS expression in lateral root junctions, similar to root staining shown in GUS analysis of AtLOJ (Lateral organ junctions), a gene argued to be associated with organ demarcation (Prasad et al., 2005). As roots grow primarily at their apex, it is postulated that this basal region has ceased to grow and would support the RT-qPCR data of AtDRM1 and AtDRM2 expression in mature, non-growing tissues. Similarly, findings associating DRM1/ARP transcript expression with mature or non-growing root tissue have been described in pea and B. rapa (Lee et al., 2013; Stafstrom et al., 1998). The lack of root staining in plants containing the constructs AtDRM1entprom:GUS, AtDRM2500bpprom:GUS and AtDRM2entprom:GUS may be a result of the root-specific expression motif being independent of any motifs associated with expression in other non-growing tissue types. An additional explanation for the lack of AtDRM1entprom:GUS expression may be the consequence of repression blocking positive regulation of the root-specific motif via cis-activation upstream of the 500 bp fragment.

Expression of AtDRM2entprom:GUS in the guard cells of stomata in young, developing tissue was also observed. DRM1/ARP expression has been associated with abiotic stress responses (Govind et al., 2009; Hwang et al., 2005; Lee et al., 2013; Stafstrom, 2000) and guard cell staining may represent involvement in this kind of stress-response process. As ABA responses are characteristic of guard cells, the promoter sequences
were screened for ABA-related motifs. Characterised ABA-related motifs were identified in both AtDRM1 and AtDRM2 promoter sequences, including motifs for binding of ATHB5 (Johannesson et al., 2001) and ABI4 (Niu et al., 2002). Therefore, it is likely that the motifs controlling the GUS expression associated with the guard cell staining in the AtDRM2 construct remain to be characterised. Alternatively, AtDRM2 might be involved in ABA-independent early phase signalling for stomatal closure (Montillet et al., 2013). As no staining was seen in AtDRM2 500bp:GUS, it is likely that regulatory motifs required for AtDRM2 expression are located outside the 500 bp region, including any guard cell-specific motifs. DOF transcription factors have roles in diverse processes including light, phytohormone and defence responses, seed development and germination and as such exhibit guard cell transcriptional expression (Suh et al., 2005; Yanagisawa, 2002). The biologically characterised DOF transcription factor target motif (Yanagisawa and Schmidt, 1999) was identified in both the truncated and putative full length promoter fragments for both AtDRM1 and AtDRM2, suggesting it is not responsible for this expression, or that a repressive element is upstream of the 500 bp fragment.

2.4.5 The DRM1/ARP family: more than dormancy

The expression data for AtDRM1 and AtDRM2 genes indicate that neither is associated with purely dormant, meristematic tissue, but instead with tissues which are not growing, whether having received a cue to grow and being held back, or not. As such, these genes are unlikely to be involved solely in dormancy, but are instead associated with a broader no-growth role, whether that be via involvement in cell elongation or cell division.

AtDRM1 and AtDRM2 exhibited particularly high relative expression levels in non-growing, mature tissue at the base of inflorescence bolts. Inflorescence bolts grow acropetally, with both cell division and cell elongation having ceased outside 7 cm from the shoot apical meristem (Suh et al., 2005). These data complement findings from pea, where transcripts accumulated in mature stem and root tissue (Stafstrom et al., 1998). Similarly, in hypocotyl and root elongation studies in R. pseudoacacia and B. rapa, transcripts accumulated in non-elongating cells, as defined by an inverse correlation of DRM1/ARP expression with the growth marker RPL27 (Lee et al., 2013; Park and Han, 2003). Arabidopsis plants over-expressing either of these B. rapa family members
showed a reduction in seedling hypocotyl length compared with the wild-type, probably as a result of reduced cell elongation, when grown in dark conditions (Lee et al., 2013). This further supports an association between the presence of the *DRM1/ARP* transcript and periods of reduced cell elongation and/or cell division.

To further this case, low mRNA expression was displayed in 6-day-old seedlings that were undergoing growth in terms of cell elongation of cotyledons and hypocotyls, but also cell division to form new organs such as leaves and flowers. To highlight the importance of using a combination of techniques to describe a true global expression profile, while this sample (as well as the day 16 sample) showed relatively low expression levels in the RT-qPCR analysis, GUS analyses revealed distinct staining in the tips of cotyledons and young leaves. Leaves grow via cell division primarily from the base, and therefore the tip of the organ is the first part of the tissue to stop growing by division (Donnelly et al., 1999; Ferjani et al., 2007) - another association between sites of ‘no growth’, predominantly relating in this instance, to cell proliferation, and *AtDRM1/AtDRM2* expression. RT-qPCR analyses in *B. rapa* showed increasing expression of *BrDRM1* and *BrARP1* with aging in cotyledons, reaching a maximum in senescing cotyledon tissue (Lee et al., 2013).

Over-expression of *AtDRM1* or *AtDRM2* in *Arabidopsis* produced a subtle developmental retardation, evidenced by over-expressing lines having fewer leaves on any given day throughout the vegetative phase. This also meant that throughout subsequent development, these plants lagged behind wild-type, reaching anthesis later and they were physically shorter throughout floral phase. This difference in height did not reflect an overall dwarfing of plants. However, as the mutant lines did eventually catch up, as shown in the uniformity of height 1 week post-anthesis, i.e. at a developmentally equivalent timepoint. When a snapshot of bolt number was taken at the same numbers of days post-sowing (i.e. different developmental stages), no differences were exhibited between over-expression lines and wild-type. This finding was consistent with the determinate nature of *Arabidopsis* growth, whereby plants will continue to produce branches at a common rate with wild-type, until the threshold height for branching to cease has been reached.

Over-expression of the SWI/SNF chromatin remodelling gene *AtCHR12* also produces a very subtle developmental retardation phenotype (Mlynárová et al., 2007). Of note is that this phenotype is only visible in plants that have been exposed to a stress and that
this is dosage-dependent. These plants also have increased transcript levels of \textit{AtDRM1} and their knockout mutants have a reduction in \textit{AtDRM1} levels (Mlynárová et al., 2007), suggesting that \textit{AtDRM1} is acting downstream of \textit{AtCHR12} in a stress-induced growth arrest pathway. A similar hypersensitivity to environmental conditions was evident with the \textit{AtDRM1/ARP} stable homozygote over-expression lines, as demonstrated by variability in phenotypes upon replication of experiments.

Often gene over-expression work is complemented with analyses of knock-out lines. RNAi and insertion mutation was used to down-regulate \textit{AtDRM1} and \textit{AtDRM2}, respectively, with no phenotypic alterations evident (Lee et al., 2013). Upon targeting of these genes by amiRNA, only down-regulation lines exist possibly implying that true knock-outs are lethal (Chapter 4).

Furthermore, \textit{DRM1/ARP} family members have been shown to respond to a number of abiotic factors including cold and salt treatments in pea (Stafstrom, 2000), \textit{Capsicum annuum} (Hwang et al., 2005); and \textit{B. rapa} (Lee et al., 2013). However, a more in-depth assessment of their response to various dosages and periods of incubation is required (Chapter 3).


Chapter 2  

AtDRM1/ARP family members

2.5 Conclusions

AtDRM1 and AtDRM2, and their homologues in other species, have been the focus of most research to date in the field of the DRM1/ARP family, much of which involved their association with dormant meristematic tissues at the transcript level. In this study all five Arabidopsis DRM1/ARP family members were analysed, producing distinct transcription expression profiles, with expression of all members clearly detected in non-meristematic tissues, suggesting that their functions are not purely dormancy-related. Indeed, two family members exhibited floral-specific expression profiles. A possible gene duplication event producing a Brassicaceae-specific clade was evident, with Arabidopsis members AtDRM1 and AtDRM2 also showing very similar global expression profiles, suggesting possible functional redundancy within individual species of the clade.

Over-expression of these genes yields plants with general growth retardation, with no obvious effect upon the plant’s final morphology.

Because of the predicted highly disordered nature of AtDRM1/ARP family members, combined with IDPs’ general prevalence in stress response pathways (Garay-Arroyo et al., 2000; Mouillon et al., 2006), it is postulated that this family may have roles as hub proteins or chaperones in signalling cascades or transcriptional regulation. Indeed with these findings in mind, DRM1/ARP family members may have roles in chaperoning proteins critical to the no-growth phase in tissues, possibly in response to stress. A more in-depth study of the response of this family to various abiotic factors is therefore required.

These findings provide evidence that DRM1/ARP family members are not involved exclusively in maintenance of dormancy; rather they are more likely to be concomitantly present because of the inherent growth cessation associated with dormant tissues.
3 Regulation of *AtDRM1* and *AtDRM2*

Investigations into the regulation of *AtDRM1* and *AtDRM2* were undertaken during the course of this PhD project and the findings presented as a paper entitled: ‘*DRM1* and *DRM2* expression regulation: potential role of splice variants in response to stress and environmental factors in *Arabidopsis*’, submitted to the journal Molecular Genetics and Genomics.

Unless otherwise stated, all experiments and analysis were undertaken by the candidate, Georgina Rae. IDP predictions and analysis were undertaken in collaboration with Vladimir Uversky. GUS histochemical analyses were undertaken by Georgina Rae during her Masters project (Rae, 2009).

Georgina Rae wrote the entire manuscript and critical reading of the manuscript was provided by Vladimir Uversky, Karine David and Marion Wood.
**DRM1 and DRM2 expression regulation: potential role of splice variants in response to stress and environmental factors in Arabidopsis**

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**Abstract**

Plants ability to respond to their environment is critical to their survival due to their sessile nature. This can be in response to standard conditions such as seasonal changes in temperature and light leading to dormancy, or more unusual circumstances such as increased salt conditions. One gene family with increasing associations with stress conditions at the transcript level is the DRM1/ARP gene family. Traditionally correlated with dormancy, increases in transcript levels in response to various treatments have also been reported in various species. As alternative transcript splicing is common in stress conditions, the splice variants of *AtDRM1* and *AtDRM2* were assessed further in this study. A previously undescribed splice variant of *AtDRM1* (*AtDRM1.6*) is introduced in this work. *In silico* analyses of predicted protein sequence of all splice variants showed that all variants retain the predicted intrinsically disordered nature. Transcriptional studies of *AtDRM1* and *AtDRM2* in response to a wide range of abiotic, physical and hormonal treatments showed that *AtDRM1.6* is differentially regulated at the transcriptional level compared with other splice variants. Promoter analyses demonstrated *AtDRM1* light regulation via the upstream promoter sequence.


3.1 Introduction

Plants are sessile organisms and as such have a well defined developmental program, but also are able to sense and respond to their environment quickly. Indeed, plants need to be able to respond to a number of stresses, abiotic and biotic, to ensure their continued existence. Generally, a plant will minimise growth upon perception of unsuitable conditions and only return to growth when optimal conditions return (Rohde et al., 1999), with the exception of submergence stresses (Arber, 1920).

One process which severely impacts a plant’s survival in response to abiotic factors such as light and cold, as well as endogenous biotic factors such as hormonal regulators, is dormancy release. Dormancy is defined as the period of no visible growth occurring specifically in meristematic tissues (Lang et al., 1987). Endodormancy, or winter dormancy in perennials such as kiwifruit and grape (Lang et al., 1987), protects the plants from adverse abiotic conditions such as reduced light hours and extreme cold in temperate climates.

Decisions such as release from winter dormancy, and resource expenditure for processes including vegetative growth and floral initiation, are all tightly controlled by the environment, as well as the action of hormones at both the transcriptional and translational levels.

*DORMANCY-ASSOCIATED GENE-1/AUXIN-REPRESSED PROTEIN (DRM1/ARP)* is often used as a genetic marker for dormant meristematic tissues. *DRM1/ARP* was first associated with dormancy through decapitation studies carried out in pea (Stafstrom et al., 1998). Upon decapitation of the apical bud, lower dormant buds were released from paradormancy. *PsDRM1* transcript levels are high in dormant buds located below the shoot apical meristem, but are no longer detectable in these buds by 6 hours after decapitation, only increasing again as the next most apical bud triggers paradormancy upon the lower buds. Similar decapitation studies in *Arabidopsis* (Tatematsu et al., 2005), and kiwifruit using hydrogen cyanamide (HC) as an ecodormancy releasing agent (Wood et al., 2013), show a similar transcriptional profile, linking *DRM1/ARP* expression with dormant tissue.
Transcriptional analyses of all five Arabidopsis DRM1/ARP family members following decapitation suggest that the decapitation transcriptional profile is not conserved across all family members, with AtDRM1, AtDRM2 and AtDRM4 showing the characteristic reduction in expression post-decapitation while in contrast AtDRM3 was induced post-decapitation (Chapter 2; Tatematsu et al., 2005). Furthermore, AtDRM3 and AtDRM5 showed a floral-specific profile, suggesting potential functional divergence in the Arabidopsis DRM1/ARP family.

The role(s) of hormone(s) in the regulation of DRM1/ARP family remains to be fully elucidated. The first family member identified, FaλSAR5, was from a cDNA library of strawberry (Fragaria ananassa) fruit deprived of auxin (Reddy and Poovaiah, 1990). Since this discovery, conflicting data have emerged for auxin being a transcriptional repressor of DRM1/ARP family members. Supporting data for auxin repression has been reported in pea PsDRM1 (Stafstrom, 2000) and black locust, RpARP (Park and Han, 2003). However, data from Elaegnus umbellata (EuNOD) (Kim et al., 2007) and Pyrus pyrifolia ARP1 (PpARP1) and PpARP2 (Shi et al., 2013) suggests an induction of these DRM1/ARP homologues, in response to exogenous auxin treatment. Further studies will be required to understand these data.

Scarce data currently exist considering DRM1/ARP levels in response to other hormones. Abscisic acid (ABA), which has been linked to induction and maintenance of dormancy in poplar (Rohde et al., 2002), has been shown to induce PsDRM1 transcript expression (Stafstrom, 2000); salicylic acid (SA) reduced transcript expression of PpARP1 and PpARP2 in mesocarp discs (Shi et al., 2013); while gibberellic acid (GA) had no effect (Stafstrom, 2000).

As already mentioned, light also influences dormancy status in mersitem tissue, as well as other non-meristematic morphological features of the plant. The light signalling mutant phyb, in both Arabidopsis and Sorghum (Sorghum bicolor) exhibits a reduced bud outgrowth phenotype (Finlayson et al., 2010; Kebrom et al., 2010; Kebrom et al., 2006) with a contemporaneous increase in DRM1/ARP expression (Finlayson et al., 2010; Kebrom et al., 2010; Kebrom et al., 2006). Expression of TBL1/BRC1, a gene involved in branching suppression (Aguilar-Martínez et al., 2007; Finlayson, 2007), is also increased. However, in tbl1/brc1 mutants DRM1/ARP is unaffected, despite the increased branching phenotype where we might have expected a decrease in
DRM1/ARP expression (Finlayson, 2007). This suggests that either these genes are acting in independent pathways in dormancy release, as is seen in the flowering regulation system which has multiple pathways (reviewed in Andrés and Coupland, 2012); or that DRM1/ARP is acting upstream of TBL1/BRC1 (Finlayson, 2007).

Further to a possible role in light signalling, transcriptional up-regulation of DRM1/ARP family members in response to a diverse range of abiotic factors, including cold, salt, heat and drought have been described in various species: pea (Stafstrom, 2000); Capsicum annuum (Hwang et al., 2005); Brassica rapa (Lee et al., 2013) and peanut (Arachis hypogaea) (Govind et al., 2009). A study considering the effect of cold treatment in conjunction with auxin application (Park and Han, 2003), showed that RpARP transcript expression is induced in the presence or absence of auxin, providing evidence for the cold stress superseding any auxin response.

Genes involved in stress responses often undergo alternative splicing (reviewed in Mastrangelo et al., 2012; Ner-Gaon et al., 2004). Splicing variation of mRNA transcripts is a phenomenon which amplifies the number of possible protein products of any given gene, with 22–30% of intron-containing genes estimated to undergo splice variation in Arabidopsis (Barbazuk et al., 2008; Campbell et al., 2006; Wang and Brendel, 2006). Types of variations include exon skipping; unspliced introns; alternative donor/acceptor; alternative terminal exon; transcripts either beginning or ending within an intron and intron retention (Ner-Gaon et al., 2004). However, intron retention is the most frequent at >30% (Ner-Gaon et al., 2004). Splicing variation, particularly intron retention, is prominent in stress response pathways (reviewed in Mastrangelo et al., 2012; Ner-Gaon et al., 2004), with intron-retained isoforms in kiwifruit showing transcriptional responses to stress conditions (Wood et al., 2013). AtDRM1 is also represented in splice variation databases (Ner-Gaon et al., 2004). However, a thorough analysis of transcriptional profiles of these isoforms has not been previously described.

Recently, kiwifruit and Arabidopsis DRM1/ARP family members have been predicted to be intrinsically disordered proteins (IDPs), including both AtDRM1 and AtDRM2 (Chapter 2; Wood et al., 2013). IDPs do not have stable tertiary structure under physiological conditions but can at least partially fold with a change in their environment, whether due to changes in the physiological conditions or due to
interaction with an appropriate binding partner (Dunker et al., 2001; Uversky and Dunker, 2010). These types of proteins have already been linked with roles in stress responses in plants. For example, the late embryo abundant (LEA) family of proteins has been linked to abiotic stress tolerance (Garay-Arroyo et al., 2000; Mouillon et al., 2006). Also, plant-specific GRAS proteins (named after the first three members: GIBBERELLIC ACID INSENSITIVE (GAI), REPRESSOR OF GAI (RGA) and SCARECROW (SCR)) that are known to play critical and diverse roles in plant development and signalling, often acting as integrators of signals from multiple plant growth regulatory inputs, were shown to possess long disordered N-terminal domains of great functional importance (Sun et al., 2010; Sun et al., 2011). Another family of IDP’s, the heat-shock proteins (HSP), are integral to a plant’s response to abiotic stress through their role as molecular chaperones in planta, facilitating protein refolding (reviewed in Wang et al., 2004). Due to the ability of IDPs to bind with multiple partners, they are often part of protein interaction networks where they play a number of crucial regulatory roles (Dosztányi et al., 2006; Dunker et al., 2005; Ekman et al., 2006; Haynes et al., 2006; Patil and Nakamura, 2006; Singh and Dash, 2007; Singh et al., 2007; Uversky, 2011; Uversky, 2013b; Uversky et al., 2005).

These data, as well as multiple lines of evidence showing transcript expression outside of purely dormant meristematic tissue, provide increasing evidence that DRM1/ARP s are involved in responding to sub-optimal conditions, whether they be abiotic or biotic.

In this work, AtDRM1 splice variants have been analysed across Arabidopsis development, as well as in response to a number of abiotic factors. Due to the high sequence similarity between AtDRM1 and AtDRM2, as well as the conservation in transcript expression profiles (Chapter 2), this work has been expanded to include the analysis of AtDRM2. The resulting proteins of all splice variants of AtDRM1 and AtDRM2 are predicted to be IDPs. A more in-depth analysis of AtDRM1 and AtDRM2 transcripts in response to varying salt treatments was undertaken as well as promoter analyses focusing on light treatments.
3.2 Methods

3.2.1 Sequence alignments

All multiple sequence alignments were performed with the Geneious Alignment program as part of the Geneious Pro 5.3.4 software, using an opening penalty of 12 and an extension penalty of 3 (http://www.geneious.com).

3.2.2 Bioinformatic analysis of Arabidopsis DRM1/ARP family: primary sequence analyses, disorder prediction and promoter analysis

The order/disorder of DRM1/ARP proteins was predicted using PONDR® (Predictor Of Natural Disordered Regions) FIT (Xue et al., 2010). Output is represented by real numbers between 1 (ideal predictor of disorder) and 0 (ideal prediction of order). An arbitrary threshold of ≥0.5 was used to identify disorder.

The CH-CDF plot analyses were performed as previously described (Huang et al., 2012; Mohan et al., 2008; Wood et al., 2013).

An α-helix-forming molecular recognition feature predictor (α-MoRFs-II) based on PONDR® prediction and a large positive data set (Cheng et al., 2007; Oldfield et al., 2005) was used to identify putative α-MoRFs.

Putative proteolytic cleavage sites identified by the PEST motif were identified using epestfind (http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::epestfind). PEST motifs are defined as hydrophilic stretches of at least 12 amino acids length with a high local concentration of critical amino acids.

The web-based phosphorylation predictor DISPHOS 1.3 (http://www.dabi.temple.edu/disphos/pref.html) was used to predict phosphorylation of serine, threonine and tyrosine residues in a query sequence as part of the PONDR® website (http://www.pondr.com). The Arabidopsis thaliana group predictor was used to minimise the misclassification of residues.

Arabidopsis sequence identifier codes were used to interrogate the PhosPhAt 4.0 database (http://phosphat.mpimp-golm.mpg.de/index.html) for the identification of
phosphorylated and oxidated residues from databases (Durek et al., 2010; Heazlewood et al., 2007).

Sequence upstream of the 5' UTR of AtDRM1 and AtDRM2, but downstream of the neighbouring gene’s annotated UTR was retrieved from TAIR (The Arabidopsis Information Resource: http://www.arabidopsis.org/) and designated ‘entire promoter sequence’ (AtDRM1entprom; AtDRM2entprom). Sequences were analysed using PLACE, a database of plant cis-acting regulatory DNA elements (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999) and putative regulatory motifs of interest annotated on to the sequence.

### 3.2.3 Plant growth conditions

All plants used in this work were Arabidopsis thaliana (Columbia ecotype background). Seeds were soaked for 30 min in sterile water, followed by 5 min sterilisation in 95% ethanol with a subsequent 5 min incubation in 4% sodium hypochlorite, 0.0001% triton X-100 solution. Sterilised seeds were then washed at least three times in sterile water. For soil-grown plants, imbibed seeds were placed on to moist filter paper and incubated in darkness at 4°C for 2–4 days. Seeds were then sown on to soil irrigated with 0.16% Dimilin® 25W (Veg Gro Supplies).

For plants grown on plates, seeds were sterilised and then transferred to ½ MS media plates. Plates were grown in a light and temperature controlled growth room (12 h light (50 µEm⁻²s⁻¹), 12 h dark, at 22°C).

For plants grown in flasks, seeds were sterilised and stratified for 2 days in darkness at 4°C. Seedlings were grown in ½ MS liquid media in flasks on a shaking platform at ~80 rpm room (12 h light (50 µEm⁻²s⁻¹), 12 h dark, at 22°C).

For plants grown on rockwool, seeds were placed on to moist filter paper and incubated in darkness at 4°C for 2–4 days. Seeds were then sown either directly onto rockwool for transcript analysis, or onto an agar plug (½ MS) for phenotypic analysis, in rockwool saturated with hydroponics media (KNO₃ 1.25 mM, Ca(NO₃)₂.4H₂O 1.5 mM, KCl 5 mM, C₁₀H₁₂FeN₂NaO₈ 72 mM, KNO₃ 1.25 mM, KH₂PO₄ 0.5 mM, MgSO₄.7H₂O 0.75 mM, H₂BO₃ 50 mM, MnSO₄.4H₂O 10 mM, ZnSO₄.7H₂O 2.0 mM, CuSO₄.5H₂O 1.5 mM, (NH₄)₆Mo₇O₂₄.4H₂O 0.075 mM, KCl 50 mM, C₆H₁₃NO₄.S.H₂O 0.5 mM; adjusted to pH 5.7 with orthophosphoric acid). Rockwool was saturated with hydroponics media
every week to maintain nutrient status required for plant growth. Plants were grown in long day conditions (18 h light/6 h dark).

3.2.4 Plant material for expression analyses

3.2.4.1 Wild-type expression profile

Tissue of wild-type plants grown on rockwool was harvested throughout plant development (day 6; day 16; day 22 vegetative phase: mature leaves = 4 oldest, basal leaves, young leaves: 4 youngest, apical leaves, axil region: stem from the rosette portion of the plant, including node, internode axillary bud tissue; day 36 mature/floral phase: low bolt, basal 2 cm of primary inflorescence bolt; top bolt, apical 2 cm of primary inflorescence bolt; flowers: entire floral region of primary bolt) for total RNA extraction. Time points were selected as they covered Arabidopsis development from young seedlings to mature plants. For day 6 and day 16 samples, approximately 100 and 20 plants, respectively, were harvested. For all other samples, tissue was harvested from 6 plants and pooled. The experiment was repeated in its entirety for biological replicates (AtDRM1.1, AtDRM1.2, AtDRM1.3, AtDRM1.4 and AtDRM2: n=3; AtDRM1.6: n=2).

3.2.5 Abiotic stress screening

Seedlings were grown in ½ MS liquid in flasks, until day 6 (T₀). Where an agonist was added (Appendix 12), flasks remained in the same conditions after T₀. After a 6 h treatment, seedlings were harvested by pouring the entire contents of the flask through filter paper to remove excess liquid and transferred to liquid N₂ for snap freezing. Experimental samples represented pooled samples of ≥50 seedlings per treatment.

For the wounding treatment, seeds were sown directly onto soil and grown to a mature vegetative phase (no visible inflorescence bolt). The largest leaf of a plant was wounded with a single cut using sterile scissors. No tissue was removed in the wounding process. Entire plants were harvested 24 h post-wounding and soil rinsed from roots with water prior to snap freezing in liquid N₂ and stored at -80°C, if required. Experimental samples represented pooled samples of four plants per treatment.

For screening, data is representative of one biological replicate. Only where relative changes in transcript expression of the treatment >1.5 times compared with controls were findings discussed further. For the salt time-course the experiment was repeated in
its entirety for biological replicates (AtDRM1.1, AtDRM1.2, AtDRM1.3, AtDRM1.4 and AtDRM2: n=3; AtDRM1.6: n=2).

3.2.6 RNA Isolation and Quantitative RT-qPCR

RNA isolation and quantitative RT-qPCR were completed as described previously (see §2.2.4). The primer sequences used in this study are described in Appendix 7.2. All RT-qPCR products were verified by sequencing.

3.2.7 Promoter analysis

Cloning and Arabidopsis transformations for development of promoter:GUS lines were completed as described previously (see §2.2.6). GUS histochemical analyses were completed as described previously (see §2.2.7).

3.2.8 Statistical analysis

All statistical analyses were carried out using the GenStat® 14th Edition software (www.genstat.co.uk).
3.3 Results

3.3.1 *AtDRM1* has six splice variants

TAIR annotation predictions indicate that the gene *AtDRM1* has five splice isoforms (*AtDRM1.1, AtDRM1.2, AtDRM1.3, AtDRM1.4* and *AtDRM1.5*), with each supported by the presence of at least one expressed sequence tag (EST) in the database. An additional previously undescribed splice variant, *AtDRM1.6*, has been identified in this study (Figure 3.1A). Splice variant 5 has a shift in an intron/exon boundary compared with other isoforms and is the only isoform that was never detected at the transcript level in this study (Appendix 7.14). As such, it was removed from subsequent analyses.

