Regulation of Seed Germination by *MFT*, *MEE26*, and *CYP89A2* in *Arabidopsis thaliana*

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ABSTRACT

Germination is the process by which quiescent seeds transition to seedlings. To optimize germination over time, seeds enter a dormant state, which is the inability of a complete viable seed to go through germination even in optimal conditions. In many plant species, seed dormancy can be broken by prolonged storage (after-ripening) or imbibition at low temperatures (stratification). Germination can be affected by several factors such as light. Plants perceive light through a group of photoreceptors that detect Red (R) and Far-red (FR) light. R light such as sunlight promotes germination, however, FR light (shade light found under the canopy) inhibits germination. Light also affects the signalling of phytohormones such as Abscisic Acid (ABA) and Gibberellic Acid (GA). A high ABA/GA ratio is known to be involved in inducing and maintaining seed dormancy. The gene *MOTHER-OF-FT-AND-TFL1* (*MFT*) also affects germination. MFT has been previously shown to promote primary dormancy in Arabidopsis. In this thesis, the function of MFT in after-ripened seeds was investigated and the results indicate that it also promotes dormancy in after-ripened Arabidopsis seeds treated with FR light. This is consistent with the fact that *MFT* expression has been previously shown to be promoted by FR light. The role of two other genes in the regulation of seed dormancy and germination in both freshly harvested and after-ripened seeds was investigated. They are *Cytochrome P450 89A2* (*CYP89A2*) and *Maternal Effect Embryo Arrest 26* (*MEE26*), which are both regulated by MFT. Expression of *CYP89A2* was up-regulated by FR light. Though it is promoted by MFT, phenotypic analysis revealed that freshly harvested seeds of CYP89A2-overexpressor (oe) lines promote germination. This is also the case for after-ripened seeds of CYP89A2oe lines treated with FR light. *MEE26* is also promoted by FR light. MEE26oe and silenced lines indicate that it promotes dormancy in freshly harvested seeds and represses germination in after-ripened seeds treated with FR, even though *MEE26* is strongly repressed by MFT. This work therefore provides new insight into the function of these genes and shows that the regulation of germination is a complex interaction of different factors.

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DECLARATION

I declare that this thesis is a presentation of my original work and some collaborative work with Fabian Vaistij (his specific contributions are indicated in the text). Some of this work (the chapter *on mft-2)* has been published in in the Proceedings of the National Academy of Sciences of the United States of America (PNAS). This work has not previously been presented at any other university. All sources are acknowledged as Referenced.

Chapter 1: Introduction

1.1 Short Synopsis

In flowering plants, double fertilization occurs through a process in which the pollen tube brings two sperm cells into the female gametophyte, which is found in the mature ovule. A sperm will fertilize a haploid egg cell to form the diploid zygote and the other sperm will fertilize a diploid central cell to make the triploid endosperm. The zygote goes through several cell divisions and processes of differentiation. After fertilization, seed development takes place in which there is a simultaneous development of the zygote and the endosperm to form the mature seed. In Arabidopsis, seed development consists of two stages, which are embryogenesis and maturation (Baud *et al*, 2002). During embryogenesis the embryo's general structure is formed, and the endosperm is also developing alongside the embryo. The development of the endosperm occurs in three phases with the last phase being degeneration. During degeneration, the endosperm becomes a thin layer surrounding the embryo. It supplies the embryo with nutrients, for example, sucrose is transported from the endosperm to the embryo (Baud *et al*, 2005; Chen *et al*, 2015) to be stored as carbon in the form of Triacylglycerol (TAG) lipids, and thus the embryo increases in size, and post-germinative growth is fuelled (Baud *et al*, 2002; Penfield et al, 2004). The maturation phase takes place as soon as the embryo completes morphogenesis (Wobus *et al*, 1999). During maturation, the embryo undergoes growth arrest and reserves are later accumulated in the form of TAG lipids and proteins. The breakdown of these reserves will then provide nutrients during germination until the seedling can photosynthesize and grow photo-autotrophically (Baud *et al*, 2002). In late maturation, seeds become quiescent and tolerant to desiccation as severe water loss occurs. After seed development, the seed has an exterior layer made up of dead tissues called the testa. Under this lies the endosperm, which surrounds the embryo.

The dry seed can become quiescent or dormant. Quiescent seeds can germinate when provided with the right environmental conditions needed for growth, however, dormant seeds will resume germination when they are exposed to hormonal, environmental, metabolic or some form of physical conditions (Raghavan, 2002). These adaptation traits are essential in order to prevent viviparity or pre-harvest sprouting. Viviparity takes place when seeds that are still inside fruits and attached to the mother plant

germinate. Embryos of these seeds do not go through a period of quiescence or dormancy, instead they germinate right after the embryo development stage (Sussex,1975; Raghavan, 2002). During wet conditions, some mature cereal grains germinate in the ear or on the panicle just prior to harvesting. This is known as pre-harvest sprouting (PHS). PHS decreases the yield and quality of grains, thereby resulting in severe economic losses (Fang and Chu, 2008).

1.2 Germination and its Significance

Germination occurs when a quiescent seed develops into an active seedling. Delayed germination allows time for the dispersal of seeds and it ensures that offspring propagate under suitable conditions (Wang *et al*, 2016). Germination starts when the dry seed absorbs water and ceases after the protrusion of the embryonic axis (Bewley and Black, 1994). Germination is known as a process that involves the rupture of the testa, after which the endosperm splits open. The micropylar endosperm then ruptures and the radicle protrudes (Leubner-Metzger, 2003; Liu *et al*, 2005).

Germination occurs in three phases. In phase I, when Arabidopsis seeds initially absorb water, mucilage is released from the seed. This mucilage is made up of rhamnogalacturonan pectin and cellulose found in the external part of the seed and forms a layer that is water soluble. There is also an internal layer of mucilage that is connected to the testa through microfibrils of cellulose (Windsor *et al*, 2001). Mucilage is known to help seeds stick to surfaces and on animals, which aids in seed dispersal (Mummenhoff *et al*, 2004). Due to the fact that the mucilage is extremely hydrophilic and reduces loss of water, it also allows germination to occur in environments of osmotic and saline stress (Yang *et al*, 2010).

When water is absorbed by the cells of dry seeds it causes some temporary changes in the structure of the seeds as it expands and undergoes a transformation in size and shape (Robert *et al*, 2008). Arabidopsis wild-type (WT) seeds have a prolate spheroid shape subsequent to imbibition. Changes in membrane structures also occur. Instantly, solutes and low molecular weight metabolites escape out to the nearby solution. This causes the components of the phospholipid membrane to transform from the gel-like structure to the normal moistened and liquid crystal-like structure (Crowe and Crowe, 1992). This final stable structure of the membrane curtails further solute leakage, which can hasten the rate of germination by decreasing the concentration of inhibitors of germination that would otherwise prevent

it (Matilla *et al*, 2005). However, leakage is also an indication that damage has been caused to membranes and/or cellular regions due to the rapid and non-uniform intake of water (Powell and Matthews, 1978). Seeds take care of the damage that occurs during dehydration, maturation as well as rehydration by carrying out several repair mechanisms during imbibition. Repair of membranes, proteins, as well as DNA repair takes place during this time.

The enzymes and structures necessary for metabolic activity are all inside the seed. They partially survive the desiccation phase at the end of maturation and they are activated by imbibition, which triggers respiration upon a steep intake of oxygen. The activities of the glycolytic and pentose phosphate pathways all begin in phase I, and stimulation of the enzymes of the Krebs cycle follows (Nicolas and Aldasoro, 1979).

Everything the seed needs to synthesize proteins, except polysomes, exists inside the embryo of the dry seed. Immediately after rehydration of the seed, some ribosomes are used to create polysomal protein complexes. Initially, the extant ribosomes synthesize proteins, thus the number of single ribosomes reduce. New ribosomes are synthesized and used after polysomal assembly (Dommes and Van der Walle, 1990). Transcription of new mRNA takes place, and these encode proteins that are vital for normal cellular metabolism, such as activities that support growth maintenance (Bewley and Marcus, 1990).

During phase II of germination, the intake of water by the seed decreases. Furthermore, changes that take place in seed size and shape also decreases. The level of water remains stable inside the seed. The length of time that this phase takes can vary. In Arabidopsis, testa rupture occurs in phase II (Liu *et al*, 2005) and this is followed by endosperm rupture in phase III and another burst of oxygen intake arises (Botha *et al*, 1992; Bewley and Black, 1994). The embryonic radicle protrusion through the surrounding structures is an indication of the termination of germination and the commencement of seedling growth. Cell division in the radicle may occur during this protrusion. The synthesis of DNA in the cells of the radicle after germination also takes place (Osborne and Boubriak, 1994). The protrusion of the radicle is driven by turgor pressure, which weakens the cell walls surrounding the embryonic axis (Cosgrove,

1997). A few possibilities for the start of radicle growth are available. One could be that a rise in turgor pressure could lead to extension of the cells of the radicle (Welbaum and Bradford, 1990). Another possibility is that because the cell walls of the radicle are extensible, this can allow the cells to elongate (Bewley, 1997).

1.3 Factors Affecting Germination

1.3.1 Phytohormones:

Phytohormones are molecules that exist in low micromolar concentrations inside the plants. Their levels, signalling, as well as their interactions, function in controlling the process of germination (Kucera *et al*, 2005). Phytohormones have various vital functions in plants such as regulating response to stimuli as well as controlling various signal transduction pathways in abiotic stress. They also play a role in controlling various developmental processes in plants, the maintaining of seed dormancy and promotion of germination (Davies, 2010).

Abscisic Acid

In addition to regulating growth, ABA has different roles to play in plants. During embryogenesis, ABA promotes dormancy and suppresses precocious germination. It is produced in maternal tissues and after that in embryos and endosperms, in smaller quantities (Nambara and Marion-Poll, 2003). ABA controls the production of important seed storage proteins and lipids in the middle stage of embryogenesis. In late embryo development, ABA promotes desiccation tolerance in seeds and this allows them to be able to survive the dry state for an extended time period (Finkelstein *et al*, 2002). In freshly harvested seeds, the endosperm is known to repress germination. This is because enhanced levels of ABA are released from the endosperm (Lee *et al*, 2010). During the vegetative phase, ABA also functions in regulating the response of plants to different types of stress such as drought and osmotic imbalance. Three stages of development have been observed after conducting physiological studies on how ABA inhibits the growth of the radicle. ABA does not prevent phase I in which the outer testa ruptures, nor phase II in which radicle extension takes place. However, it does prevent phase III in which the radicle penetrates through the inner testa and endosperm (Bewley and Black, 1985).

The C terminal domain of the ABI1 protein has Protein Phosphatases 2C. It is involved in counteracting kinases as well as transmitting ABA signalling inside guard cells for regulation of stomata (Leung *et al*, 1994). *ABI2* gene product is 80% identical to ABI1 protein and also encodes a Protein Phosphatases 2C protein that negatively regulates ABA responses. The *abi1* and *abi2* mutants have an effect on vegetative responses as well as in the closure of the stomata in response to ABA. Furthermore, both mutants have reduced seed dormancy and a reduced prevention of growth by ABA.

ABI3 is part of the B3-domain protein family, which also includes FUSCA3, Leafy Cotyledon 2 of Arabidopsis and Viviparous 1 of maize (Stone *et al*, 2001). ABI3 is a transcriptional activator that directly targets genes that code for seed storage proteins as well as Late Embryonic Abundance (LEA) proteins (Parcy *et al*, 1994; Baybrook *et al*, 2006). ABI3 acts through ABA Responsive Elements (ABRE) found in the promoters of genes that respond to ABA (Hobo *et al*, 1999), and mainly functions in embryonic ABA signalling, thus it is highly expressed in embryos. ABI3 activity is dominant in the late stages of embryo development, and its expression decreases during the process of germination (Parcy *et al*, 1994). After germination and before vegetative growth, ABA recruits late embryogenesis programs that are able to allow arrested germinated embryos to acquire osmotolerance. This takes place during a very limited time after stratification. These embryos continue to stay viable, quiescent and osmotolerant in the presence of ABA (Lopez-Molina *et al*, 2002). This is an adaptive trait of Arabidopsis plants that helps in increasing their rate of survival during drought. ABI3, ABI5, and late embryogenesis genes are needed during this time window. ABI3 is important in growth arrested embryos as it acts upstream of *ABI5* for its expression (Lopez-Molina *et al*, 2002). However, ABI3 functions only in the presence of ABA, because without ABA, the expression of *ABI3* cannot arrest growth. The *abi3* is a recessive mutant that has a weak *abi3* allele, and studies have shown that mutant embryos cannot complete the maturation stage and that they go straight into a germinative programme whilst in the seeds without allowing desiccation tolerance to develop or dormancy induction to occur (Nambara *et al*, 1995).

ABI4 is known to be part of the Apetala2 domain transcription factor family and is known to also act as a transcriptional regulator that targets effector genes that are involved in seed maturation and storage

reserves, some signalling proteins and transcription factors. ABI4 is required in signalling of ABA in the course of seed development and maturation (Finkelstein *et al,* 1998). It is also involved in regulating plants response to glucose (Laby *et al,* 2001), mobilising lipids from embryos (Penfield *et al,* 2006), and retrograde signalling of chloroplasts (Koussevitzky *et al,* 2007). The expression of *ABI4* is controlled by ABA, glucose, and trehalose (Ramon *et al,* 2007). It is up-regulated in seeds during maturation and in seedlings during post germinative growth arrest (Arroyo *et al*, 2003). Seeds of *abi4* loss-of-function-alleles have higher levels of resistance to osmotic and salt stress and have seedling that grow prematurely (Quesada *et al,* 2000).

ABI5 is a member of the basic leucine zipper (bZIP) family of transcription factors and acts as a transcriptional regulator of genes that contain ABRE in their promoters. ABI5 signals stimulate ABA responses in plants (Lopez-Molina and Chua, 2000). In the presence of ABA, there is an activation of *ABI3,* which increases the levels of ABI5, thereby causing hypersensitive response. Overexpression of *ABI5* causes hypersensitivity to ABA in plants, and consistent with this the *abi5* mutant is insensitive to ABA. In WT plants, ABI5 also functions in triggering a growth arrest of germinated embryos that is dependent on ABA. During this time the accumulation of *ABI5* occurs. ABI5 is known to control the expression of *AtEm1* and *AtEm6* (hydrophilic proteins that are essential for the development of desiccation tolerance) during post germinative growth arrest by binding to their promoters, which have an ABRE (Nakamura *et al*, 2001). In the absence of ABA and in the process of vegetative growth, *ABI5* is degraded through the 26S proteasome machinery (Lopez-Molina, 2001).

Many genes are up-regulated in response to ABA. Factors in ABA signalling for expression of such genes include ABRE, ABA Responsive Elements Binding Proteins (AREB)/ABRE Binding Factors (ABF), Pyrabactin Resistant 1 (PYR1)/PYR1-Like Proteins (PYL)/Regulatory Components of ABA Receptor (RCAR), SNF1 Protein Kinase 2 (SnRK2), and group A 2C-type Protein Phosphatases (Nakashima and Yamaguchi-Shinozaki, 2013).

In the presence of ABA, PYR1, PYL and RCAR bind ABA and then interact with PP2C in order to stop the activity of protein phosphatases. Group A PP2C has nine members, which include ABI1 and

ABI2 (reviewed in Hirayama and Shinozaki 2007). In the default state, SnRK2 is autophosphorylated and thus activated. Group A PP2C deactivate SnRK2 through the process of dephosphorylation (Fujii *et al*, 2009). Thus PYR1/PYL/RCAR play a role of negatively regulating PP2C and positively regulating SnRK2, and this is very significant in ABA signalling.

The bZIP gene products including *ABI5* are present in the nucleus. In order to activate the AREB/ABF type proteins, post-translational modification must take place. SnRK2 will phosphorylate ABI5 *in vitro* (Furihata *et al*, 2006), which will then bind to the promoters of the genes that respond to ABA. The target genes are known to have a *cis*-element known as an ABRE or an ABRE and a Coupling Element (CE) in their promoters, this leads to the up-regulation of their expression (reviewed in Yamaguchi-Shinozaki and Shinozaki 2006). For example, *9*-cis-*epoxycarotenoid dioxygenase* found in Arabidopsis (*AtNCED3*), is a gene that encodes an enzyme involved in the synthesis of ABA. Its expression is upregulated by dehydration and it is known that ABA is produced in response to dehydration (Behnam *et al*, 2013). The promoter of this gene contains an ABRE *cis*-element (Behnam *et al*, 2013).

Gibberellic Acid (GA)

GA controls different developmental processes in the life cycle of plants and these include the germination of seeds, expansion of leaves, elongation of stems and roots (Davies, 2004). GA synthesis is triggered when favourable environmental conditions prevail. This also triggers the expression of genes encoding enzymes, and thus, enzymes such as 1, 3 glucannase (Leubner-Metzger *et al*, 1995) and 1,4 mannan endohydrolase (Bewley 1997) are released. These enzymes hydrolyse the cell walls to allow the radicle to break though the endosperm (Wu *et al*, 2001).