*AtDRM2* also has two TAIR isoform predictions, differing by inclusion of an additional six bases at the 5' end of the second exon in *AtDRM2.2* compared with *ADRM2.1*, and the presence of two introns in the 3' UTR sequence of *AtDRM2.1* (Figure 3.1C).
No splice isoforms of \textit{AtDRM1} retain intron 1; while isoforms 3 and 6 retain intron 3 which is lacking in isoforms 1, 2 and 4. Compared with isoform 1, isoform 4 has lost intron 4 at the 3' end of the sequence resulting in modification of the C-terminal protein sequence due to an associated frameshift. Variants 2 and 3 differ in the presence/absence of intron 3 in the 3' UTR, downstream of the in-frame stop codon, the resulting protein of which is designated AtDRM1.2/.3. An additional splice variant designated \textit{AtDRM1.6} was also identified in this study. \textit{AtDRM1.6} is the only isoform to retain intron 2 of the \textit{AtDRM1} gDNA and is predicted to result in a truncated protein (Fig 3.1A & B).
All splice variants retain the phosphorylation of residues 28 and 29 as identified in the phosphorylated protein database PhosPhAt 4.0 (Fig 3.1B).

**3.3.2 All AtDRM1 splice variants are predicted to be IDPs**

To assess whether the changes in the C-terminal protein sequence affected the structural predictions of the different AtDRM1 splice variants, particularly the recent IDP predictions (Chapter 2), intrinsic disorder predictions for each of the AtDRM1 splice variants were carried out (Figure 3.2A). Similarly, the same predictions were carried out for AtDRM2 splice variants to assess the effect of retention of intron 2 on their structural predictions (Figure 3.2A).
Figure 3.2. Disorder analysis of Arabidopsis DRM1 and DRM2 splice variants.

A) PONDR® analysis of disorder distribution profiles associated with splice variants of Arabidopsis DRM1 and DRM2 protein. B) Disorder analysis: combined CH-CDF plot of Arabidopsis DRM1 and DRM2 splice variant proteins. The CH-CDF plot is sectioned into four distinct quadrants as described in Huang et al., (2012). Quadrant 1 (Q1): rare proteins: predicted to be disordered by CH and ordered by CDF analyses; quadrant 2 (Q2): ordered proteins: predicted to be ordered proteins by CH and CDF analyses; quadrant 3 (Q3): mixed proteins: predicted to be ordered proteins by CH and disordered proteins by CDF comprising of proteins that contain both ordered and disordered regions; and quadrant 4 (Q4): disordered proteins: predicted to be disordered by both CH and CDF analyses. y axis: distance of each AtDRM1 protein from major boundary line in CH plot (positive for disordered, negative for ordered or small number of disordered); x axis: distance of each AtDRM1 protein from boundary line in CDF plot (positive for ordered, negative for disordered).
A Divergence of PONDR®-FIT profile was seen between splice variants of AtDRM1 after ~95 aa. AtDRM1.1, AtDRM1.4 and AtDRM1.6 all showed a similar profile, retaining the C-terminal intrinsically disordered region (IDR). AtDRM1.2/3 was the only variant which showed an additional minimum at the C-terminal sequence suggesting a loss of disorder at the C-terminus (Figure 3.2A).

The CH-CDF plots were used to assess the potential degree of disorder, combining predictions by cumulative distributions function curves (CDFs) and by charge-hydropathy plots (CHs) (Huang et al., 2012). All splice variants fell into quadrant 3 (Q3) (Figure 3.2B).

Changes in splicing of AtDRM1 had no effect upon the spatial localisation of the predicted α-molecular recognition features (α-MoRFs), with a single α-MoRF present in the C-terminal sequence of all isoforms (~77–110 aa). By contrast, a predicted PEST motif (predicted site of proteolytic cleavage) is lost in splice variant AtDRM1.6 (103–117 aa) (Figure 3.1B).

AtDRM2 splice variants were also subjected to PONDR®-FIT analysis (Figure 3.2) and α-MoRF predictions (Figure 3.1D). Both isoforms are predicted to be IDPs with almost identical PONDR®-FIT profiles. AtDRM2.1 has three predicted α-MoRFs (1–18 aa, 22–39 aa and 67–86 aa), while isoform sequence variation results in the loss of a predicted N-terminal α-MoRF in AtDRM2.2 (only 10–27 aa and 69–86 aa).

No cysteine residues are found in either AtDRM1 or AtDRM2 splice variant protein sequence (Figures 3.1C & D).

### 3.3.3 AtDRM1 transcription exhibits tissue-specific splicing

To determine if the different splice variants of AtDRM1 were differentially expressed in Arabidopsis and could provide information on the function of the gene, we used variant-specific primers for RT-qPCR analyses (Appendix 7.2; Figure 3.3). Due to the high sequence similarity, primers were unable to be designed targeting the individual AtDRM2 splice variants.
Figure 3.3. Transcript expression profiles across development in wild-type plants.

Graphs show the target/reference ratio for AtDRM1.1, AtDRM1.2, AtDRM1.4 and AtDRM1.6 over a developmental series in wild-type plants. SEM are represented in the error bars. Samples were normalised to the reference genes (SAND: At2g28390, TIP41-L: At4g34270 and UBC9: At4g27960). Values were calibrated to the day 16 sample prior to calculating the mean of biological replicates.
Transcript expression was detected for all splice variants of \textit{AtDRM1} except \textit{AtDRM1.5}, as well as for \textit{AtDRM2}. Expression levels for \textit{AtDRM1.3} were outside of the sensitivity range of this assay leaving only profiles for splice variants \textit{AtDRM1.1}, \textit{AtDRM1.2}, \textit{AtDRM1.4} and \textit{AtDRM1.6} with reproducible results. \textit{AtDRM1.1}, \textit{AtDRM1.2}, \textit{AtDRM1.4} and \textit{AtDRM2} showed higher levels of transcript expression in the low bolt sample and a constitutive profile across all other samples. In contrast, the highest transcript expression of \textit{AtDRM1.6} was detected in the day 6 sample with the low bolt sample exhibiting comparable \textit{AtDRM1.6} expression levels to day 16, axil, and floral samples. Expression of \textit{AtDRM1.6} in leaf samples (young or mature) and the top bolt sample was barely detectable.

### 3.3.4 Effects of various abiotic stresses on \textit{AtDRM1} and \textit{AtDRM2} expression

In order to assess the response to abiotic stress of \textit{AtDRM1} splice variants and \textit{AtDRM2}, a preliminary screen of a range of abiotic factors including physiological conditions, synthetic chemicals and hormones was undertaken (Figure 3.4).
Figure 3.4. Expression profile of wild-type plants across an abiotic treatment screen.

Graphs show the fold-change of treatment/control for AtDRM1.1, AtDRM1.2, AtDRM1.4, AtDRM1.6 and AtDRM2 over an abiotic treatment series in wild-type plants. Samples were normalised to the reference genes (EF1α: At1g07940 and SAND: At2g28390). To calculate the fold-change treatment/control, the ratio of treatment target/reference ratio to the relevant control sample’s target/reference ratio was calculated. Fold-change values <1.5 were disregarded as background.
Not all transcripts assessed were reliably detectable across this series. *AtDRM1.1, AtDRM1.2, AtDRM1.4, AtDRM1.6* and *AtDRM2* were all detectable. However, *AtDRM1.3* was on the outer limits of sensitivity of the assay and therefore data was not included. In addition, *AtDRM1.5* was never detected across this series.

The *AtDRM1* splice variants and *AtDRM2* showed opposing responses to different temperature conditions. *AtDRM1* splice variants were all induced at 32°C, and down-regulated at 4°C; while the opposite was observed for *AtDRM2*. An increase in expression was detected for all transcripts exposed to dark, wounding, HC and MG132, whilst silwet induced expression of *AtDRM1.1, AtDRM1.2, AtDRM1.4* and *AtDRM2*, while reducing expression of *AtDRM1.6* (Figure 3.4).

While the sugar fructose induced all transcripts, closely related molecules sucrose and glucose produced more variable results. No change greater than 1.5-fold was detected for sucrose in *AtDRM1.2* or *AtDRM1.4*, while *AtDRM1.1* and *AtDRM1.6* showed an induction and *AtDRM2* was repressed. Glucose induced all transcripts except *AtDRM1.2*, for which there was no significant change in expression. Mannitol induced transcript expression in *AtDRM1.1* and *AtDRM2*, but not in *AtDRM1.2* or *AtDRM1.4*.

Salt treatment caused an induction in all transcripts except *AtDRM1.2* and *AtDRM1.6*, where no change greater than 1.5-fold was detected. Similarly, cyclohexamide was associated with a significant down-regulation of all transcripts except *AtDRM1.2*.

Kinetin caused down-regulation of all transcripts except *AtDRM1.6*, which was induced. Similarly, GR24 treatments caused down-regulation of all transcripts except *AtDRM1.6*, which exhibited no change. By contrast, GA-3 caused no change in *AtDRM1.1, AtDRM1.6* and *AtDRM2*, but was associated with a down-regulation in transcript levels for *AtDRM1.2* and *AtDRM1.4*. IAA treatment caused no change in *AtDRM1.2*, but reduced transcript expression of *AtDRM2, AtDRM1.1* and *AtDRM1.4* and induced expression of *AtDRM1.6*. Similarly, all transcripts except *AtDRM1.4*, for which there was no change, were down-regulated with methyl jasmonate (MJ) treatment. All transcripts except *AtDRM1.6* and *AtDRM2* were down-regulated with ABA treatment. *AtDRM1.2* and *AtDRM1.6* showed no change with SA treatment, while *AtDRM2* was down-regulated, and *AtDRM1.1* and *AtDRM1.4* were up-regulated. Etephon treatment was associated with a down-regulation of all transcripts assessed, except *AtDRM1.4*. 
3.3.5 Response to salt treatment

Exposure to excessive salt concentrations was chosen as an abiotic stress for further analyses, which is not directly related to dormancy release. This is in contrast to many hormones and physiological treatments, including auxin; cytokinin; strigolactone; cold and wounding. Furthermore, increases in both AtDRM1 and AtDRM2 transcripts were recorded with salt treatment in the abiotic stress screening (Figure 3.4). Initially, salt treatment conditions were optimised for dosage (Appendix 7.5), followed by transcriptional analyses of AtDRM1 splice variants and AtDRM2 over a 2-day period after treatment with salt (Figure 3.5).
Figure 3.5. Temporal expression profiles of AtDRM1/ARP’s in the presence of salt.

Graphs show the target/reference ratio for AtDRM1.1, AtDRM1.2, AtDRM1.4, AtDRM1.6 and AtDRM2 over a 100 mM NaCl-treated time-course in wild-type pooled seedlings. SEM are represented in the error bars. Samples were normalised to the reference genes (to Apt2: At1g80050, Ef1a: At1g07940, and UBC: At4g27960) and were calibrated to $t_0$ (immediately before treatment).
Not all transcripts assessed were reliably detectable across this series, as previously observed (Figures 3.3 & 3.4). *AtDRM1.1, AtDRM1.2, AtDRM1.4, AtDRM1.6* and *AtDRM2* were all detectable. However, as previously observed, *AtDRM1.3* was on the outer limits of the sensitivity of the assay and therefore the data were not included. In addition, *AtDRM1.5* expression was not detected across this series of samples.

In the control samples, all detectable transcripts displayed a biphasic profile with a peak in transcript expression at 30 min and 2 days post-treatment, except *AtDRM1.6* which peaked at 2 days, but was relatively lowly expressed through the first 3 h after treatment.

Expression analyses of all *AtDRM1* splice variants showed a different profile in response to salt treatment compared with *AtDRM2*. *AtDRM1.1* showed an immediate down-regulation with salt treatment at 10 and 30 min after the salt treatment, which was no longer evident 1 h post-treatment, followed by an induction at 3 h. Both *AtDRM1.2* and *AtDRM1.4* showed highly conserved profiles of an induction with salt treatment at either 1 h or 3 h, respectively, until 12 h post-treatment. *AtDRM1.6* exhibited a rapid induction by 10 min post-treatment which was no longer evident at 3 h. Compared with the other transcripts assessed, *AtDRM1.6* showed a parallel increase in both the control and salt-treated samples at 6 h and 12 h.

*AtDRM2* expression was similar to *AtDRM1.1*, with a down-regulation with salt treatment at 30 min post-treatment, followed by an induction at 1 h and no change after this between treated and non-treated samples.

### 3.3.6 Visualisation of temporal and spatial expression of *AtDRM1* and *AtDRM2* in response to different light regimes

In order to visualise the effect that different light regimes have upon the mRNA expression of *AtDRM1* and *AtDRM2*, promoter:*GUS* lines expressing *GUS* under the 500 bp promoter fragment (*AtDRM1*500bp<sub>prom</sub>:*GUS* and *AtDRM2*500bp<sub>prom</sub>:*GUS*) or putative entire promoter fragment of either *AtDRM1* (*AtDRM1<sub>entprom</sub>:*GUS*) or *AtDRM2* (*AtDRM2<sub>entprom</sub>:*GUS*) were studied alongside 35S:*GUS* (positive control) and wild-type negative controls (Figure 3.6). Light grown samples were grown for 6 days in light conditions (12 h light / 12 h dark). Dark grown seedlings were grown for 6 days in continuous darkness. A light regime of 3 days light (12 h light / 12 h dark) followed by
3 days continuous darkness was included in this experiment. The aim was to gain insight into possible effects of seedling development as a result of etiolation in the continuously dark grown seedlings rather than a direct response to light conditions.
Figure 3.6. GUS histochemical analysis of *AtDRM1* and *AtDRM2* promoter:GUS lines.

The positive control (35S:GUS) exhibited constitutive expression (Figures 3.6M–O), with the exception of the absence of staining in the positive control petiole (Figure 3.6M). No staining was detected in the negative (wild-type) control line (Figures 3.6P–R).

Staining was observed in cotyledon tips and in the axil region in all lines except AtDRM2500bp:GUS for which no staining was visible (Figures 3.6A–I). Both AtDRM1entprom:GUS and AtDRM1500bp:GUS showed a significant change in the staining profile of dark grown seedlings compared with the light grown seedlings (Figures 3.6C, D & F).

With dark treatment, AtDRM1entprom:GUS exhibited staining over the entire cotyledon, down the apical hook and extending to over the apical portion of the hypocotyl (Figure 3.6F). This represents an increase in the AtDRM1 mRNA spatial profile compared to light grown seedlings, which showed discrete staining in the tips of cotyledons and the axil regions only (Figure 3.6D). In addition, when AtDRM1entprom:GUS was grown under light/dark conditions, an intermediary staining pattern was visible, with staining visible at cotyledon tips, in the axil region, in the vasculature and possibly in guard cells of the stomata, in particular associated with the cotyledon margins (Figures 3.6D & E).

AtDRM1500bp:GUS displayed a distinct area of expression in dark grown seedlings compared with AtDRM1entprom:GUS under identical conditions (Figures 3.6C & F). Conversely, the dark grown samples of AtDRM1500bp:GUS showed a more extensive staining pattern compared with light grown samples, with staining visible across the distal portion of the cotyledons (Figures 3.6A & C). As observed with AtDRM1entprom:GUS, an intermediary staining pattern was also exhibited within AtDRM1500bp:GUS seedlings grown under a light/dark regime, with staining visible at cotyledon tips and in the axil region, but with a slightly broader pattern than displayed in the light grown samples (Figures 3.6A & B).

No changes in the GUS expression profiles were displayed across the three light treatments for either of the lines carrying AtDRM2 promoter fragments (AtDRM2entprom:GUS or AtDRM2500bp:GUS) (Figures 3.6G–L).
3.3.7 Promoter sequence analysis

Potential *cis*-regulatory elements were identified in the promoter sequences of *AtDRM1* and *AtDRM2* (all sequence upstream of the 5’ UTR until the next annotated gene coding sequence) were identified using the online PLACE tool (http://www.dna.affrc.go.jp/PLACE/) (Higo et al., 1999) (Figure 3.7).
Figure 3.7. Map and alignment of conserved regulatory elements detected in sequence upstream of the 5' UTR in AtDRM1 and AtDRM2.

W-Box (purple); ARR1-binding element (gold); DOF-binding element (green); GT-1 motif (grey); MycC-binding element (light blue); ATB2-binding element (blue); BELL homeodomain-binding element (red); Evening element motif (lime green); Site II element (black); CBF/DREB-binding element (pale pink) and SV40 core enhancer element (turquoise). Arrow direction represents strand upon which motif was identified (forward = sense; reverse = antisense).
Chapter 3  Regulation

As At\textit{DRM2}_{500bprom}-\textit{GUS} lines show no expression across normal development (Chapter 2), it is of interest to assess which motifs are found in the remainder of the \textit{AtDRM2} entire promoter sequence, as well as through the entirety of the \textit{AtDRM1} promoter sequence, which may influence \textit{GUS} expression in transgenic lines.

Of those conserved regulatory elements presented, the ARR1-binding element, GT-1 motif, the Site II element and the SV40 core enhancer element were not identified in \textit{AtDRM2}_{500bprom} promoter sequence. By contrast, the W-Box motif, the DOF-binding element, the MycC-binding element, the ATB2-binding element, the BELL homeodomain-binding element, the Evening element motif and the CBF/DREB-binding element were identified both within the \textit{AtDRM2}_{500bprom} promoter sequence, as well as either upstream of it within \textit{AtDRM2} or in the promoter sequence of \textit{AtDRM1}.

The W-box motif, ARR1-binding element and the GT-1 motif were all identified in sequence associated with detectable \textit{GUS} expression (\textit{AtDRM1}_{entprom} and \textit{AtDRM2}_{entprom}, after the initial 500 bp fragment) (Figure 3.6).

In contrast, DOF-binding elements; MycC-binding elements; ATB2-binding elements; BELL homeodomain-binding element; and Evening elements are all located in both \textit{AtDRM1} and \textit{AtDRM2} promoter sequences, including both of the 500 bp fragments.

Site II elements and SV40 core enhancer elements are specific to the \textit{AtDRM1} upstream regulatory sequence. The CBF/DREB-binding element was identified in \textit{AtDRM1}_{entprom} and \textit{AtDRM2}_{500bprom}.
3.4 Discussion

3.4.1 The implications of alternative splicing on AtDRM1 and AtDRM2

Alternative splicing of one gene into multiple transcripts is a means of increasing levels of regulation and control in a biological system. While five isoforms have been annotated in the TAIR database for AtDRM1, this study introduces an additional intron-retained isoform, AtDRM1.6. Splice variants AtDRM1.1, AtDRM1.4 and AtDRM1.6 all display differences in the coding sequence of the cDNA and subsequent differences at the protein level. By contrast, the resulting predicted mature protein of variants AtDRM1.2 and AtDRM1.3 are the same, despite differences at the cDNA level. It is feasible, however, that such splice variants may exhibit a modification in their transcript stability or translational efficiency and thus its biological relevance, if any (Gebauer et al., 1998). Evidence including the shifted intron/exon boundary; lack of detection at the transcript level; that it is represented by only one cDNA clone in TAIR and that to date no additional supporting data has been documented, suggests that splice variant AtDRM1.5 may be artefactual or particularly rare. Intron retention in AtDRM1.6 results in a truncated protein and the loss of a C-terminal putative PEST motif. PEST sites are predicted targets for ubiquitination and subsequent proteolytic cleavage (Rechsteiner and Rogers, 1996), potentially leading to an increased stability of the AtDRM1.6 protein. While alternative splicing of AtDRM1 results in changes to the C-terminus of the predicted proteins, all splice variants retain their predicted IDP status, with the previously described C-terminal α-MoRF still present (Chapter 2).

AtDRM2 also has two isoforms AtDRM2.1 and AtDRM2.2, described in TAIR, which vary by the inclusion of two additional amino acids in the coding region (intron 2). While still being predicted IDPs, AtDRM2 splice variation results in the presence or absence of an N-terminal α-MoRF. α-MoRFs are putative protein:protein interaction regions (Cheng et al., 2007; Hsu et al., 2013; Mohan et al., 2006; Oldfield et al., 2005; Vacic et al., 2007), therefore this loss might represent the loss of the ability to bind to a partner. This loss may be further amplified by the characteristic promiscuity of IDPs (Dosztányi et al., 2006; Dunker et al., 2005; Ekman et al., 2006; Haynes et al., 2006; Patil and Nakamura, 2006; Singh and Dash, 2007; Singh et al., 2007; Uversky et al., 2005) such that AtDRM2.2 may have lost the ability to bind a number of interacting partners with this two amino acid addition.
Assessment of these AtDRM1 splice variants across plant development demonstrated that AtDRM1.1, AtDRM1.2 and AtDRM1.4 share a similar profile of constitutive expression with a relative higher expression level in the non-growing tissue of low bolt samples. This is consistent with previous studies of AtDRM1 and AtDRM2 (Chapter 2).

The newly identified additional AtDRM1 splice variant AtDRM1.6 did not exhibit the conserved expression profile observed with the other splice variants of AtDRM1 and AtDRM2. Instead AtDRM1.6 lacked the relative increase in expression in low bolt samples, with highest expression detected in 6-day-old seedlings, possibly facilitating functional diversity within the AtDRM1 gene.

3.4.2 Plant hormones associated with dormancy transcriptionally regulate AtDRM1 and AtDRM2

Both dormancy and more generally plant development are highly regulated by the presence, absence and crosstalk of various hormones. Previous studies have shown that DRM1/ARP family members are likely to have roles outside of dormancy associated with meristematic tissues (Chapter 2; Lee et al., 2013; Park and Han, 2003). Indeed, Arabidopsis plants over-expressing either AtDRM1 or AtDRM2 exhibit a subtle retardation of overall development (Chapter 2).

In this study it was shown that hormones related to changes in dormancy, including auxin, strigolactone and cytokinin, all transcriptionally regulated both AtDRM1 splice variants and AtDRM2. AtDRM1.6 exhibited opposing transcriptional responses compared with the other splice variants of AtDRM1 in response to a number of hormones assessed.

The DRM1/ARP family, in particular the ARPs, are so called due to down-regulation of their expression by auxin (Kim et al., 2007; Park and Han, 2003; Reddy and Poovaiah, 1990) and this was confirmed for AtDRM1.1, AtDRM1.4 and AtDRM2. The presence of auxin-responsive motifs in the upstream regulatory sequence of both genes may facilitate this regulation. By contrast, AtDRM1.6 was induced with auxin treatment, supporting previous work in Elaeagnus umbellata and Pear (Kim et al., 2007; Shi et al., 2013). These conflicting data may reflect differences in the type and age of tissue assessed; or in light of the differences between AtDRM1 splice variant responses, may be indicative of splice isoforms having different functional roles in the plant which are being controlled, in this case by auxin.
In terms of meristematic tissues such as axillary buds, auxin and cytokinin generally have opposing roles, such that high levels of one hormone is concomitant with low levels of the other. By contrast, the transcriptional profiles of AtDRM1 splice isoforms (except AtDRM1.6) and AtDRM2 exhibited down-regulation with treatment with both auxin and cytokinin. This is likely a reflection of the type of tissue being assessed - young seedling tissue which is not meristematic. In non-meristematic organs such as stems, auxin becomes a growth-promoting hormone, specifically promoting growth via cell expansion/elongation. DRM1/ARP transcript expression has been shown to be inversely correlated with sites of cell elongation (Lee et al., 2013; Park and Han, 2003), therefore that AtDRM1 and AtDRM2 are in many cases down-regulated by auxin and cytokinin may provide further evidence for a role for these genes in preventing growth by cell elongation.

By contrast, GR24 treatment, the synthetic analogue of the branching inhibitor strigolactone, resulted in down-regulation of all AtDRM1 splice variants (except AtDRM1.6 which showed no difference) and AtDRM2. DRM1/ARP transcripts are up-regulated in dormant axillary buds (Stafstrom et al., 1998; Tatematsu et al., 2005). Therefore, if expression of AtDRM1/AtDRM2 showed some causative relationship with the dormancy status of the tissue, we would have expected an induction of AtDRM1 and AtDRM2 expression by strigolactone. The fact that it is down-regulated suggests either that DRM1/ARP is involved in a strigolactone-independent pathway; or that Arabidopsis seedlings, upon which this assay was completed, respond differently to strigolactone than axillary buds of mature plants.

### 3.4.3 Dark induction of AtDRM1 and AtDRM2

Due to the sessile nature of plants, the ability to perceive different light conditions and respond accordingly is critical to their survival. As DRM1/ARP gene expression is up-regulated in the light perception mutant, phyb, which exhibits increased hypocotyl elongation and reduced branching (Finlayson et al., 2010; Kebrom et al., 2010; Kebrom et al., 2006), it was first important to confirm that DRM1/ARP expression is up-regulated in dark etiolated seedlings. Indeed, transcription of both AtDRM1 splice variants and AtDRM2 was induced by dark growth conditions in seedlings.

Seedlings grown under continuous dark exhibit the classic etiolated phenotype with reduced cotyledons and elongated hypocotyls and are developmentally different from
light-grown seedlings. As such determining whether any modifications in mRNA expression, illustrated by a change in either or both temporal and spatial GUS expression, is due directly to the developmental stage, light regime or a combination of both is difficult.

Histochemical analyses showed light grown expression profiles were conserved with those described previously for all AtDRM1 and AtDRM2 promoter sequence constructs, with GUS expression induced in tips of cotyledons and the axil region of 6-day old seedlings of all constructs except AtDRM2\textsubscript{500bpprom:GUS} (Chapter 2). GUS expression was induced in seedlings grown in continuous darkness when driven by the AtDRM1\textsubscript{entprom} only, despite the presence of the IBOX motif, generally identified in regulatory sequence of light responsive genes (Giuliano et al., 1988), in all the promoter sequences used in this study. The absence of the expanded localisation of staining in the AtDRM2\textsubscript{entprom:GUS} lines in dark conditions, compared with AtDRM1\textsubscript{entprom:GUS} lines, may indicate a divergence in roles relating to cotyledon expansion in young seedlings.

In accordance with microarray studies in poplar (Wilkins et al., 2009), promoter:GUS lines grown in light/dark growth conditions did not have an expression profile similar to that of the dark-grown samples. Instead, an intermediary developmental phase elicited an intermediary spatial expression distribution, suggesting that the resulting expression profile may be due to the differences in plant development as opposed to a light response per se. However, by contrast wild-type seedlings grown in light conditions for 6 days and exposed to only 6 hours of darkness exhibited perturbation of both AtDRM1 and AtDRM2 transcript levels (Figure 3.4), without a concomitant visible morphological change (data not shown). The binding site of AtBZIP53, a transcription factor implicated in reprogramming of asparagine metabolism in response to low energy stresses including dark (Dietrich et al., 2011), has been identified in the promoter sequence of both AtDRM1 and AtDRM2 (Chapter 2). Therefore, the lack of dark response in AtDRM2\textsubscript{entprom:GUS} may be due to the inclusion of a binding site for some inhibitory protein for this dark response pathway.