The significance of GA can be seen in the *ga1-3* mutant, which contains a *GA1* gene deletion. GA1 encodes an enzyme that is needed in the first step of GA synthesis (Sun and Kamiya, 1994). The *ga1-3* shows a phenotype that is deficient in GA. Such phenotypes include dark green leaves and severe dwarfism (Silverstone *et al*, 2001). Further impaired developments are defects in root growth, decrease in apical dominance, and lack of flowering during short days (Wilson *et al*, 1992). Without GA treatments, the seeds of this mutant fail to germinate (Dill and Sun, 2001).

GA works to promote germination by triggering degrading of the DELLA proteins through the 26S proteasome machinery. There are five *DELLA* genes, namely, *RGA, GAI, RGL1, RGL2,* and *RGL3* involved in GA signalling. Four of them (RGA, GAI, RGL2 and RGL3) function in repressing seed germination (Cao *et al*, 2005).

RGA and GAI have 80% amino acid similarity (Dill and Sun, 2001). By studying the null alleles of *rga24* and *gai-t6*, in the *ga1-3* mutant background, it was observed that RGA and GAI have functions that overlap in suppressing vegetative growth. Examples of such vegetative growth are leaf expansion, apical dominance and stem elongation. GAI and RGA are also involved in regulating floral induction. It is further observed that the contribution of RGA to this suppression is greater than GAI. Furthermore, when the RGA and GAI function is removed from *ga1-3*, this does not restore floral development or seed germination. This indicates that neither of these proteins function greatly in regulating floral development or seed germination (Dill and Sun, 2001).

Dormant seeds store high levels of ABA due to their inability to suppress RGL2 expression. Piskurewicz *et al*, (2008) shows that RGL2 triggers the synthesis of ABA as well the activity of ABI5. Furthermore, Piskurewicz and Lopez-Molina (2009) show that RGL2 and RGL3 are involved in preventing testa rupture in the presence of high ABA and low GA levels, however, RGL3 does this at a lower extent than RGL2. This can be due to the fact that in high ABA and reduced GA conditions the expression of *RGL3* mRNA is induced, but this is to a lower extent compared to *RGL2*. Reduced levels of GA prevent endosperm rupture, because this condition causes an increase in RGL2 levels, which in turn causes an increase in endogenous ABA.

Dry seeds have low levels of GA, however, after imbibition, GA levels rise (Debaujon and Koornneef, 2000). GA signalling takes place when it binds to Arabidopsis GID1-Like receptors, which then stimulate interaction of the GID1-GA with the DELLAs. This will cause the DELLA proteins to undergo conformational changes, which will be recognized by the F-box protein SLEEPY1 (SLY1). This leads to the ubiquitination of the DELLA (Griffiths *et al*, 2006). The Arabidopsis SLY1 and rice GID2 belong to the E3 Ubiquitin of the SCFSLY1 and SCFGID2 complex respectively. The *sly1* and

gid2 mutants are known to have an accumulation of DELLA proteins and, thus, have stunted growth (Piskurewicz *et al*, 2008). They are also not responsive to GA. Studies have shown that both SLY1 and GID2 function in recruiting DELLA for degradation (Sasaki *et al*, 2003). SPINDLY1 (SPY1) is a protein that negatively regulates GA signalling. It has been shown to have the same sequence with O-Linked N-acetylglucosamine transferase (OGT) in mammals (Thornton *et al*, 1999). In animals, OGT can change proteins by glucosylation of residues of Ser/Threonine. These residues compete with kinases for regions where phosphorylation can take place (Wells *et al*, 2001). SPY1 functions similarly to OGT in vitro, and it is also known to target DELLA proteins (Daviere and Archard, 2013).

12-cis-Oxophytodeinoic Acid (OPDA)

Oxylipins are different bioactive metabolites that are made through peroxidation of membrane lipids (Mosblech *et al*, 2009). Jasmonic Acid (JA) controls stress response and development in plants. OPDA is a precursor of JA. JA synthesis takes place through the octadecanoid pathway, from polyunsaturated fatty acids released by lipases inside plastid membranes (reviewed by Schaller and Stintzi, 2008). The introduction of molecular oxygen is catalysed by 13-lipoxygenase and this produces 13-hydroperoxy derivatives which are then transformed into OPDA. This process occurs through Allene Oxide Synthase (AOS) and Allene Oxide Cyclase (AOC) and it ends the segment of the pathway that takes place in the plastids.

JA as well as other metabolites such as Methyl Jasmonates (MeJA) and Jasmonyl-L-Isoleucine (JA-lle) are all known as jasmonates. Jasmonates and OPDA are oxylipins that act as phytohormones and play a role in regulating response to biotic and abiotic stress in addition to growth and development of plants such as growth of roots, coiling of tendrils, senescence, development of glandular trichomes and reproduction (Koo and Howe, 2009). In wheat grains that are dormant, JA is known to promote the loss of dormancy (Jacobsen *et al*, 2013). When MeJA is applied, the ABA biosynthetic gene is suppressed, thereby reducing ABA levels in imbibed embryos before germination. In Arabidopsis, when matured seeds are given exogenous treatment of JA at elevated levels, there is suppression of germination, however, OPDA is a more effective and stronger repressor of germination (Dave *et al*, 2011). This

repression of germination by OPDA has been demonstrated in the *comatose* (*cts*) mutant seeds. In these seeds, a mutation occurred in a gene encoding an ATP Binding Cassette (ABC) transporter protein, also known as Peroxisomal Defective 3 (PED3) and ABC-Transporter1 (PXA1) (Footitt *et al*, 2002). This ABC transporter protein imports OPDA to the peroxisome where JA is synthesized, thus, *cts-2/pxa1-1* mutants have an accumulation of OPDA and have low germination frequencies compared to WT (Dave *et al*, 2011). This can be explained, at least partially, by the fact that OPDA increases the protein levels of ABI5. It also acts through RGL2 and MOTHER-OF-FT-AND-TFL1 (MFT), a dormancy promoting transcription factor, to prevent germination (Dave *et al*, 2016). ABA and MFT also promote its accumulation.

Although OPDA has some similar signalling properties to JA, there are some differences. An example is that OPDA promotes the expression of some genes whose expressions are not up-regulated by JA. The *opr3* plant (deficient in OPDA reductase isoform needed to synthesize JA) that lack JA are still able to resist insect and fungal attack. This shows that without JA, OPDA can still control defence response (Stinzi *et al*, 2001). Some of these mutant plants that are deficient in JA accumulation demonstrate male sterility due to a delay in anther dehiscence and only MeJA (not OPDA) can restore the fertility (Stinzi and Browse, 2000). This indicates that JA is responsible for controlling the development of the anther.

The expression of genes induced by OPDA is through two signalling routes, the first is part of the JA signalling pathway and is dependent on CORONATINE INSENSITIVE 1 (COl1) (Stintzi *et al*, 2001). COl1 is also known to be responsible for many responses that are JA dependent. Both JA and OPDA can start the COl1 signalling pathway and response to wounding will need the combined activity of both JA and OPDA. During the signalling of JA, COl1 turns into a SCFCOl1 complex which acts as an E3-type ubiquitin ligase (Xu *et al*, 2002). In the course of wounding or treatment with JA, there is an enhanced expression of Jasmonate Zim Domain (JAZ) proteins, which are recruited by COl1 and degraded by the 26S proteasome machinery. The JAZ proteins negatively control MYC2 (a transcription factor-like protein) which activates responses to jasmonates. When JAZ is degraded,

transcription factors like MYC2 are released and they bind to the JA responsive elements, which are located on the promoters of JA responsive genes (Chini *et al*, 2007).

The second signalling route is independent of CO11 and is thought to be similar to the signalling mechanism exhibited by phytoprostanes. Phytoprostanes and OPDA have the same structure, biological effect, and possibly sharing similar functions as well. They are known to be involved in increasing the levels of secondary metabolites in plant systems, up-regulate genes involved in stress response and inhibit oxidative stress in cells (Thoma *et al*, 2003). Both OPDA and phytoprostanes (not JA) are called cyclopentenones. Also, they both have a structure called an α, β-unsaturated carbonyl, and thus they belong to the group of Reactive Electrophyllic Species (RES). The cyclopentenone ring has been suggested to be the main determinant of biological activity (Almeras *et al*, 2003). The promoter of the genes whose expression was induced by the A_1-type phytoprostanes (PPA_1) was analysed by Mueller (2008) and the result of the analysis revealed that they contain a TGACG (TGA) motif. These motifs have sites that the TGA transcription factor can bind to (Lam *et al*, 1989). Because there is a similarity in the control of gene expression by both PPA1 and OPDA, Mueller (2008) also shows that OPDA also interacts with TGA to control gene expression.

1.4 Seed Dormancy in Arabidopsis

The levels of plant hormones are important in controlling seed dormancy and germination. All the hormones named above are involved in regulating seed dormancy and germination, however, alterations in the ratio of ABA and GA, i.e., an elevated ABA:GA ratio is the primary factor in initiating primary dormancy (Ali Rachedi *et al*, 2004). ABA is produced during seed development and this results in primary dormancy, which prevents germination. In WT, ABA is present at the outset of seed imbibition and then reduces. This is due to ABA catabolic gene from the CYP707A family (Okamoto et al, 2006). This shows that the levels of ABA can be changed at various stages of seed development. Due to the fact that nurfluorazon, an ABA inhibitor, is able to promote germination (Debeaujon and Koornneef, 2000), de novo ABA can be seen as an essential element in the maintaining dormancy in imbibed seeds (Ali Rachedi *et al*, 2004).

Dormancy occurs when a seed is unable to germinate under any environmental conditions specific to the species that would have otherwise been favourable for its germination (Chahtane *et al*, 2016). This optimizes germination over time. It also prevents vivipary and pre-harvest sprouting (PHS). Vivipary has been associated with low levels of ABA in seeds. Viviparous mutants such as *viviparous-5* (*vp5*) and *vp2* are found in maize and are known to have reduced levels of ABA. These mutants do not undergo seed maturation and are also not able to start seed dormancy, however, by applying ABA to the growth medium, dormancy as well as desiccation tolerance is reinstated (Hable *et al,* 1998). The maize *vp1* and *abi3* show similar phenotypes such as germinating prematurely and insensitivity to abscisic acid (Wilkinson *et al,* 2002).

Others such as wheat seeds experience PHS. An elevated expression of ABA biosynthetic genes not only increases dormancy levels but also prevents pre-harvest sprouting (PHS) (Fang and Chu, 2008). Even though the direct spraying of ABA onto plants can be possible, the regulation of endogenous levels of ABA is much more effective. Increasing the levels of nine-*cis*-epoxycarotenoid dioxygenase (NCED), an enzyme involved in the biosynthesis of ABA, has been utilized in the prevention of PHS of Arabidopsis seeds (Chernys and Zeevaart, 2000).

Freshly harvested Arabidopsis seeds are said to be in a state of primary dormancy. Primary seed dormancy is an adaptive trait that arises in the mother plant during the process of seed development (Baskin and Baskin, 2004). Most seeds that are freshly harvested exhibit this trait and dormancy breakage mechanisms will have to be carried out to trigger germination. After losing primary dormancy, seeds can undergo secondary dormancy, which is the decrease in seeds' germinability. It occurs after harvest or dispersal of seeds. In the field secondary dormancy can be seen as part of a recurring changes in the seeds' level of dormancy that takes place in a seasonal pattern. Factors such as temperature, lack of oxygen and light, as well as the presence of volatile inhibitors can all induce secondary dormancy.

Based on the classification system developed by Marianna G. Nikolaeva, (1967), a comprehensive system was proposed by Baskin and Baskin, (2004), which contains five classes of dormancy in seeds.

These are Physiological Dormancy (PD), Morphological Dormancy (MD), Morphophysiological Dormancy (MPD), Physical Dormancy (PY), and Combinational Dormancy (PY and PD).

The first class of dormancy, Physiological Dormancy (PD), is located in many gymnosperms and angiosperm seeds, and it is the main type of dormancy in the field. Most seed models like *Arabidopsis thaliana* undergo this class of dormancy. Three levels of PD exist, and these are deep, intermediate, and non-deep. These levels depict the embryos' growth behaviour when it is removed from the seed (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006).

- PD Deep: The level of dormancy is deep and, thus, embryos that are removed from the seeds either do not grow or form seedlings that are abnormal. Application of GA cannot break this level of dormancy, however, long period of cold and warm stratification is essential in triggering germination.
- PD Intermediate: In this level, embryos excised from the seeds can grow into normal seedlings, however, GA treatment is needed to break dormancy. Two to three months of cold and warm stratification can also be used to trigger germination, and after-ripening can shorten this period.
- PD Non-deep: As the level of dormancy is not very deep, embryos removed from these seeds can grow into normal seedlings. To trigger germination, seeds can be treated with GA, and a few days of cold and warm stratification as well as after-ripening can be used.

Coat dormancy refers to dormancy that is imposed on the seed by the structures covering the embryo, thus non-dormant embryos are able to germinate when removed from dormant seeds. Coat is a term that is used to refer to the testa (seed coat), endosperm, and the pericarp. The endosperm serves as a blockade to the protruding radicle. For germination to occur, the walls around the micropylar endosperm have to be weakened so that the radicle can emerge and extend (Leubner-Metzger, 2003). The mechanical resistance by the testa and endosperm is stronger than the pressure exerted by the growing embryo and this is the root cause of PD non-deep (Koornneef *et al*, 2002; Leubner-Metzger, 2003).

Apart from the dormancy related processes occurring in zygotic tissues, the seed coat is also involved in regulating dormancy. It is known to regulate water absorption (Wyatt, 1977), oxygen intake

(Corbineau & Come, 1993) or other solutes that leak out of the seed that could otherwise prevent germination (Edwards, 1968). In doing this, the seed coat is able to inhibit germination. In Arabidopsis, mutants that contain changes in the pigmentation or development of their seed coat demonstrate decreased levels of dormancy when grown in the glasshouse (Debeaujon *et al*, 2000). For example, *transparent testa (tt)* mutants have lower levels of tannins inside the internal coverings of their seed coats and have been observed to have elevated germination frequency (Debeaujon *et al*, 2000, 2003). This phenotype was inherited from maternal tissues, which indicates that seeds with pigmentation that is highly intense are less permeable and more dormant.

When the temperature is reduced in the period of seed set, matured Arabidopsis seeds will exhibit increased levels of primary dormancy (Fenner, 1991). MacGregor *et al* (2014) showed that this leads to an up-regulation in the expression of genes that code for enzymes that function in the pathways of flavonoids and procyanidin synthesis in the seed coat. Mutants whose seed coats are extremely permeable and/or have reduced concentrations of procyanidin have decreased levels of dormancy after being exposed to low temperatures during maturation.

Morphological Dormancy (MD) is the second class of dormancy in which embryos are not fully developed, however, the cotyledons and hypocotyl-radicle are well distinguished (Baskin and Baskin, 1998). When given time, the embryos will germinate without the use of any treatment. An embryo that is underdeveloped causes a delay in germination because these embryos need time to grow to a significant size before germination can take place (Baskin and Baskin, 2004). Most of the time, in this type of dormancy, the seeds are also regulated by PD, thereby inhibiting germination and seedling establishment. These types of seeds have Morphophysiological Dormancy (MPD). Seeds with this type of dormancy require longer periods for embryo growth and germination to occur compared to seeds having MD. MPD consist of eight levels (table 1) depending on the need for temperature and the ability of GA to break dormancy (Baskin and Baskin, 2004).

Types of MPD	Temperature needed to Break Seed Dormancy	GA Overcomes Dormancy
Non-deep Simple	warm and cold stratification	yes
Intermediate Simple	warm and cold stratification	yes
Deep Simple	warm and cold stratification	yes/no
Deep Simple Epicotyl	warm and cold stratification	yes/no
Deep Simple Double	Cold and warm and cold	?
Non-deep Complex	Cold	yes
Intermediate Complex	Cold	yes
Deep Complex	cold	no

Table 1.1: **Types of MPD**: Eight types of MPD as well as the temperature needed to break them, and the GA needed to overcome dormancy (Baskin and Baskin, 1998)

Physical Dormancy (PY) is the third class of dormancy in which water movement is restricted by water impermeable seeds or fruit coats. This dormancy is caused by a water-resistant seed coat. In the field as well as in laboratory conditions, PY can be broken by creating a cavity called a water gap inside the seed coat. This will permit water to get to the embryo. Evidence has occurred that the use of heating to disrupt the seed coat can break PY in some plant species (Morrison *et al*, 1998). The fifth class of dormancy is Combinational Dormancy (PY and PD) which consists of a water-resistant seed coat and, in addition, there is a component of physiological dormancy. The physiological part of this dormancy is most of the time a non-deep level (Baskin and Baskin, 1998). Seeds with CD will need to be after-ripened in dry storage to break dormancy. They will also need to be stratified for a few weeks and later imbibed to break PY.

1.4.1 Mechanisms of Dormancy Breakage

In Arabidopsis, seed dormancy can be broken by carrying out processes that promote germination. Such processes are after-ripening, and cold treatment (also called stratification).