### 3.4.4 Stress-induced intron retention in AtDRM1/ARP family members

Stress-responsive genes are over-represented in alternative splicing databases (Kazan, 2003; Wang and Brendel, 2006), with intron-retention being the major form of alternative splicing in *Arabidopsis* and the form of alternative splicing which is most
prevalent in stress responses (Ner-Gaon et al., 2004). Indeed, *AtDRM1* and *AtDRM2*, which both undergo alternative splicing, are transcriptionally regulated by various stress treatments. *AtDRM1* splice variants and *AtDRM2* showed varying responses after treatment with hormones generally associated with stress: ABA, SA and MJ.

Temperature effects on expression were opposite between *AtDRM1* splice variants and *AtDRM2*, with *AtDRM1* isoforms induced at 32°C and down-regulated at 4°C, and the opposite exhibited by *AtDRM2*. This contradicts previous findings in pea (*PsDRM1*) (Stafstrom, 2000), *Capsicum annuum* (*CaARP*) (Hwang et al., 2005) and *B. rapa* (*BrDRM1* and *BrARP1*) (Lee et al., 2013), which all showed induction with cold treatment. Heat shock treatment of *B. rapa* led to an increase in *DRM1/ARP* family member transcripts (Lee et al., 2013). In this study treatment with mannitol was used to induce osmotic stress, an abiotic factor closely linked with drought conditions. Research undertaken in peanut showed an induction of *DRM1/ARP* homologue expression in drought treated plants (Govind et al., 2009) similar to that exhibited by the mannitol-treated *Arabidopsis* seedlings, possibly reflecting activation of similar pathways with either treatment. Indeed, the binding element of the CBF/DREBs (Svensson et al., 2006; Xue, 2002), associated with cold and drought responses was identified in the upstream promoter sequence of *AtDRM1*, but also in *AtDRM2*500bprom (Mehrnia et al., 2013). This element has been described as over-represented in promoters of the target genes of the cell-proliferation and branching enhancing ERF BUD ENHANCER (EBE) genes, suggesting that *DRM1/ARP* may be a direct target of this transcription factor (Mehrnia et al., 2013). Other sugars treatments assessed also exhibited an induction of *AtDRM1* splice variants, contradicting findings from *Arabidopsis* and black locust (Gonzali et al., 2006; Park and Han, 2003).

Among all the *AtDRM1* splice variants, *AtDRM1.6* retains the most introns (retaining introns 2–5) and as such may be used as an indicator of stress-induced intron retention. Therefore, if intron-retention in *AtDRM1* is linked to stress conditions we could expect this transcript to be up-regulated with stress treatment. This was not obviously the case, either meaning that the splicing is stress-independent, or that the timing of abiotic stress screen has not captured the stress response and a time-course is required. Stress-induced intron retention has previously been described in the *DRM1/ARP* family in kiwifruit (Wood et al., 2013). Moreover, the *DRM1/ARP* family member identified from the salt tolerant *Arabidopsis* relative *Thellungiella halophila* is most closely related to
AtDRM1.2/3, which itself undergoes intron-retention, suggesting that this isoform might be responding to stress. That relatively conserved alternative splicing is maintained across species suggests that conserved splice variation occurs even when the transcriptional machinery is not failing, as suggested by the constant expression levels of the reference genes and no reports of transcriptional machinery breakdown in the literature despite many stress-related arrays.

This association between AtDRM1 and AtDRM2 transcription and stress, combined with the predicted intrinsic disorder of the resulting proteins, is similar to that described in another plant protein family, the heat shock proteins. These proteins have important roles in protein refolding in response to abiotic stresses, including extreme heat, salinity, water and oxidative stress, and have also been shown to be IDPs (reviewed in Wang et al., 2004). This molecular chaperoning function is prevalent in IDPs (Tompa and Csermely, 2004). As DRM1/ARP family members are increasingly being linked with abiotic stress responses and are predicted to be IDPs it is possible that this family have roles as molecular chaperones also.

### 3.4.5 Temporal regulation of AtDRM1/ARP

Transcriptional cascades include direct responders and indirect responders further downstream and as such the timing of the transcript analyses becomes critical. This is highlighted by the time course analysis in response to salt which was carried in this study. Salt was an abiotic factor of interest as it is not traditionally associated with dormancy status as is the case for light and temperature. Our initial screen had shown an induction of AtDRM1.1, AtDRM1.4 and AtDRM2, but not AtDRM1.2 and AtDRM1.6.

The biphasic AtDRM1 and AtDRM2 transcriptional profiles in the control plants across the time-course highlighted that both genes are undergoing some circadian regulation, as has been described previously (Schaffer et al., 2001), therefore comparisons were made between salt-treated and control plants at a given time-point only.

Over the time-course both AtDRM1.1 and AtDRM2 showed an immediate down-regulation with salt treatment, prior to subsequent induction. By contrast, AtDRM1.2 and AtDRM1.4 exhibited an induction which was maintained across the time course. AtDRM1.6 was induced in response to salt treatment as early as 10 min after salt treatment. These divergent profiles could reflect varying roles for these isoforms at
different stages after treatment, with \textit{AtDRM1.6} having a role in the initial response, then \textit{AtDRM1.2} and \textit{AtDRM1.4} being involved later on in the stress response cascade; or could be indicative of temporally overlapping functional redundancy between variants. This finding also suggests the discrepancies in transcriptional responses of \textit{DRM1/ARP} family members and splice variants which are prevalent in the literature may well be antagonized by differences in protocols, in particular, assaying tissues after different periods of treatment or growth conditions, as well as different organs entirely.

A temporary response to stress conditions can also be observed at the phenotypic level. Indeed, over-expression of the \textit{CHROMATIN-REMODELLING-12 (CHR12)} gene in \textit{Arabidopsis} induced temporary growth arrest in stress conditions only with a concomitant induction of \textit{AtDRM1} expression (Mlynárová et al., 2007). While morphological differences have been observed in plants over-expressing \textit{DRM1/ARP} family members (Chapter 2; Lee et al., 2013), the subtle nature of these changes combined with difficulties described replicating the data suggest that \textit{DRM1/ARP} over-expression lines might also exhibit stress-mediated phenotypic differences. No differences in growth habit were detected in over-expression lines in response to salt treatments (Appendix 7.16).
3.5 Conclusions

The *Arabidopsis* DRM1/ARP family has five members, with the family’s complexity further compounded by the presence of transcriptionally detectable splice variants. While splice variants exhibited relatively conserved transcriptional responses to a plethora of factors, in many instances including hormonal and temporal responses to salt treatment *AtDRM1.6* differed, possibly representing differing roles for this intron-retention variant in the plant. Moreover, the resulting differences in protein sequence may provide opportunities for differences in binding patterns and protein degradation. Subtle temporal differences in the transcript profiles of *AtDRM1* splice variants may reflect varying functions throughout the stress response signalling cascade or overlapping functional redundancy. Responses to such a vast array of factors also provide further evidence for a wider role for this gene family, outside of dormancy maintenance in meristematic tissues.

Both alternative splicing as well as IDP’s characteristic role as hub proteins suggests that the DRM1/ARP gene family is contributing greatly to the phenotypic plasticity of the *Arabidopsis* proteome (Mastrangelo et al., 2012; Pietrosemoli et al., 2013; Romero et al., 2006). This is particularly important for survival of plants in response to abiotic factors due to their sessile nature. Indeed, a protective role for DRM1/ARP family members through cessation or minimisation of growth in non-permissive conditions is emerging.
Chapter 4

4 Down-regulation of AtDRM1 and AtDRM2

4.1 Introduction

The function of the DRM1/ARP family has so far not been fully elucidated. Routinely used as a molecular marker for dormancy status in meristematic tissue, increasing evidence is emerging for a broader role of DRM1/ARP family members in non-growing tissue and therefore it is hypothesised that this gene may have a role in maintaining the no-growth status of these tissues. Over-expression of AtDRM1 or AtDRM2 results in a subtle developmental retardation (Chapter 2). A complementary approach to study the function of these genes is to knock-out or down-regulate the expression of the gene of interest. Based upon the current literature, in the case of DRM1/ARP we hypothesise that a down-regulation in gene expression would lead to an increase in growth of mutant plants. In particular, an increase in bud outgrowth and/or increased germination efficiency would be anticipated.

4.1.1 Previous work

4.1.1.1 T-DNA mutants

Previous database/stock centre searches including mining of the Arabidopsis stock centres (the Nottingham Arabidopsis Stock centre (NASC), the RIKEN Bioresource Center, the Arabidopsis Biological Resource Center (ABRC) and the INRA-Versailles Genomic Resource Center) yielded few available T-DNA mutants of AtDRM1 and AtDRM2. Very recently, one available T-DNA insertion line of AtDRM2 (SALK_054451) was analysed (Lee et al., 2013). However, the T-DNA was located in the 3’ UTR sequence and no differences in phenotype were observed compared with wild-type plants (Lee et al., 2013).

4.1.1.2 RNAi lines

An additional source of Arabidopsis mutant lines is from the AGRIKOLA project (Arabidopsis Genomic RNAi Knock-out Line Analysis) which utilised high-throughput cloning to generate RNAi hairpins from gene-specific sequence tags (GSTs) identified by the Complete Arabidopsis Transcriptome Microarray (CATMA) consortium (Hilson et al., 2004). Previous work analysing an AGRIKOLA (Hilson et al., 2004) RNAi line
targeting \textit{AtDRM1} (N213376; \textit{AtDRM1\textsubscript{AGRIKOLA}} RNAi) (Rae, 2009), produced a number of subtle morphological changes compared to wild-type (Table 4.1).

### Table 4.1. Comparison of \textit{AtDRM1\textsubscript{AGRIKOLA}} RNAi phenotype compared to wild-type.

<table>
<thead>
<tr>
<th>Trait</th>
<th>\textit{AtDRM1\textsubscript{AGRIKOLA}} RNAi cf. WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Germination efficiency</td>
<td>No change</td>
</tr>
<tr>
<td>Root length in 6 day old seedlings</td>
<td>Increased</td>
</tr>
<tr>
<td>Relative hypocotyl length in light/dark</td>
<td>No change</td>
</tr>
<tr>
<td>Vegetative phase rosette diameter (day 22)</td>
<td>No change</td>
</tr>
<tr>
<td>Time to flower (rosette leaf number)</td>
<td>Decreased</td>
</tr>
<tr>
<td>Height in long days (day 36)</td>
<td>Increased</td>
</tr>
<tr>
<td>Height in short days</td>
<td>Increased</td>
</tr>
<tr>
<td>Total bolt number in long days (day 36)</td>
<td>Increased</td>
</tr>
<tr>
<td>Total bolt number in short days</td>
<td>No change</td>
</tr>
<tr>
<td>Bolt number after inflorescence bolt decapitation</td>
<td>No change</td>
</tr>
</tbody>
</table>

Down-regulation of \textit{AtDRM1} was associated with an increase in root length; a reduction in time to flower as determined by number of rosette leaves at bolting; an increase in bolt number and primary bolt height in long days and an increase in bolt number only in short day conditions. RT-qPCR analysis of \textit{AtDRM1} down-regulation in this mutant line across development highlighted that \textit{AtDRM1} was not consistently down-regulated across plant development, throughout its lifecycle (Figure 4.1). Considerable down-regulation was only observed at day 16 and in leaf and bolt tissue. Moreover, at day 6 when \textit{AtDRM1\textsubscript{AGRIKOLA}} RNAi lines showed an increased root length, down-regulation of the gene was not observed. Similarly, down-regulation was not observed consistently in the general axil region from which increased numbers of inflorescence bolts emerge in these mutants, possibly a result of dilution of the axillary bud-derived signal due to inclusion of surrounding tissue in the sample.
Recently, the only published attempt at knocking-out \textit{AtDRM1}, an RNAi generated by the group to \textit{AtDRM1} (\textit{AtDRM1} RNAi), showed no phenotypic differences compared with wild-type plants (Lee et al., 2013). This work was carried out alongside analysis of SALK\textunderscore054451 introduced earlier. However, the degree of down-regulation in these \textit{AtDRM1} RNAi and SALK\textunderscore054451 lines was not shown (Lee et al., 2013). To date, no absolute knock-out lines have been described and all of the lines discussed display only a down-regulation in the levels of \textit{DRM1}/\textit{ARP} transcript compared with wild-type.

\textbf{4.1.1.3 Artificial micro RNA (amiRNA)}

Due to the absence of null mutants available in the \textit{Arabidopsis} T-DNA collections and the inefficiency of \textit{AtDRM1\textsubscript{AGRIKOLA}} RNAi in down-regulating the expression of
AtDRM1, an alternative strategy was applied: artificial miRNA (amiRNA) (Schwab et al., 2005). Artificial miRNAs are small, 21mer RNAs which can be designed to target single or multiple genes using the DICER/RISC degradation pathway in the same manner as RNAi (Schwab et al., 2005). The key difference however, is that the long fold-back hairpin of RNAi allows multiple possible siRNAs to be formed. Our lack of understanding on the DICER binding position to the hairpin structure, as well as the parameters by which targeting is defined, means that the siRNAs specific target sequence within the RNAi hairpin sequence is unknown (Ossowski et al., 2008). By contrast, the 21mers of amiRNA maximise targeting of very specific sequences and the parameters for miRNA target selection are described (Ossowski et al., 2008). In addition, because they are always generated from the same sequence, they can be optimised successfully.

This approach has been used successfully to down-regulate a variety of genes, including genes with roles in virus resistance (Niu et al., 2006; Qu et al., 2007), leaf polarity (Alvarez et al., 2006) and regulation of flowering (Mathieu et al., 2007).

The general experimental strategy for development of amiRNA lines is described in Figure 4.2 (Rae, 2009).
Figure 4.2. Experimental strategy for cloning and amiRNA line development.

Work carried out during the course of this PhD project for additional lines is shaded in grey.
The web-based design tool WMD – Web MicroRNA Designer (http://wmd.weigelworld.org/cgi-bin/mirnatools.pl?page=1) was used to propose a series of candidate 21mer miRNA target sequences (Schwab et al., 2006). A benefit of the amiRNA approach is that the software enables reproducible targeting of multiple family members simultaneously. However, attempts to generate amiRNA constructs that would target all five family members in Arabidopsis (AtDRM1; AtDRM2; AtDRM3; AtDRM4 and AtDRM5) failed (Rae, 2009). Instead only AtDRM1 and AtDRM2 could be targeted simultaneously. Therefore amiRNA constructs were developed targeting AtDRM1 (AtDRM1 amiRNA), AtDRM2 (AtDRM2 amiRNA), or both simultaneously (AtDRM1/AtDRM2 amiRNA) (Rae, 2009). In order to minimise the risk that results would be affected by off-targeting, two constructs (designated A and B for each target) were developed targeting each of AtDRM1, AtDRM2 and both AtDRM1 and AtDRM2 simultaneously. Moreover, analysis of two constructs for the same target allows comparison of any effect differences in the respective levels of gene silencing exhibited by each has on the phenotype.

Available stable, homozygote amiRNA lines were screened for down-regulation using RT-qPCR and a summary of the available stable, homozygote amiRNA lines with down-regulation confirmed is provided in Table 4.2.
Table 4.2. Summary of original down-regulation lines targeting AtDRM1/ARP family members.

This table is based on data from Rae (2009).

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Construct designation</th>
<th>Number of stable homozygote lines assessed for down-regulation of the target gene (line identifier)</th>
<th>Lines verified as down-regulated for the target gene(s) to be used for subsequent analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtDRM1</td>
<td>(A) 4 (1, 2, 3, 7)</td>
<td>1, 2, 7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(B) 0</td>
<td>none available</td>
<td></td>
</tr>
<tr>
<td>AtDRM2</td>
<td>(A) 0</td>
<td>none available</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(B) 1 (1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AtDRM1/AtDRM2 simultaneously</td>
<td>(A) 6 (2, 3, 4, 5, 6, 7)</td>
<td>4, 6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(B) 3 (1, 2, 5)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

In order to ensure the robustness of any phenotype, a minimum of three independent lines would be assessed at the transcript level for each of the constructs available. Sufficient numbers of lines had been assessed and confirmed for down-regulation of the target gene(s) for AtDRM1 amiRNA (A), AtDRM1/AtDRM2 amiRNA (A) and AtDRM1/AtDRM2 amiRNA (B) (Table 4.2), therefore additional lines were generated during this project for AtDRM1 amiRNA (B); AtDRM2 amiRNA (A) and AtDRM2 amiRNA (B).

4.2 Aims of this chapter

The aim of this chapter is to characterise the available AtDRM1 and AtDRM2 mutant lines under both standard developmental conditions and in response to salt stress, an abiotic factor which is not directly related to dormancy release, but that does cause retardation of growth. Gene-specific amiRNA targeting either AtDRM1, AtDRM2 or both AtDRM1 and AtDRM2 simultaneously, were the primary focus of this work, as well as analysis of an available FLAG T-DNA insertion line.
4.3 Methods

4.3.1 Plant growth conditions

Plants were grown as previously described (see §2.2.3).

4.3.2 Plant material for expression analyses

Plants were grown and sampled as previously described (see §2.2.5).

4.3.3 RNA Isolation and Quantitative RT-qPCR

RNA isolation and quantitative RT-qPCR were completed as described previously (see §2.2.4).

4.3.4 Cloning and *Arabidopsis* transformation

Sequence verified constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation for transformation into wild-type *Arabidopsis* plants (ecotype ‘Columbia’) via floral dip method (Clough and Bent, 1998).

4.3.5 Screen for stable homozygote inserts

T1 seeds were screened for primary transformants on kanamycin (50 µg/mL) selection. The ratio of resistant:sensitive T2 individuals on kanamycin selection was then scored. Independent lines with a 3:1 ratio of resistant plants to sensitive plants (≥95% confidence based on a Chi square statistical analysis) were designated as single-copy insertion events and resistant lines maintained. Stable homozygote lines with putative single copy insertions were those that grew with 100% resistance to kanamycin selection at the T3 generation. These lines were selected for further analysis.

4.3.6 Screen for FLAG insertion mutants

To confirm the identity of the mutant, seeds were grown on selection plates with media supplemented with kanamycin (50 µg/mL).
4.3.7 Germination and hypocotyl assays

Seedlings were grown on plates as described previously (see §2.2.3) in a random block design, in either standard light conditions (see §2.2.3) or in the dark (maintaining all other growth conditions except complete darkness). At day 6, seedlings were scored for germination and then transferred to a fresh plate for photographing. Hypocotyl length was then measured using the ImageJ image processing programme (http://rsbweb.nih.gov/ij/index.html). Hypocotyl length was defined as the length from the base of cotyledons to the top of the roots.

4.3.8 Salt Assay

Plants were grown and sampled as has been previously described (Appendix 7.15).
4.4 Results

4.4.1 Additional AtDRM1 insertion mutant - FLAG 405-F02

In addition to the previously described insertion mutant (SALK_054451; see §4.1.1.1), a further insertion mutant which according to the TAIR annotation was targeting the coding sequence of AtDRM1 (exon 2; FLAG 405-F02) was identified. Kanamycin-resistant seedlings were selected, indicating that the T-DNA was present. Expression analysis was undertaken using primers spanning the predicted insertion site (Appendix 7.2) to determine whether AtDRM1 was down-regulated in the mutant line (Figure 4.3).

![Diagram of AtDRM1 with FLAG insertion](image)

**Figure 4.3.** Down-regulation assessment of AtDRM1 FLAG 405-F02 line.

A) Schematic representation of FLAG-405F02 predicted insertion event in the AtDRM1.2 gene (representing all AtDRM1 splice variants) indicating primer binding sites spanning insertion in exon 2. B) Graph illustrating the target/reference ratio for AtDRM1 RT-qPCR expression analysis in vegetative rosette tissue of an AtDRM1 FLAG line, compared to wild-type (Ws). SEM of 4 technical replicates are represented in the error bars. Samples were normalised to the reference genes SAND and UBC9.
Chapter 4

Down-regulation

Expression analysis of the mutant line (background Ws) compared to wild-type Arabidopsis (ecotype Ws) showed that the targeted transcript was not down-regulated (Figure 4.3) and no phenotypic differences were observed in preliminary screening, therefore this line was deemed unsuitable for further analysis.

4.4.2 Development of amiRNA lines

The inconsistencies associated with $AtDRM1_{AGRIKOLA}$ RNAi in down-regulating $AtDRM1$ expression and the lack of null Arabidopsis mutants available in T-DNA collections meant that a different approach to down-regulating $AtDRM1/ARP$’s was required. Artificial miRNA constructs have been generated and assessed for down-regulation of the target gene prior to the PhD project. However, these lines were not fully analysed and insufficient independent lines were initially generated, except for the construct targeting both $AtDRM1$ and $AtDRM2$ simultaneously (Table 4.2; see §4.1.1.3).

Binary vectors containing the sequence verified $AtDRM1$ amiRNA (B), $AtDRM2$ amiRNA (A) and $AtDRM2$ amiRNA (B) constructs were used to generate transgenic Arabidopsis. Primary transformants were selected on kanamycin with 10-12 independent lines obtained for all constructs. Independent lines exhibiting putative single copy insertion events were obtained for all constructs (Table 4.3). Three-four putative single insertion event lines were screened in the subsequent T3 generation for homozygosity at the T-DNA loci.
Table 4.3. Screening for single copy insertions in T2 amiRNA lines.

Expected number of sensitive plants is calculated based on the given number of resistant plants. (*) denotes lines used for total RNA extraction for RT-qPCR analysis.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Line</th>
<th>Sensitive plants</th>
<th>Resistant plants</th>
<th>Expected number of sensitive plants</th>
<th>Chi Statistic (2dp)</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AtDRM1 amiRNA (B)</strong></td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>2</td>
<td>0.50</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.25</td>
<td>0.25</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7</td>
<td>54</td>
<td>15.25</td>
<td>4.46</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>4*</td>
<td>27</td>
<td>107</td>
<td>33.5</td>
<td>1.26</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>0.00</td>
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<td></td>
<td>7</td>
<td>7</td>
<td>60</td>
<td>16.75</td>
<td>5.68</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>19</td>
<td>70</td>
<td>22.25</td>
<td>0.47</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>9*</td>
<td>9</td>
<td>37</td>
<td>11.5</td>
<td>0.54</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>10*</td>
<td>21</td>
<td>43</td>
<td>16</td>
<td>1.56</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3</td>
<td>7</td>
<td>2.5</td>
<td>0.10</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>12*</td>
<td>21</td>
<td>64</td>
<td>21.25</td>
<td>0.00</td>
<td>99%</td>
</tr>
<tr>
<td><strong>AtDRM2 amiRNA (A)</strong></td>
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<td>4</td>
<td>10</td>
<td>3.5</td>
<td>0.07</td>
<td>99%</td>
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<tr>
<td></td>
<td>2</td>
<td>7</td>
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<tr>
<td></td>
<td>3*</td>
<td>11</td>
<td>45</td>
<td>14</td>
<td>0.64</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100</td>
<td>0</td>
<td>25</td>
<td>225.00</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>11</td>
<td>74</td>
<td>21.25</td>
<td>4.94</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>6*</td>
<td>20</td>
<td>38</td>
<td>14.5</td>
<td>2.08</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>7*</td>
<td>12</td>
<td>53</td>
<td>16.25</td>
<td>1.11</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>50</td>
<td>116</td>
<td>41.5</td>
<td>1.74</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>20</td>
<td>1</td>
<td>5.25</td>
<td>41.44</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>10*</td>
<td>25</td>
<td>85</td>
<td>27.5</td>
<td>0.23</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>8</td>
<td>58</td>
<td>16.5</td>
<td>4.38</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>12*</td>
<td>19</td>
<td>182</td>
<td>50.25</td>
<td>19.43</td>
<td>Not significant</td>
</tr>
<tr>
<td><strong>AtDRM2 amiRNA (B)</strong></td>
<td>1*</td>
<td>57</td>
<td>222</td>
<td>69.75</td>
<td>2.33</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>2*</td>
<td>52</td>
<td>117</td>
<td>42.25</td>
<td>2.25</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>35</td>
<td>20</td>
<td>13.75</td>
<td>32.84</td>
<td>Not significant</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>37</td>
<td>85</td>
<td>30.5</td>
<td>1.39</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>6*</td>
<td>14</td>
<td>62</td>
<td>19</td>
<td>1.32</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>14</td>
<td>31</td>
<td>11.25</td>
<td>0.67</td>
<td>99%</td>
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<td></td>
<td>8*</td>
<td>7</td>
<td>39</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>3</td>
<td>7</td>
<td>2.5</td>
<td>0.10</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>2.00</td>
<td>99%</td>
</tr>
</tbody>
</table>

As a number of lines have been developed prior to this work, a summary table compiling Table 4.2 and Table 4.3 is presented as Table 4.4.
Table 4.4. Summary of available stable homozygote down-regulation lines targeting AtDRM1/ARP family members.

Lines in white were characterised prior to this project (hereafter referred to as original lines). Lines in grey boxes were developed during this project (hereafter referred to as additional lines).

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Line name</th>
<th>Strategy/Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtDRM1</td>
<td>FLAG 405-F02</td>
<td>Insertion mutant</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 RNAi (Lee et al., 2013)</td>
<td>RNAi</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 AGRIKOLA RNAi</td>
<td>Agrikola RNAi</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (A) 1</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (A) 2</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (A) 3</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (A) 7</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (B) 4</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (B) 9</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (B) 10</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1 amiRNA (B) 12</td>
<td>amiRNA</td>
</tr>
<tr>
<td>AtDRM2</td>
<td>SALK_054451 (Lee et al., 2013)</td>
<td>insertion mutant</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (A) 3</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (A) 6</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (A) 7</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (A) 10</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (B) 1</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (B) 2</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (B) 6</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM2 amiRNA (B) 8</td>
<td>amiRNA</td>
</tr>
<tr>
<td>AtDRM1 and AtDRM2 simultaneously</td>
<td>AtDRM1 RNAi/SALK_054451 (cross) (Lee et al., 2013)</td>
<td>RNAi and insertion mutant</td>
</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (A) 2</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (A) 3</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (A) 4</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (A) 5</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (A) 6</td>
<td>amiRNA</td>
</tr>
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<td></td>
<td>AtDRM1/AtDRM2 amiRNA (A) 7</td>
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</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (B) 1</td>
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</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (B) 2</td>
<td>amiRNA</td>
</tr>
<tr>
<td></td>
<td>AtDRM1/AtDRM2 amiRNA (B) 5</td>
<td>amiRNA</td>
</tr>
</tbody>
</table>

4.4.3 Confirmation of down-regulation of the amiRNA targeted genes

Total RNA was extracted from 14-day old seedlings (confirmed as homozygous for a single insertion of the T-DNA at the T3 generation), as well as from wild-type plants grown concurrently and used for transcript expression verification (Figure 4.4).
Chapter 4 Down-regulation

Figure 4.4. Transcript expression of AtDRM1 and AtDRM2 in amiRNA transgenic lines.