After-ripening

After–ripening is the long-term storage of freshly harvested matured seeds at room temperature. It is carried out in order to release dormancy and promote germination in dormant seeds. A factor that influences after-ripening is temperature. The storage of seeds in dry and warm environment can hasten the after-ripening process in arid climates (Baskin and Baskin, 1998). For instance, the dormant Columbia (*Col)* and Landsberg (*ler)* ecotypes of Arabidopsis can easily germinate after undergoing after-ripening for up to 2 weeks at a temperature of 20ºC to 25ºC (Koornneef *et al*, 2000). Moisture content of the seed also influences after-ripening, thus it is inhibited in dry seeds. After-ripening happens at reduced moisture content (< 0.10 g H_2O/g dry weight) (Bewley *et al*, 2013), in which it is thought that water is unavailable for biochemical reactions (Vertucci and Farrant, 1995)

It has been suggested that the process of after ripening involves protein oxidation by Reactive Oxygen Species (ROS), which are produced in the dry state (Kranner et al., 2006, 2010b). The activity of antioxidant enzymes is limited due to insufficient water, thus the seed will depend on antioxidant molecules, like Glutathione (GSH), in order to be protected from oxidative damage. GSH is oxidized by ROS to its dimer (GSSG), which increases during after-ripening (Kranner and Grill, 1993).

Dry seeds contain mRNA that existed during maturation and also survived desiccation, and thus, are called long lived transcripts (Rajjou *et al*, 2004). Many of these transcripts have been discovered in Arabidopsis by transcriptome analysis. Furthermore, it has been observed that the genes of these transcripts have ABRE, which indicates that ABA is necessary during the maturation stage (Nakabayashi *et al*, 2005; Nambara *et al*, 2010). The endosperm and embryo store various distinct transcripts in the course of seed development (Le *et al*, 2010). Analysis of dry seeds in two Arabidopsis ecotypes, Columbia (Col) and Cape Verde Island (Cvi), show that they have similar transcripts. Furthermore, stored mRNA of Late Embryogenesis Abundant (LEA) genes form the majority of the transcripts. This indicates that in addition to preparing seeds for germination, transcripts of dry seeds reflect seed maturation process (Kimura and Nambara, 2010).

Stratification

Once seeds are imbibed, there is a change in their response to low temperature. To trigger germination in dormant seeds, they are imbibed and stored at temperatures as low as 4ºC (Bewley and Black, 1994). This process is called stratification. It is thought that this mimics the period during autumn and winter when the soil is moist and cold and, thus, seeds inside the soil gradually lose dormancy in preparation for germination in spring. In Arabidopsis, stratification for 2-4 days at 4ºC is sufficient to trigger germination. The hormonal process that occurs during stratification is not well known, however, it has been shown that in Arabidopsis the content of bioactive GA4 increased in stratified seeds. This is partly due to increased expression levels of GA biosynthesis genes, like AtGA3ox1, which is needed for stimulation of germination in cold treated seeds. (Yamauchi *et al*, 2004).

Light

Phytochromes function in inducing germination through the perception of light and this occurs because light stimulates the production of GA (Derkx and Karssen, 1993). Light also stimulates the responsiveness of plants to GA (Derkx and Karssen, 1993). It triggers germination in plants that have cold induced dormancy (Donohue *et al*, 2007). During maturation, seed dormancy is induced by cool temperatures in the *hy2-1* mutant, which is deficient of a functional chromophore that is found in the five phytochromes (Donohue *et al*, 2007). Hayes and Klein (1974) show that the ratio of R/FR light that the mother plant experiences at the time of maturation has an effect on the germination rate of the mature seed.

1.5 Effect of Light Quality on Germination

Light signalling by phytochromes controls the germination of seeds. Phytochromes are photoreceptors that are able to perceive light and use it in plant development. There are five phytochromes in Arabidopsis designated A – E (Figure 1.1) (as reviewed by Li *et al*, 2011). Under the canopy, shading by other plants changes the quality of light. Light that is transmitted or reflected from the surrounding vegetation is rich in far-red (FR) light wavelength and deficient in red (R) light wavelength, because it

is absorbed by chlorophyll and carotenoid to be used in the process of photosynthesis (as reviewed by Li *et al*, 2011).

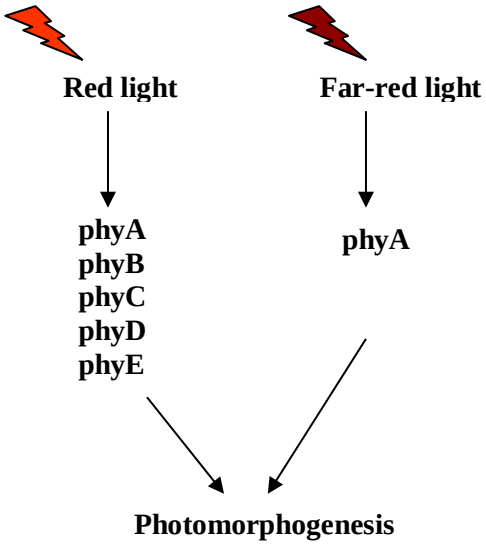


Figure 1.1: **The five phytochromes in Arabidopsis**: Phytochromes A-E are able to detect both R and FR light and use it for plant growth (photomorphogenesis).

Phytochrome A (phyA) and phyB are both able to perceive FR and R light. Phytochromes can switch reversibly between the two forms, the biologically active (Pfr) and the inactive (Pr) forms (Shinomura *et al*, 1996). The Pfr form perceives FR light to become the Pr form, whilst the Pr form perceives R light to become the Pfr form (Figure 1.2).

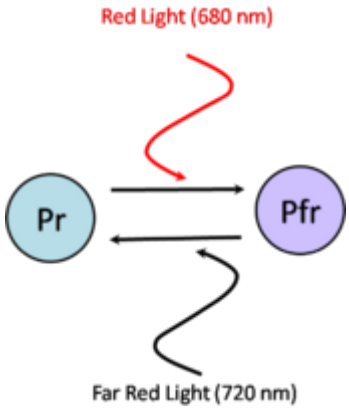


Figure 1.2 **Photoreversibility of Phytochromes**: Phytochromes can switch between two conformations, Active (Pfr) and Inactive (Pr) (www.2009.igem.org)

Hence, R and FR light activate and deactivate phyB respectively, however, R light or during daytime phyA is deactivated and FR light or darkness (night time) activates phyA (Kircher *et al*, 2011). Both

Pfr phyA and phyB promote germination. When seeds are in the dry state, they are irresponsive to changes in light, but, upon imbibition, they are able to perceive the different light cues (Dechaine *et al*, 2009). PhyB accumulates upon imbibition, however, PhyA accumulation in seeds occurs a few hours after imbibition (Casal *et al*, 1998). Under experimental conditions, when seeds are initially exposed to a pulse of R or FR, this will activate or deactivate phyB before the accumulation of phyA. If this is followed by another pulse of FR light later during imbibition, phyB will be deactivated, but, at the same time, phyA is being activated. Later, an R pulse will act to activate both phyA and phyB. This would be equivalent to continuous white light as it is rich in R (Figure 1.3).

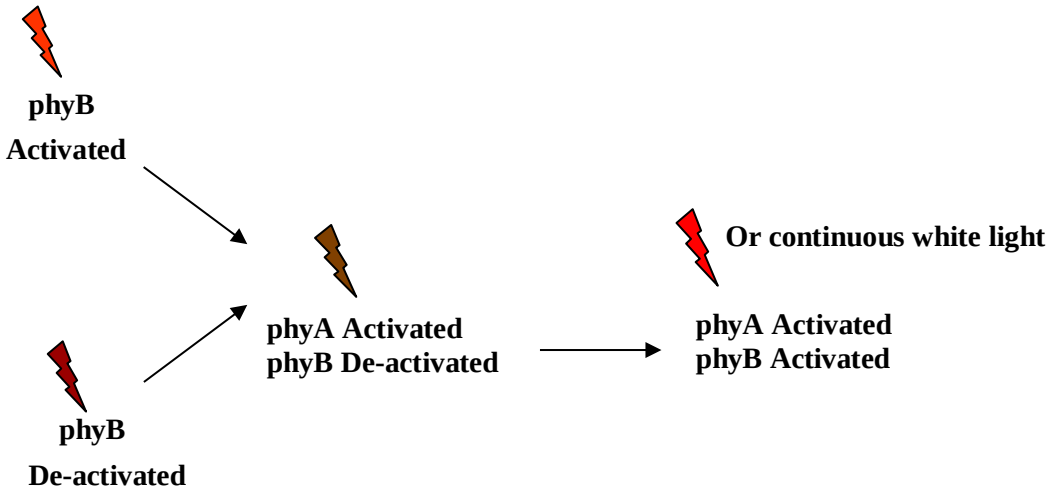


Figure 1.3: **Process of Activation and deactivation of phyA and B:** a pulse of R or FR will activate or deactivate phyB before the accumulation of phyA. Another pulse of FR light later during imbibition will deactivate phyB while phyA is being activated. A R pulse or continuous white light will activate both phyA and phyB.

The mode of action of phyA and phyB in controlling seeds germination has been separated into three different catagories; Low Fluence Response (LFR), Very Low Fluence Response (VLFR), and High Irradiance Response (HIR). LFR is the most common mode of action of phytochromes in most plants and is controlled by phyB. In the LFR, a great part of the Pr is converted to Pfr form, therefore, it needs a greater amount of R light fluence. FR light cannot induce LFR mode of action (Casal *et al*, 2014) and cancels it by decreasing the amount of Pfr phyB made by R light. Thus, LFR are photoreversible by R:FR light. The induction of VLFR can occur through exposure to R or FR light, however, high irradiance of FR light is needed to get to saturation point (Botto *et al*, 1996). In VLFR mode of action, a small proportion of Pfr is needed to reach saturation. HIR involves the exposure to high intensities of

light for longer periods of time. De-etiolation of seeds in all types of light quality is regulated by HIR. The phyA HIR is more intensive under FR conditions (Franklin et al., 2007).

In the natural environment, the seedling is in complete darkness before it emerges from the soil and litter above, thus the VLFR mode of action is needed to start the initial stages of de-etiolation. After the seedling grows above the soil and overtops the litter, the LFR of phyB will be needed to continue de-etiolation. However, if there is presence of canopy, the HIR of phyA will be needed to complete de-etiolation (Yanovsky *et al*, 1995).

Once phyA/phyB are activated, they interact with a set of proteins in the group of basic-helix-loop-helix (bHLH) transcription factors called Phytochrome Interacting Factors (PIFs) (Leivar and Quail, 2011). The proteins of bHLH are a superfamily of transcription factors located in all eukaryotes, however, they have been thoroughly characterized in mammals (Ledent and Vervoort, 2001). In Arabidopsis, Toledo-Ortiz *et al*, (2003) identified 147 genes encoding bHLH. The bHLH domain is made up of sixty amino acids and has two regions. One region is the basic region, which has 15 amino acids, and the other is the HLH region. The basic region is found at the N-terminus end and operates in binding of DNA. The other region, the HLH, is situated at the C-terminus of the domain. Furthermore, HLH is mostly made up of hydrophilic residues and plays a role in dimerization (Ferre-D'Amare *et al*, 1994). The bHLH recognizes binding sites with a DNA sequence consisting of six nucleotides. This sequence is termed the E-box (5-CANNTG-3). The E-box consists of various types, but the G-box, 5-CACGTG-3, is very common (Toledo-Ortiz *et al*, 2003).

There are fifteen members of the PIF subfamily in Arabidopsis (Leivar and Quail, 2011). The first PIF protein that was discovered was PIF3 through yeast-two-hybrid screen (Ni *et al*, 1998). This was followed by the discovery of PIF4, and PIF1, PIF5, PIF6 and PIF7, which were discovered later through sequence homology (Huq and Quail, 2002; Oh *et al*, 2004). Most bHLH proteins bind to cis-elements in a consensus sequence called the E-box, however, PIF proteins bind to the G-box. The entire group of PIF proteins contain a conserved motif in the N-termini (Leivar and Quail, 2011). This motif is known as the active phyB-binding motif. In addition to this motif, PIF1 and PIF3 also have an active

phyA-binding motif. However, compared to PIF3, PIF1 has greater binding affinity to phyA (Al-Sady *et al*, 2006).

When seeds that are imbibed detect light, the activated phytochromes travel to the nucleus and transfer the light signal partly through the destruction of a bHLH transcription factor named PIF1. PIF1 has an adverse regulatory effect on seed germination (Oh *et al*, 2004, 2006). PIF1 binds to the promoters of at least 166 genes. The genes that PIF1 targets are those involved in cell wall loosening (Oh *et al*, 2009) and the hormone signalling genes like the DELLAs, as well as ABA Response Transcription Factors like *ABI3* and *ABI5*. PIF1 prevents germination through the induction of *GAI* and *RGA* as well as *ABI3* and *ABI5* expressions (Park *et al*, 2011). PIF1 is involved in the regulation of the metabolism of hormones, thereby regulating their signalling. It down-regulates the expression of GA synthetic genes like *GA-3-Oxidaes1 (GA3ox1)* and *GA3ox2* and promotes GA catabolic genes, thus reducing GA levels. Furthermore, PIF1 promotes the expression of ABA synthetic genes and suppresses ABA catabolic genes, which increases ABA levels (Park *et al*, 2011). However, PIF1 doesn't directly link to hormone metabolic genes, even though they have G-box elements (Oh *et al*, 2007).

SOMNUS (SOM) is a CCCH zinc finger protein that has unknown molecular function. These groups of proteins are not well defined because some of them control the stability of RNA, others control the processing of RNA, and some control transcription (Hudson *et al*, 2004). PIF1 directly binds to the promoter of *SOM*, thus, SOM acts downstream of PIF1 and regulates hormone metabolic genes (Kim *et al*, 2008). SOM suppresses germination by promoting genes that synthesize ABA and inhibiting genes that synthesize GA. However, SOM does not activate the up-regulation of *GAI* and *RGA* in imbibed seeds (Park *et al*, 2011).

R light inhibits elongation of hypocotyl/stem, thus mutants of phyB have elongated hypocotyls in response to R light (Reed *et al*, 1993). Seedlings that are grown in the dark show a photomorphogenic development in which resources and nutrients are concentrated in hypocotyl elongation rather than development of the roots and cotyledon. This allows for the hypocotyls to elongate and search for light that is needed for photoautotrophic development (Josse and Halliday, 2008). This skotomorphogenic

growth is controlled by PIFs, as the PIF quadruple mutants, *pif1pif3pif4pif5 (pifq),* all show a photoautotrophic growth system (Shin *et al,* 2009). The dark grown *pifq* has a gene expression pattern similar to WT grown in R light (Shin *et al,* 2009). In dark grown seedlings, phyA is present in copious amounts, however the quantity of phyB is higher in seedlings grown in the light (Park *et al,* 2011).

1.6 Genetic Factors that Affect Germination

Delay of Germination 1 (*DOG1*)

DOG1 belongs to a small novel gene family that is found only in plants. Through the study of *DOG1* structure, Bentsink *et al* (2006) were able to identify four other genes from this family, and they are known as *DOG1-Like 1-4*. *DOG1* is greatly expressed in seeds, it is expressed at the beginning of seed development and the level elevates at the final stages of seed development. The transcripts of *DOG1* continue to exist in after-ripened seeds but become non-existent in dormant and after-ripened imbibed seeds (Bentsink *et al,* 2006).

Transcription Elongation Factors S-II (TFIIS) (Liu *et al,* 2011) and histone monoubiquitination (Liu *et al,* 2007) all function in enhancing *DOG1* expression. DOG1 also goes through alternative splicing which is carried out by Nine-Teen-Complex-Related Proteins (NTR1) and is also needed for increased *DOG1* transcript levels (Dolata *et al,* 2015). Four splicing variants have previously been identified (Bentsink *et al,* 2006) and recently, Nakabayashi *et al,* (2015) has identified a fifth one which reserves most of the *DOG1* transcripts. Alternative splicing is needed for the level of DOG1 proteins to increase, as single protein isoforms are unable to increase effectively in seeds. DOG1 can also undergo self-binding and this is required for it to function effectively. The difference in its capability to self-bind is reflected in the varied depths of dormancy in the different Arabidopsis backgrounds.

The discovery of *DOG1* occurred through its identification as an important Quantitative Trait Locus (QTL) for seed dormancy (Alonso-Blanco *et al,* 2003). Its regulation of seed dormancy in seeds that have reduced levels of dormancy is not dependent on ABA (Graeber K *et al,* 2014), however, primary seed dormancy occurs though the activity of both ABA and DOG1 (Nakabayashi K, *et al,* 2012). The *dog1* mutant has high levels of germination frequency. The high dormancy levels in freshly harvested

seeds are also linked to increased expression of *DOG1*, which has been observed in both laboratory and natural settings. Some environmental conditions, such as decreased temperatures at the time of seed maturation, increase dormancy levels and this has been associated with high expression levels of *DOG1* (Chiang *et al,* 2011). Dekkers *et al* (2016) have shown that DOG1 is involved in promoting seed maturation through its interaction with *ABI3.* Southern European Arabidopsis Accessions whose native habitat consist of warm winters higher than 4^{o} C, are more dormant and have enhanced *DOG1* expression compared to those in the north that experience winter below 4^{o}C (Chiang *et al,* 2011). The DOG1 protein is stable but inactive in the process of after-ripening (Nakabayashi K, *et al,* 2012).

Spatula (SPT)

Spatula is a member of the basic/helix-loop-helix (bHLH) superfamily of proteins. Even though SPT shares some similarities with the PIFs and the Phytochrome Interacting Factor-Like (PILs), they are also different in that *SPT* does not have an Active Phytochrome-binding (APB) domain (Reymond *et al,* 2012). *SPT* is expressed in many parts of the plant including leaf structure.