Graph illustrating the target/reference ratio for AtDRM1 and AtDRM2 in T3 homozygous 14 day old seedlings, compared to wild-type. Independent transgenic lines are labelled with construct designation in brackets, followed by line number. ACTIN2 and Unknown Protein (At2g32170) were used as reference genes and data. SEM of 4 technical replicates are represented in the error bars.

Down-regulation of AtDRM1 transcript was exhibited in all AtDRM1 amiRNA (B) lines assessed compared with wild-type. AtDRM2 was not down-regulated in any of these lines except line 10. Similarly, AtDRM2 transcript levels were down-regulated in all AtDRM2 amiRNA lines (including construct A and B). In addition, AtDRM1 transcript levels exhibited some off-target down-regulation, with down-regulation exhibited in AtDRM2 amiRNA (A) lines 3, 6 and 7 and AtDRM2 amiRNA (B) line 6.

As the AtDRM1\textsubscript{AGRIKOLA} RNAi line previously assessed showed inconsistent down-regulation across development (Figure 4.1), it was of interest to look at the down-regulation of the amiRNA lines across development. AtDRM2 amiRNA (B) line 1; AtDRM1/AtDRM2 amiRNA (A) line 4 and AtDRM1/AtDRM2 amiRNA (B) line 2 were selected as lines of interest based upon previous anecdotal differences in bolt number and plant height and analysed by RT-qPCR (Figure 4.5).
Transcripts were detected in all tissues assessed. *AtDRM2* transcript was down-regulated in *AtDRM2* amiRNA compared to wild-type in all tissues assessed; while *AtDRM1* showed no major changes in expression across this series as was expected. Both *AtDRM1* and *AtDRM2* transcripts were down-regulated in *AtDRM1/AtDRM2* (A) amiRNA compared to wild-type, except *AtDRM2* in low bolt tissue. *AtDRM1* was consistently down-regulated in *AtDRM1/AtDRM2* (B) amiRNA. However, despite being a target of the amiRNA, *AtDRM2* was only down-regulated in day 6, day 16, young leaf and flower tissue, but not in mature leaf, axil, low bolt and top bolt tissue.

Figure 4.5. Expression levels of *AtDRM1* and *AtDRM2* across development in amiRNA lines.

Graphs illustrating the target/reference ratio for *AtDRM1* (white bars; A, C and E) and *AtDRM2* (grey bars; B, D and F) over a developmental series in wild-type plants (plain bars) compared to transgenic lines (lined bars): *AtDRM2* amiRNA (B) line 1 (A, B); *AtDRM1/AtDRM2* amiRNA (A) line 4 (C, D) and *AtDRM1/AtDRM2* amiRNA (B) line 2 (E, F). SD of 4 technical replicates are represented in the error bars. Samples were normalised to the reference genes *EF1α* and *SAND*. 
Despite some variation in the efficiency of down-regulation of the amiRNA across development, available amiRNA lines were analysed for their phenotype. While additional lines were being generated, phenotypic characterisation of the original lines was undertaken. As no consistent differences were observed, only adult plant height and branching were studied as a screen for the additional amiRNA lines being generated.

The proportion of seeds germinated after 6 days was assessed for AtDRM1/ARP amiRNA lines compared to wild-type (Figure 4.6).

![Figure 4.6. Seed germination in AtDRM1/ARP amiRNA lines.](image)

Wild-type seeds showed >95% germination across all biological replicates. No AtDRM1/ARP amiRNA lines demonstrated a reproducible and consistent difference in the proportion of germinated seeds in all three biological replicates and between
independent lines. However, *AtDRM1* amiRNA (A) line 2, *AtDRM1/AtDRM2* amiRNA (A) line 4 and *AtDRM1/AtDRM2* amiRNA (B) line 2 exhibited a statistically significant reduction in the proportion of germinated seed in 2 out of 3 biological replicates. No differences in germination were observed in any replicates for *AtDRM2* amiRNA (B) line 1 or *AtDRM1/AtDRM2* amiRNA (A) line 6. Conversely, *AtDRM1* amiRNA (A) line 1 exhibited a statistically significant increase in biological replicate 1 (white) and a statistically significant reduction in biological 3 (dark grey).

As the transcript of *DRM1/ARP* family members has been associated with non-elongating tissue (Lee et al., 2013; Park and Han, 2003), the hypocotyl length of both light- and dark-grown *AtDRM1/ARP* amiRNA lines was assessed and compared to wild-type (Figure 4.7).
Figure 4.7. Phenotypic analysis of 6 day old amiRNA plants.

The hypocotyl length was measured for AtDRM1/ARP amiRNA lines after growth in light (white bars; A, C and E) and dark (grey bars; B, D and F) conditions. Independent transgenic lines are labelled with construct designation in brackets, followed by line number. Lined bars represent wild-type samples. Three independent biological replicates are shown for light and dark growth conditions. All data sets were subjected to a one-way ANOVA test followed by Tukey’s test for multiple comparisons (95% confidence), with SEM represented in error bars (n = ≥16).

Growth under light conditions yielded a reduction in the hypocotyl length for all AtDRM1/AtDRM2 amiRNA lines compared to wild-type in 2/3 replicates assessed. No conserved effects upon growth were observed for any other lines under light conditions. Conflicting trends were exhibited under dark growth conditions. One biological replicate showed increased hypocotyl length for most amiRNA lines compared to wild-type (Figure 4.7B), while the opposite was seen in another replicate (Figure 4.7F). The additional biological replicate in seedling grown in darkness exhibited no statistically significant changes in growth compared with wild-type.

130
As *DRM1/ARP* transcript is associated with bud dormancy (Stafstrom et al., 1998; Tatematsu et al., 2005) and elongation (Lee et al., 2013; Park and Han, 2003) available lines were sown on rockwool and their mature floral phase phenotype was scored, including the number and height of inflorescence bolts (Figure 4.8).

![Figure 4.8. Phenotypic analyses of adult amiRNA plants.](image)

The primary bolt height (A & B) and number of inflorescence bolts >1cm (C & D) of 40 day old plants (N ≥ 6). Original lines (A & C) and additional lines (B & D) were analysed in separate experiments. Lined bars represent wild-type samples. Independent transgenic lines are labelled with construct designation in brackets, followed by line number. All data sets were subjected to a one-way ANOVA test followed by Tukey’s test for multiple comparisons (95% confidence), with SEM represented in error bars.
Overall, amiRNA lines did not show any major statistically significant alterations in either primary bolt height or inflorescence bolt number, although a trend towards increased bolt height was evident in the original lines (Figure 4.8). Exceptions to this were evident in individual lines, but these were not conserved across biological replicates (Figure 4.8; Appendix 7.16). For example, AtDRM1 amiRNA (A) line 3 had an increase in plant height in one replicate (Figure 4.8); however this was not evident in another replicate (Appendix 7.16).

As DRM1/ARP family members are historically linked with changes in transcript expression in response to dormancy release by decapitation, it was of interest to consider the effect that down-regulation of these genes might have upon bud outgrowth in response to decapitation (Figure 4.9).

Figure 4.9. Inflorescence bolt number post-decapitation in amiRNA plants.
(A) Number of bolts prior to decapitation (grey bars). (B) Number of bolts ten days after decapitation (white bars). Independent transgenic lines are labelled with construct designations in brackets, followed by line number. Lined bars represent wild-type samples. All data sets were subjected to a one-way ANOVA test followed by Tukey’s test for multiple comparisons (95% confidence), with SEM represented in error bars.
Prior to decapitation, no differences were observed in bolt number for any amiRNA lines compared with wild-type plants. Upon decapitation, no significant differences were observed in the number of bolts which subsequently grew out for amiRNA lines compared with wild-type plants.

### 4.4.5 Assessment of AtDRM1/ARP down-regulation lines in response to salt treatment

Both AtDRM1 and AtDRM2 have been shown to be transcriptionally regulated in response to a range of abiotic treatments including salt in a temporal fashion, possibly indicative of these genes having a role in immediate responses to initiate protective processes (Chapter 3). Whether the presence of AtDRM1/ARP transcript provides protection against salt stress and its absence increases susceptibility to the stress, was assessed using the AtDRM1/ARP down-regulation lines. AtDRM1\textsubscript{AGRIKOLA} RNAi and AtDRM2 amiRNA (B) line 1 were chosen as representative lines for further analysis as both had exhibited phenotypic alterations in previous work.

Seedlings of down-regulation lines were grown on plates with media supplemented with salt to assess any resulting changes in growth habit of these plants (Figure 4.10). 0 mM NaCl was included as a negative control. 100 mM NaCl was used as it is routinely used in salt treatment assays in the literature. 40 mM NaCl and 1 M NaCl provided a minimal and maximum stress (respectively), compared with the 100 mM NaCl sample.
Figure 4.10. Artificial miRNA lines grown on varying salt concentrations.

Six day old seedlings were transferred to ½ MS plates supplemented with either 0 mM NaCl, 40 mM NaCl, 100 mM NaCl, or 1 M NaCl and grown for further 12 days before analysis. In order to minimise interplate-variation effects, lines were distributed across four different plates and a composite image generated.

The growth of wild-type seedlings on all concentrations of NaCl was stunted compared with seedlings grown on standard media (0 mM NaCl). Growth retardation was dose-dependent, with seedlings grown on 100 mM NaCl exhibiting significantly greater growth retardation than plants grown on 40 mM NaCl (Figure 4.11). In addition, wild-type plants grown on 100 mM NaCl exhibited photo-bleaching. 1 M NaCl appeared to
be lethal for wild-type seedlings with no additional growth detectable after transfer to the treatment plates and significant photo-bleaching (Smillie and Nott, 1982). Compared to wild-type, no reproducible differences in the growth response were detected in either $AtDRM1_{AGRIKOLA}$ RNAi or $AtDRM2$ amiRNA (B) line 1 at any concentration of salt assessed (Figure 4.10). As $AtDRM1_{AGRIKOLA}$ RNAi and $AtDRM2$ amiRNA (B) line 1 are not null mutants and exhibit residual down-regulated transcript expression (Figure 4.1 & 4.5), it was of interest to assess the transcriptional response to salt treatment of these lines compared to wild-type by RT-qPCR (Figure 4.11). The transcript response was assessed immediately prior to treatment, at 6 h post-treatment (prior to any observable gross phenotypic response) and at 12 days after treatment (when a gross phenotypic response was evident).
Figure 4.11. Transcript expression levels in down-regulation lines compared with wild-type in the presence of salt.

Graphs show the target/reference ratio of *AtDRM1* (top) and *AtDRM2* (bottom) normalised to the reference gene *ACTIN2* over a plate-based salt treatment series. Standard deviation of technical replicates are represented in error bars.

*T*<sub>0</sub> provides a baseline, confirming down-regulation in both *AtDRM1 AGRIKOLA* RNAi and *AtDRM2* amiRNA (B) line 1 compared to wild-type prior to any treatment. In wild-type seedlings, both *AtDRM1* and *AtDRM2* exhibited no difference in transcript accumulation compared with *T*<sub>0</sub> when transferred to plates with no salt. At *T*<sub>6h</sub> both genes showed increases in transcript levels with increasing NaCl concentrations, with the exception of *AtDRM2* with 1 M NaCl which returned to pre-treatment (*T*<sub>0</sub>) levels.
Wild-type seedlings maintained similar transcript levels to those seen at T\(_{6h}\) at T\(_{12d}\) at the different NaCl concentrations.

At 6 h post-treatment, an increase in both target transcripts (\(AtDRM1\) in \(AtDRM1\)\_AGRIKOLA RNAi and \(AtDRM2\) in \(AtDRM2\) amiRNA (B) line 1) was exhibited with 0 mM NaCl which contrasts with wild-type where no difference in transcript accumulation was evident. This T\(_{6h}\) timepoint provides the baseline for assessing any transcript accumulation relating to the salt treatment alone.

At 6 h post-treatment \(AtDRM1\) transcripts in \(AtDRM1\)\_AGRIKOLA RNAi seedlings accumulated in a dose-dependent fashion, similar to that exhibited by wild-type except for with 1 M NaCl, where \(AtDRM1\) transcript levels returned to T\(_{0}\)-equivalent levels - even lower than the T\(_{6h}\) baseline for salt treatment. The increase in \(AtDRM1\) transcript expression with 40 mM NaCl and 100 mM NaCl brought transcript levels to slightly higher than wild-type, over-riding any down-regulation of the transcript compared to wild-type within those samples. At 12 days post-treatment, \(AtDRM1\) transcripts in \(AtDRM1\)\_AGRIKOLA RNAi remained at T\(_{0}\)-equivalent levels, contrasting with the profile of wild-type seedlings.

In \(AtDRM2\) amiRNA (B) line 1 \(AtDRM2\) transcripts also showed a dose-dependent increase in expression at 6 h post-treatment, similar to the wild-type profile. Moreover, the same return to T\(_{0}\)-equivalent \(AtDRM2\) transcript levels was evident with 1 M NaCl. NaCl-associated \(AtDRM2\) transcript accumulation was also not evident at 12 days post-treatment in \(AtDRM2\) amiRNA (B) line 1.
4.5 Discussion

4.5.1 Availability and quality of AtDRM1/ARP down-regulation resources

Thorough ongoing mining of the available Arabidopsis mutant databases has yielded few mutants targeting the coding sequence of either AtDRM1 or AtDRM2. Only very recently the first publication describing down-regulation lines of AtDRM1 and AtDRM2 has emerged. An RNAi construct was used to target AtDRM1, while an available SALK insertion line was used as a representative AtDRM2 down-regulation mutant. No phenotypic differences were observed in any of the mutant lines, including a cross of the two lines, compared with wild-type across development (Lee et al., 2013). More pertinent to this argument is that the level of down-regulation was not shown for these lines, making it difficult to draw conclusions on the efficacy of the line with any confidence.

An available FLAG insertion line was also assessed but showed no transcript down-regulation despite being kanamycin-resistant. While cases of translational inhibition (reduction in target proteins via transcript targeting) in insertion mutants exist without down-regulation at the transcript level (Bezanilla et al., 2005; Monte et al., 2003; Ossowski et al., 2008), this could not be clarified for this FLAG line. Confirmation of AtDRM1 protein down-regulation could be completed by comparing mutant protein extract with wild-type protein extract on a Western Blot and using antibodies targeting AtDRM1 to detect a reduction in AtDRM1 protein levels. However, antibodies generated in this PhD project did not show sufficient binding sensitivity to make this differentiation possible, indeed successful binding of the antibody was evident in over-expression lines only (Chapter 5). For this reason the FLAG line was not used for further work.

By comparison, AtDRM1\textsubscript{AGRIKOLA} RNAi exhibited a number of phenotypic changes compared with wild-type plants. However, down-regulation was not conserved across development, suggesting a link between the construct and phenotypic difference is more complex than purely transcript expression levels. Moreover, some evidence of the endogenous induction of AtDRM1 transcript in response to salt surpassing wild-type levels in AtDRM1\textsubscript{AGRIKOLA} RNAi was also seen. Indeed, AtDRM1\textsubscript{AGRIKOLA} RNAi exhibited a parallel dose-dependent increase in AtDRM1 transcription levels with increasing salt concentrations at 6 hours post-treatment (with the exception of 1 M salt).
The issue of the presence of residual target transcript is eliminated in null mutants where targeted transcripts are consistently totally undetectable.

Many studies do not confirm down-regulation across a treatment series, raising the question: is inconsistent down-regulation more prevalent than currently reported? Significant variability in silencing efficacy exists with RNAi technologies in plants (reviewed in McGinnis, 2010). RNAi may not induce any post-transcriptional silencing, down-regulation being lost in later generations and insufficient silencing to breach threshold levels of the target gene for wild-type functionality are all issues with the technology which have arisen in previous studies.

Reliable knock-out lines are routinely generated in mice, yeast and Eschericia coli for example, using homologous recombination. However, this technology remains to be optimised in higher plants such as Arabidopsis and therefore other approaches are required.

Development of amiRNA technology targeting AtDRM1 and AtDRM2 had been commenced prior to these PhD studies, although further lines were required in order to determine the robustness of the phenotype and genotype of the mutant lines targeting AtDRM1, AtDRM2, or both simultaneously. In this work a number of additional lines were generated, although none exhibited an absolute knock-out with target transcripts readily detectable using RT-qPCR. AtDRM2 amiRNA was more efficient at down-regulating the target transcript across a range of conditions, including development salt treatment, than AtDRM1_AGRIKOLA RNAi. Indeed amiRNA has been shown to be more effective at down-regulating a gene involved in Arabidopsis virus-resistance compared with hair-pin RNAi previously (Qu et al., 2007). When attempting to target both AtDRM1 and AtDRM2 simultaneously however, for both constructs developed, only AtDRM1 was consistently down-regulated across development, highlighting the difficulty associated with generating reliable down-regulation resources for the AtDRM1/ARP gene family.

A number of reasons have been proposed addressing why silencing might not be successful in Arabidopsis amiRNA lines (Ossowski et al., 2008). The WMD construct design software is optimised for producing short-interfering RNAs (siRNAs) with favourable intrinsic properties (Schwab et al., 2006). However, many studies on miRNA precursors have focused on mammalian systems and applicability of these findings to plant systems is less characterised (Schwab et al., 2006). Similarly, the accessibility of
the target site complementary to the miRNA is a problem in mammalian systems (Ameres et al., 2007; Overhoff et al., 2005; Schubert et al., 2005) which may also be an issue in plant systems (Ossowski et al., 2008).

Both AtDRM1AGRIKOLA RNAi and the various amiRNA lines generated utilise the 35S CaMV promoter for ‘constitutive’ expression of the constructs. GUS histochemical analyses showed that the 35S CaMV promoter does not provide ubiquitous expression, particularly in the low bolt area (Figure 2.5M). This may explain the lack of down-regulation exhibited in the low bolt region of AtDRM1/AtDRM2 (A and B) amiRNA (Figure 4.5).

While null mutants of AtDRM1 or AtDRM2 are not currently available for analyses and the available down-regulation lines show inconsistencies, phenotypic effects may still occur due to translational inhibition (Bezanilla et al., 2005; Monte et al., 2003; Ossowski et al., 2008).

Due to inconsistencies in down-regulation and phenotypic characteristics, experiments were incrementally and systematically altered in order to attempt to identify contributing sources of variation and eliminate such variables. For this reason, many experiments undertaken during the development and characterisation of AtDRM1/ARP down-regulation resources were completed as a preliminary screen.

### 4.5.2 Effects of putative negative feedback regulation on down-regulation systems

A further issue affecting the efficiency of amiRNA constructs discussed in Ossowski et al., (2008) is the reduction in transcript levels resulting from amiRNA being compensated for in planta with an increase in the rate of transcription, otherwise known as negative feedback regulation. This might explain why target transcripts are not being consistently down-regulated for both amiRNA and RNAi lines.

For example, when both AtDRM1 and AtDRM2 are targeted by amiRNA simultaneously, down-regulation of transcription of AtDRM2 is not displayed in the low bolt sample compared with wild-type. The low bolt was the site of highest AtDRM2 expression in the wild-type development profile (Chapter 2, Figure 2.4). That this aspect of the expression profile is even displayed in down-regulation lines suggests that it is of
particular importance for the plant to have \textit{AtDRM2} expression in the non-growing low bolt tissue and therefore may have pathways in place to regulate accordingly.

A reduction in hypocotyl length was observed for amiRNA constructs targeting both \textit{AtDRM1} and \textit{AtDRM2} simultaneously. However, no significant difference was observed when either gene was targeted individually, suggesting possible redundancy between the two genes. Interestingly, this is similar to that described in \textit{Arabidopsis} plants over-expressing homologues of \textit{BrDRM1} and \textit{BrARP1}, from the closely related species \textit{B. rapa}, where hypocotyls were shorter (Lee et al., 2013). Both over-expression and down-regulation of a gene resulting in the same phenotypic effect has been described previously in rice \textit{H-TYPE THIOREDOXIN (OsTRXh1)} (Zhang et al., 2011). Combined with the lack of conserved down-regulation evident in transcriptional studies, it is possible that this finding is indicative of some negative feedback regulation. Both the down-regulation and over-expression lines would need to be grown concurrently in order to clarify this.

\textbf{4.5.3 Do null mutants exhibit embryonic lethality?}

The absence of null mutant lines for \textit{AtDRM1} and \textit{AtDRM2} despite numerous attempts, could reside in the very nature of the protein encoded by these genes. The DRM1/ARP family of proteins are predicted to be IDP’s (Wood et al., 2013) including \textit{Arabidopsis} family members (Chapter 2), a protein structure which is commonly associated with roles as hub proteins (Dosztányi et al., 2006; Dunker et al., 2005; Ekman et al., 2006; Haynes et al., 2006; Patil and Nakamura, 2006; Singh and Dash, 2007; Singh et al., 2007; Uversky et al., 2005). If this characteristic is also the case for DRM1/ARP proteins, the lack of null mutants might reflect lethality in such lines. Similarly, the small size of the coding genes of \textit{AtDRM1} and \textit{AtDRM2} (1356 bp and 856 bp, respectively; proteins of 11.55 – 14.40 kD) may have an effect, providing a smaller region into which insertion events can occur, therefore reducing the chance of the required insertion event occurring. However, null mutants are available for similar sized proteins, for example knock-out insertion mutants have been described for the chloroplast-targeted DnaJ proteins, \textit{AtJ8}, \textit{AtJ11} and \textit{AtJ20} (8.3-, 17.8- and 23.4-kD, respectively) (Chen et al., 2010). Indeed insertion events in the protein-coding region of a gene results in a null mutant at least 90% of cases (Wang, 2008).
An alternative hypothesis for the lack of absolute knock-outs is that null mutations result in an embryo lethal phenotype. In order to avoid this effect, amiRNA constructs are in the process of being developed which are driven by an ethanol-induced promoter. Down-regulation of *AtDRM1/AtDRM2* will be tightly controlled temporally possibly allowing induction of complete knock-out of the target transcript at the developmental stage for which its presence is not absolutely critical.

**4.5.4 Conflicting phenotypic evidence for a role in prevention of growth**

A mutant’s phenotype is described by way of a comparison with wild-type plants grown as controls within the same experiment. Differences were observed between wild-type samples of different biological replicates in the analyses of 6-day-old seedlings and adult plants (e.g. primary bolt height of 121.8 cm compared with 190.2 cm), possibly a reflection of subtle differences in the growth conditions. While biological replicates are included in experiments to allow for this situation and therefore statistical analyses are undertaken only within biological replicates, the variation may still obscure a change in the phenotype of mutant lines. One example of this was the relatively short hypocotyl length of dark-grown wild-type seedlings in one biological replicate (Figure 4.7B, compared with Figure 4.7D & 4.7F). Across the three replicates an increase and a decrease in hypocotyl length was observed, suggesting there are likely some further environmental conditions which remain to be identified having an impact on these plants in the dark. This is highly possible as *AtDRM1* and *AtDRM2* are highly regulated at the transcriptional level by numerous abiotic factors (Chapter 3).

No conserved statistically significant differences were observed in adult (40-day-old) amiRNA lines compared with wild-type, in terms of plant height or the number of inflorescence bolts. This contrasts with previous work using the *AtDRM1* AGRIKOLA RNAi line, which showed an increase in plant height and branching (day 36) (Rae, 2009). Indeed, Lee et al., (2012) described no phenotypic change in 42-day-old mutant plants, although the images provided suggest a reduction in height similar to that exhibited by their over-expression lines. As no actual measurements were provided further clarification is not possible. During this project amiRNA lines assessed did however show a trend towards increased primary bolt height, the consistency of which was likely to be affected by stochastic environmental factors.
These inconsistencies could be a result of all currently available data being ‘snapshots’ of plant morphology at a given time-point/age of plant, as opposed to a developmentally equivalent stage. A developmental retardation has been described in lines over-expressing AtDRM1 or AtDRM2 (Chapter 2), with fewer leaves on any given day through the vegetative phase, plants reaching anthesis later and physically shorter plants through floral phase. Notably, no difference in overall plant height was observed when measurements were taken at the same age of plant, suggesting the differences in height were related to differences in developmental stage and not a dwarfing phenotype. As the AtDRM1_AGRIKOLA RNAi line exhibited a decreased time to flower, indicative of undergoing more rapid development than wild-type, with a concomitant increase in plant height and branching at day 40, it is possible that reducing AtDRM1/AtDRM2 transcript expression could result in a similar opposing trend to that of the over-expression lines.

As AtDRM1/ARP transcript expression is associated with dormancy status in meristematic tissues (Chapter 2; Finlayson, 2007; Gonzalez-Grandio et al., 2013; Stafstrom et al., 1998; Tatematsu et al., 2005; Wood et al., 2013), it was of interest to assess how the down-regulation lines would respond upon release of paradormancy by decapitation. While no phenotypic differences were conserved across lines, two of the four lines targeting AtDRM1 alone exhibited an unexpected reduction in bud outgrowth after decapitation. No significant differences were observed when AtDRM2 was targeted, or both genes simultaneously. This is similar to the transcriptional analyses carried out during the course of this project which showed down-regulation of AtDRM1 upon decapitation, but conflicting results in AtDRM2 (Chapter 2). This finding could be indicative of functional divergence of these two genes. However, previous work has shown a conserved response to decapitation in AtDRM1 and AtDRM2 (Tatematsu et al., 2005). Further studies including additional lines will clarify this point.

4.5.5 Putative effects of unintended stresses during standard growth conditions

AtDRM1/ARP amiRNA lines showed a general reduction in the proportion of seeds that had germinated after 6 days, possibly indicative of a reduction in seed viability in amiRNA lines. AtDRM1 and AtDRM2 transcript expression has been linked with expression levels of the SWI/SNF chromatin remodelling gene AtCHR12 (Mlynárová et
al., 2007), suggesting that these genes might be involved in an epigenetically regulated pathway. Furthermore, evidence is emerging for a transgenerational stress memory in *Arabidopsis* mediated via epigenetics (Boyko and Kovalchuk, 2010; Ding et al., 2012; Paszkowski and Grossniklaus, 2011), such that the phenotype of offspring can be affected by the inherited epigenetic status passed on by parents based on the stress conditions in which they were grown. Indeed, *Arabidopsis* seed germination is promoted by epigenetic transcriptional activation (Neff, 2012), therefore alterations in chromatin remodelling, for example, may impact on the seed’s viability depending on the growth conditions of the plant from which is was harvested.

Not only is the transcript expression of *AtDRM1* and *AtDRM2* down-regulated in *atchr12* knockout mutants (Mlynárová et al., 2007), parallels are also evident in the phenotypic characterisation of *AtDRM1/ARP* and *atchr12* mutants. Mlynárová et al., (2007) published that “the rare and unpredictable occurrence of growth arrest under controlled growth conditions may reflect a stochastic exposure to unintended stress”. Indeed, throughout development of *AtDRM1/ARP* mutant resources these plants often appeared more susceptible to issues including aphid infestations, mildew and compromised watering than other plants being grown around the same time in the same location, including over-expression lines.

In order to assess whether *AtDRM1/ARP* down-regulation lines were more susceptible to stress conditions plants were grown on plates and transferred to media supplemented with varying concentrations of salt at 6 days old. As discussed earlier, the transcriptional profile of target genes (*AtDRM1* in *AtDRM1_AGRIKOLA* RNAi and *AtDRM2* in *AtDRM2* amiRNA (B) line 1) in these conditions was similar to that observed in wild-type plants. However, these plants showed no obvious gross phenotypic changes under these conditions. It is proposed that *AtCHR12* plays a role priming the plants for a growth-arrest response (Mlynárová et al., 2007). If *AtDRM1/ARP* is acting downstream of *AtCHR12* in this pathway, any phenotypic differences may also be particularly subtle and changes to the experiment might be required. More subtle experiments could be carried out by challenging seedlings with high (near lethal) concentrations of salt for a shorter period of time and then assessing the recovery of these plants on standard media.

Indeed, it is also possible that any priming or protective role for *AtDRM1/ARP* is not all or nothing, but rather is a threshold issue and residual expression in these lines is
providing sufficient basal levels of transcript for any protective function of the gene products to be maintained.
4.6 Conclusion

Very few down-regulation resources are available targeting \textit{AtDRM1} or \textit{AtDRM2}; indeed no true null mutants have been described to date. For this project, amiRNA lines were generated and their phenotypes characterised. Variation in the effectiveness of amiRNA has been described previously with an estimated 75\% of constructs successfully down-regulated their target gene(s) (Ossowski et al., 2008). Alternatively, inefficiency of down-regulation, combined with an unexpected shortening of the hypocotyl in down-regulation lines, similar to that described in over-expression lines (Lee et al., 2013), provides two lines of evidence for \textit{AtDRM1} and \textit{AtDRM2} being under negative feedback regulation. In addition, that the reduced hypocotyl length was observed in only those lines where both \textit{AtDRM1} and \textit{AtDRM2} were down-regulated, and not when either gene was down-regulated individually, suggests that in this instance these genes may be functionally redundant. By contrast, in adult plants with either \textit{AtDRM1} or \textit{AtDRM2} down-regulated, a trend towards taller plants was observed, consistent with the hypothesis that \textit{DRM1/ARP}’s have a role in growth prevention.

Inherent difficulties in the development and maintenance of down-regulation lines and lack of consistent phenotypic differences compared with wild-type plants could be indicative of absolute knock-outs being lethal for the plant. By contrast, this observation could also suggest these genes have a role similar to that hypothesised for \textit{AtCHR12}, in priming plants for growth in stress conditions.

Clarification of the \textit{AtDRM1} and \textit{AtDRM2} protein levels in these mutants may provide clarity on whether translational inhibition has occurred and therefore whether they are likely to be an effective tool in the future.
5 Biochemical analyses of AtDRM1 and AtDRM2

5.1 Introduction

While regulation of gene transcription is very important, the functional unit is often the protein molecule. Analysis of the protein localisation, structure, post-translational modifications, stability and identifying interacting partners are all essential to fully understand the function of the gene at the protein level. For this reason it was of interest to study the proteins coded for by the AtDRM1 and AtDRM2 genes.

Expression of protein in heterologous systems, followed by purification of the product in a soluble form is in many cases the first step for this type of approach. If successful, the purified protein can be used to generate specific antibodies to the protein of interest, as well as be used for structural characterisation. Many expression systems are bacteria-based and can be improved by optimising the codons used to suit this organism (reviewed in Gustafsson et al., 2004), although other systems have been also developed including insect-based (Kost et al., 2005) and wheat-germ-based systems (Morita et al., 2003). To facilitate purification, tags are fused to either the N- or C-terminus of the target sequence and the construct transformed into bacterial cells, enabling high-throughput expression of the protein.

In order to determine interacting partners, purify proteins and conduct localisation studies, antibodies are an essential tool. Antibodies can be either monoclonal or polyclonal. Polyclonal antibodies are a mixture of antibodies binding to various epitopes on a protein and are generated during an organism’s immune response representing a range of epitope affinities and specificities and can therefore often be used for many different applications. By contrast, monoclonal antibodies are generated from a single cell line and therefore can be associated with superior target specificity. This specificity can also limit the success of these antibodies for different applications dependent on their binding site. However, monoclonal antibodies are relatively expensive compared with polyclonal antibodies meaning that polyclonal antibodies are favoured in many research applications.
As previously mentioned, expression can be regulated post-transcriptionally and post-translationally. This can occur in a number of ways, including targeting the protein for proteolytic cleavage and post-translational modifications to the resultant protein (discussed in Chapter 1). Protein degradation can occur both *in vivo* and *in vitro*, with the latter having major implications for working with proteins in the laboratory.

Proteins usually function by interacting with other proteins (protein:protein), acting upon a substrate, or in the case of gene regulation, by binding to DNA (protein:DNA). Protein:protein interactions are particularly common and form the basis of the growing field of ‘interactomics’ (reviewed in Lievens et al., 2010). Identifying a protein’s binding partner(s) can provide insight into the function of a gene. There are many approaches that can be used, all with their advantages and disadvantages. One that has been used extensively is Yeast-2-Hybrids (Y2Hs).

The aim of this chapter was to study AtDRM1 and AtDRM2 at the protein level. Attempts to express the proteins, generate antibodies, analyse the stability of the protein and assess protein:protein interactions will be described.
5.2 Methods

5.2.1 Protein visualisation

Denaturing gel electrophoresis (SDS Polyacrylamide Gel Electrophoresis (PAGE)) was used to separate proteins based on molecular size prior to visualisation via Coomassie Blue staining or Western Blot. Buffers used for these approaches are described in Table 5.1.

**Table 5.1. Buffers used for protein visualisation techniques.**
All solutions were made up to the required volume with sterile water.

<table>
<thead>
<tr>
<th>Name</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x SDS loading buffer (Laemmli)</td>
<td>75 mM Tris-HCl (pH 6.8), 2% (w/v) sodium dodecyl sulphate (SDS), 0.03% (w/v) bromophenol blue, 1 mM EDTA, 10% (v/v) glycerol, 100 mM DTT</td>
</tr>
<tr>
<td>1x MES running buffer</td>
<td>50 mM MES, 50 mM Tris base, 3.5 mM SDS, 1 mM EDTA</td>
</tr>
<tr>
<td>1x Towbin transfer buffer</td>
<td>192 mM glycine, 25 mM Tris, 0.05% SDS, 20% (v/v) methanol</td>
</tr>
<tr>
<td>TBS + T20</td>
<td>10 mM Tris Base, 150 mM NaCl, 0.0001% Tween20, adjust to pH 8.0 with HCl</td>
</tr>
<tr>
<td>Blocking Solution</td>
<td>TBS + T20, 5% non-fat milk powder</td>
</tr>
<tr>
<td>Antibody solution</td>
<td>Antibody at the appropriate concentration in blocking solution</td>
</tr>
</tbody>
</table>

5.2.1.1 Denaturing gel electrophoresis

Proteins were extracted in Laemmli buffer (unless otherwise stated) therefore the protein preparation could not be quantified using standard approaches. Instead, in order to ensure equal loading of protein gels, equal amounts of tissue were ground in liquid nitrogen. Equal amounts of protein samples were resuspended in 1x Laemmli buffer (Table 5.1) and heated to 95°C for 3 min. Cell debris was removed from the solution by centrifugation at 10,000 x g in a bench-top microfuge for 2 min. Supernatants were
taken for SDS-PAGE analysis. Up to 15 μL of sample was loaded into wells of commercially available NuPAGE® Novex® Bis-Tris Mini Gels (Invitrogen) loaded in an XCell SureLock™ Mini-Cell (Invitrogen) according to the manufacturer’s instructions. Gels were run at a constant voltage of 200 V for 35 min in 1x MES running buffer (Table 1). Unless otherwise specified, Precision Plus Protein™ Dual Colour Standards (BioRad) was used as the protein standard.

Following electrophoresis, gels were either stained with Coomassie Blue (SimplyBlue™ SafeStain) according manufacturer’s instructions, or transferred to a PVDF membrane for Western Blotting.

5.2.1.2 Western Blotting

Immobilin-P™ PVDF (Millipore) membrane was rinsed for 30 s in methanol, then both the gel and PVDF membrane were incubated with gentle shaking for 30 min in 1x Towbin transfer buffer (Table 5.1). Proteins were electroblotted onto PVDF membrane at 5V for ≥5 h, using the semidry transfer cell Trans-Blot SD (BioRad), between blotting paper soaked in 1x Towbin transfer buffer.

For immunodetection of the target protein, the blot was first incubated in blocking solution (Table 5.1) either overnight at 4°C, or for 2 h at room temperature. The blot was incubated for 1 h in the primary antibody solution, followed by 3 x 5 min washes in TBS + T20 at room temperature with gentle agitation to remove any unbound antibodies. The blot was then incubated for 1 h in the secondary antibody solution, followed by 3 x 5 min washes in TBS + T20 at room temperature with gentle agitation. Protein bands were visualised by incubating the blot in 1-Step BCIP/NBT alkaline phosphatase detection reagent (Sigma). To prevent over-staining, the reaction was stopped by immersing the blot in sterile water upon the bands reaching satisfactory intensity.

The antibodies used in this work are summarised in Table 5.2.
**Table 5.2: Summary of antibodies used.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody</th>
<th>Concentration of primary antibody</th>
<th>Secondary antibody</th>
<th>Concentration of secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>His&lt;sub&gt;6&lt;/sub&gt;-tag</td>
<td>Anti-His&lt;sub&gt;6&lt;/sub&gt; (Roche)</td>
<td>1:250</td>
<td>Anti-mouse IgG-Alkaline phosphatase (Sigma)</td>
<td>1:1000</td>
</tr>
<tr>
<td>AtDRM1</td>
<td>Anti-AtDRM1 (this work)</td>
<td>1:500</td>
<td>Anti-rabbit IgG-Alkaline phosphatase (Sigma)</td>
<td>1:1000</td>
</tr>
<tr>
<td>AtDRM2</td>
<td>Anti-AtDRM2 (this work)</td>
<td>1:500</td>
<td>Anti-rabbit IgG-Alkaline phosphatase (Sigma)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

### 5.2.2 Protein expression

#### 5.2.2.1 Autoinduction method for production of recombinant proteins in E. coli

For development of protein expression constructs, the Champion™ pET200 Directional TOPO® Expression Kit with BL21 Star™ (DE3) One Shot® Escherichia coli cells was used according to manufacturer’s instructions. Wild-type Arabidopsis cDNA was used as a template for amplification using the Pfu DNA Polymerase (Stratagene) as per manufacturer’s instructions and gene-specific primers (pGR0001 and pGR0003; Appendix 7.17). Amplified fragments were analysed on a 1% agarose gel using the 1Kb+ DNA ladder molecular size marker (Invitrogen) and the PCR products purified using the Zymoclean™ gel DNA recovery kit (Zymo Research) according to manufacturer’s specifications. The purified PCR product was directionally cloned into the pET200/D-TOPO vector (Invitrogen) and transformed into BL21 Star™ (DE3) One Shot® E. coli (Invitrogen) according to manufacturer’s instructions. BL21 Star™ (DE3) (genotype: F<sup>ompT</sup> hisD<sub>B</sub> (r<sup>B</sup> m<sup>B</sup>) gal dcm rne131 (DE3)) were transformed according to manufacturer’s instructions with sequence verified constructs free of errors and in-frame with the fusion tag. Protein fusions were expressed from BL21 Star™ (DE3) One Shot® cells transformed with the relevant expression construct using the previously described autoinduction approach (Studier, 2005).
5.2.2.2 **Overlap/extension cell-free protein expression**

pET30A/AtDRM1 codon-optimised was used as a template for amplification using the iProof™ Polymerase system (BioRad) as per manufacturer’s instructions using the iProof™ HF buffer, with the addition of 1.9 mM MgCl₂. The PCR program had a 30 s denaturation step at 98°C followed by 30 cycles of denaturation at 98°C for 10 s, primer annealing at 63°C for 10 s and extension at 72°C for 30 s. The final step was an additional 10 min elongation at 72°C. Amplified fragments were analysed on a 1% agarose gel using the 1Kb+ DNA ladder molecular size marker (Invitrogen) and the PCR products were purified using the Zymoclean™ gel DNA recovery kit (Zymo Research) according to manufacturer’s specifications.

The remainder of this work was carried out according to Kralicek et al., 2011.

5.2.3 **Protein stability assay**

Plants were grown in flasks, as previously described (see §3.2.3), for 6 days prior to harvesting and snap freezing in liquid N₂. Frozen tissue was ground to a fine powder in liquid N₂ using mortar and pestle. Using a reverse time-course to allow gel loading at the same time, aliquots of ~100 mg ground tissue were incubated with extraction buffer (100 mM KPO₄ (pH 7.8); 1 mM EDTA; 1% Triton X-100; 10% glycerol; 1 mM DTT) only, 10 µM MG132 (resuspended in protein extraction buffer), protease inhibitor cocktail solution (cOmplete mini EDTA-free; Roche) (1 tablet in 10 mL extraction buffer), or a combination of treatments (10 µM MG132 and protease inhibitor cocktail). Protein was visualised using Western Blots.

5.2.4 **Y2H**

5.2.4.1 **Development of Y2H constructs**

For development of Y2H constructs, wild-type Arabidopsis cDNA was used as a template for amplification using iProof™ High-Fidelity DNA Polymerase (BioRad) according to manufacturer’s instructions using the iProof™ HF buffer, with the addition of 1.9 mM MgCl₂. Amplified fragments were analysed on a 1% agarose gel using the 1Kb+ DNA ladder molecular size marker (Invitrogen) and the PCR products were purified using the Zymoclean™ gel DNA recovery kit (Zymo Research) according to manufacturer’s specifications. The purified PCR product was directionally cloned into
the pENTR™/D-TOPO vector (Invitrogen) according to manufacturer’s instructions. Constructs verified by sequencing were sub-cloned using the Gateway® system (Invitrogen) into the required bait or prey vector.

5.2.4.2 Development of Y2H screening cDNA library
Total RNA was extracted via the Pine Tree method (Chang et al., 1993) and mRNA prepared using the FastTrack™ MAG mRNA isolation kit (Invitrogen). The mRNA was then used to generate the library using the Cloneminer™ II cDNA Library Construction kit (Invitrogen) according to the manufacturer’s protocol.

5.2.4.3 Yeast transformation

Express transformation for candidate screening
Yeast to be transformed (PJ69-4α or PJ69-4a) were grown overnight on rich medium (D-glucose or dextrose 20 g/L, yeast extract 10 g/L, bacto peptone 10 g/L, adenine 20g/L and agar 17g/L, adjusted to pH 6.0). Cells were removed from media using a sterile 10 µL loop, resuspended in water and pelleted by centrifugation at 1000 x g for 1 min. The pellet was washed twice in sterile water, followed by two washes in LiAc/TE solution (LiAc 0.1 M, 100 mM Tris, 10 mM EDTA, pH 8.0). Cells were then resuspended in 250 µL fresh LiAc/TE solution. Twenty-five µL cells were mixed by gentle inversion of the tube with 25 µg Salmon Sperm DNA solution (Invitrogen); 0.3 to 0.5 µg of plasmid and 150 µL of polyethylene glycol (PEG) 40%, LiAc/TE solution. The cell mixture was incubated for 20 min at 30°C, then 15 min at 42°C. Cells were subsequently washed in sterile water, pelleted at 1000 x g for 2 min, resuspended in WO media (Yeast nitrogen base (without aa and without ammonium sulphate) 1.7 g/L, ammonium sulphate 5 g/L, D-glucose or dextrose 20 g/L, adjusted to pH 6.0) and plated on the appropriate selection plate (WO media supplemented with agar 17 g/L, adenine (ADE) 20 mg/L, uracil (URA) 20 mg/L, lysine (LYS) 30 mg/L, methionine (MET) 20 mg/L and either +/- histidine (HIS) 10 mg/L, tryptophan (TRP) 20 mg/L or leucine (LEU) 60 mg/L).

Standard transformation for library screening (for 1mL transformation reaction)
Yeast carrying the bait construct were grown overnight in 25 mL selective liquid media at 28-30°C at 220 rpm. 10 mL of the overnight culture was used to inoculate a further
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Biochemical Analyses

100 mL rich media (D-glucose or dextrose 20 g/L, yeast extract 10 g/L, bacto peptone 10 g/L and adenine 20 g/L. Adjusted to pH 6.0) which was grown in the same conditions until OD_600 reached ~1. The entire culture was pelleted by centrifugation at 1500 x g for 5 min. The pellet was washed twice in water, then twice in LiAc/TE solution, and cells resuspended in 500 µL fresh LiAc/TE solution. Fifty µL cells were mixed by gentle inversion of the tube with 50 µg Salmon Sperm DNA solution (Invitrogen); and 0.3 to 1 µg of plasmid. After incubation of the mixture for 10 min at 30°C, 300 µL of polyethylene glycol (PEG) 40%, LiAc 0.1 M, TE solution was added and the tube inverted to mix. The cell mixture was incubated for 30 min at 30°C, then 20 min at 42°C. Cells were subsequently washed in sterile water, pelleted at 1000 x g for 2 min, resuspended in WO media and plated on the appropriate selection plate.

5.2.4.4 End-point PCR

Platinum® taq DNA Polymerase (Invitrogen) was used according to manufacturer’s instructions with the Y2H cDNA library as a template. Products were visualised on a 1% agarose gel.
5.3 Results

5.3.1 Heterologous expression of AtDRM1

Obtaining large amounts of purified proteins is the first step in many biochemical analyses. For this work the AtDRM1.2/.3 splice isoform coding sequence was used as it was readily detected at the transcript level.

5.3.2 AtDRM1 expression in bacterial system

Bacterial expression systems are regularly used to express high quantities of heterologous proteins. For most aspects of this work the Champion™ pET Expression System (Invitrogen) was used. Expression of the gene of interest is driven by the bacteriophage T7 promoter modified to include a lac operator sequence. This requires the presence of T7 RNA Polymerase, which is provided in the E.coli strain used (BL21 Star™ (DE3) One Shot®, Invitrogen).

5.3.2.1 Overall strategy

IPTG induction of protein expression usually commences upon the E.coli reaching the log phase of replication, requiring constant monitoring of the cell culture replication rate. One approach which eliminates this requirement is the auto-induction system (Studier, 2005), where a combination of glucose and lactose are incorporated into the induction media instead of IPTG, leading to an induction of gene expression for the protein of interest.

The Champion™ pET Expression System (Invitrogen) uses a polyhistidine (His$_6$) tag to facilitate purification of the expressed protein in a metal-chelating system such as a nickel column and detection using commercially available anti-His antibodies. The 3D structure of a protein can induce steric interference which can impact upon folding of the recombinant protein and therefore accessibility of the tag for binding. For this reason, fusion tags can be located at either the N- or C-terminus of the protein of interest. As the structure of AtDRM1 is not known, both versions were constructed. A C-terminal His$_6$-tagged construct was already available in the form of pET30A/AtDRM1$_{codon-optimised}$, an expression construct for AtDRM1 sequence optimised for bacterial codon usage (Figure 5.1) (generated by GenScript; Marion Wood, Plant
and Food Research, Mt Albert). Due to the lack of protein data an additional N-terminal His$_6$ fusion recombinant protein construct was developed.

**Figure 5.1. Sequence alignment of native AtDRM1 and codon-optimised AtDRM1.**

Alignment of coding sequences for native AtDRM1 compared with codon-optimised AtDRM1, with associated translations.

### 5.3.2.2 Expression of AtDRM1 in bacteria

The AtDRM1 CDS sequence was amplified from wild-type *Arabidopsis* cDNA. The PCR product was directionally cloned into the pET200/D-TOPO vector (Invitrogen) and transformed into *E. coli*. Positive clones were identified by PCR on colonies and verified by sequencing.

Sequence verified clones of pET200/AtDRM1, pET30A /AtDRM1$_{\text{codon-optimised}}$ and the pET200/D/LacZ positive control plasmid (provided in the pET200/D-TOPO vector kit, Invitrogen) were transformed into BL21 Star™ (DE3) One Shot® Cells (Invitrogen). Auto-induction of protein expression was undertaken, with cells grown for 67 h and expression assessed by Western Blot of total cell extract using anti-His$_6$ antibodies (Figure 5.2).
Figure 5.2. Western Blot of bacterial expression of AtDRM1.

Western Blot showing the total cell samples (soluble and insoluble combined) for pET200/AtDRM1, pET30A/AtDRM1\textit{codon-optimised} and pET200/D/LacZ (positive control), using the Anti-His\textsubscript{6} antibody for protein detection. Lane 1 represents protein standards, while subsequent lanes show expression reaction products for each of the constructs. Black asterix shows expected size of recombinant AtDRM1 or AtDRM1\textit{codon-optimised}. White asterix indicates LacZ positive control band.

LacZ positive controls undertaken in parallel with either construct both generated the expected product at 121kD indicating the auto-induction protocol was successful. No products in the order of ~12-14kD were detected in either pET200/AtDRM1 or pET30A/AtDRM1\textit{codon-optimised} reactions, signifying that His\textsubscript{6}-tagged AtDRM1 was not expressed in \textit{E. coli}.

The same approach was attempted for six kiwifruit DRM1/ARP homologue proteins with no success (Rae & Wood; data unpublished), suggesting that the bacterial expression system used in combination with His\textsubscript{6}-tags approach was not suitable for the expression of DRM1/ARP recombinant proteins.
5.3.2.3 *Protein expression in cell-free system*

Due to a consistent lack of AtDRM1 protein induction, an alternative approach using a cell-free system was attempted. A PCR-directed cell-free approach has been designed to optimise protein expression using a range of nine fusion tags, allowing quick assessment of the tags, without the usual requirement of development of individual expression constructs for each (Kralicek et al., 2011). Cell-free systems utilise the macromolecular machinery for transcription and translation found in cell extracts, together with added amino acids and energy-rich molecules enabling synthesis of proteins (Katzen et al., 2005). Furthermore, this approach utilises the cell-free systems ability to use PCR-amplified DNA as a template for protein synthesis, allowing rapid screening of tags (1-2 days) in microlitre quantities. A series of overlap/extension PCRs are carried out to generate the cell-free expression template used for cell-free protein synthesis to identify the best fusion construct as judged by production of the greatest amount of soluble protein (approach is described in Figure 5.3). For this work, a gene-specific forward primer (pGR0055) was designed which included the TEV protease site and the N-terminus of the codon-optimised AtDRM1 sequence. The version of the gene sequence optimised for *E.coli* expression (pET30A/AtDRM1, codon-optimised) was used as a template for generation of the TEV-target gene-His$_6$-T$_7$terminator DNA fragment as this type of cell free system is extracted from *E. coli* and use of codon-optimised sequences has been shown to improve its success previously (Chumpolkulwong et al., 2006).
Figure 5.3. Schematic of the overlap/extension PCR approach used for the generation of a panel of fusion tagged AtDRM1 PCR products for cell free protein expression.

The TEV-codon optimised DRM1-His$_6$-T$_7$ terminator DNA fragment is generated using pGR0055 and CF4 primers. A set of two overlap/extension PCRs are then carried out using gel extracted T$_7$ promoter-fusion tag-TEV and TEV-target gene-T$_7$ terminator DNA fragments. The two primer sets (CF1/CF4 and CF3/CF2) generate complementary overhangs which ligate in the cell-free system to produce a circular, vector-like template (Wu et al., 2007). Figure modified from (Kralicek et al.).

This work was undertaken in collaboration with Andrew Kralicek (Mt Albert, Plant and Food Research). PCR generation of the TEV-DRM1-T$_7$ terminator DNA fragment was undertaken by Georgina Rae, while the remainder of the protocol, including overlap/extension PCR and cell-free protein synthesis were undertaken by Andrew Kralicek. Initial screening was carried using small volumes (50 µL), the reaction centrifuged and both the soluble fraction (supernatant) and an entire reaction mixture, including the insoluble protein (reaction) were analysed on a polyacrylamide denaturing gel (Figure 5.4).
Figure 5.4. Coomassie Blue gel of SDS-PAGE analysis of AtDRM1\textsubscript{codon-optimised} -fusion tag panel cell free protein synthesis.

4-12% SDS-PAGE gel showing the completed cell-free expression reactions for the different fusion-tagged AtDRM1\textsubscript{codon-optimised}\textsuperscript{-}His\textsubscript{6} constructs. For both gels, the first lane represents protein standards (Precision Plus Protein\textsuperscript{™} standards (BioRad)), while subsequent lanes show expression reaction products for each of the fusion-tags, including total reaction (R) and supernatant (S). His\textsubscript{6}: 6x histidine; T7tag: N-terminal 16 amino acid residues from the T7 bacteriophage gene 10 protein; GB1: B1 immunoglobulin binding domain of protein G; PpiB: Peptidyl-prolyl cis-trans isomerase B; Trx: Thioredoxin; MBP: Maltose-binding protein; CAT: N-terminal ten amino acid residues of Chloramphenicol acetyltransferase; OMP: outer membrane protein A signal sequence peptide, 21 amino acids; GST: Glutathione-S-transferase; NusA: N-utilisation substance A. Black asterix denotes successful expression products of predicted molecular size.

SDS-PAGE analysis shows that T7tag-, CAT- and OMP-tagged constructs produced bands of sufficient quality to be visualised by Coomassie Blue staining in their reaction mixture (but not their supernatant). All products were approximately 15kD, consistent with the predicted size of the AtDRM1 protein (AtDRM1.2: 14.4kD) plus the tags (T7tag: 2.2kD; CAT: 2.3kD and OMP: 3.1kD). His\textsubscript{6}; GB1-; PpiB-; TRX-; MBP-; GST- and NusA-tagged constructs showed no bands indicative of successful cell-free protein synthesis in either the entire reaction or supernatant lanes.
5.3.2.4 Summary of protein expression attempts for AtDRM1

Overall, a number of approaches for AtDRM1 protein expression were attempted and the results are summarised in Table 5.3.