Studies previously carried out show that SPT functions in controlling the size of plant organs. It works to repress the growth and development of some plant organs. The *spt* mutants have long hypocotyls, bigger leaves and cotyledons, but when *SPT* is overexpressed, the size of these organs is reduced (Josse *et al,* 2011). In different organs, the size difference could be either as a result of changes in the number of cells or changes in the size of cells. It could also be due to both. In cotyledons, SPT plays a role in suppressing the expansion of the cotyledons in a manner similar to the DELLAs, even though the DELLAs down-regulate *SPT* protein accumulation (Josse *et al*, 2011). In the leaves, SPT functions in preventing cell division. It is able to do this by controlling the growth of the meristematic region of leaves, thus the *spt* has widespread meristems compared to WT (Ichihashi *et al*, 2010).

There is also a high expression of *SPT* in the Root Apical Meristem (RAM) (Groszmann *et al,* 2010). The *spt* mutant has lengthened roots due to the rise in number of cortical cells around the diameter of the root (Makkena and Lamb, 2013), which suggests that SPT functions to suppress growth all over the root. This, however, does not affect the general root pattern. SPT is needed for development of tissues

that come from the carpel margin, and it is also essential for the development of the transmitting tract as well as the septum, style and stigma (Alvarez and Smyth, 1999).

SPT also functions in controlling the response of plants to low temperatures (Penfield *et al*, 2005). During the daytime, when temperatures are cool, SPT works to regulate germination and growth and there is no effect on growth when temperatures are warmer. Temperatures during the daytime have an influence on vegetative growth and SPT works in integrating growth and temperatures in the daytime. SPT has been previously shown to function in the process of primary seed dormancy, which is consistent with the findings of Belmonte *et al* (2013) indicating that the transcript abundance of *SPT* increases during seed development.

The Graham laboratory carried out further studies to investigate the activity of SPT in the process of seed dormancy and germination. Among other lines, Vaistij *et al* (2013) studied *spt-2* in Landsberg (*Ler*) ecotype of Arabidospsis. After performing germination assays, the *spt-2* mutant was observed to have a very dormant phenotype. Reduced levels of endogenous ABA or constitutive activation of GA response pathways does decrease this extreme level of dormancy in this ecotype. Measurement of endogenous levels of ABA and GA were carried out on *spt-2*, and the results showed that the levels of ABA were increased in dry and imbibed seeds, however, levels of GA were reduced in dry seeds, but no changes were detected in imbibed seeds. In the *spt-2* mutant, the levels of ABA and GA in dry seeds reflect its highly dormant phenotype. Expression of ABA and GA signalling genes was analysed in freshly matured dry seeds in order to find SPT regulated genes. The results indicate that there is an increased level of ABI4 and RGA in the *spt-2* mutant. Transcriptomic data were analysed from freshly harvested stratified seeds of *Ler* that were sampled 1 day after imbibition. There is an elevated expression of *SPT* during this time in *Ler* seeds that are germinating (Penfield *et al*, 2005). The transcriptomic analysis involved two sets of comparisons and the results from these analyses show that, in both comparisons sets, 10 genes had their profiles changed in a similar way. *MFT* was one of them and it has 3 G-boxes in its promoter. It is down-regulated by SPT. Furthermore, ChIP-qPCR analysis using SPTmyc establishes that *MFT* is a direct target of SPT, but this interaction has not been detected in germinating seeds. It was, thus, suggested that the high level of dormancy of the *spt-2* mutant was

due to the up-regulation of the expression of *MFT, RGA,* and *ABI4* and this supersedes the reduced expression of *ABI5* and *RGL3.* In *Ler,* the up-regulation of the expression of *RGA* in *spt-2* could further strengthen the dormancy-repressing route.

MOTHER-OF-FLOWERING LOCUS -FT-AND-TERMINAL FLOWER1-TFL1 (MFT)

The Phosphatidyl Ethanolamine Binding Proteins are a conserved set of proteins that are found in all eukaryotes. In Arabidopsis, the members of this group that are well characterized are FLOWERING LOCUS (FT), TERMINAL FLOWER1 (TFL1), and MFT. FT and TFL1 are known to act in controlling the timing of flowering (Kardailsky *et al,* 1999), however, MFT is responsible for controlling seed germination (Nakamura *et al,* 2011; Xi *et al,* 2010). It is localized in the nucleus and, thus, works as a transcription factor to regulate *ABI5* expression by suppressing it in non-dormant seeds. Nakamura *et al* (2011) carried out a study in wheat in order to detect genes that played a role in seed dormancy. This was done by growing seeds in different temperatures and selecting genes that were differentially expressed in the embryos. A homolog of *MFT* (TaMFT) was identified in wheat whose expression was elevated when seeds were matured at lower temperatures. Mapping analysis was carried out and revealed that MFT, which was on chromosome 3A, occurs together with the quantitative trait locus (QTL) QPhs.ocs-3A.1 that was associated with seed dormancy. After carrying out complementation analysis, it was observed that in T1 seeds an up-regulation of *MFT* caused lower germination frequencies. Moreover, the premature germination of underdeveloped embryos was repressed by the transient overexpression of *MFT* driven by the ubiquitin promoter of maize. All this indicates that the function of MFT in wheat is to repress seed germination.

Xi *et al,* 2010 carried out a study in Arabidopsis that reveals that during seed germination, the phytohormones ABA and GA control *MFT.* The application of ABA in germinating seeds causes the elevated expression of *MFT* in the embryo. Seeds of *mft-2* mutant show a phenotype that is hypersensitive to ABA and thus has lower germination frequency compared to WT. *MFT* expression is induced by ABI5 and suppressed by ABI3, however, MFT supresses *ABI5*. Moreover, RGL2 induces

the expression of *MFT*. Thus, Xi *et al*, 2010 shows that MFT promotes germination in after-ripened Arabidopsis seeds, whilst Nakamura *et al*, 2011 shows that it represses germination in wheat.

Vaistij *et al* (2013) showed that MFT functions in promoting dormancy during the process of seed development as seeds of *mft-2* mutant from Ler and Col have higher frequencies of germination compared to WT. After ripened seeds of Ler are more sensitive to exogenous ABA compared to WT. This has been previously reported for Col seeds by Xi *et al*, 2010. Thus, it was deduced that MFT functions in promoting dormancy during seed development and also to promote germination in after-ripened imbibed seeds exposed to ABA applied exogenously.

To understand the mechanism by which MFT functions, the Graham laboratory carried out transcriptomic analysis on three pairs of *mft-2* vs WT comparisons in order to detect genes that were deregulated in the *mft-2* mutant. These three pairs of comparisons were: (i); freshly matured dry seeds developed at 15^{o}C; (ii) freshly matured (developed at 15^{o}C) imbibed seeds in the dark for 7 days at 4^{o}C; and (iii) imbibed seeds in the dark for 7 days at 4^{o}C. For each of these conditions, the comparisons were between WT and *mft-2* (Figure 1.4).

The seeds were set under these different conditions because the level of germination is influenced by the temperature at which seeds develop as well as stratification (Figure 1.4). For example, primary dormancy is induced in seeds that are developed at low temperatures (e.g., 15^{o} C). In the second comparison, although the seeds have been set at low temperature, they have been imbibed and stratified, there will be some level of germination. However, the last comparison will have higher germination frequencies compared to the other two comparisons because they have been developed at a higher temperature and have also been imbibed and stratified. This helped to identify genes that were deregulated in the same way in the *mft-2* background in the different environmental conditions. This indicates that those genes are directly controlled by MFT and not the different environmental conditions.

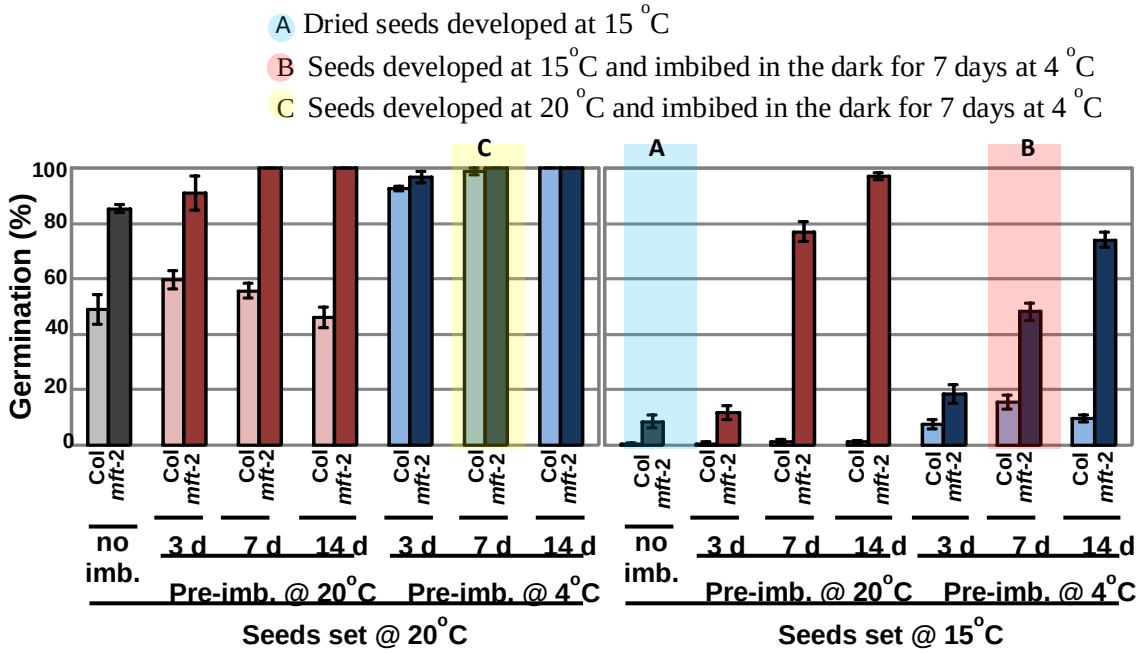


Figure 1.4: **Germination levels of seeds developed at different temperatures**: i) freshly-mature (developed at 15 °C) dry seeds (pair A); ii) freshly-mature (developed at 15 °C) imbibed seed in dark for 7 days at 4 °C (pair B); and iii) freshly-mature (developed at 20 °C) imbibed seeds in dark for 7 days at 4 °C (pair C). The seeds were set under these different conditions because the level of germination is influenced by the temperature at which seeds develop as well as stratification.

The analysis revealed that in all three comparisons, there were a common set of genes that were up-regulated and down- regulated in the *mft-2* mutant, and this was independent of the environmental conditions at which the RNA samples were collected for the experiment. The genes were then decreased to 29 and 38 respectively by choosing those in which the deregulation of expression was at least 2-fold in all three pairs of comparisons. The results of the transcriptomic analysis showed that *Maternal Effect Embryo Arrest 26 (MEE26)* was at the top of list of genes that were up-regulated in the *mft-2* mutant and that *Cytochrome P450 89A2* (*CYP89A2*) was at the top of the list of genes that were down-regulated in *mft-2* (see table 1 and 2 in Appendix 1).

1.7 Aim of This Thesis

The aim of this research is to first investigate the role that MFT plays in after-ripened seeds treated with FR/R light. Furthermore, this thesis will also study how MEE26 and CYP89A2 function in the control of seed dormancy and germination. This could further shed light on the reduced dormancy phenotype of the *mft-2,* and thereby explain the mode of action of MFT. Since *MEE26* is up-regulated in the *mft-2* background (MFT suppresses *MEE26*) and *CYP89A2* is promoted by MFT, it is therefore hypothesized that MEE26 will promote germination and CYP89A2 will promote dormancy.

CHAPTER 2: METHODOLOGY

2.1 Plant and growth conditions

The plants that were used were the *Arabidopsis thaliana* (Col background), wild type (WT), overexpressing lines of *MEE26* and *CYP89A2, MEE26* silencing (MEE26IR) lines, *CYP89A2* T-DNA insertion lines, *mft-2* (SALK_147675) mutant lines (Xi *et al,* 2010), *som-3* (SALK_008075) (Kim *et al,* 2008), *pif1-1*(as described in Huq *et al,* 2004), as well as the *abi5-7* (as described in Saavedra *et al*, 2010) mutant were all in the *Col* ecotype. The *della4* mutant is as described in Cao *et al,* (2005) and is in the *Ler* background. Two *CYP89A2* T-DNA insertion lines, Gabi Kat GK-169E08 (*cyp89A2*–1) and Salk-082987(*cyp89A2*-10) were obtained from Nottingham Arabidopsis Stock Centre (NASC). In table 2.1 homozygous Salk lines were confirmed by PCR using primers (*cyp89A2-10*) and *cyp89A2-1* were primers used for German Plant Genomic Program (Gabi-Kat) lines. Seeds used were harvested from plants that were grown in the greenhouse with lighting to provide a 16hr photoperiod with a temperature of 20-22 ^{o}C. All genotypes were grown together.

2.2 DNA Extraction

To make DNA extraction buffer, 10.27 g of sucrose and 1M Tris-HCL pH 7.5 was added to distilled water. Thirty ml of NaCl (1M stock) was also added to this after which it was autoclaved to 121ºC for 1 hour. A small section of leaf from the plant was cut and put inside 1.5 ml Eppendorf tubes. 100 µL of DNA extraction buffer was added to it. DNA lysis was carried out by using a small plastic pestle to grind the leaf inside the Eppendorf tube, after which another 100 µL of sucrose solution was added. It was immediately heated at 95-100 ºC, after which it was spun at 13000 rpm for 1min, and later 50-100 µL of supernatant was transferred into another 1.5 ml Eppendorf tube.

2.3 Identifying Transgenic Homozygous Lines

Transgenic lines designed to over-express (OE) or silence (IR) *MEE26* and *CYP89A2* were made in Arabidopsis Col ecotype. MEE26oe and MEE26IR lines were made in wild-type and *mft-2* backgrounds respectively, whilst CYP89A2oe was made in both wild-type and *mft*-2 background. This was done by using the CaMV-35S promoter of the binary vector pFGC5941. The MEE26-OE, MEE26-IR and

CYP89A2oe vectors were used to transform Arabidopsis Col plants by Agrobacterium floral dipping process.

T2 seeds from T1 primary independent transgenic plants were plated on ½ Murashige and Skooge (MS) and selected for BASTA (Non-selective herbicide Glufosinate, the trade name is BASTA). Batches with 3:1 ratio of BASTA resistant plants indicative that they carry only one copy of the insertion were kept and resistant seedlings transferred to soil to produce T3 seeds. Pools of T3 seeds were selected on ½ Murashige and Skooge (MS) + BASTA media plates. Those that were 100% BASTA resistant indicated that the mother plant was homozygous for the T-DNA insertion. These homozygous T3 seeds from every line were planted in soil and placed in the greenhouse to grow and form the T4 stock seeds.

2.4 Identifying Homozygous Lines in MEE26IR *Cross Pollinated With WT*

Siliques, leaves and any open flowers were removed from the selected bud of the selected stem of the mother plant (MEE26IR lines). Tweezers were used to also take away the sepals, petals and the six stamens around this bud without removing the pistil. A newly opened flower was chosen from WT *Col* plant and tweezers were used to squeeze close to the pedicel so as to remove the flower. The male anthers from the flowers of WT were then brushed on the female stigmas from the flowers of MEE26IR lines. A coloured tape was wrapped around the stem of the MEE26IR plant that was cross pollinated. An indication of the success of the crosses is the elongation of the siliques. Cross pollination produced normal seed set and the resulting F1 seeds from cross pollinated lines were grown in soil. They were left to self-pollinate and produce F2 seeds which were plated on ½ MS and Basta and selected for Basta resistance/ sensitivity. The resistant ones indicated that they still have the transgene in the F2. These resistant seedlings were selected and transferred to P40 trays. Later, DNA was extracted from the leaves of the seedlings. MEE26IR primers (table 2.1) were used to PCR genotype for lines that had no T-DNA amplification indicating that they were WT. The mother plants were homozygous for the transgene.

2.5 R and FR Light Treatment of Seeds

After-ripened seeds of WT and *mft-2* were sterilized and sown on water agar plates (0.9% wt/vol) to create three replicates of WT seeds to be treated with FR/R light and another three replicates to be treated with FR light. This was also repeated for *mft-2* seeds. The plates were left to imbibe in the presence of white light for three hours, after which they were placed in the light treatment box and LED irradiated with FR (4.5 μmol $m^{-2} \cdot s^{-1}$) and R (20 μmol $m^{-2} \cdot s^{-1}$). The following treatments were given.

i. FR/R- 5 mins pulse of FR light followed by another 5 mins pulse of R light

ii. FR- 5 mins pulse of FR light

After light treatment, the plates were wrapped with foil in the presence of green light and placed in the growth cabinet. RNA extraction was performed 1 day after imbibition. cDNA was synthesized and analysed by qPCR.