<table>
<thead>
<tr>
<th>Coding sequence to be expressed</th>
<th>Vector</th>
<th>Tag</th>
<th>Location of tag</th>
<th>Expression system</th>
<th>Protein expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtDRM1</td>
<td>pET200 (Invitrogen)</td>
<td>His₆</td>
<td>N-terminus</td>
<td>E.coli</td>
<td>No</td>
</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
<td>pET30a</td>
<td>His₆</td>
<td>C-terminus</td>
<td>E.coli</td>
<td>No</td>
</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
<td>n/a</td>
<td>His₆</td>
<td>N-terminus</td>
<td>Cell-free</td>
<td>No</td>
</tr>
<tr>
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<td>T7tag</td>
<td>N-terminus</td>
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<td>Yes*</td>
</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
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<td>Gb1</td>
<td>N-terminus</td>
<td>Cell-free</td>
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</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
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<td>PpiB</td>
<td>N-terminus</td>
<td>Cell-free</td>
<td>No</td>
</tr>
<tr>
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<td>Trx</td>
<td>N-terminus</td>
<td>Cell-free</td>
<td>No</td>
</tr>
<tr>
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<td>MBP</td>
<td>N-terminus</td>
<td>Cell-free</td>
<td>No</td>
</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
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<td>N-terminus</td>
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<tr>
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<td>N-terminus</td>
<td>Cell-free</td>
<td>Yes*</td>
</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
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<td>GST</td>
<td>N-terminus</td>
<td>Cell-free</td>
<td>No</td>
</tr>
<tr>
<td>AtDRM1_codon-optimised</td>
<td>n/a</td>
<td>NusA</td>
<td>N-terminus</td>
<td>Cell-free</td>
<td>No</td>
</tr>
</tbody>
</table>

(*') denotes insoluble protein

5.3.3 Antibody development

Antibodies specific to a protein of interest provide a tool enabling a wide range of protein studies of both native and recombinant proteins. Commercially developed antibodies were not available targeting either AtDRM1 or AtDRM2, therefore an attempt to generate antibodies to each protein has been undertaken in this study. The original aim was to use the purified protein to generate antibodies. However, as soluble recombinant protein was not expressed, an alternative approach exploiting synthetic peptides derived from AtDRM1 and AtDRM2 genes was used. A summary of this process is provided in Figure 5.5.
5.3.3.1 Antibody design and generation

Antibodies targeting AtDRM1 and AtDRM2 were designed and generated in collaboration with GL BioChem (Shanghai) Ltd.

To minimise potential peptide sequence errors, the risk of which increases with peptide length, synthetic peptides are generally designed to be 11-15 aa. A number of criteria are followed in order to design peptides to be used for raising antibodies: I) sequences must be sufficiently unique to minimise the risk of non-specific binding; II) the epitope should target a region of the protein it will be able to access, therefore the sequence should be hydrophilic and surface-oriented; III) the risk of targeting an internal region of the protein can be further reduced by targeting terminal sequence (either N-terminus or C-terminus) and IV) the amino acid composition will also affect the success of a
chosen peptide, with certain amino acids affecting solubility, stability and subsequent purification.

In light of these considerations, two peptides were designed (AtDRM1<sub>pep</sub>: QRKEHVALCLVGAWIK; and AtDRM2<sub>pep</sub>: CDWLYSDKTRSHK) targeting AtDRM1 and AtDRM2, respectively (Table 5.4; Figure 5.6).

### Table 5.4. Summary of antibody-binding specificities of AtDRM1 and AtDRM2 splice variant proteins.

<table>
<thead>
<tr>
<th>Gene model</th>
<th>Expected MW</th>
<th>Expected Antibody Binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtDRM1.1</td>
<td>13.41</td>
<td>Possible anti-AtDRM2</td>
</tr>
<tr>
<td>AtDRM1.2/.3</td>
<td>14.40</td>
<td>anti-AtDRM1</td>
</tr>
<tr>
<td>AtDRM1.4</td>
<td>13.81</td>
<td>Possible anti-AtDRM2</td>
</tr>
<tr>
<td>AtDRM1.6</td>
<td>12.12</td>
<td>No binding</td>
</tr>
<tr>
<td>AtDRM2.1</td>
<td>11.55</td>
<td>anti-AtDRM2</td>
</tr>
<tr>
<td>AtDRM2.1</td>
<td>11.66</td>
<td>anti-AtDRM2</td>
</tr>
</tbody>
</table>

Antibodies raised against both AtDRM1<sub>pep</sub> and AtDRM2<sub>pep</sub> are designed to target the C-terminus of the protein. AtDRM1<sub>pep</sub> targets AtDRM1.2/.3 only; while AtDRM2<sub>pep</sub> targets both AtDRM2.1 and AtDRM2.2. Some off-target binding of AtDRM2<sub>pep</sub> to AtDRM1.1 and AtDRM1.4 is possible with 9/14 and 10/14 residues, respectively, conserved with the target region. A cysteine (C) residue has been added to the N-terminus of the AtDRM2<sub>pep</sub> to facilitate conjugation with the carrier protein required for antibody generation.
Figure 5.6. Schematic of AtDRM1 and AtDRM2 proteins showing antibody binding sites.

Protein alignment of AtDRM1 and AtDRM2 splice variants. Peptides synthesised for generating antibodies are represented in turquoise for AtDRM1$_{\text{pep}}$ and red for AtDRM2$_{\text{pep}}$. Yellow bars represent homology of AtDRM2$_{\text{pep}}$ with C-terminal region of two AtDRM1 splice variants (| indicates mismatch).

As peptides on their own are in many instances unable to elicit a sufficient immune response, the synthetic peptides were conjugated to the carrier protein Keyhole Limpet Haemocyanin (KLH). Carrier proteins have an excess of antigenic residues which induce the immune response required for antibody generation.

Immune responses to a given peptide can be variable among individuals, therefore two rabbits were inoculated with each conjugated peptide and sera collected (AtDRM1$_{\text{pep}}$: R1275 and R1276; AtDRM2$_{\text{pep}}$: R1277 and R1278). Polyclonal antibodies were isolated from sera via affinity purification and verified by ELISA. Antibodies were designated anti-AtDRM1 (R1275 and R1276) and anti-AtDRM2 (R1277 and R1278) (generated from inoculation with AtDRM1$_{\text{pep}}$ and AtDRM2$_{\text{pep}}$, respectively).

5.3.3.2 Antibody verification and optimisation

In order to assess whether the antibodies could bind to their target and were sufficiently specific, protein was extracted from wild-type tissue and tissue of Arabidopsis plants...
over-expressing either AtDRM1 or AtDRM2 (Chapter 2), and assessed by Western Blot (Figure 5.7).

![Western Blot showing equal amounts of wild-type; AtDRM1 OX and AtDRM2 OX protein extract challenged with anti-AtDRM1 antibodies from R1275 (A) and R1276 (B); and anti-AtDRM2 antibodies from R1277 (C) and R1278 (D). Lane 1 represents protein standards, while subsequent lanes show total protein extract. Black asterix indicate expression of AtDRM1/AtDRM2. Peptide controls are included for R1276 (B) and R1278 (D).]

**Figure 5.7. Western Blot assessing antibody binding.**

Western Blot showing equal amounts of wild-type; AtDRM1 OX and AtDRM2 OX protein extract challenged with anti-AtDRM1 antibodies from R1275 (A) and R1276 (B); and anti-AtDRM2 antibodies from R1277 (C) and R1278 (D). Lane 1 represents protein standards, while subsequent lanes show total protein extract. Black asterix indicate expression of AtDRM1/AtDRM2. Peptide controls are included for R1276 (B) and R1278 (D).

Some antibody binding is visible for all antibodies, to varying degrees, between 10 and 15 kD as would be indicative of AtDRM1/AtDRM2 protein. Moreover, all samples show binding profiles above 25 kD. As these Western Blot’s were generated via a denaturing gel, it is unlikely that this is targeting multimers of the target protein. Instead, it is likely to be indicative of the antibodies cross-reacting with proteins not related to AtDRM1 or AtDRM2, despite bioinformatic analysis suggesting otherwise.

For R1275 (anti-AtDRM1), this binding is particularly faint suggesting epitope affinity is poor for this antibody. R1275 and R1276 (anti-AtDRM1) exhibit a single band of
antibody binding, suggesting it is specific for AtDRM1.2/3. R1277 and R1278 (anti-AtDRM2) exhibit a doublet of binding, particularly in the AtDRM1 OX sample. This is indicative of the antibody showing the predicted off-target binding of AtDRM1.1/AtDRM1.4 as well as binding AtDRM2. Having loaded equal amounts of protein, all antibodies exhibited fainter binding in the wild-type sample compared with over-expression lines, making over-expression levels of expression required for reliable detection and confirming that AtDRM1 and AtDRM2 over-expression lines are showing over-expression at the levels of both transcript and protein.

Peptide controls were included for R1276 and R1278 to confirm that the antibodies are binding to the target site. Both displayed specific binding to the relevant peptide and corresponding lack of binding to the alternate peptide, further verifying that these antibodies are binding specifically to their target sequence.

R1277 exhibits an additional band between 25-37 kD based upon the protein standards. While this could be indicative of an AtDRM2 doublet being maintained despite the denaturing conditions, it is more likely that this is suggestive of cross-reaction with an unrelated protein, since there is no increase in this unknown protein’s expression in the AtDRM1 and AtDRM2 over-expression lines. Moreover, both AtDRM1 and AtDRM2 lack any cysteine residues, which are integral for disulfide bonds that may result in dimer formation.

Overall, due to the low binding efficiency exhibited by R1275 and R1276, the antibodies raised to AtDRM2pep (R1277 and R1278) were chosen for further work with R1277 arbitrarily chosen for further optimisation (henceforth referred to as anti-AtDRM2).

Binding specificity can be further confirmed using a peptide competition assay. Peptides are incorporated into the primary antibody solution, competing out the available antibodies such that they are unavailable to bind to the target protein on the membrane. Anti-AtDRM2 was subjected to the peptide competition assay (Figure 5.8).
Figure 5.8. Summary of peptide competition assay for antibody R1277.
Western Blots showing anti-AtDRM2 (R1277) antibody binding to AtDRM2 OX protein extract incorporated with varying proportions of the associated target peptide (AtDRM2_{pep}) or a negative control peptide (AtDRM1_{pep}). Lane one of each gel represents the protein standard, while lane 2 represents the protein extract. Two replicates of the experiment were performed and a composite image generated. Black asterix indicates binding of AtDRM2.

Two replicates of this experiment were carried out and exhibited matching profiles across the treatments. Anti-AtDRM2 binding was confirmed when only the antibody was used for protein detection. When an off-target peptide was included, binding was still visible. Inclusion of the target peptide (AtDRM2_{pep}) at either a ratio of 10:1 (antibody:peptide) or 1:1 removed binding. This suggested that the peptide was binding available antibody molecules making them unavailable for binding to proteins on the membrane.

5.3.4 Protein stability

Both AtDRM1 and AtDRM2 predicted proteins include predicted PEST motifs which may be involved in targeting these proteins for proteolytic degradation (Chapter 2; Appendix 7.5). To assess whether these proteins are degraded in vitro, a crude protein sample was extracted from 6-day-old AtDRM2 over-expressing seedlings (Chapter 2) and was incubated with either no proteolytic inhibitors, MG132 which is known to
block degradation of proteins by the proteasome, or with a protease inhibitor cocktail targeting serine-, cysteine- and metallo-proteases, or a combination of treatments across a time-course (Figure 5.9A). Tissue from AtDRM2 over-expression lines was used in order to optimise protein detection in Western Blots, such that any reduction in protein (due to degradation) would be readily detectable. Due to the relatively low binding efficiency of the antibody, at the limits of sensitivity of the Western Blot, these data were particularly faint, although reproducible across three replicates. Therefore a schematic representation of these results is provided below (Figure 5.9B).

A cross-reacting protein 25-37kD provides evidence of consistent loading, acting as an internal control for stable proteins.

**Figure 5.9. Western Blot showing AtDRM2 stability.**

A) Western Blot analysis of AtDRM2 OX total protein extract over 2 h without any protective additives; with MG132; with protease inhibitor targeting serine-, cysteine- and metallo-proteases (protease I); or with both MG132 and the protease cocktail inhibitor (combo). Anti-AtDRM2 was used for the primary challenge. For both Western Blots, lane 1 represents the protein standard. T0 provides a loading control in lane 2 of both gels. Black asterix highlights expected size for AtDRM2. Positive peptide control (AtDRM2_pep) is shown in the final lane (white asterix). B) Schematic representation of reproducible relative binding pattern.

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T₀ provides a baseline of detectable protein in the extract. Indeed, AtDRM2 protein is detectable immediately after extraction (T₀), but is no longer detectable 30 min post-extraction (or thereafter), unless a proteolytic inhibitor is included in the solution (MG132 or protease inhibitor cocktail). One hour post-extraction, all proteolytic inhibitors are preventing protein degradation, but by 2 hours after extraction, only the protease inhibitor cocktail provides sufficient protection. No additive effect is exhibited by combining MG132 and the protease inhibitor cocktail.

5.3.5 Protein:protein interaction studies using yeast-2-hybrids

Initially it was planned to undertake Co-Immunoprecipitation studies to assess protein:protein interactions. Despite exhibiting sufficient specificity, the low binding of these antibodies meant that they were not suitable for such binding studies. Moreover, the inability to reproducibly detect AtDRM1 or AtDRM2 protein from a wild-type extract meant that purification of the protein from crude extracts using these antibodies was not worth pursuing. Therefore an alternative approach was chosen: yeast-2-hybrids (Y2H). A combination of candidate gene and cDNA library assays were undertaken.

5.3.5.1 Candidate binding analysis

Selection of candidate genes

Yeast-2-hybrid assays allow a rapid assessment of direct protein:protein interactions for proteins of interest. As transcriptional expression of AtDRM1 and AtDRM2 has traditionally been associated with periods of dormancy it was of interest to assess whether either protein bound to AtBRC1, an integrator of the MAX/CCD/DAD branching release pathway at the bud level (reviewed in Chapter 1) (Aguilar-Martínez et al., 2007; Finlayson, 2007).

Yeast-2-hybrid library screening in cotton (Gossypium hirsutum) using Gh14-3-3h as a bait led to the identification of a homologue of AtDRM1 as a potential interactor (Zhang et al., 2010). However, the clone retrieved was a partial cDNA and was not further analysed by Zhang and collaborators. 14-3-3 proteins are associated with defects in light sensing and response (Mayfield et al., 2007) therefore it was an interesting candidate to test. The Arabidopsis homologue of Gh14-3-3h (At14-3-3µ; At2g42950) was used as a candidate for AtDRM1 and AtDRM2 binding studies.
Development of constructs for candidate screening

Candidate studies involve development of bait and prey constructs for each interaction being assessed, as well as the opposite combination of the pair for verification purposes. Complementary vectors carrying candidate proteins bound to either the activation domain of GAL4 (AD), or the binding domain of GAL4 (BD), are constructed. Yeast are then generated to express both constructs. If the two proteins interact the AD and BD forms a functional GAL4 transcription factor which activates a HIS3 reporter gene, coding for IMIDAZOLE GLYCEROL PHOSPHATE DEHYDROGENASE, a histidine biosynthesis enzyme.

As none of the constructs to be used for this study were already available, constructs were designed and generated. Vectors pBD2 and pAD2 (Maier et al.) were used as bait and prey constructs, respectively, utilising the Gateway® cloning system (Table 5.5). The coding sequence of AtDRM1.2/3; AtDRM2.1; AtBRC1 (At3g18550); and At14-3-3µ (At2g42590) were amplified using gene-specific primers (Appendix 7.17) and cloned into the entry vector pENTR™/D-TOPO (Invitrogen). Upon verification these constructs were sub-cloned into both pBD2 and pAD2.

The Y2H vector expression the C-terminal section of AtBRC1 fused to the BD (pGBTK7/AtBRC1-TERC) was provided by Florian Chevalier (Centro Nacional de Biotechnologia; CSIC; Spain) (see following candidate screening section).

The different constructs and empty vectors (negative controls) were transformed into the PJ69-4 yeast strain (bait: PJ69-4α strain; prey: PJ69-4a strain) and plated on the appropriate selection media (Table 5.5).

<table>
<thead>
<tr>
<th>Bait / Prey</th>
<th>Vector</th>
<th>Selection</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bait</td>
<td>pBD2</td>
<td>auxotrophic to TRP (-TRP)</td>
<td>(Maier et al.)</td>
</tr>
<tr>
<td>Prey</td>
<td>pAD2</td>
<td>auxotrophic to LEU (-LEU)</td>
<td>(Maier et al.)</td>
</tr>
<tr>
<td>Bait</td>
<td>pGBTK7</td>
<td>auxotrophic to TRP (-TRP)</td>
<td>Clontech</td>
</tr>
</tbody>
</table>
Candidate screening

The interaction status of the proteins of interest (AtDRM1 and AtDRM2) and the candidates (AtBRC1 and At14-3-3µ), as well as homodimerisation, were assessed in both orientations of bait and prey constructs. The general experimental approach used for candidate screening is summarised in Figure 5.10.

![Figure 5.10. Summary of Y2H candidate screening approach.](image)

Transformation of haploid yeast:
- pBD constructs α-mating type; pAD constructs a-mating type

Generate diploid strains for each combination to be tested on rich medium

Select diploids on -TRP, -LEU medium

Perform the Y2H assay by plating diploids on different medium:
- Positive control for diploid yeast growth (-TRP, -LEU)
- Interactions test (-TRP, -LEU, -HIS)
- Auto-activation/strength of interaction test (-TRP, -LEU, -HIS with 3AT titration)

To test interaction between two proteins, α-mating type and a-mating type yeast containing the bait and prey, respectively were mated on rich medium. Diploid yeast were selected on -TRP, -LEU medium. An indication of positive protein: protein interaction is demonstrated by positive growth of diploid yeast on plates lacking histidine (-HIS).

In some instances, basal expression of the HIS3 reporter gene is observed and can be minimised by inclusion of 3-Amino-1,2,4-Triazole (3AT) in the media (Durfee et al., 1993; Kishore and Shah, 1988). All constructs were assessed for auto-activation by
undertaking an assay with the relevant empty vector control and plating on a 3AT range from 0 mM 3AT to 100 mM 3AT. All detected auto-activation could be removed with 3AT except that of pBD2/AtBRC1 (Figure 5.11). pAD2/AtDRM1 and pBD2/AtDRM2 exhibited auto-activation which could be removed with inclusion of 5 mM and 50 mM 3AT in the media, respectively (Appendix 7.18).

Figure 5.11. Auto-activation of AtBRC1 bait constructs.

Diploid yeast of AtBRC1 bait constructs (pBD2/AtBRC1 on left; pGBT7/AtBRC1-TERC on right) mated with the empty prey construct (empty pAD2) on a 3AT titration.

Auto-activation of pBD2/AtBRC1 could not be removed with 3AT. An AtBRC1 bait deletion construct was provided by Florian Chevalier (Centro Nacional de Biotechnologia; CSIC; Spain) with the entire N-terminal sequence, including the TCP transcription factor domain, removed (pGBT7/AtBRC1-TERC). This construct no longer exhibited auto-activation and could therefore be used for subsequent binding studies (Figure 5.11).
Having assessed the auto-activation status of all the constructs, a positive control series from the ProQuest™ Two-Hybrid System (Invitrogen) was also used. Known strong- (pEXP™22/RalGDS-wt), weak- (pEXP™22/RalGDS-m1) and not detectable- (pEXP™22/RalGDS-m2) interacting partners were mated with pEXP™32/Krev1 and the resulting diploid yeast included on all candidate screening plates (Figure 5.12).

**Figure 5.12. Control diploid yeast over a 3AT titration.**

Diploid yeast of strong- (pEXP™22/RalGDS-wt:pEXP™32/Krev1); weak- (pEXP™22/RalGDS-m1: pEXP™32/Krev1); and not detectable- (pEXP™22/RalGDS-m2: pEXP™32/Krev1) interactions on a 3AT titration.

Findings from binding studies of AtDRM1 and AtDRM2 with the candidate interaction partners AtBRC1 and At14-3-3μ are summarised in Table 5.6.
Table 5.6. Summary of Y2H candidate screen.

Grey shading indicates the combination was not assessed.

<table>
<thead>
<tr>
<th>Prey (pAD2)</th>
<th>Bait (pBD2/pGBKT7)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AtDRM1</td>
</tr>
<tr>
<td>AtDRM1</td>
<td>No binding</td>
</tr>
<tr>
<td>AtDRM2</td>
<td>No binding</td>
</tr>
<tr>
<td>At14-3-3µ</td>
<td>No binding</td>
</tr>
<tr>
<td>AtBRC1</td>
<td>No binding</td>
</tr>
</tbody>
</table>

No interactions were detected between either AtDRM1 or AtDRM2 and the candidate proteins. Furthermore, no homodimerisation was detected for either protein.

5.3.5.2 Y2H library screening

As no interactions were identified using the candidate Y2H approach, a Y2H library screen was carried out to provide a less-targeted approach. Instead of testing known proteins, a bait construct is challenged with a library of prey constructs generated from a cDNA library. A cDNA library was selected which was anticipated to include AtDRM1 based on transcriptional data (Chapter 2; Figure 2.4) and therefore it was also likely to also include binding partners.

AtDRM1.2/.3 was selected for use as bait in the Y2H library screen. A summary of the library screening approach is described in Figure 5.13.
**Development of DRM1 pDEST32 bait construct and Arabidopsis cDNA prey library**

The pENTR™/D-TOPO/AtDRM1.2/3 clone generated for Y2H candidate assays (confirmed by sequencing) was used for sub-cloning into the bait (binding domain) construct vector pDEST32, using the Gateway system (Invitrogen). Having verified the construct via restriction digest analysis, it was subsequently transformed into the PJ69-4α yeast strain.

The *Arabidopsis* cDNA prey library was kindly donated by Wei Cui (Mt Albert, Plant and Food Research). The library was generated from 3 week old seedling tissue. To verify that an interacting partner is likely to be present in a prey library, confirmation that the bait protein was present was necessary. An end-point PCR amplifying *AtDRM1*
(pGR0001 and pGR0003) and AtDRM2 (pGR0004 and pGR0006) was carried out (Figure 5.14).

![Agarose gel showing the products of an end-point PCR for detection of AtDRM1 CDS (left) and AtDRM2 CDS (right) in the AtcDNA library used for Y2H library screening. Ladders are the 1 KB Plus DNA ladder (Invitrogen). AtDRM1 +ve and AtDRM2 +ve are AtDRM1 and AtDRM2 in pENTR™-D/TOPO, respectively. Water samples represent the negative PCR control.](image)

**Figure 5.14. PCR confirmation of bait protein in cDNA library.**

Both AtDRM1 and AtDRM2 were detected in the cDNA library, suggesting that potential interacting partners may also be present.

**Auto-activation test and transformation optimisation**

In order to verify that pDEST32/AtDRM1 did not auto-activate, and to minimise background histidine production which might obscure weak interactions, a diploid yeast expressing both pDEST32/AtDRM1 and an empty prey construct (pDEST22; Invitrogen; activation domain) was generated. Resulting diploid yeast were selected by growth on -TRP, -LEU plates. An individual colony was then replicated on to plates also lacking histidine, supplemented with varying concentrations of 3AT (Figure 5.15).
Figure 5.15. Assessment of self-activation and determination of 3AT concentration required to titrate basal levels of \textit{HIS3} expression for pDEST32/AtDRM1.

Diploid yeast carrying AtDRM1 pDEST32 and empty pDEST22 plated on -LEU, -TRP (positive growth control) and -LEU, -TRP, -HIS with 0 mM 3AT; 2 mM 3AT; 5 mM 3AT; 10 mM 3AT; 25 mM 3AT; or 50 mM 3AT.

Yeast grew on plates lacking leucine and tryptophan, confirming that the yeast were diploid, carrying both pDEST32/AtDRM1 and empty pDEST22. No growth was detected on plates lacking leucine, tryptophan, and histidine, as well as those plates supplemented with any concentration of 3AT, showing that pDEST32/AtDRM1 induced no background expression of \textit{HIS3}. As a result, subsequent interaction studies were completed on -LEU, -TRP, -HIS plates without the requirement of 3AT.

The amount of prey cDNA library DNA used to transform bait can significantly impact upon the number of transformation events occurring and therefore the number of possible interactions available for screening. Yeast containing pDEST32/AtDRM1 were transformed with either 0.5 µg; 1 µg; 2 µg; 5 µg; or 10 µg of the cDNA prey library using the standard yeast transformation protocol, plated on -TRP, -LEU plates and the transformation efficiency calculated (Table 5.7).
Table 5.7. Yeast transformation efficiency analysis.

<table>
<thead>
<tr>
<th>Amount of cDNA library (µg) used to generate a 1 mL transformation product</th>
<th>Number of colonies</th>
<th>Transformation efficiency (transformants/µg)</th>
<th>Predicted number of colonies for 60 mL screen transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 µl plated</td>
<td>200 µl plated</td>
<td>300 µl plated</td>
<td>Total plated (600 µl)</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>235</td>
<td>558</td>
</tr>
<tr>
<td>2</td>
<td>166</td>
<td>183</td>
<td>315</td>
</tr>
<tr>
<td>5</td>
<td>37</td>
<td>464</td>
<td>730</td>
</tr>
<tr>
<td>10</td>
<td>301</td>
<td>956</td>
<td>1651</td>
</tr>
</tbody>
</table>

Transformation of the yeast with 10 µg of library cDNA produced the greatest predicted number of colonies for the eventual 60 mL transformation required for the actual Y2H library screen. The efficiency of this transformation was highest with 1 µg DNA used for the transformation. As there is a trade-off between the gains associated with using large amounts of DNA and the efficiency, an intermediate value of 1.5 µg of DNA per 1 mL transformation product was selected for the Y2H library screen.

**Y2H library screen**

pDEST32/AtDRM1 containing yeast in the exponential growth phase were transformed with the *Arabidopsis* cDNA library using the standard yeast transformation protocol, generating ~60 mL of transformation product. The entire transformation product was plated on -TRP, -LEU, -HIS plates and incubated at 30ºC for 1 week.

The transformation efficiency of this experiment was 821.6 transformants/µg with the total number of colonies estimated at 50 000.

Over the course of 7 days, 361 individual colonies were selected as they appeared and transferred to -TRP, -LEU, -HIS plates. By week one of incubation, all plates showed
significant background of small yeast-like growth making further selection of positives difficult. As subsequent verification of interactions was going to be undertaken, an inclusive approach was taken with all possible positive colonies selected.

Twenty colonies were still viable after 2 days growth on -TRP, -LEU, -HIS plates at 30°C, including the control diploid yeast from candidate screening which had been included on every plate. The 20 candidates were replica plated on -TRP, -LEU, -HIS plates with a 3AT titration (as well as on -TRP, -LEU to confirm yeast viability) with the results displayed in Table 5.8.

Table 5.8. Summary of Y2H library screen.

Growth is represented by a shaded grey box. No binding is represented by a white box. The predicted *Arabidopsis* protein sequence was determined using the resulting sequence as the query sequence for a TBLASTn search of the *Arabidopsis* database.

<table>
<thead>
<tr>
<th>Candidate #</th>
<th>Growth detected on -TRP, -LEU (control)</th>
<th>Growth detected on -TRP, -LEU, -HIS</th>
<th>Predicted <em>Arabidopsis</em> protein</th>
<th>In-frame?</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>none</td>
<td>At3g12320</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>7</td>
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<td>none</td>
<td>none</td>
<td></td>
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<td>none</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>none</td>
<td>At4g01150</td>
<td>no</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>At1g20340</td>
<td>yes</td>
<td>At1g77450</td>
<td>yes</td>
</tr>
<tr>
<td>13</td>
<td>At1g77450</td>
<td>yes</td>
<td>At2g34590</td>
<td>no</td>
</tr>
<tr>
<td>14</td>
<td>none</td>
<td>none</td>
<td>none</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>At1g44575</td>
<td>no</td>
<td>At1g44575</td>
<td>no</td>
</tr>
<tr>
<td>16</td>
<td>none</td>
<td>none</td>
<td>At1g44575</td>
<td>no</td>
</tr>
<tr>
<td>17</td>
<td>none</td>
<td>none</td>
<td>At1g44575</td>
<td>no</td>
</tr>
<tr>
<td>18</td>
<td>At1g44575</td>
<td>no</td>
<td>At1g44575</td>
<td>no</td>
</tr>
<tr>
<td>19</td>
<td>At4g35090</td>
<td>no</td>
<td>At4g35090</td>
<td>no</td>
</tr>
<tr>
<td>20</td>
<td>At3g11100</td>
<td>yes</td>
<td>At3g11100</td>
<td>yes</td>
</tr>
</tbody>
</table>
As all candidates exhibited growth on some level of 3AT, indicative of a possible interaction, all were subjected to a yeast colony PCR and the product sequenced. The sequence was used for a TBLASTn search of the Arabidopsis database to identify the putative binding partners. 8/20 candidates contained Arabidopsis sequences (Table 5.8). For a possible interaction to be valid, the construct must be in-frame. As this approach searches using the nucleotide sequence not the protein sequence, it was then necessary to verify that the construct was in-frame with the activation domain. Three candidates (12, 13 and 20) contained in-frame proteins, At1g20340 (DNA-damage-repair/toleration protein 112; DRT112), At1g77450 (NAC domain containing protein 32; ANAC032) and At3g11100 (MYB4 transcription factor), respectively.

In order to validate the interaction between pDEST32/AtDRM1 and these three proteins, the constructs were transformed back into yeast using the same approach as used for the candidate screening with the interaction controls run in parallel. All controls exhibited the expected profile as described in Figure 5.12. Diploid yeast grew on -TRP, -LEU plates indicating that the yeast were viable. However, no binding with AtDRM1 was detected for any of the candidates, as indicated by no growth on -TRP, -LEU, -HIS (Table 5.9).

<table>
<thead>
<tr>
<th>Prey (pDEST22)</th>
<th>Bait (pDEST32/AtDRM1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRT112 (At1g20340)</td>
<td>No binding</td>
</tr>
<tr>
<td>ANAC032 (At1g77450)</td>
<td>No binding</td>
</tr>
<tr>
<td>MYB4 TF (At3g11100)</td>
<td>No binding</td>
</tr>
</tbody>
</table>

Table 5.9. Summary of Y2H library screen candidate validation.
Chapter 5
Biochemical Analyses

5.4 Discussion

5.4.1 Viability of antibodies targeting AtDRM1 and AtDRM2

Antibodies are a tool required for a range of biochemical analyses. As soluble protein could not be expressed for antibody generation, using the various approaches presented, synthetic peptides were designed and used as an alternative inoculum. Peptide design for raising antibodies requires a number of considerations including that the target protein sequence is not hydrophobic. The amino acid residues constituting the central variable region of AtDRM1 and AtDRM2 bear little homology making them appealing targets for peptide design. However, they are composed of residues considered too hydrophobic, suggesting steric interference could become an issue with target residues likely to be buried in the folded protein and therefore unavailable for antibody binding. Furthermore, hydrophobic residues provide challenges for peptide synthesis; therefore, this region could not be targeted.

The C-terminal target sequences are composed of suitable amino acid residues for peptide synthesis and antibody targeting. However, some off target binding is expected with these sequences. AtDRM2_{pep} is likely to have some off-target binding to AtDRM5. Since this family member has only been detected at the transcriptional level in floral tissue (Chapter 2, Figure 2.4), cross-reactivity is unlikely to be an issue in other tissues being assessed. In addition, antibodies generated to AtDRM2_{pep} could exhibit cross-reactivity with AtDRM1.1 and AtDRM1.4 making the anti-AtDRM2 a slightly less targeted antibody than was originally desired.

Binding of anti-AtDRM1 and anti-AtDRM2 was confirmed with both plant protein extracts and peptide controls. Cross-reactivity of anti-AtDRM2 was assessed using a peptide competition assay. Overall, the antibodies exhibited reasonable specificity. However, their binding affinity was relatively poor, with reliable detection only associated with extracts from plants over-expressing the target protein and not in wild-type samples. This made these antibodies unsuitable to use for comparative analyses of protein expression and sub-optimal for pull-down assays (binding studies) or protein purification. The unavailability of antibodies as a resource for future work provides a major limitation for biochemical studies in the future. Therefore, upon expression or
isolation of sufficient quantities of DRM1/ARP protein, further attempts to generate antibodies using the native protein will likely be advantageous.

5.4.2 Challenges relating to the intrinsically disordered nature of AtDRM1 and AtDRM2

Intrinsically disordered proteins are defined by the lack of tertiary structure which they exhibit (Dunker et al., 2005; Dunker et al., 2001; Dunker et al., 2008; Dyson and Wright, 2005; Tompa, 2002; Uversky, 2002; Uversky and Dunker, 2010; Uversky et al., 2000; Uversky et al., 2005; Wright and Dyson, 1999), the same tertiary structure which characteristically provides proteins with protection from degradation by proteases and proteasomes (Tsvetkov et al., 2008). DRM1/ARP family members, including Arabidopsis members (Chapter 2 and Chapter 3) are predicted to be intrinsically disordered (Wood et al., 2013). In addition, predicted PEST motifs, targeting proteins for proteolytic degradation, were present in both AtDRM1 and AtDRM2, including all splice variants except AtDRM1.6, suggesting that the resulting protein of AtDRM1.6 may be more stable than other family member proteins (Chapter 3). This is supported by evidence that within 30 min of protein extraction, AtDRM2 was no longer detectable on a Western Blot. The AtDRM2 protein was protected from degradation by MG132, a proteasome inhibitor, and a protease inhibitor cocktail targeting targeting serine-, cysteine- and metallo-proteases, making AtDRM2 susceptible to degradation via both proteolytic pathways. This raises obvious challenges for working with the protein in the laboratory as while either proteolytic cleavage inhibitor provided some protection, by 2 h after extraction, the protein was barely detectable. Upon extraction, samples need to be analysed or stored at -80°C immediately. Therefore, the inability to generate soluble protein thus far could instead reflect an inability to detect the protein prior to its in vitro degradation.

It is appealing to associate the failure to express His6-tagged AtDRM1 in the bacterial system with the intrinsically disordered nature of the protein. Indeed, in addition to issues relating to protein stability, many examples exist of increased susceptibility of IDPs to protein degradation (reviewed in Suskiewicz et al., 2011), particularly during over-expression in bacteria and subsequent purification (Paz et al., 2010) including, BH3-only proteins (Hinds et al., 2007) and NEUROLIGIN 3 (Paz et al., 2008). By contrast, examples also exist of successful expression of IDPs in bacterial systems,
including the intrinsically disordered N-terminal domain of the DELLA proteins from *Arabidopsis* (Sun et al., 2010), suggesting there is not a universal method for expressing IDPs but rather the strategy needs to be chosen on a case-by-case basis.

### 5.4.3 Protein expression strategies

A number of factors need to be considered when designing a protein expression strategy, including which expression vector is used and therefore the fusion tag and transcriptional promoter used (Higgins, 1999), as well as arguably the most important factor – codon usage (Lithwick and Margalit, 2003). The codon usage of a given protein can greatly impact the success of attempts to express a protein in a heterologous system, such as bacteria (reviewed in Gustafsson et al., 2004). If a codon is rarely used in a system, the likelihood that a heterologous protein is expressed is reduced (Kane, 1995). Therefore, these rare codons can be replaced, without modifying the resulting amino acid composition, with codons more suitable for the given host. In this study, both a codon-optimised AtDRM1 construct (pET30A/AtDRM1\textsubscript{codon-optimised}) and native AtDRM1 construct (pET200/AtDRM1) were used for expressing the His\textsubscript{6}-tagged (C- and N-terminus tagged, respectively) protein in bacteria, with no success. Indeed this was also the case when attempting to express both codon-optimised and native kiwifruit DRM1/ARP family members in the bacterial system (data not shown). Therefore, codon usage was not a contributing factor to the failure of expression of the protein in this attempt.

Both *Arabidopsis* and kiwifruit approaches used either N- or C-terminal His\textsubscript{6}-tag fusions. Due to the high affinity of the amino acid histidine (HIS) with immobilised metal ion matrices (e.g. nickel column), His\textsubscript{6}-tags are a commonly used approach in protein expression and purification studies (reviewed in Terpe, 2003). His\textsubscript{6}-tags did not facilitate the expression of the DRM1/ARP proteins from either species. These constructs all included the N-terminal start methionine which can lead to a reduction in the amount of detectable protein as the promoter will drive expression from any start site immediately downstream of it, therefore AtDRM1 protein could be being expressed without the His\textsubscript{6}-tag required for detection and purification. It is unlikely that this would lead to no detectable expression, rather only a reduction would be seen. As no expression was detectable, a different tag needed to be assessed. A number of tags are available with different advantages and disadvantages (reviewed in Terpe, 2003).
Considerations such as a tag’s ability to protect the associated protein from degradation, increase protein solubility and increase yield are all important.

A PCR-directed bacteria-based cell-free expression screen was undertaken using N-terminal fusions with the AtDRM1<sub>codon-optimised</sub> sequence. This allowed screening of ten tags, including His<sub>6</sub> as a negative control in vitro. Expression was detected associated with each of three relatively small tags (2.2 – 3.1 kD) – the N-terminal 16 amino acid residues from the T7 BACTERIOPHAGE GENE 10 protein (T7tag), the N-terminal ten amino acid residues of CHLORAMPHENICOL ACETYL-TRANSFEERASE (CAT), and the OUTER MEMBRANE PROTEIN A signal sequence peptide (Omp). Despite successful expression, the product was restricted to the insoluble phase. None of the large fusion partners which often have the advantage of often increasing the solubility of the associated protein (Terpe, 2003), such as Maltose Binding Protein (40 kD), facilitated expression of AtDRM1.

The cell-free system used in this work is based on cell extracts from E.coli (Kralicek et al., 2011) which may not provide the additional components required for synthesis of soluble AtDRM1 protein. Future work should consider other expression systems such as the wheat germ-based cell free system which may provide required chaperones not found in the bacterial system preventing aggregation of expressed DRM1/ARP family member proteins. Indeed the wheat germ system uses extracts from dormant wheat seeds, providing an increased likelihood of interacting proteins being present since AtDRM1/AtDRM2 expression is high in dormant seeds (Schmid et al., 2005). Similarly, over-expressing a tagged DRM1/ARP protein in planta and extracting and purifying the resulting protein may provide the required components for generation of soluble protein. Enrichment of non-tagged DRM1/ARP protein from plant extracts may also be a viable approach due to the low mass of the protein, although the poor binding efficiency demonstrated by the available antibodies, may compromise this approach.

Alternatively, future efforts could focus on solubilising the insoluble proteins generated by the cell-free system. Common approaches include dissolving in SDS or urea. However, evidence also exists for buffer-insoluble proteins, particularly disordered proteins, being solubilised in water (Li et al., 2006). These approaches could enable solubilisation of the expressed protein and therefore facilitate further studies such as structural studies and generation of further antibodies.
The inability to express and isolate soluble protein through the course of this work means that structural characterisation by circular dichroism or nuclear magnetic resonance (NMR) spectroscopy of this protein has not been completed, leaving experimental structural characterisation of AtDRM1/ARP family members and confirmation of IDP status incomplete.

5.4.4 Possible importance of post-translational modifications for AtDRM1 and AtDRM2 function

Many IDPs are designated hub proteins through their ability to bind multiple partners (Dosztányi et al., 2006; Dunker et al., 2005; Ekman et al., 2006; Haynes et al., 2006; Patil and Nakamura, 2006; Singh and Dash, 2007; Singh et al., 2007; Uversky et al., 2005), making it of particular interest to identify any binding partners of AtDRM1. As AtDRM1 had been proposed to act upstream of the branching signal integrator AtBRC1 based on the finding that AtDRM1 transcript levels were unaffected in the buds of the highly branched Atbrc1 mutant (Finlayson, 2007) it was also a possibility that the proteins may interact with each other. That no interaction was detected between these proteins despite several attempts provides support for the alternative hypothesis that AtDRM1 is not acting upstream of AtBRC1, but rather these proteins are involved in distinct pathways. Furthermore, no interaction was detected between AtDRM1 and At14-3-3µ, as had previously been described in cotton (Zhang et al., 2010). Of note is that the originally interaction in cotton involved only a partial DRM1/ARP fragment and no reciprocal binding assessments were undertaken for this finding. This study used a full length, more biologically relevant, AtDRM1 clone and assessed the interaction with bait and prey constructs in either direction, providing strong evidence that the original finding in cotton may be a false positive.

Despite these results, it was highly likely that AtDRM1 interacts with other proteins and Y2H library screen was undertaken to identify further candidates for testing. This library screen returned no verifiable interactors. The presence of both AtDRM1 and AtDRM2 in the cDNA library was confirmed, making it highly likely that any interacting partners were also present in the cDNA library. Despite this, it is still possible that binding partners were not present in the library. In addition, the transformation efficiency for the library screen was particularly low even after several attempts at optimisation, meaning not all transcripts in the library were necessarily
transformed, therefore the entire library may not have been an accurate representation of the mRNA population of seedling tissue.

Indeed false positives are a common issue in Y2H studies (Serebriiskii et al., 2000). Three in-frame candidates emerged from the Y2H library screen in the form of DRT112 (At1g20340), ANAC032 (At1g77450) and a relatively uncharacterised MYB transcription factor (At3g11100). Both DRT112 and ANAC032 were particularly attractive candidates due to associations with ROS pathways in Arabidopsis (Desikan et al., 2000) and stress response and developmental defects (Kleinow et al., 2009), respectively. Regardless of the desirability of these potential interactors, multiple attempts to replicate the original interaction designated them false positives also. This further emphasises the need for Y2H library screens to be followed by thorough candidate verification by back-transforming into yeast for further Y2H studies as well as alternative techniques such as affinity purification before drawing conclusions on any biological relevance of the binding.

High levels of background colonies were detected on the library screening plates. 3AT is usually included in the media to prevent background. However, previous assays had shown that background growth with pDEST32/AtDRM1 was not an issue and therefore 3AT was not required. This resulted in some putative positive interactions being disguised in the background and therefore not selected for further analysis, a compounding source of reduced return of possible interactors.

Regardless of any issues associated with library screen transformation and possible background, technically some interactions could still have been detected unless the interactions require some additional binding partner which was not present or post-translational modification of the proteins which was not undertaken in the yeast system. A common example of this is that phosphorylation may be required for protein:protein interactions. As AtDRM2 is reported in experiment-based phosphorylation protein databases and AtDRM1 has predicted sites of phosphorylation (Chapter 2), it is possible that interactions involving these proteins may be phosphorylation-dependent, as is the case for interactions with plant receptor-like protein kinase (RLK)’s (Stone et al., 1994). Having proteins with the required post-translational phosphorylation modification, which is not provided in a heterologous yeast system, is critical to these studies. To circumvent this, an additional kinase can be co-expressed in the Y2H system. However,
this relies on previously determined data highlighting the exact kinase. This information has not been determined for DRM1/ARP gene family members.

A number of alternative strategies to Y2H exist for identification of protein interaction partners. Affinity purification is an approach mentioned earlier. However, the lack of high efficiency binding affinity with the available antibodies generated within the scope of this study, means that plants carrying a tagged-AtDRM1/ARP would need to be generated. Instead, bimolecular fluorescent complementation (BiFC) could be used. As it is undertaken in cell culture, *Arabidopsis* cells would be used, possibly providing the required kinase for phosphorylation of AtDRM1/ARP for interactions to occur.
5.5 Conclusion

Biochemical analysis of AtDRM1 and AtDRM2 is made difficult due to the lack of high quality antibodies with sufficient binding affinity, coupled with the rapid \textit{in vitro} degradation of the protein. With these considerations, Y2H became the most viable approach for binding studies given the available time and resources. However, the Y2H technology has its own weaknesses, namely that it is undertaken in a heterologous system which may not provide the required chaperones and/or post-translational modifications, such as phosphorylation, for protein binding, as well as its high rate of false positives.

Thorough attempts at expression of His6-tagged AtDRM1 in bacteria showed that this method is not suitable for the generation of DRM1/ARP recombinant proteins from \textit{Arabidopsis} and kiwifruit. By contrast, the cell-free system showed that small fusion tags such as CAT, OMP and NusA are unexpectedly more suitable for this protein. Future work will consider the wheat germ cell-free system using these smaller tags to express the AtDRM1 protein.

The lack of binding partners elucidated from the Y2H studies suggests that post-translational modifications may be critical to interactions involving AtDRM1 and/or AtDRM2. Further characterisation of the post-translational modifications of these proteins will provide insight into the mode of the action of these proteins.
6 Discussion and Conclusions

Plant survival in a plethora of environmental conditions is mediated by their ability to respond quickly, by carefully regulating their development. Dormancy release is one developmental phase which impacts upon a plant’s architecture to facilitate survival in their given conditions. To improve our understanding of this process at the molecular and physiological levels molecular markers can be used. Historically, the association between DRM1/ARP transcript level increases and dormancy in meristematic tissues made this family a useful tool as a molecular marker for dormancy in plants. However, its biological function remains unknown. In an attempt to improve the understanding of the in planta role of Arabidopsis dormancy-associated genes, AtDRM1 and AtDRM2, further investigations were undertaken in this PhD.

This research contributes to our understanding of the entire Arabidopsis DRM1/ARP family, with a particular focus on AtDRM1 and AtDRM2. Transcriptional studies undertaken across plant development as well as in response to various treatments provide insight into the possibly diverse roles engaged in by family members. A specific role in temporary growth arrest emerged with studies of mutant plants either over-expressing or down-regulating either gene. Biochemical analyses were also undertaken and will form the basis of future work.

This chapter begins with a consideration of how these data align with the traditional focuses of research for this gene family. This is followed by a discussion on the emerging hypothesised function of AtDRM1 and AtDRM2 and the challenges associated with studying these genes and their resulting proteins. Finally, suggestions for future work based on these PhD findings will be presented.

6.1 Moving on from historical themes in DRM1/ARP research

Historically, two primary themes emerge from the literature around DRM1/ARP family members: a putative repression by auxin and use as a marker for dormant tissues.

Firstly, DRM1/ARP has been described as repressed by auxin, hence many family members having the name ‘auxin-repressed protein’. In this study, AtDRM1 and
AtDRM2 were repressed by IAA after 6 h treatment, except for one splice variant, AtDRM1.6, which was induced (Chapter 3; Figure 3.4). While in previous work in Arabidopsis (Rae, 2009) both induction and repression of AtDRM1 and AtDRM2 transcripts was exhibited in Arabidopsis seedling tissue over a time-course. In general, conflicting evidence for this finding has emerged with assays undertaken on different tissues, at different times and with different auxin analogues at a wide range of concentrations, making it particularly difficult to tease apart the discrepancies in transcriptional responses. It is noteworthy though that auxin has opposing effects on growth in the different tissues of a plant. For example, in apical dominance auxin and subsequent downstream signals inhibit growth by preventing the initiation of axillary bud outgrowth. Conversely, auxin becomes necessary for the stem to grow upon dormancy release of the axillary bud. Similarly, in expanding leaves, auxin promotes growth. This seemingly opposite difference provides a warning that consistency and focus in experimental design is going to be imperative for identifying a role, if any, for auxin in regulating DRM1/ARP family members. Overall however, no trends in the transcriptional profiles of family members in response to auxin treatment are evident, suggesting that DRM1/ARP family members are not directly regulated by auxin.

The second major historical focus of DRM1/ARP research has been their routine use as molecular markers for dormancy in meristic tissues. Since DRM1/ARP transcript was shown to decrease upon apical bud decapitation in pea in 1998 (Stafstrom et al.), similar results have been shown in Arabidopsis in response to decapitation (Chapter 2; Figure 2.4B; Tatematsu et al., 2005), as well as in kiwifruit in response to dormancy release via application of HC (Wood et al., 2013). Moreover, DRM1/ARP transcript has been used as a marker in mutant lines with reduced or increased bud outgrowth in a variety of species. While it has proven suitable for studies of the branching mutants phyb, axr1-12, max2 and tin; no change was evident in buds of the branching mutants brc1 or 35S:YUCCA (reviewed in Chapter 1). This suggests either that AtBRC1 is involved in a DRM1/ARP independent branching response; or that DRM1/ARP is not directly involved in specific meristematic tissue outgrowth at all. In support of both hypotheses, preliminary results from crosses indicate that over-expression of AtDRM1 cannot suppress the increased branching brc1 phenotype (data not shown) and no interaction between either AtDRM1, or AtDRM2 and AtBRC1 proteins was shown during this work (Chapter 5; Table 5.6). These data suggest that DRM1/ARP family
members are not involved in dormancy specifically, but rather the presence or lack of their transcripts is associated with a concomitant growth response in the particular tissue.

6.2 Complexity of the DRM1/ARP family

A robust and thorough bioinformatic analysis has been undertaken of the Arabidopsis DRM1/ARP family during this work. This is the first time the entire family has been studied in Arabidopsis and has provided a deeper understanding of the relationships between family members, their splice isoforms and the nature of their respective predicted proteins. The DRM1/ARP protein family can be divided into two main clades based on the presence (Clade I) or absence (Clade II) of a conserved C-terminal domain, with Clade II further divided based upon presence of a partially conserved C-terminal domain (Chapter 2; Figure 2.1). Family members can be identified from all available plant genomes assessed, including monocotyledons such as rice, dicotyledons such as Arabidopsis and including the primitive plant Selaginella moellendorffii, a member of the earliest known vascular division (McElwain and Willis, 2002). That the protein has been maintained throughout the plant kingdom, with a relatively high level of conservation, makes this gene a prime candidate for having a critical function in plants.

As species have evolved to suit diverse ecological niches and gained complexity, ploidy events have provided the requisite raw genetic material upon which evolution can act. Indeed, the copy number of this gene has expanded in a number of species with one copy identified in the primitive S. Moellendorffii, compared with twelve copies identified in rice (Wood et al., 2013) and five family members identified in Arabidopsis. That these additional copies of the gene have been maintained provides not only raw genetic material allowing adaptation of additional genes to new function, but also enables plants to develop functional redundancy in critical genes required for plant development and survival. Indeed, AtDRM1 and AtDRM2 are members of a Brassicaceae-specific sub-clade and their developmental transcript expression profiles are very similar (Chapter 2; Figures 2.1B and 2.4), suggesting these genes may have arisen as a result of gene duplication event after the divergence of the Brassicaceae family.
Not only are there five DRM1/ARP family members in *Arabidopsis*, the complexity of this family is further compounded by the introduction of splice variants. Variation in splicing provides increased, often functional, diversity in the proteome, with more than 30% of intron-containing genes in *Arabidopsis* estimated to be alternatively spliced (Barbazuk et al., 2008; Campbell et al., 2006; Wang and Brendel, 2006). Steps towards understanding the complexity of this highly conserved family and its splice variants, some of which have already been described in other species (Wood et al., 2013), was undertaken during this project. It was shown that both *AtDRM1* and *AtDRM2* undergo alternative splicing, with a previously undescribed splice variant of *AtDRM1* (*AtDRM1.6*) discovered through the course of this project (Chapter 3; Figure 3.1). The alternative splicing exhibited by *AtDRM1* transcripts is known as intron retention and is prevalent in stress conditions (reviewed in Mastrangelo et al., 2012; Ner-Gaon et al., 2004), in accordance with the variable transcriptional profiles exhibited in response to various stresses (Chapter 3; Figure 3.4).

All *Arabidopsis* family members are predicted to be intrinsically disordered proteins (Chapter 2; Figure 2.2) – a relatively recently recognised group of proteins which lack tertiary structure under standard physiological conditions (Wright and Dyson, 1999). Moreover, all splice variants retain their predicted intrinsically disordered nature in their predicted protein sequence (Chapter 3; Figure 3.2). Intrinsically disordered proteins are prevalent in stress response pathways (Garay-Arroyo et al., 2000; Mouillon et al., 2006) and often play roles as hub proteins or in signalling cascades (Iakoucheva et al., 2004; Minezaki et al., 2006a). This potential promiscuity in binding provides opportunities for these proteins to have diverse roles in different tissues as well as different phases of development. Indeed, in light of the diverse transcriptional responses exhibited by the different splice variants to a plethora of stress treatments, it is possible that the range of binding partners will facilitate functional responses to these different conditions.

6.3 *AtDRM1* and *AtDRM2* in temporary growth arrest outside of dormancy

Mutant analyses undertaken for this project showed a trend towards down-regulation of *AtDRM1* and/or *AtDRM2* being associated with a concomitant increase in primary bolt height (Chapter 4; Figure 4.8), although this remains to be proven conclusively. Over-
expression of *AtDRM1* or *AtDRM2*, by comparison, did show a decrease in plant height at any given day (Chapter 2; Figure 2.6). Through the course of this project, data emerged from the close relative of *Arabidopsis*, *B. rapa* (Lee et al., 2013). Over-expression of *BrDRM1/ARP* genes was associated with a general reduction in growth, including reduced plant height, supporting the association between *DRM1/ARP* family members and no growth states in plants. A subtle but important difference was observed in over-expression lines analysed in this project compared with those of *B. rapa*. As mentioned earlier, over-expression of *B. rapa DRM1/ARP* genes was associated with a decrease in bolt height at 30 days old. However, this phenotype could be indicative of a difference in growth capability or a difference in developmental stage. When developmentally equivalent time-points were assessed in this project (1 week post-anthesis), it became clear that the height difference was no longer present (Chapter 2; Figure 2.6), meaning the phenotype was indicative of a delay in development.