2.6 RNA Extraction and Synthesis of cDNA

RNA extraction was carried out on freshly harvested, after-ripened and dry seeds, as well as seeds imbibed for 24 hours. To make XT buffer, 0.2 M sodium borate, 30 mM EGTA, 1% SDS, 1% sodium deoxycholate, 2% polyvinylpyrollidone, 10 mM DTT, and 1% octylphenoxypolyethoxyethanol (IGEPAL pH 9.0) were added to distilled water. This was autoclaved after allowing it to stand for 24-48hrs. The following was added to every 3.5 ml of XT buffer; 0.07 g PVP, 5.39 mg DTT, and IGEPAL up to 1% final conc. 150 mg of dry/imbibed seeds were weighed and kept in 1.5 ml Eppendorf tubes and put in liquid nitrogen. They were ground to powder and extracted with frozen mortar and pestle and 250 μL of XT buffer was added. They were decanted to the 1.5 ml Eppendorf tubes and thawed. Another 250 μL of XT buffer was added. They were then treated with 20 μL of proteinase K, vortexed, and incubated at 42 ^{o}C for 90 mins. 0.5 ml was pipetted into a new Eppendorf tube and 40 μL of 2 M Kcl was added and mixed. They were then incubated on ice for an hour. The Eppendorf tubes were placed in a centrifuge and spun at 13000 rpm 4 ^{o}C for 20 mins, after which the supernatant was placed into a new Eppendorf tube and 180 μL of 8 M LiCl was added and mixed. RNA was then precipitated at -20 ^{o}C and left overnight. RNA was collected by spinning at 13000rpm 4 ^{o}C for 20 mins and supernatant

was discarded. Purification of RNA was done by following the manufacture's protocol in the Qiagen RNeasy kit. DNase treatment of RNA samples was carried out by using 1-8 μL of RNA, 1 μL of RQ1 RNase-Free DNase 10X Reaction Buffer, 1 μL of RQ1 RNase-Free DNase. Nuclease free water was added to this until it reached a final volume of 10 μL. The treatment continued by following the instructions from the protocol provided by Promega. The synthesis of First-strand cDNA was carried out by adding 1μL of OligodT and 1μL of dNTP Mix (10 mM each). This was heated to 65 ^{o}C for 5 mins and chilled briefly on ice. It was then spun briefly and the following were added; 4 μL of 5X First Strand Buffer, 2 μL 0.1M DTT, 1 μL RNase Out. This was mixed by pipetting and was then incubated at 42^{o}C for 2 mins. 1 μL of Superscript II Reverse Transcriptase (Invitrogen) was added and mixed, after which it was incubated at 42 ^{o}C for 50 mins. Finally, the reaction was inactivated by warming it up at 70 ^{o}C for 15 mins.

2.7 Gene Expression Analysis Using QPCR

PCR was carried out using iQ SYBR Green Super mix as well as the My iQ Real-Time PCR detection system (BioRad) in accordance with the instructions of the manufacturer. For normalization, the constitutively expressed Actin (ACT2) gene was used. cDNA was diluted in 180 μL of distilled water and 2 ml of this was used, along with the MFT, MEE26, or CYP89A2 primers (table 2.1) depending on the gene to be studied.

2.8 Sterilization of Seeds

150ml of distilled water was added to a beaker and 4.5ml of HCL was pipetted into it. Into this was added half of Precept Disinfectant Tablets (bleach) from FisherScientific. Seeds were put into 1.5ml of Eppendorf tubes and placed on a rack with the lids open. This and the beaker was placed inside a plastic storage container. The storage container was closed and left to stand in a fume cabinet for 3 to 4hrs.

2.9 Germination Assays of Freshly Harvested Seeds

Seeds were harvested from brown siliques after which they were sieved, and experiments were carried out within 24 hours after harvesting. After-ripened seeds were used six to eight weeks after dry storage. To carry out germination assays, 50 to 100 sterilized seeds were plated on 0.9 (w/v) water agar plates. They were then placed in growth cabinets under controlled environmental conditions with constant light (150 µmol m^{-2} s^{-1}) at a temperature of 20 °C. Germination was scored after imbibing seeds for seven days. Where necessary seeds were stratified in the dark after sowing in water agar plates. They were covered with three sheets of foil paper and placed in the refrigerator (3 ^{o}C – 5 ^{o}C) for three days. They were then placed in growth cabinets with constant light (150 µmol m^{-2} s^{-1}) at a temperature of 20 °C and germination was scored after seven days.

2.10 Germination Assays of After-ripened Seeds Treated with Light

Seeds were sterilized and sown on water agar plates so that each batch of seeds from different plants are given three light treatments; FR/R, FR and FR-FR. The plates were left to imbibe in the presence of white light for three hours, after which they were placed in the light treatment box and LED irradiated. The following treatments were given.

i. FR/R- 5 mins pulse of FR light (4.5 µmol m^{-2}·s^{-1}) followed by another 5 mins pulse of R light (20 µmol m^{-2}·s^{-1})

ii. FR - 5 mins pulse of FR light

iii. FR-FR – 5 min pulse of FR light, followed by another 2 hours of FR light 2 days later.

All plates were wrapped in three layers of foil in the presence of green light and placed in the growth cabinet for 5 days under controlled environmental conditions at a temperature of 20 °C. Germination was scored after imbibing seeds for five days. After two days of imbibing in the dark, seeds treated with FR-FR were removed and given 2 hours FR light treatment. They were then wrapped again in three layers of foil and left in the growth cabinet. Germination was scored 3 days later.

2.11 Hypocotyl Elongation Experiment of Dark Grown Seedlings

Seeds were sterilized and plated on ½ MS media after which they were covered in three sheets of aluminium foil and stratified for three days at 4°C. The plates were removed on the fourth day and treated with 1 hour pulse of R light to induce germination. They were wrapped in foil again and placed in the growth cabinet in a standing position. Phenotypes were scored 5 days after imbibition in the dark by using a ruler to measure in cm the length of the hypocotyls of ten seedlings for each plant. This was converted in mm and the average length was calculated.

2.12 Phytohormone Extraction

After-ripened seeds (80-100 mg) of WT and *mft-2* were sterilized and sown on water agar plates to create 3 replicates of WT seeds to be treated with FR/R and another 3 replicates to be treated with FR light three hours after exposure to white light. All plates were wrapped in three layers of foil in the presence of green light and placed in the growth cabinet to imbibe for 12 hours under controlled environmental conditions at a temperature of 20 °C – 22 °C. Another set of WT seeds were given the same treatment and imbibed in the dark for 24 hours after light treatment. This was repeated for *mft-2* mutant seeds.

1ml extraction solvent (99:1 isopropanol:acetic acid) was pipetted into 2ml microfuge tube. 10µl of internal standard mix for GA and ABA (5µg/ml stock d2-GA4 and d6-ABA) 10 µl of Prostaglandin A1 internal standard for oxylipins (2 µg/ml stock) was added to the extraction solvent. These tubes were later kept on ice. Seeds were ground using the GenoGrinder and transferred to the tube containing 1ml solvent and internal standard. Another 900 µl of extraction solvent was added to the 2 ml microfuge tube after which they were vortexed and left overnight on a shaker at a speed of 250 rpm in a cold room in the dark. They were then centrifuged at 12000 rpm for 5 minutes at 4°C. Supernatant (1 ml at a time) was put in a screw-cap vial and dried in a speed vac. Samples were re-extracted with 1 ml of extraction solvent and placed in the rotating wheel in the cold room for 1 hour at 250 rpm, after which they were put in the centrifuge at 12000 rpm for 5 minutes at 4°C. Supernatant was dried once again in the screw-cap vial. Before analysis, 50 µl of methanol was pipetted into the dried extract and this was transferred

to a tapered vial and crimped for analysis. This was analysed by injecting 2 µL onto an ultraperformance liquid chromatography (UPLC)-MS, which has an Acquity UPLC system (Waters) connected to a Finnigan LTQ ion trap mass spectrometer (Thermo Electron). This analysis was carried out by Dr. Swen Langer, a technical specialist in the Centre of Excellence in Mass Spectrometry, University of York.

2.13 Seed Permeability Assay

Using the methods described in MacGregor *et al* (2014), freshly harvested seeds of WT, *mft-2* mutant, and CYP89A2oe were put in a 1% (w/v) solution of 2,3,5-triphenyltetrazolium chloride (Sigma Aldrich, cat. no. T8877-5G) in a 96-well plate to create three replicates of each set of seeds. They were then incubated in the dark at 30 ^{o}C for 48 hours. After moving the well plate from the incubator, a black velvet cloth was placed under the plate and a picture was taken using a digital camera.

2.14 Statistical analysis

Asteriks in figures specify the outcomes of two tailed Student t test that was carried out in Microsoft Excel. This was carried out to compare treatments, overexpressors, or mutant to the controls they correspond to.

cyp89A2-10 **LP**	**5' CCA TCA GTA GGC TGC TTC TTG 3'**
cyp89A2-10 RP	**5' GAG ATA GGG AGG GAT TCA GTG 3'**
LBb	**5' ATT TTG CCG ATT TCG GAAC 3' LBb**
c*yp89A2-1* For	5' TTA CAA TAT CTC CGA CTC GTC AAA 3'
cyp89A2-1 **Rev**	5' TCT CTT ACG CAG AAT CAG 3'
	GABI Pac LB 106 5' TTA CAA TTG AAT ATA TCC TGC CG 3'
	GABI Pac RB 106 5' GAC AGG ATA TAT TGG CGG GTA AAC 3'
cyp89A2-1 **For1**	5' CAC CTC TGT CGG CGG CGG GT 3'
cyp89A2-1 **Rev1**	5' GAG GAT ATC ATA GAA GCC AT 3'
cyp89A2-1 **For2**	5' TAC CGC ATC AGG CGC CAT TC 3'
cyp89A2-1 **Rev2**	5' CCA CTG TCG GCA GAG GCA TC 3'
	03144/35St GABI PAX 161 RB 5'GTG GAT TGA TGT GAT ATC TCC 3'
	08409 GABI PAC 161 LB 5'ATA ATA ACG CTG CGG ACA TCT ACA TTTT 3'
MEE26IR RP	5' CCG ATG AGC TTT ACA CTC TCG 3'
MEE26IR LP	5' TCT AAC CGA AAG CAC CAT GTC 3'
LBb	5' ATT TTG CCG ATT TCG GAAC 3' LBb
Actin For	5' TGA GAG ATT CAG ATG CCC AGA ACT 3'
Actin Rev	5' TGG ATT CCA GCA GCT TCC AT 3'
MFT For	5' ATC ACT AAC GGC TGC GAG AT 3'
MFT Rev	5' CGG GAA TAT CCA CGA CAA TC 3'
MEE26 For	5' TGA TCA GAA GCC GAG TGA TG 3'
MEE26 Rev	5' AAC GTA AAG CCA GGG 3'
CYP89A2 For	5' GTT AGG GAA CGA TCG GCA AC 3'
CYP89A2 Rev	5' TCC GTT ACC TCC TGA CCC TA 3'

Table 2.1: Sequence of Primers used in PCR and Q-PCR

CHAPTER 3: MFT Plays A Crucial Role In The Far-Red Light Mediated Seed Repression Of Germination

3.1 Introduction

Phosphatidylethanolamine-binding proteins (PEBP) are 21 kDa in size and were first identified in the brains of bovines (Bernier and Jollès, 1984). They are found in all taxa including bacteria, plants and animals (Bradley *et al*, 1996). The structure of the PEBP comprises of a single domain and a β fold. The domain is made up of two β sheets that are oriented in opposite directions and contain a Greek-key topology. It also has a C-terminus with an $\alpha\beta\alpha$ component. The two β sheets interact together via a hydrophobic interphase (Serre *et al*, 1998). Serre *et al* (1998) performed a crystallographic study to better understand the PEBP family and their functions. The study revealed that a single cavity was found close to the surface of the protein. It is made up of residues of the C-terminal helix as well as two regions known as CR1 and CR2 that connect each other. This small cavity appears to be the ligand binding site and the CR1 and CR2 regions have conserved residues in the whole PEBP family.

Even though the PEBP family broadly exists in all species, its function is still under investigation. There is still some uncertainty on whether it plays the same roles or different roles in all species. However, studies have shown that it does work with the machinery of cell-signalling (GTP binding protein), and it is situated in regions where cell growth takes place, i.e., developing brain and inflorescence meristem of flowering plants. It also has the ability to attach to biological membranes. All these signify that these proteins could function in modifying membrane structures during the process of cell growth, or they could act as messengers amidst cell membrane and cytosol. Furthermore, they are capable of binding hydrophobic ligands (Banfield *et al*, 1998).

The number of PEBP genes totally varies throughout all the species and this indicates the different roles played by the members of the family. In Arabidopsis, the PEBP family proteins consist of six members. These are FLOWERING LOCUS T (FT) and TWIN SISTER OF FT (TSF), which belong to the FT-like clade, TERMINAL FLOWER 1 (TFL1), BROTHER OF FT AND TFL1 (BFT) and

ARABIDOPSIS THALIANA CENTRORADIALIS (ATC), which are in the TFL1-like clade, and MOTHER OF FT AND TFL1 (MFT), which is within the MFT-like clade (Karlgren *et al*, 2011).

In plants, the genes of the PEBP family are involved in controlling the processes that are involved in the change from the vegetative stage to the reproductive stage and are also known to function in determining the architecture of plants (Kardailsky *et al*, 1999). In Arabidopsis, the most studied and well-known PEBP proteins are FLOWERING LOCUS T (FT) and TERMINAL FLOWER1 (TFL1). In Arabidopsis, *FT* and *TWIN SISTER OF FT* (*TSF*) are FT-like genes that are known to play a role in floral activation. Their mutants have been observed to have a delay in flowering time. During long days, the expression of *FT* and *TSF* are induced in the companion cells of the phloem. There is a partial genetic redundancy between *FT* and *TSF* in initiating flowering time. Also, *CONSTANS (CO)*, which encodes double B-box zinc-finger nuclear proteins, controls *FT*. *CO* is involved in connecting both temperature and light in the process of controlling flowering (Huq *et al*, 2000). The induced expression of *CO* results in the up-regulation of *FT* inside companion cells of the phloem (An *et al*, 2004) as well as the veins of leaves (Takada and Goto 2003) and this strongly promotes flowering (Samach *et al*, 2000). When *FT* or *CO* are ectopically expressed, this can lead to early flowering (Kardailsky *et al*. 1999). The control of flowering occurs through a photoperiodic pathway involving the transcripts of *GIGANTEA (GI)-CO-FT* to be expressed (Fujiwara *et al*, 2008).

Reports indicate that FT is a transferrable signal transducer called 'florigen' and that it can move from the phloem to the shoot apical meristems (SAM) (Jaeger and Wigge 2007) where it interacts with, *FLOWERING LOCUS D* (*FD*) which is a b-ZIP transcription factor (Abe *et al*, 2005). This causes an FT/FD complex that then binds to *APETALA1 (AP1)* to trigger the start of flowering (Abe *et al*, 2005). Transcripts of *TFL1* are located in the SAM, however studies have revealed that the proteins of TFL1 are capable of moving to lateral areas where they have an interaction with FD in order to repress *AP1* and *LFY*. This prevents floral initiation and also inhibits the inflorescence meristem from transforming into a flower (Ahn *et al*, 2006). Thus, FT and TFL1 are movable proteins that interact with FD and function significantly in the regulation of floral meristem and the formation of inflorescence shoot. BROTHER OF FT AND TFL1 (BFT) functions in controlling *FT-FD* interaction and could present an

adaptation system that adjusts photoperiodic flowering in environments with high salinity (Ryu *et al*, 2014). MFT is known to play a role in primary seed dormancy (Vaistij *et al*, 2013).

Tao *et al*, (2014) identified and characterized an *MFT*-like gene *JcMFT1* (an ortholog of *Arabidopsis MFT* from *Jatropha*) and its promoter was cloned and characterized in Arabidopsis seeds. The results revealed that *JcMFT* expression in *Jatropha* seeds and its promoter in Arabidopsis seed was up-regulated by ABA and this could mean that *JcMFT* has a G-box as well as an RY repeat in its promoter. Similarly, Li *et al*, (2014) discovered *GmMFT*, an *MFT* homolog in soybean, and showed that its expression increases in response to ABA and GA3 during seed germination. Xi *et al*, (2010) indicates *MFT* expression is up-regulated in response to ABA, furthermore, *mft-2* mutant is hypersensitive to ABA. In germinating seeds, ABI3, ABI5, and RGL2 control the expression of *MFT*.

Tao *et al* (2014) performed a GUS assay, which showed that *MFT* is highly expressed inside transgenic Arabidopsis seeds, similarly, Li *et al*, (2014), showed that *GmMFT* is also highly expressed in soybean seeds. Belmonte *et al*, (2013) carried out a study that described gene activity in various regions of Arabidopsis seeds from fertilization to maturity. This was done by dissecting sections of seeds using a Laser Microdissection system. RNA was extracted from them and analysed. Figure 3.1 shows the parts of a seed tissue where *MFT* is expressed. It is mainly expressed in the endosperm, but there is also high expression in the embryo and the seed coat, and low expression in the chalazal seed coat. Furthermore, MFT protein is localized in the nucleus (Xi *et al*, 2010).

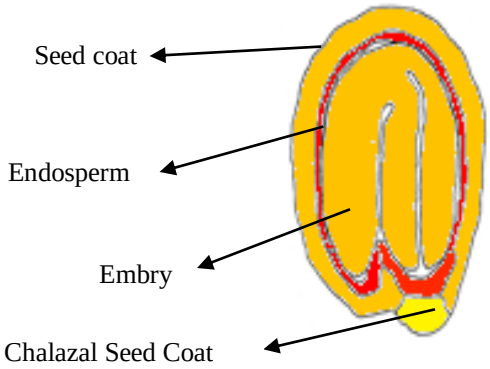


Fig 3.1: **Absolute expression of *MFT* in seed tissue**; Red colour indicates the maximum absolute expression level of this gene or very close to the maximum (main expression), orange corresponds to high expression i.e expression levels very close to the maximum absolute expression, and yellow indicates no expression or low expression i.e. absolute expression levels close to zero (Belmonte et al. 2013; eFP browser)

Because the sequence of *MFT* is highly homologous to *FT* and *TFL1*, Yoo *et al*, (2004) carried out a study to investigate if MFT functions in regulating flowering time in a similar manner to FT or TFL1. Gain-of-function and loss-of-function mutants were used, and the results indicate that when *MFT* is over-expressed flowering is enhanced. However, a loss of function mutant produces no observable phenotype. This shows that MFT promotes flowering and plays a redundant role in controlling period of flowering in Arabidopsis. Similarly, Hou and Yang (2016) also show that in the pteridophyte *Adiantum capillus-veneris*, the expression of *AcMFT* is controlled in a way similar to *FT* during long and short-day conditions. *AcMFT* was ectopically expressed in Arabidopsis and this promoted floral transition and also this partially complemented the delayed flowering in *ft-1* mutant. This shows AcMFT acts similarly to FT in controlling flowering time. However, expression pattern and transgenic analysis in studies that were later performed showed that MFT greatly functions in seed development more than the regulation of flowering time (Chardon and Damerval, 2005). Analysis performed by fluorometric GUS indicated the promoter of *JcMFT* was mostly active during mid and late stages of seed development, however, its activity gradually decreased after germination (Tao *et al*, 2014).

On the other hand, Chen *et al* (2014) carried out a study that shows that expression of *FT* is also related to reduced dormancy in Arabidopsis. Results from this study revealed that previous and current temperatures that have been experienced by the mother plant pass through to the FT locus inside phloem of siliques. FT then prevents the production of proanthcyanidins in fruits and this affects tannin accumulation in the seed coat. An altered tannin accumulation could result in reduced dormancy levels. *FLOWERING LOCUS C* (*FLC*), a gene that prevents flowering, acts downstream of FT during the process of seed dormancy. Chen and Penfield (2018) showed that maternal FLC was needed for the regulation of seed dormancy by FT. This indicates that the regulation of seed dormancy by FT and FLC occurs either in the process of carpel development or just after fertilization and this takes place in response to temperature changes.

Li *et al* (2014) showed that when *GmMFT* was ectopically expressed in Arabidopsis it prevented seed germination at an early phase, and thus it was proposed that *GmMFT* negatively regulates seed germination. Zhenghong *et al*, 2016 carried out a study to investigate the function of *HbMFT*, a homolog

of *MFT* in *Hevea brasiliensis.* The study revealed that overexpression of *HbMFT* in Arabidopsis seeds prevented germination and seedling growth. *HbMFT* mainly acts in regulating seed maturation and development of the stamen, however, it is a negative regulator of seed germination and seedling growth, as well as flowering. Nakamura *et al* (2011) show that in wheat, TaMFT acts to promote dormancy and Vaistij *et al* (2013) show that *MFT* plays an important role in promoting dormancy during seed development in Arabidopsis. On the other hand, Xi *et al*, 2010 showed that in *Arabidopsis*, MFT promotes germination in imbibed after-ripened seeds with exogenous and endogenous ABA.

The Graham lab has been involved in the study of the role of MFT in primary dormancy in the model plant *Arabidopsis.* The study reveals that *MFT* works to promote dormancy in freshly harvested seeds. Germination of after-ripened seeds depends on the quality of light, and it is known that FR light suppresses it. Therefore, this chapter will investigate whether the promotion of dormancy by MFT in after-ripened seeds is mediated by light.

3.2 Results

Far-red light induces MFT expression in a PIF1and SOM dependent manner

In the Graham Laboratory it was previously determined that the expression of *MFT* was dependent on PIF1 and SOM (Vaistij *et al*, 2018). This led to the hypothesis that the expression of *MFT* should increase in the presence of FR light. To test this hypothesis, RNA was extracted from WT seeds 24 hours after being treated with light. One light treatment included two short (5 minutes) and consecutive FR and R light pulses (FR/R) and the other treatment involved only a short 5 minutes pulse of FR (Figure 3.2a and 3.2b). After this, RNA was isolated, and cDNA was produced. Gene expression was analysed though qPCR. Figure 3.2c shows that there was approximately fifty times more expression of *MFT* in seeds treated with FR than those treated with FR/R light. Therefore, FR promotes the expression of *MFT*. The Graham lab had previously analysed the expression of *MFT* in the FR-light pathways. Their results showed that *MFT* expression was promoted by PIF1, the DELLAs, ABI5 and SOM (Vaistij *et al*, 2018).

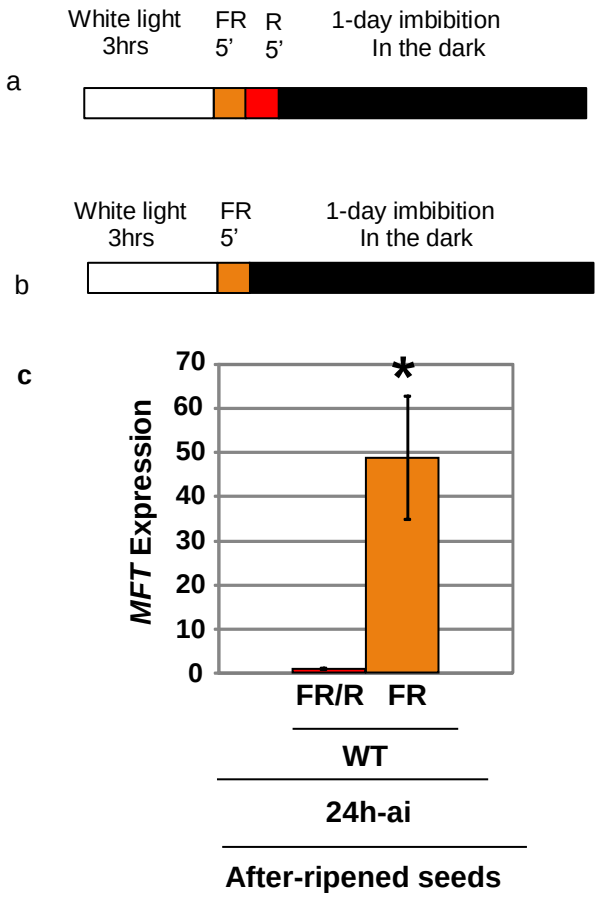


Figure 3.2: **Relative Expression of *MFT***. The schematic illustration of the plan of experiment (a, b) Relative *MFT* expression in after-ripened seeds of WT treated with FR/R and FR light(c). Gene expression represents fold-change in WT seeds treated with FR/R light. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to WT seeds treated with FR/R.

MFT represses germination in after-ripened seeds treated with light

The *MFT* expression profile led to the assessment of germination of seeds treated with light. Seeds of WT and *mft-2*, a loss–of–function mutant which is a TDNA insertion in the Columbia (Col) background, were treated with i) two short (5 minutes) and consecutive FR and R light treatments, ii) only one short FR pulse which deactivates phyB and represses germination, and iii) a first short FR pulse and a second long (2 hours) FR pulse (FR-FR) which activates phyA and partially compensates for the deactivation of phyB (Figure 3.3a). The first FR/R pulses do not affect phyA because it accumulates only after longer periods of imbibition. Furthermore, it needs longer FR exposure to be activated, i.e., it is less sensitive to light.

After-ripened seeds of WT and *mft-2* treated with FR/R all had high levels of germination (Figure 3.3b) because R light eliminates the repression of germination by FR light. As expected, the germination level

of WT seeds is reduced when treated with FR light, however, seeds of *mft-2* had higher levels of germination (there is no repression of germination). After treatment with FR-FR the germination level of WT seeds increased, and *mft-2* seeds germinated more than WT. This result indicates that MFT also functions to repress germination in after-ripened seeds treated with FR and FR-FR light.

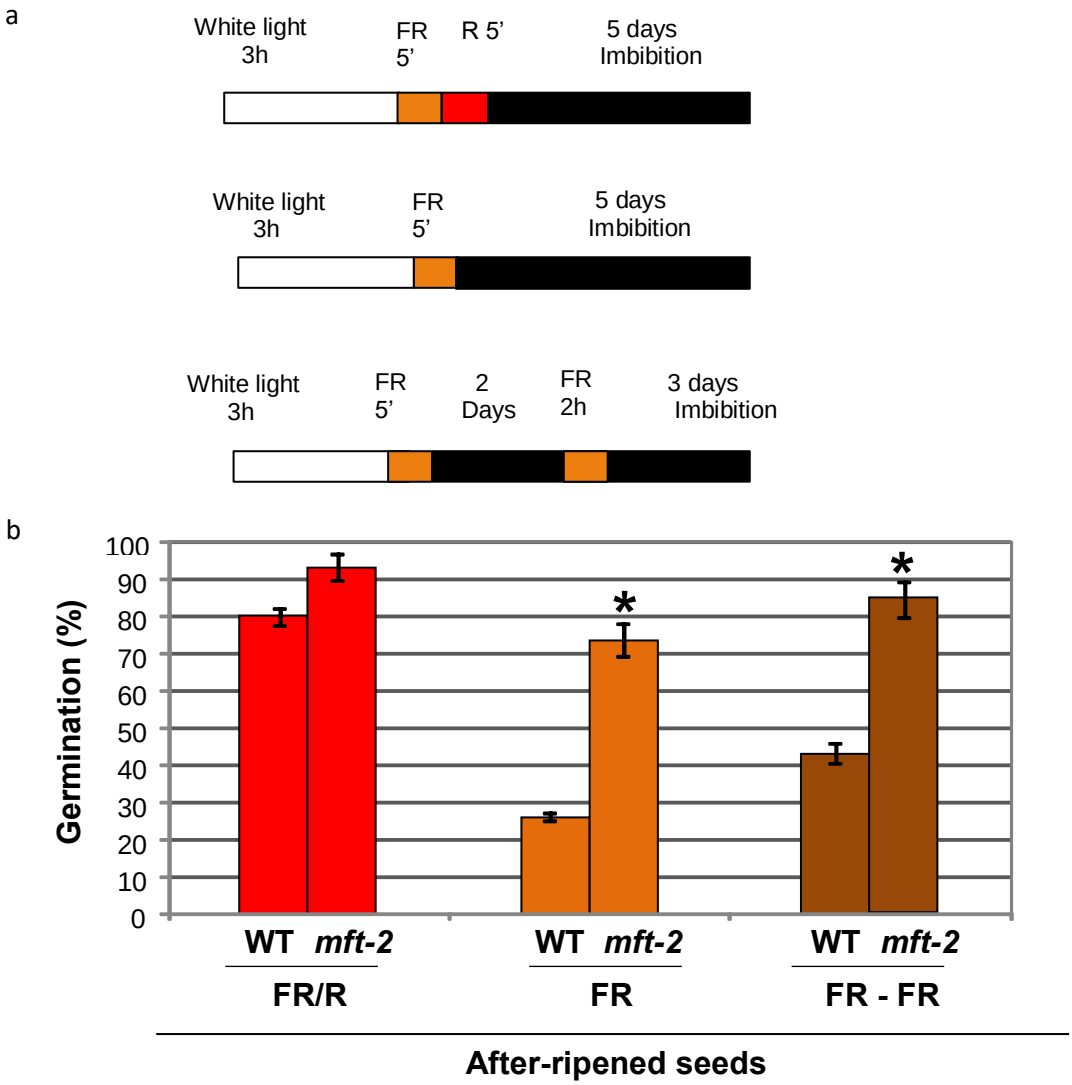


Fig 3.3: **Germination Assay of After-ripened Seeds** (a) Schematic illustration of plan of experiment (b) Germination WT and *mft-2* after-ripened seeds on water agar plates treated light; 5 min pulse of FR followed 5min pulse of Red (FR/R), only 5 min pulse of FR (FR), 5 min pulse of FR followed by 2hrs pulse of FR 2 days later (FR-FR). Germination was scored 5 days after imbibition. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

In wheat, *TaMFT* has been shown to be an important element in the promotion of dormancy (Nakamura *et al,* 2011). The wheat study used mature freshly harvested seeds that were non-dormant. Vaistij *et al,* (2013) have also shown that MFT promotes primary dormancy in Arabidopsis. This was confirmed by

carrying out germination assays on freshly harvested seeds of the *mft-2* and WT (Col). Results from this study revealed that seeds of the *mft-2* background germinated approximately twice more than WT (Figure 3.4). This shows that MFT promotes dormancy.

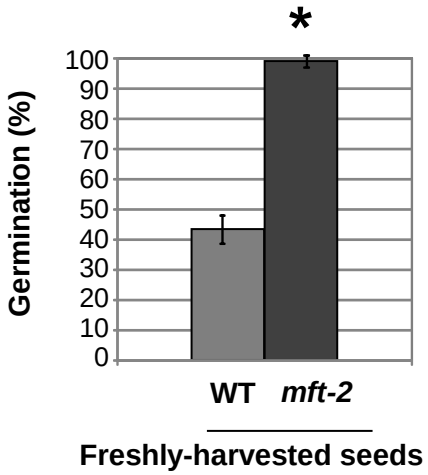


Figure 3.4: **Germination assay of freshly harvested seeds.** Stratified WT(Col) and *mft-2* seeds on water agar plates 7 days after imbibition (DAI). Error bars represent SD of at least four determinations. Asterisks over the bar specify statistically significant difference ($P < 0.05$) compared to its corresponding WT.

ABA and OPDA Cannot Explain the *mft-2* Phenotype

In order to obtain an insight into how MFT operates in repressing germination, relative levels of endogenous ABA and OPDA were measured in after-ripened and imbibed WT and *mft-2* seeds 12 hours and 24 hours after treatment with light. Figure 3.5 shows that WT seeds treated with FR light had higher levels of endogenous ABA (Vaistij *et al*, 2018), as previously demonstrated (Kim *et al*, 2008). This is because FR light impairs the degradation of endogenous ABA, which leads to its accumulation (Lee *et al*, 2012). This validates the experiment. Furthermore, *mft-2* seeds treated with FR/R and FR and imbibed for 12 hours had a greater than 2-fold increase in ABA compared to their corresponding WT. A similar increase in ABA can be observed in *mft-2* seeds treated with FR and imbibed for 24h compared to its corresponding WT. Seeds of *mft-2* treated with FR/R and imbibed for 24 hours had a 2-fold increase in endogenous levels of ABA compared to their corresponding WT. This is similar to what is described in freshly harvested dry seeds. It showed that, counter-intuitively, there is more ABA in *mft-2* mutant seeds (Vaistij *et al*, 2013).

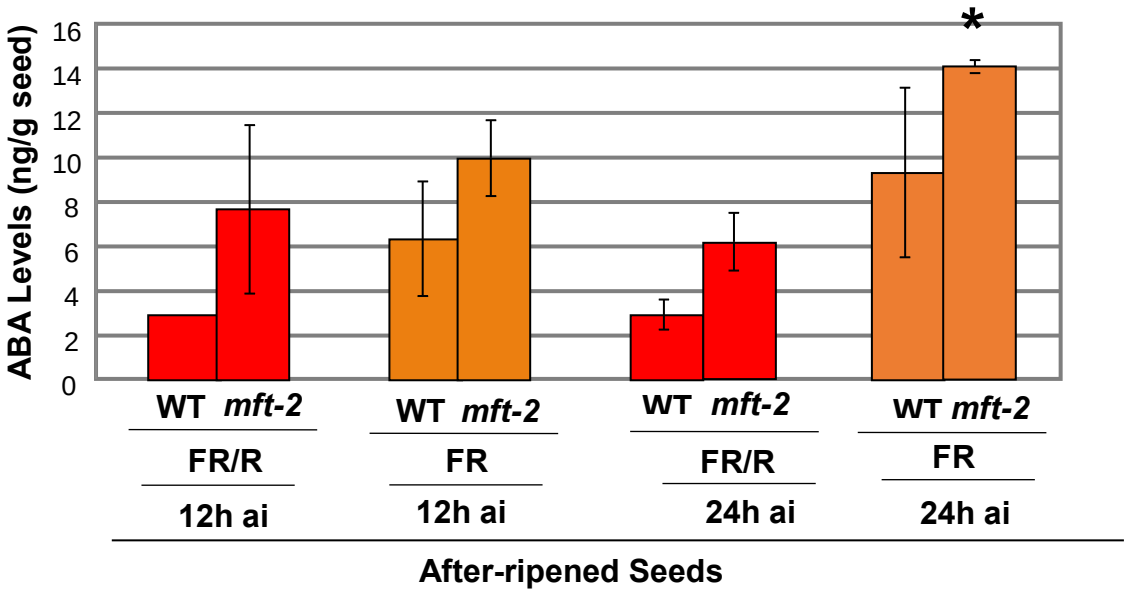


Figure 3.5: **Endogenous ABA levels in After-ripened seeds**. ABA levels in WT and *mft-2* imbibed and after-ripened seeds (12-24h ai) treated with FR/R and FR light. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to its corresponding WT.