It is possible that this developmental lag is reminiscent of the stress-induced growth arrest responses observed in mutants of *AtCHR12* (Mlynárová et al., 2007). Stochastic environmental factors may be inducing growth arrest in both over-expression lines and wild-type plants which is being amplified in the *AtDRM1/AtDRM2* over-expression lines causing the retardation.

Previous work in dissected hypocotyl segments of black locust described a negative association between *DRM1/ARP* transcript and growth status of tissue segments, in particular cell elongation (Park and Han, 2003). This association with a no growth status expands on a putative role for *DRM1/ARP* family members in dormancy maintenance in meristematic tissues, separating *DRM1/ARP* from purely meristematic tissue-associated functions. Indeed, the findings from this study and others using hypocotyl tissue provides evidence for *DRM1/ARP* mutants having an affected growth capacity, while the finding from the mature plant studies could reflect subtle differences in function of these genes in different tissues of the plant.

Overall, the *DRM1/ARP* family is not involved exclusively in the maintenance of dormancy; rather they are more likely to be concomitantly present due to the inherent growth cessation associated with dormant tissues. That transcriptional responses are in some cases detectable as early ten minutes after stress treatment suggests that any role may be temporally specific to the early modulation of plant growth.
6.4 A role for *AtDRM1* and *AtDRM2* in protective stress responses

Traditionally correlated with dormancy, increases in *DRM1/ARP* transcript levels in response to various physical, chemical and endogenous factors have also been reported in various species. That *AtDRM1* and *AtDRM2* transcripts are almost always detectable, while the protein is readily degraded (Chapter 5; Figure 5.9), could be indicative of the transcript being accumulated to prime plants for a quick stress response. Possibly mediated either by an activated form of the protein, or different splice variants, similar to the priming response which has been described for the response to biotic stress (reviewed in Conrath et al., 2006).

As alternative transcript splicing is common in stress conditions, the transcript accumulation of splice variants of *AtDRM1* was assessed further in this study, showing that *AtDRM1.6* is differentially regulated at the transcriptional level compared with other splice variants, particularly in response to hormones (Chapter 3; Figure 3.3, 3.4 & 3.5). Temporally, *AtDRM1.6* showed a transcriptional increase as early as ten min after salt treatment, while other splice variants took 30 min to 3 hours to exhibit a response (Chapter 3; Figure 3.5), suggesting that alternative splicing might be producing proteins with distinct roles associated with the time since exposure to the stress. It is also feasible that the rapid transcriptional response of *AtDRM1.6* meant that detecting the transcript is often missed since few experiments target such a short time-course.

Despite inherent difficulties associated with procuring true knock-out lines of *AtDRM1/ARP* family members, it was demonstrated that down-regulation lines mimicked the transcriptional profile of wild-type plants in the early salt stress response (Chapter 4; Figure 4.11). This could be indicative of these genes being involved in a negative feedback regulation system, whereby upon detection of low levels of transcript under certain conditions, a signal is sent to induce transcription. If this is the case, *DRM1/ARP* must be of particular importance in this early phase response as the plant attempts to maintain transcript levels for possible translation, priming the plants for the protective action of *AtDRM1* and *AtDRM2*.

Further complexity is evident when both *AtDRM1* and *AtDRM2* are targeted for down-regulation. A reduction in the length of hypocotyls was shown when plants were grown in the light (but not in the dark-grown plants) (Chapter 4; Figure 4.7). This finding
conflicts with a direct association of these transcripts with prevention of cell elongation. Although that the phenotype was only seen in light-grown seedlings and was no longer detectable in dark grown seedlings suggests that this response is overcome in etiolation conditions, in which both genes are known to be transcriptionally up-regulated (Figures 3.4 & 3.6). Finally, this phenotype was not detected in lines targeting only AtDRM1 or AtDRM2 individually, indicative of functional redundancy between the two genes.

Epigenetics is garnering increasing attention in the genetics community as an additional layer of regulation and therefore complexity for organisms (reviewed in Henderson and Jacobsen, 2007). A number of modifications to the packaging of the DNA strand can affect the transcriptional status of the gene. AtDRM1 appears to be acting downstream of AtCHR12 (discussed in §2.4.5), a SWI/SNF-type chromatin remodelling gene (Mlynárová et al., 2007). In general, SWI/SNF-type remodelling proteins form a complex with chromatin allowing transcription factors to access promoters of the target genes (Cristofaro et al., 2001). Therefore, AtCHR12 could be epigenetically regulating AtDRM1, facilitating transcription to occur in response to stresses.

One of the common responses to both biotic and abiotic stresses is the formation of reactive oxygen species (ROS) (Pham and Desikan, 2012; Piterková et al., 2013). ROS has also been associated with developmental processes, including bud dormancy. Application of HiCane® is routinely used to release bud dormancy in kiwifruit and grape and evidence is emerging for this acting via a ROS-mediated pathway (Halaly et al., 2008). These data allow formation of the current hypothesis: that DRM1/ARP proteins might be acting in a general stress response pathway, such as that mediated by reactive oxygen species (ROS), specifically those involved in the early modulation of growth prevention signals. However, this remains to be determined.

### 6.5 Challenges in studying AtDRM1/ARP

Through the course of this project some challenges arose which made standard functional characterisation approaches difficult. Central to the analyses of many genes is analyses of knock-out mutants. Despite relatively exhaustive attempts to source or generate them, it was not possible to obtain knock-out mutants to either AtDRM1 or AtDRM2 during this project. The lack of null mutants for either AtDRM1 or AtDRM2 provides a challenge for analyses as without breaching the critical minimal threshold
amount of transcript, the function of the gene product may remain unaffected. As it is possible that absolute knock-out of AtDRM1 and/or AtDRM2 expression is embryo lethal, development of ethanol-inducible amiRNA lines is underway. The available down-regulation lines were notoriously difficult to work with, with anecdotal examples of increased susceptibility to poor growth conditions such as aphid infestation experienced. This provides support for a protective role for AtDRM1 and AtDRM2 in sub-optimal conditions, despite no alteration in survival of either over-expression or down-regulation lines compared with wild-type in salt-stressed conditions.

All AtDRM1/ARP family members are predicted to be IDPs (Chapter 2; Figure 2.2), as are all splice variants of AtDRM1 and AtDRM2 (Chapter 3; Figure 3.2). At the protein level, the predicted IDP nature of these proteins has major implications for working on these proteins. Such proteins are often subject to increased levels of degradation as was demonstrated in this thesis. AtDRM2 is readily degraded in vitro via both proteases and proteasome-mediated degradation (Chapter 5; Figure 5.9). This makes assessment at the protein level difficult as protein visualisation needs to happen immediately after protein extraction for meaningful data to be retrieved. Moreover, the antibodies generated in this project showed low binding efficiency. Without reliable antibodies we were not able to validate down-regulation of mutant lines at the protein level. Similarly, protein expression studies across development or in response to various stress treatments may have provided critical insight into what is happening in the regulation of this gene and its resulting proteins. Alternative strategies, including Co-Immunoprecipitation for binding studies, are difficult without reliable antibodies.

### 6.6 Proposed model for the role of AtDRM1 and AtDRM2 in plants

A model for the proposed action of AtDRM1 and AtDRM2 in Arabidopsis has been generated by linking the findings from this project with the model presented earlier (Chapter 1, Figure 1.4) (Figure 6.1).
DRM1 and DRM2 transcription is regulated by endogenous, physical and chemical factors, producing transcripts which are alternatively spliced in the spliceosome. It is hypothesised that transcription is facilitated by chromatin remodelling mediated by an active form of CHR12. DRM1/DRM2 proteins are readily degraded by proteases as well as in the proteasome and this could be associated with growth in plants. When plants perceive the required stress signal, DRM1/DRM2 protein may be post-translationally modified (PTM), in response to stress-induced ROS signalling, allowing interaction with various unknown proteins (X, Y, Z and Q). Upon interaction with a binding partner, DRM1/DRM2 are able to function in the temporary growth arrest required upon the immediate perception of the sub-optimal growth conditions.
This study, in conjunction with findings in the literature, show that DRM1 and DRM2 are almost always detectable at the transcript level, although these levels fluctuate across development and in response to various treatments including physical, hormonal and chemical. This model suggests that upon perception of physical and chemical stress, transcription of DRM1/DRM2 is increased through activated-CHR12-mediated chromatin remodelling allowing access to the promoter sequence by the required transcription factors. Upon transcription, transcripts are targeted to the spliceosome for splicing into a range of splice variants. The resulting proteins are readily degraded by proteases and targeting to the proteasome allowing growth, preventing build-up, but also maintaining a background level of expression so that upon perception of a ‘stress’ it is primed to respond to the threat quickly. If the protein is not degraded, protein modifications will take place, possibly mediated via ROS in response to endogenous, physical or chemical factors, which facilitate binding to a range of interacting partners due to the IDP nature of the protein. ROS can induce transcriptional cascades as well as post-translational modifications and therefore may be affecting the regulation of the family members at multiple levels. Splice variation may enhance diversity allowing binding to be developmental phase specific. Similarly, the increasing complexity may enable responses to the plethora of factors which plants need to respond to in order to ensure their survival.

Upon expression of AtDRM1 and AtDRM2 and putative post-translational modifications, active protein appears to have roles in the immediate growth arrest associated with stress conditions in various tissues. In combination with the available literature, this research has clearly demonstrated that the association between this gene family and growth suppression is related to stress rather than dormancy per se, as it is not restricted to meristematic tissue. This is an important observation in that, by definition, dormancy is associated with meristematic tissue only (Lang et al., 1987).

What is not emphasised in Figure 6.1 is the increasing complexity which is provided by the presence of splice variants, the intrinsically disordered nature of AtDRM1/ARP proteins allowing possible binding of multiple partners and the putative post-translational modifications of these proteins. Each additional layer of variation provides increasing opportunities for survival of Arabidopsis plants.
6.7 Future work

This study has provided the basis for a number of future research directions, including epigenetic, transcriptional and biochemical studies.

As there are indications that AtDRM1/ARP family members may be epigenetically regulated by AtCHR12, further studies of this level of regulation are necessary. Chromatin-Immunoprecipitation is an immunological approach which could be used. However, more recently fluorescence resonance energy transfer (FRET)-based approaches are being used to assess the chromatin structure in specific regions of DNA in real-time (Yang and Narlikar, 2007). This FRET-based approach could be used to compare samples with or without AtCHR12 expression to assess the resulting accessibility of the AtDRM1 promoter.

To determine whether AtDRM1/ARP proteins are functioning via ROS pathways in planta, available AtDRM1/ARP mutant lines could be grown under ‘standard’ and ROS-inducing ‘stress’ conditions and the resultant ROS profile assessed using ROS-detecting fluorescent dyes and confocal microscopy. If a difference is evident in the ROS profile for AtDRM1/ARP mutants, future work developing transgenic lines crossing AtDRM1/ARP mutants with known ROS mutants could follow, to address dependency on ROS sources for phenotypes observed.

Finally, post-translational modifications of proteins in response to various signals can have a significant impact on the function of the protein. Many proteins are modified in such a fashion in the presence of ROS. Y2H studies undertaken during this PhD suggest that post-translational modification is pertinent in the mode of action of the AtDRM1/AtDRM2 protein (Chapter 5). This is supported by bioinformatic data which shows that these proteins are predicted to be phosphorylated, with AtDRM2 even identified in the PhosPhAt4.0 database of phosphorylated proteins (Appendix 7.5 and Figure 3.1). Tagged AtDRM1/ARP constructs could be transformed into Arabidopsis plants enabling over-expression of a recombinant protein which can be purified from total protein extract of the transgenic plants and analysed using Mass Spectrometry to determine the profile of any modified proteins. Moreover, by comparing plants grown under standard and stress conditions (that induce ROS); insight into the function of these modifications may be provided. Upon identification of the specific residues undergoing post-translational modification via mass spectrometry, these residues could
become candidates for future mutational studies including assessment of residues for protein stability and binding.

If post-translational modifications are detected, this will provide further evidence that AtDRM1/ARP proteins might rely on post-translational modifications for binding with interacting partners. IDPs often have a role as hub proteins, therefore further work to identify interacting partners will likely yield interesting results. Over-expression of tagged AtDRM1/ARP proteins in planta will also provide an opportunity for alternative protein interaction studies, namely Co-Immunoprecipitation. By utilising an in planta system, issues associated with potential phosphorylation are circumvented.
6.8 Conclusions and significance

The main goal of this PhD thesis project was to expand the knowledge of the biological function of *Arabidopsis DRM1* and *DRM2*. This has been achieved through: thorough characterisation of the transcriptional profiles of all five *Arabidopsis DRM1/ARP* family members, as well as splice variants of *AtDRM1*; characterisation of over-expression and down-regulation lines; a deeper understanding of the predicted nature of the resulting proteins of these transcripts, in particular that it is likely to be intrinsically disordered and that it is readily degraded *in vitro*; attempts at bacterial-based approaches for expressing soluble protein and attempts at identification of interacting partners. The *DRM1/ARP* gene family remains an exciting family with significant potential if the issues clearly highlighted are overcome. The results obtained here provide a foundation for: further studies into the epigenetic regulation of *AtDRM1/ARP* family members; investigation into the relationship between *AtDRM1/ARP* family members and ROS and characterisation of post-translationa modifications of the protein.

In addition to providing insight into some fundamental questions of biology, members of the *DRM1/ARP* have potential commercial applications as family members are increasingly being associated with growth control in both standard and sub-optimal conditions. Indeed these data suggest that *DRM1/ARP* family members are suitable candidates for studies of plant growth and development in response to stress conditions. Agriculture concerns around providing sufficient high quality food for the world’s population, particularly in light of climate change and the modification to the environment crops are grown in, make understanding these processes critical to working towards a solution. Further understanding of proteins involved in plant stress responses, such as the *DRM1/ARP* family, will facilitate development of more stress-tolerant crops through a deeper understanding of the molecular mechanisms underpinning these stress responses.
7 Appendices
### 7.1 Conceptual translation and GenBank ID of putative full-length DRM1/ARP candidates from available plant species

<table>
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<tr>
<th>Species</th>
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<th>Abbreviated ID</th>
<th>Conceptually Translated Protein Sequence</th>
<th>Reference</th>
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<td><em>Paeonia suffruticosa</em></td>
<td>ABW74471</td>
<td>PsARP</td>
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<td><em>Physcomitrella patens</em> subsp. <em>Patens</em></td>
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<td>PpDRM1</td>
<td>MGLLQLWDDTVAGPPQPEKGLKRLREEQLDAGLPVVFPGTSEIPC SFSSNLAELEGLSTLCAVREKDVRWVSFHPDGEMSGNSAEFEGVAAPNPSVYDWDVLSSDTRRSRR</td>
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<td>PsDRM4</td>
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<td>Park and Han, 2003</td>
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<td><em>Robinia pseudoacacia</em></td>
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<td>RpARP</td>
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<td>ZmDRM1</td>
<td>MGILLDQLWLDDTVAGPRPDGSGLGGLKRYASFPSSSSSSAVVPPSTEAGAAAGSFGSAGSAVPSVRPS现货APASAPSLGTPPIFPAAGGRKRSNSRATTPGTSQPPPEV</td>
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# 7.2 Oligonucleotide primers for RT-qPCR

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<th>Sequence (5’-3’)</th>
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<td>Actin-F</td>
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<td>Actin-F</td>
<td>GGTAACATTGTGCTCAGTGTTGG</td>
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<td>pGR0065</td>
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<tr>
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<td>UBC9-R</td>
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<td>pGR0063</td>
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<td>Apt2-R</td>
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<td>Ef1α-R</td>
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<td>pGR0052</td>
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<td>pGR0074</td>
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<td>pGR0076</td>
<td>TGCTTCTCCCGGTCAGGCAATCC</td>
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<td>pGR0072</td>
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### 7.3 Oligonucleotide primers for cloning

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<th>Primer Name</th>
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<td>AtDRM1 full-length gDNA for over-expression</td>
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<td>KFBB0229</td>
<td>AGCTATGGAACACTTTGTTGAGGCTGCA</td>
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<td>AtDRM2 500bpprom for GUS</td>
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<td>AtDRM1 entprom for GUS</td>
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<td>KFBB0243</td>
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## 7.4 Multiple sequence alignment of a range of plant DRM1/ARP family member sequences

Alignment used for the construction of the phylogenetic tree displayed in Figure 2.1B.
7.5 Predictive analysis of primary protein sequences of *Arabidopsis* DRM1/ARP family members

Predicted α-MoRFs are represented as green arrows. Predicted PEST proteolytic cleavage sites are represented as gold arrows. Sites of phosphorylation predicted by DISPHOS are represented as yellow blocks, while those predicted by PhosPhAt 4.0 are represented by light blue blocks. Biologically confirmed sites of phosphorylation and oxidation from the PhosPhAt 4.0 database are represented in purple and red blocks, respectively. Cysteine residues are represented gray blocks.
7.6 Analysis of disorder distribution profiles associated with a range of DRM1/ARP family proteins

1) Combined representation of disorder profiles for a range of DRM1/ARP family proteins from different species. 2-6) Different classes based upon the similarity of their disorder profiles.
7.7 Disorder analysis: combined CH-CDF plot of a range of DRM1/ARP family proteins

The CH-CDF plot is sectioned into four distinct quadrants as described in Huang et al. (2012). Quadrant 1 (Q1) rare proteins: predicted to be disordered by CH and ordered by CDF analyses; quadrant 2 (Q2) ordered proteins: predicted to be ordered proteins by CH and CDF analyses; quadrant 3 (Q3) mixed proteins: predicted to be ordered proteins by CH and disordered proteins by CDF comprising of proteins.
that contain both ordered and disordered regions and quadrant 4 (Q4) disordered proteins: predicted to be disordered by both CH and CDF analyses.

y axis: distance of each DRM1/ARP protein from major boundary line in CH plot (positive for disordered, negative for ordered or small number of disordered); x axis: distance of each DRM1/ARP protein from boundary line in CDF plot (positive for ordered, negative for disordered).

A) AtDRM1/ARP family members only; B) AtDRM1/ARP family members as well as a range of DRM1/ARP family proteins from different species (sequences selected based on what was included in the sequence alignments).
7.8 Map and alignment of conserved regulatory elements related to decapitation response detected in sequence upstream of start codon in *AtDRM1/ARP* family members

SRE-like binding elements (brown); Up1 element (pink); and Up2 elements (light pink). Arrow direction represents strand upon which motif was identified (forward = sense; reverse = antisense).
7.9 Map and alignment of regulatory elements detected in sequence upstream of 5' UTR in \textit{AtDRM1} and \textit{AtDRM2}

CaRG consensus motif predicted by PLACE (gold); DOF target predicted by PLACE (mint green); ABA-responsive elements predicted by PLACE (turquoise); BZIP predicted by Meme and TOMTOM (purple); ABI4 predicted by Meme and TOMTOM (green); ATHB5 predicted by Meme and TOMTOM (red); and DOF target predicted by Meme and TOMTOM (lemon). Arrow direction represents strand upon which motif was identified (forward = sense; reverse = antisense).
7.10 Confirmation of over-expression lines at transcript and protein level

Transgenic *Arabidopsis* plants harbouring 35S:AtDRM1 gDNA or 35S:AtDRM2 gDNA were generated and taken through to stable homozygous lines using kanamycin resistance as selection.

Plate-grown seedlings were assessed by RT-qPCR at day 14, confirming over-expression of all lines except AtDRM2 OX4. (Undertaken prior to the start of this PhD).

A) Confirmation of transgenic line over-expression at transcript level.

*Actin2* was used as a reference gene, with data calibrated to the control (empty vector) SEM of four technical replicates are represented in the error bars. SE is representative of four technical replicates.

While it is of interest whether an over-expression line is indeed showing an increase at the transcript level, it is of functional relevance also to show an increase in protein level of the targeted gene.
Antibodies were developed from chemically synthesised peptides, by Gl Biochem Ltd, targeting the C-terminal sequence of AtDRM1.2/3 and AtDRM2 (Chapter 5). Plants were grown to maturity; crude protein was extracted from low bolt tissue and samples analysed by Western blot. This involved grinding fresh samples in 1x laemmli buffer (including DTT), incubating samples at 95°C for min, pelleting the mixture by centrifugation and then harvesting the supernatant. Equal quantities of protein were loaded on Invitrogen pre-cast 12% Bis-Tris gels and run in MES buffer. Proteins were transferred to PVDF membrane using Towbin buffer; then challenged with 1:500 dilution of the antibody; washed 3x in blocking solution; then challenged with an anti-rabbit alkaline phosphatase labeled secondary antibody and washed a further 3x. Antibody binding was visualized using 1-Step NBT/BCIP (Sigma).

![Western blot of DRM1/ARP over-expressing lines.](image)

Antibodies targeting AtDRM1 and AtDRM2 simultaneously showed that compared with wild-type tissue, tissues from lines over-expressing either AtDRM1 or AtDRM2 gDNA fragments both have higher levels of their target protein specifically, with only wild-type levels of the other family member visible.
7.11 Additional biological replicate of phenotypic analyses of \textit{AtDRM1} and \textit{AtDRM2} over-expression lines

(A) Mean leaf number of individual \textit{AtDRM1} and \textit{AtDRM2} over-expressing lines from day 12 until day 18. Inset graph shows mean number of leaves at day 12. (B) Mean number of days to anthesis of \textit{DRM1/ARP} over-expressing lines, as scored by visible presence of petals in floral buds. (C) Mean height of plant (cm) at 29 days post-sowing. (D) Mean number of inflorescence bolts at 29 days post-sowing. (E) Mean number of inflorescence bolts at 1 week post-anthesis. (F) Mean height of plant (cm) at 1 week post-anthesis. All data sets were subjected to a one-way ANOVA test followed by Tukey’s test for multiple comparisons (95% confidence), with SEM represented in error bars (n ≥10).
### 7.12 Concentrations of solutes and solvents used for abiotic stress treatments

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<th>Final concentration of solvent</th>
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<td>sucrose</td>
<td>10 mM</td>
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<td>water</td>
<td>n/a</td>
</tr>
<tr>
<td>Auxin</td>
<td>IAA</td>
<td>10 μM</td>
<td>EtOH</td>
<td>0.001% v/v</td>
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<td>Salicylic acid</td>
<td>SA</td>
<td>0.5 μM</td>
<td>EtOH</td>
<td>0.000005% v/v</td>
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<tr>
<td>Etephon</td>
<td>etephon</td>
<td>7 μM</td>
<td>water</td>
<td>n/a</td>
</tr>
<tr>
<td>Methyl jasmonate</td>
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<td>water</td>
<td>n/a</td>
</tr>
<tr>
<td>Strigolactone</td>
<td>GR24</td>
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<td>acetone</td>
<td>0.0001% v/v</td>
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<tr>
<td>Abscissic acid</td>
<td>ABA</td>
<td>10 μM</td>
<td>NaOH</td>
<td>500μM</td>
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</table>
7.13 *AtDRM1* splice variants at cDNA and protein levels, including the putatively artefactual *AtDRM1.5*

7.14 Salt treatment assay optimisation

**AtDRM1**

- **0 mM NaCl**
- **40 mM NaCl**
- **100 mM NaCl**
- **1 M NaCl**

**AtDRM2**

- **0 mM NaCl**
- **40 mM NaCl**
- **100 mM NaCl**
- **1 M NaCl**

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**Appendices**

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Wild-type seeds were sterilised and stratified for 2 days in darkness at 4°C. Individual seeds were sown onto ½ MS plates and grown for 6 days before being transferred to ½ MS plates supplemented with either 0 mM NaCl, 40 mM NaCl, 100 mM NaCl or 1 M NaCl (T₀).

A) Graphs show the target/reference ratio from RT-qPCR for *AtDRM1* and *AtDRM2* over a salt treatment optimisation series in wild-type plants. SD of technical replicates are represented in the error bars. Samples were normalised to the reference gene (*ACTIN2: At3g18780*). *AtDRM1* and *AtDRM2* were detected in all samples and remained constant at all three time-points assessed with 0 mM salt treatment (control). *AtDRM1* and *AtDRM2* showed an increase in transcript expression levels with increasing concentrations of salt, with the exception of *AtDRM2* with 1 M NaCl treatment which showed no induction in the presence of salt and remained at pre-treatment levels over the time-points assayed. Between 6h and 12 days treatment there was no difference in expression levels for either gene. Seedlings were not sampled after 12 days with 1 M NaCl as this concentration was lethal.

B) Wild-type plants after 12 days grown on varying concentrations of NaCl (0 mM NaCl, 40 mM NaCl, 100 mM NaCl, and 1 M NaCl) across four different plates to reduce any inter-plate variation effects. WT survival was assessed 12 days after transfer to treatment. Wild-type plants showed a physical response to salt treatment, compared to no treatment, at all concentration of NaCl applied. 1 M NaCl was lethal for plants indicated by no additional growth after transfer and lack of green colour, whilst both 40 mM and 100 mM NaCl yielded viable plants indicated by growth and green colour. Based upon these observations 100 mM was selected for subsequent salt treatment work.
Appendices

7.15 *AtDRM1* and *AtDRM2* over-expression lines grown on varying salt concentrations

Plants over-expressing either *AtDRM1* or *AtDRM2* were grown as for Appendix 7.15. No changes in appearance were evident for over-expression lines when compared with wild-type plants.
7.16 Additional phenotypic analyses of adult amiRNA plants

The primary bolt height (top) and number of inflorescence bolts >1cm (bottom) of 44 day old plants (N = ≥ 3). All data sets were subjected to a one-way ANOVA test followed by Tukey’s test for multiple comparisons (95% confidence), with SEM represented in error bars.
### 7.17 Oligonucleotide primers for protein expression and Y2H cloning

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<th>Name</th>
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<td>AtDRM1 forward with CACC overhang</td>
<td>CACCATGGTGTCTGTAGAGAAGCA</td>
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<td>AtDRM1 reverse with stop</td>
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<td>T7-reverse</td>
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</tr>
<tr>
<td>pGR0055</td>
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<td>CF4</td>
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<td>-PO4-CAA AAA ACC CCT CAA GAC CCG</td>
<td>(Kralièek et al., 2011)</td>
</tr>
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7.18 Autoactivation of constructs for candidate Y2H assay

Diploid yeast of pAD2/AtDRM1 and pBD2/AtDRM2 bait constructs mated with empty prey constructs (empty pBD2 and empty pAD2, respectively) on a 3AT titration.
References


References


(Euphorbia esula L.) implicates a role for ethylene and cross-talk between photoperiod and temperature. *Plant Molecular Biology* **81**, 577-593.


Kleinow, T., Himbert, S., Krenz, B., Jeske, H. and Koncz, C. (2009). NAC domain transcription factor ATAF1 interacts with SNF1-related kinases and silencing of


References