Figure 3.6 shows that there was no significant increase in the level of endogenous OPDA in WT seeds treated with FR compared to those treated with FR/R in both 12h and 24h after light imbibition. In all conditions, there was no significant difference in endogenous OPDA levels in WT and *mft-2* seeds (Figure 3.6). Altogether, the results show that germination of the *mft*-2 seeds under FR light conditions was not due to a decrease in the level of the germination repressing phytohormones ABA and OPDA.

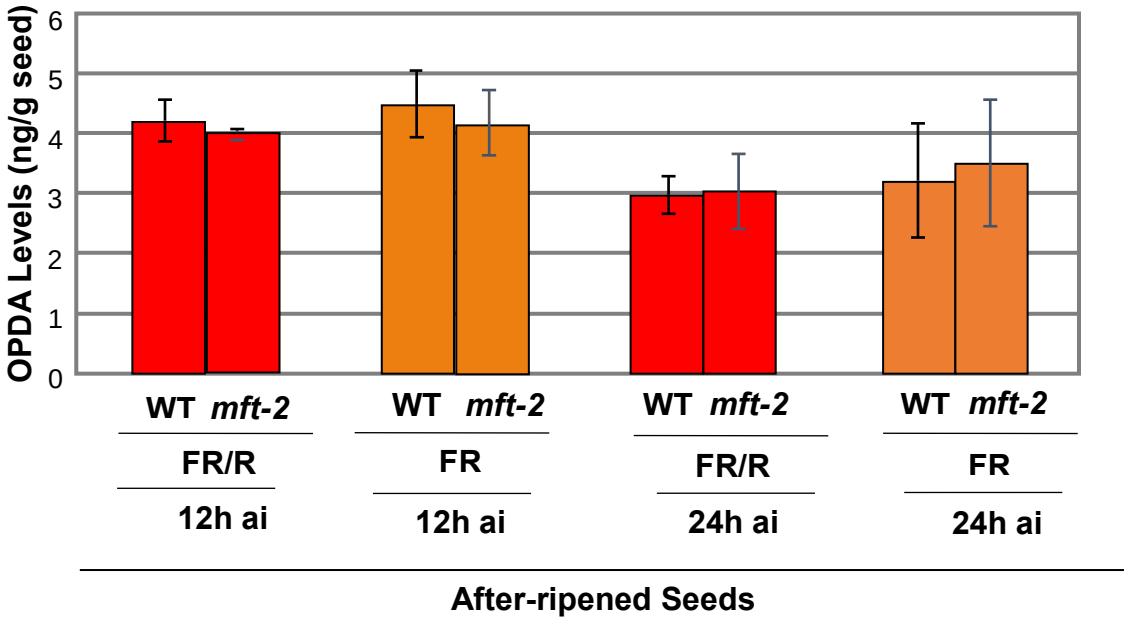


Figure 3.6: **Endogenous OPDA levels in After-ripened seeds**. OPDA levels in WT and *mft-2* imbibed and after-ripened seeds (12-24h ai) treated with FR/R and FR light. Error bars represent SD of at least three determinations.

MFT Promotes Hypocotyl Elongation in Dark-Grown Seedlings

The fact that the expression of *MFT* is regulated by light led to the decision to investigate if it regulates light signalling in seedlings. *MFT* expression was induced by FR light and PIF1, an important FR light component that also functions in hypocotyl elongation of dark grown seedlings. Thus, it was hypothesized that MFT will promote hypocotyl elongation. In this experiment, seeds were grown in the dark because PIF1 also functions in the dark (its ubiquitination by phyB is cancelled) (Leivar *et al,* 2008) similar to FR light conditions. Seedlings of SPT overexpressing transgenic line (*35S:SPT*) have been included in this experiment to be used as a tool for comparison, as it has an elongated hypocotyl when grown in the dark and is hypersensitive to FR light (Penfield *et al,* 2005).

To test hypocotyl elongation, WT, *mft-2,* and *35S:SPT* were treated as illustrated in figure 3.7a. As expected, the seedlings of *35S:SPT* had longer hypocotyls than WT. Hypocotyls of *mft-2* were shorter when compared to WT and *35S:SPT* (Figure 3.7b). Taken together this result indicates that *MFT* promotes hypocotyl elongation in the dark and this validates the hypothesis.

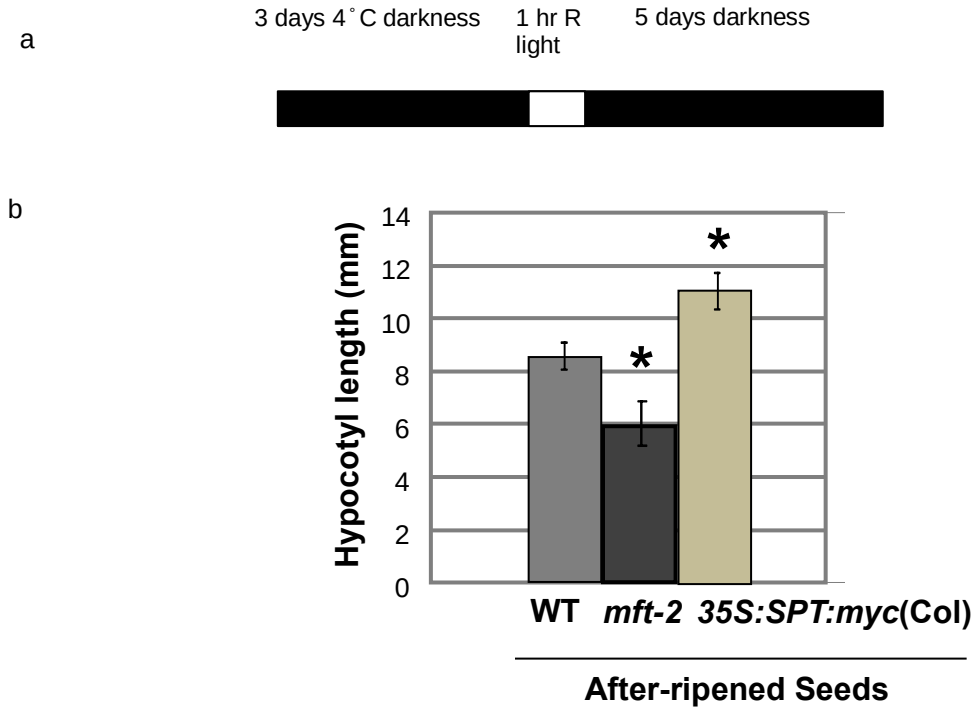


Figure 3.7: **Hypocotyl Length of Dark Grown seedlings**: (a) schematic illustration of the plan of experiment (b) seedling morphology of dark-grown seeds of WT, *mft-2,* and *35S:SPT.* Error bars represent SD of at least ten determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

3.3 Discussion

Germination of after-ripened seeds is dependent on the quality of light and it is known that FR light suppresses germination. MFT also supresses germination in both freshly-harvested and after-ripened seeds. It was hypothesized that the suppression of germination by MFT in after-ripened seeds is mediated by light. The *MFT* expression profile indicated that it is induced by FR light. Furthermore, after assessing germination of WT and *mft-2* seeds treated with light, it was observed that under FR and FR-FR, *mft-2* seeds hd higher germination frequency compared to WT. This validates the hypothesis and indicates that MFT is required for the FR inhibition of germination.

The Graham lab had previously shown that the induced expression of *MFT* by FR light is in a PIF1 and SOM dependent manner, hence the reduced expression of *MFT* in *pif1* and *som-3* mutants. This is in agreement with the fact that MFT, PIF1 and SOM all act to negatively regulate seed germination. PIF1 works in the FR signalling pathway by promoting ABA-response transcription factors like *ABI5,* and the DELLA genes. Previous work in the Graham lab showed that *MFT* is also promoted by ABI5 and the DELLAs, which are all repressors of germination. This shows that *MFT* fits in the FR light signalling pathway (Figure 3.8).

PIF1, SOM and DELLA mutants germinated under FR light and this could be due to the fact that they all regulate *MFT*. However, Piskurewicz *et al,* (2009) demonstrated that *abi5* mutants do not germinate under FR light, even though ABI5 promote *MFT*. This suggests that the decreased expression of *MFT* in the *abi5* is not enough to stimulate germination. This shows that it is more complex than the model proposed in figure 3.8.

Levels of endogenous ABA were measured in *mft-2* seeds in order to investigate the process by which MFT operates to repress germination. Surprisingly, the results show that the *mft-2* mutant had higher levels of ABA compared to WT. A similar experiment was previously conducted in freshly matured dry seeds by Vaistij *et al,* (2013) and a 2-fold increase was reported for *mft-2* mutant compared to WT. Xi *et al,* (2010) reported that MFT negatively regulates ABA signalling through the suppression of *ABI5*. Thus, after-ripened seeds of *mft-2* mutant have low germination frequencies with the application

of exogenous ABA, i.e., they are hypersensitive to ABA. This might imply that MFT proteins work to render seeds insensitive to ABA. However, the high germination rates of after-ripened *mft-2* seeds treated with FR light would not have been possible if the mutant was hypersensitive to endogenous ABA, especially in consideration of the fact that FR light induces ABA, hence *mft-2* should germinate less than WT under FR light. Furthermore, *mft-2* seeds have more ABA, thus if they were hypersensitive to ABA, they wouldn't germinate more than WT.

Levels of OPDA have also been measured in the *mft-2* mutant. The result in figure 3.6b shows that there was no difference in the endogenous levels of OPDA in *mft-2* compared to WT in FR/R and FR light treatment conditions. Altogether, this indicates that MFT does not repress germination in freshly harvested and after-ripened seeds by regulating ABA and OPDA, thus they cannot explain the less dormant phenotype of the *mft*-2 mutant. This, and the fact that ABA and OPDA promote *MFT* expression, indicates that MFT acts downstream of the ABA and OPDA signalling pathways.

The experiment on hypocotyl elongation of seedlings grown in the dark shows that *mft-2* seedlings had shorter hypocotyls compared to WT, and this indicates that *MFT* promotes hypocotyl elongation in dark grown seedlings. This does not come as a surprise because *MFT* expression is promoted by PIF1, which in turn promotes hypocotyl elongation of dark grown seedlings. When seeds are grown in the dark or treated with FR light, phyB is repressed which in turn suppresses PIF1. PIF1 suppresses germination, but promotes hypocotyl elongation (Leivar *et al,* 2008; Shin *et al,* 2009). Elongated hypocotyls are essential for seedlings grown in the dark to search for light (Josse and Halliday, 2008). *MFT* and PIF1 have similar roles or functions in that they both repress germination and promote hypocotyl elongation.

The study on *MFT* has resulted in the formation of a model (Figure 3.8). FR promotes the accumulation of PIF1, which in turn promotes *SOM.* PIF1 also promotes the DELLAs and elevates ABA levels, which stimulate *ABI3* and *ABI5*. The expression of *MFT* is induced by PIF1, *SOM,* the DELLA genes, OPDA and ABA transcription factors, especially *ABI5.*This shows that in the FR light signalling pathway, MFT is acting in conjunction with, but perhaps not exclusively to, PIF1, *SOM,* the DELLAs, ABA and

OPDA to act as a potent dormancy promoter in Arabidopsis. Furthermore, it could also be working with PIF1 to promote hypocotyl elongation in dark grown seedlings.

In conclusion, these data shows that MFT promotes dormancy in freshly harvested seeds and represses germination in after-ripened seeds. FR light mediates the repression of germination by MFT in after-ripened seeds. In addition to this, it promotes hypocotyl elongation in dark grown seedlings, and thus shares a similar phenotype with PIF1. The *mft-2* phenotype cannot be explained by phytohormones like ABA, OPDA and RGL2. Thus, further investigation needs to be performed to shed more light on this.

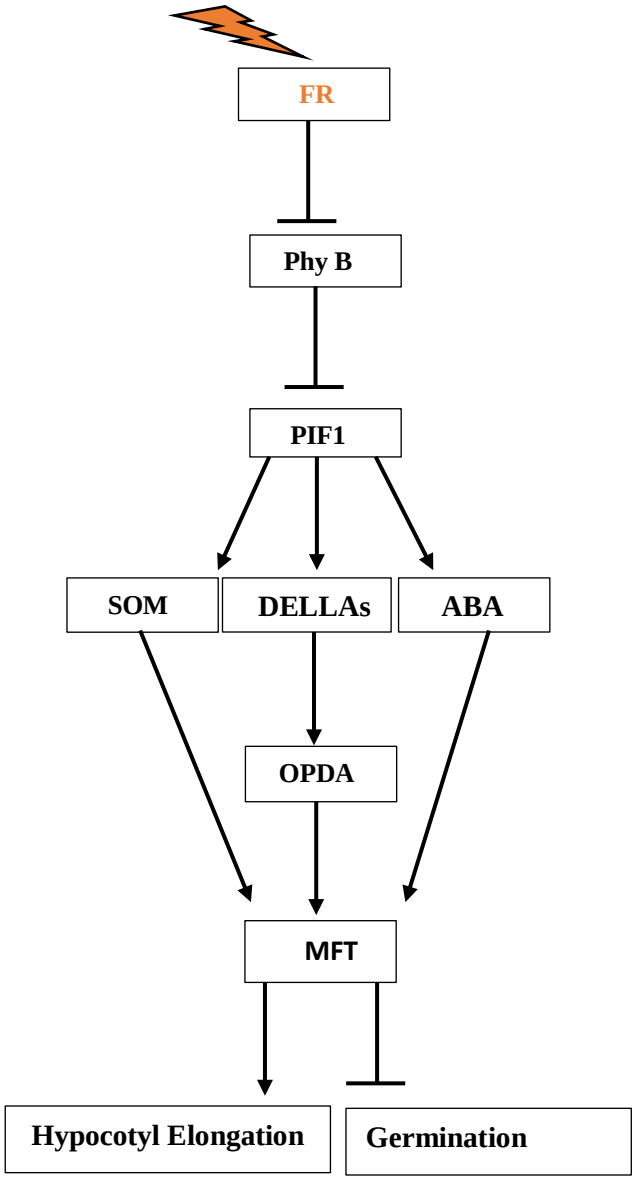


Figure 3.8: **A Proposed model illustrating how *MFT* fits into the FR light signalling pathway**. The results showed that MFT promotes dormancy in after-ripened seeds treated with FR light. This is consistent with the fact that PIF1, *SOM,* ABA, OPDA and the DELLAs which are all negative regulators of germination all promote *MFT.* This leads to suppression of germination and promotion of hypocotyl elongation.

CHAPTER 4: MEE26 is a Positive Regulator of Far-red Light Mediated Seed Dormancy and is Repressed by MFT

4.1 Introduction

The Graham laboratory performed transcriptomic analysis in order to understand how MFT functions. The analysis was carried out on three pairs of *mft-2* vs WT comparisons (i. freshly matured dry seeds developed at 15^{o}C; ii. freshly matured (developed at 15^{o}C) imbibed seeds in the dark for 7 days at 4^{o}C; and iiii. imbibed seeds in the dark for 7 days at 4^{o}C to identify genes that were deregulated in the *mft-2* mutant). For each of these conditions, the comparisons were between WT and *mft-2*. In the results of the transcriptomic analysis, *Maternal Effect Embryo Arrest 26 (MEE26)* was the first gene in the list that was up-regulated in the *mft-2* mutant. In the initial comparison (freshly matured dry seeds developed at 15^{o}C) , the expression of *MEE26* in *mft*-2 was 383 times more than its expression in WT. There is 53 and 20-fold increased expression of *MEE26* in *mft-2* compared to WT in the second and third comparisons respectively (see table 1 in appendix 1). Furthermore, compared to all other genes in the list, *MEE26* had the lowest level of expression in WT. The next gene down the list is AT4G24480, which is part of the Mitogen Activated Protein Kinase Kinase Kinase (MAP3K), a serine/threonine specific protein kinase (Adam-Phillips *et al,* 2004). AT4G24480 is known to be involved in protein phosphorylation (Ichimura *et al*, 2002). In the first comparison, AT4G24480 is expressed 29 times more in *mft-2* compared to WT. There is approximately 12 and 5.5 times more expression of AT4G24480 in *mft-2* compared to WT in the second and third comparisons respectively.

Arabidopsis contains approximately 70 Maternal Effect Embryo Arrest (MEE) genes (Pagnussat *et al,* 2005) which are part of the Gametophyte Maternal Effect (GME) class. GME are expressed in the gametophyte and are vital for embryo or endosperm development. Many mutants that have abnormalities in embryo sac development as well as other reproductive activities in Arabidopsis are called GME mutants (Pagnussat *et al,* 2005). Apart from the *mee* mutants, which form the largest group of GME mutants, *fis* class of mutants and the *capulet* mutants also form part of this group of mutants (Ngo *et al,* 2012).

Mutations that are inherited from the mother and affect the subsequent generation are known as Maternal Effect. Maternal Effect genes act predominantly during early development in many animal species (Riechmann and Ephrussi, 2001), however, in plants this is uncertain due to 'alteration of generations'. The life cycle of plants interchanges between two generations: a haploid gametophyte and a diploid sporophyte. The most prominent generation in angiosperms is the sporophyte (the flowering plant). The gametophytes go on to form cells enclosed by sporophytic tissues. The pollen grains are the male gametophytes and they form in the anther, whilst the embryo sac, the female gametophyte, forms inside the ovules. A diploid zygote forms after fertilization of the egg cell takes place in the embryo sac. This creates the sporophytic generation. Therefore, maternal contributions can take place in flowering plants from the diploid sporophyte and the haploid female gametophyte (Chaudhury and Berger, 2001). The maternal sporophyte influences the transfer of nutrients to the endosperm and also controls the size of seeds (Garcia *et al*, 2005), thus, when any defects occurs in this process, it leads to maternal sporophytic effect on development of seeds (Gutierrez-Marcos *et al*, 2004). The maternal gametophytic tissues control the activities of genes expressed in the gametophyte, which are essential for the development of the endosperm and the embryo. Analysis of publicly available data (Belmonte *et al*, 2013; and Vseed website) showed that, in newly developed seeds, *MEE26* is mainly expressed in the embryo with low expression in the endosperm, seed coat, and chalazal seed coat (Figure 4.1).

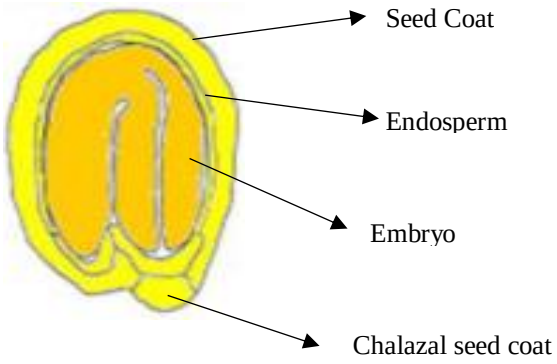


Figure 4.1: ***MEE26* expression in seed tissue**; Orange corresponds to high expression i.e expression levels very close to the maximum absolute expression value, and yellow indicates no expression or low expression i.e. absolute expression levels close to zero (Belmonte et al. 2013; eFP browser)

There is little research or detailed documentation on *MEE26*, and therefore, its role in the process of seed dormancy and germination is yet to be discovered, however, it has been mentioned in a publication

by Pagnussat *et al* (2005). Forward genetic screening was performed using transposon (D) populations in order to identify the genes that are involved in female gametogenesis and early development of the embryo. After double fertilization occurs, the female gametophyte produces the diploid embryo and the triploid endosperm, which further develops to form the mature seed. A large number of mutants having defects in female transmission were studied and approximately 130 mutants were characterized phenotypically, and the genes that were mutated were determined. This provided information on the roles the genes play in female gametophyte development and the alteration to sporophytic generation.

This study by Pagnussat *et al* (2005) discovered 8 mutants that were arrested at later stages of embryo development and had a minor delay in the development of the embryo sac and a maximum of 12 hours delay in fertilization. They also had varying levels of endosperm development. Further analysis confirmed that the embryo arrest observed was due to mutations in the female gametophytes and these mutants carrying defects in embryogenesis were named as MEE mutants. The genes that were disrupted in the female gametophyte mutants were grouped according to the phenotypes they produced and *MEE26* was in the group having an arrested endosperm development phenotype.

The female gametophyte keeps a strong contact with the sporophyte for its protection and nourishment as it develops. Studies have shown that the development of saprophytic tissues is also necessary for the occurrence of double fertilization and for seeds to form. However, there is insufficient information on the probability of female gametophyte functioning as a controller of activities of genes in the sporophyte. Thus, Armenta-Medina *et al* (2013) carried out research in order to investigate how the gametophyte regulates the surrounding sporophytic tissues of the ovule. Global expression profiling of the ovules of WT was compared with a mutant not having female gametophyte, *nozzle/sporocyteless* (*spl*), of Arabidopsis in order to find genes that are suppressed by the female gametophyte inside sporophytic tissues. Genes that were overrepresented in the mutant ovule in comparison to WT were identified and *MEE26* was among the genes whose expression was induced in *spl* ovules in comparison to wild-type. Previously, Johnston *et al*, (2007) had used microarray-based comparisons to identify *MEE26* among 527 genes that could be upregulated in gametophytic mutant *coatlicue* (*coa*), which also contained ovules lacking a female gametophyte.

MEE26 was also mentioned in another study carried out by Kinoshita *et al,* 2010. In this study, the process of early growth in which mature seeds grow into young seedlings was examined. This normally takes place within a limited time window of 48 hrs after seed have been stratified. In this time, osmotic stress or the application of exogenous ABA that triggers osmotic stress could occur and this could severely hinder growth (Lopez-Molina *et al,* 2001). Embryos whose growth is arrested express genes that encode for late embryonic proteins like Late Embryogenesis Abundant (*LEA*) genes such as *AtEm1* and *AtEm6*. These genes keep the seed osmotolerant in the presence of osmotic stress or exogenous ABA (Lopez-Molina *et al*, 2001 and 2002). In order to comprehend the physiological and developmental functions of this short period, Kinoshita *et al,* (2010) used genetic screening to identify mutants in seedling establishment that were insensitive to ABA. The *growth insensitive to ABA3* (*gia3*) was identified from this genetic screening. This mutant provided a novel signalling pathway that is dependent on ABA and is also molecularly and phenotypically separate from ABI5. It was discovered, through transcriptomic analysis, that throughout early seedling growth the expression of *MEE26* was promoted by ABA in WT and *abi5*, however, its expression was not induced in *gia3*. This indicates that GIA3 promotes *MEE26.* Analysis of *abi5* mutant seeds demonstrated that ABA triggered expression of *MEE26* is independent of ABI5. Furthermore, the GIA3 dependent expression of *MEE26* is also independent of ABI5.

In the present study, the role of MEE26 in seed dormancy/germination was investigated using silencing and overexpressing lines. It was hypothesized that because MFT represses *MEE26*, then MEE26 could function in promoting germination, thereby explaining the *mft*-2 phenotype.

4.2 Results

FR Promotes Expression of MEE26

The results of the transcriptomic findings were first validated. RNA was extracted from freshly harvested seeds of WT and *mft-2,* cDNA was made and *MEE26* expression was analysed by using quantitative RT-PCR. There was approximately 35 times more expression of *MEE26* in *mft-2* than in

WT seeds (Figure 4.2). The result of the RT-PCR shows that MFT represses *MEE26*, which confirms the findings of the transcriptomic analysis.

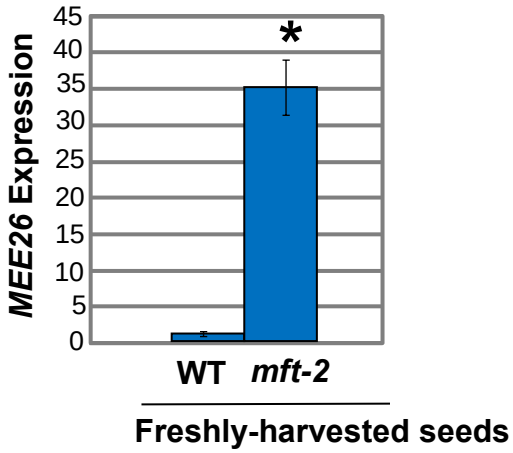


Figure 4.2: **Relative *MEE26* expression.** *MEE26* expression in freshly harvested seeds of WT (Col) and *mft-2*. Gene expression represent fold change to WT. Error bars represent SD of at least three determinations. Asterisks over the bar specify statistically significant difference ($P < 0.05$) compared to its corresponding WT.

In the previous chapter, it was shown that the expression of *MFT* in WT (Col) is up-regulated by FR light in comparison to FR/R light. Since FR promotes *MFT*, and MFT in turn suppresses *MEE26*, it was hypothesized that FR will also suppress *MEE26*. To test this hypothesis, *MEE26* expression was assessed in after ripened seeds of WT and *mft-2* treated with FR/R and FR as described in chapter 3 (Figure 3.5). Seeds of *mft-2* mutant treated with FR/R light showed an approximately 60-fold increase in expression of *MEE26* compared to WT seeds treated with FR/R light (Figure 4.3a). This is in agreement with the results in freshly harvested dry seeds (Figure 4.2) and the transcriptomic analysis and indicates that MFT represses *MEE26*. Surprisingly, there was approximately a 15-fold increase of *MEE26* in WT seeds treated with FR light compared to WT seeds treated with FR/R light (Figure 4.3a). This invalidates the hypothesis and shows that *MEE26* is promoted by FR light. In the *mft-2* mutant, there was approximately 6 times more expression of *MEE26* in seeds treated with FR light compared to those treated with FR/R light (4.3b). This indicates that MFT hinders the induced expression of *MEE26* by FR light.

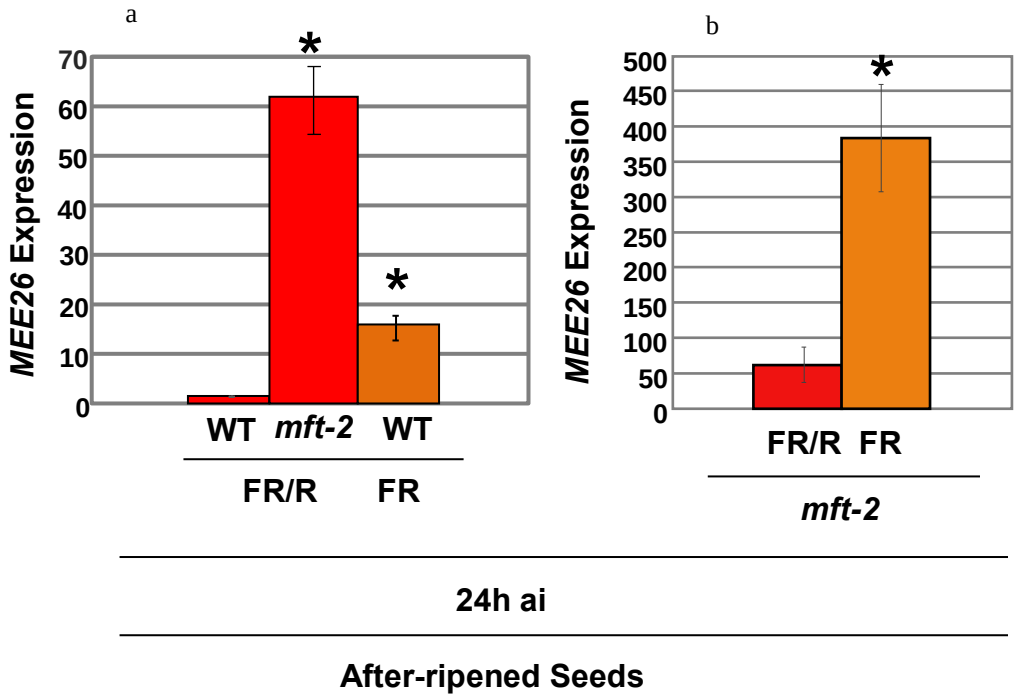


Figure 4.3**: Relative *MEE26* expression in after-ripened seeds**. *MEE26* expression in after-ripened WT and *mft-2* seeds treated with FR/R and FR light and imbibed for 24h. Panels a and b are the same analyses represented in different scales. Gene expression represent fold-change to the lowest value. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

The fact that *MEE26* expression is induced by FR light prompted the decision to check if it is also induced by other FR-pathway factors, like PIF1. Figure 4.4a shows that there was approximately four-fold increase in expression of *MEE26* in WT compared to *pif1-1,* this therefore indicates that PIF1 promotes *MEE26.* Because PIF1 works by promoting the expression of positive ABA signalling component genes and negative GA signalling component genes in the FR signalling pathway, the expression of *MEE26* was analysed in *abi5-7* and *della4* (*gai-6rga-2rgl1-1 rgl2-1* quadruple-mutant). There was approximately 13 times lower expression of *MEE26* in *abi5-7* mutants compared to WT and 5.5 times lower in *della4* quadruple-mutant. (Figure 4.4b and 4.4c). This indicates that the ABA-response transcription factors and the DELLA proteins both induce *MEE26* expression. *MEE26* expression was also analysed in the *som-3* mutant. PIF1 also promotes *SOM,* thus, it was decided to check expression of *MEE26* in *som-3* mutant. Surprisingly, there was approximately four-fold increase of *MEE26* expression in *som-3* compared to WT (Figure 4.4d). This indicates that SOM represses

MEE26 expression. Taken together, the promotion of *MEE26* by FR is dependent on PIF1 but not on SOM.

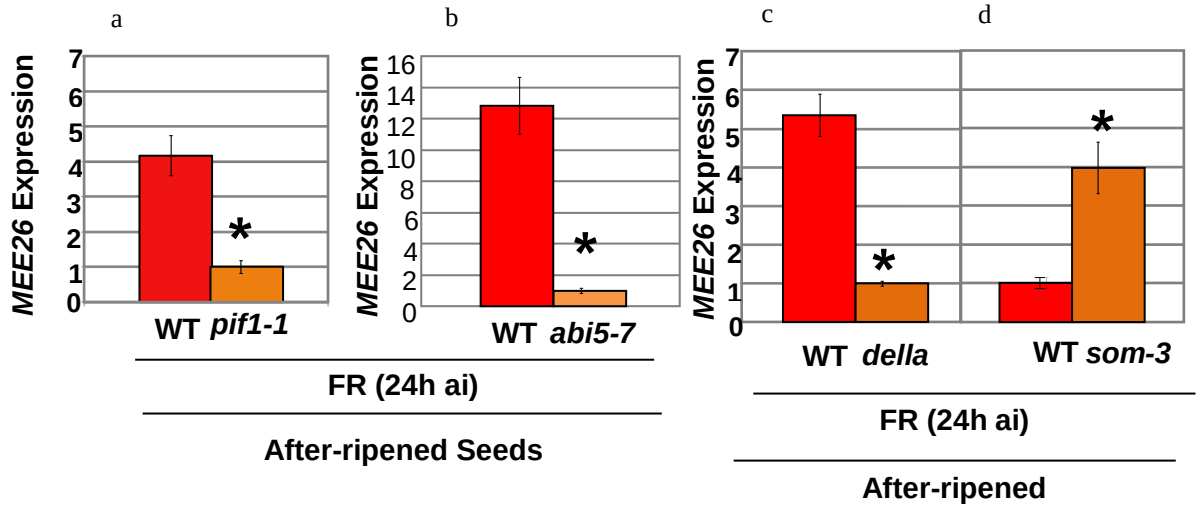


Figure 4.4: **Relative *MEE26* expression** in after-ripened seeds of (a) *pif1-1,* (b) *abi5-7,* (c) *della4,* and (d) *som-3* mutants treated with 5' pulse of FR light. Seeds were collected 24h after imbibition (ai). Gene expression represent fold-change to the lowest value Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

MEE26 Promotes Germination

Analyses of *MEE26* Silenced Lines

In order to investigate the probable role that MEE26 performs in the process of seed germination, *mee26* mutant seeds should be studied. However, there were no available *mee26* mutants and there are no T-DNA insertion lines available in The Arabidopsis Information Resource (TAIR). Hence prior to my arrival in the Graham laboratory, transgenic lines were made and designed to silence *MEE26* by RNAi through the expression of an inverted repeat (IR) of a specific region of the *MEE26* ORF (see appendix 2). Because *MEE26* is up-regulated in the *mft-2* mutant, the IR transgenic lines were made in the *mft-2* background in order to investigate if the high expression of *MEE26* could explain the low dormant phenotype of *mft-2*.

To confirm that *MEE26* was indeed silenced in these transgenic lines, its expression was assessed in freshly harvested dry seeds of 5 independent homozygous lines. This was compared to *mft-2,* because these transgenic lines were made in the *mft-2* background. Figure 4.5 shows that these lines had various levels of *MEE26* silencing ranging from 40-80%. It should be noted that the transgenic plants showed

no obvious growth or developmental phenotypes from the time that they were sown to the point of harvesting.

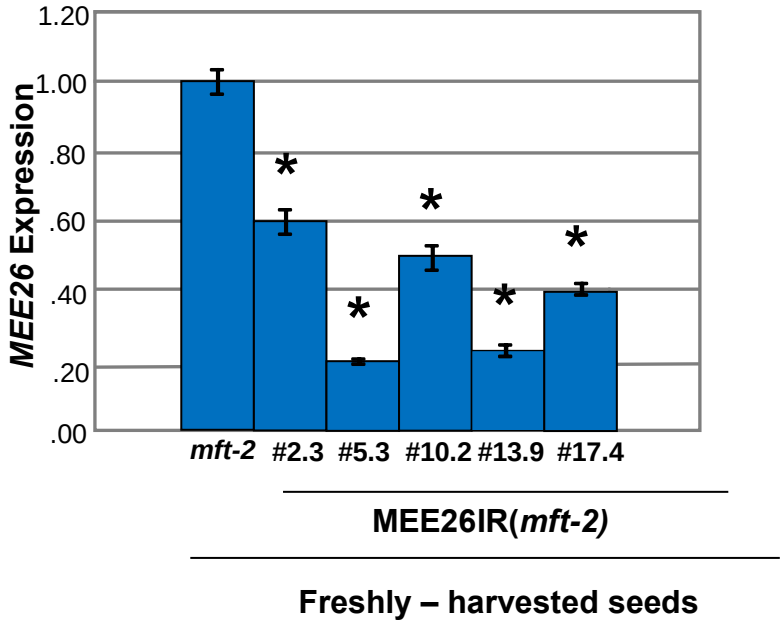
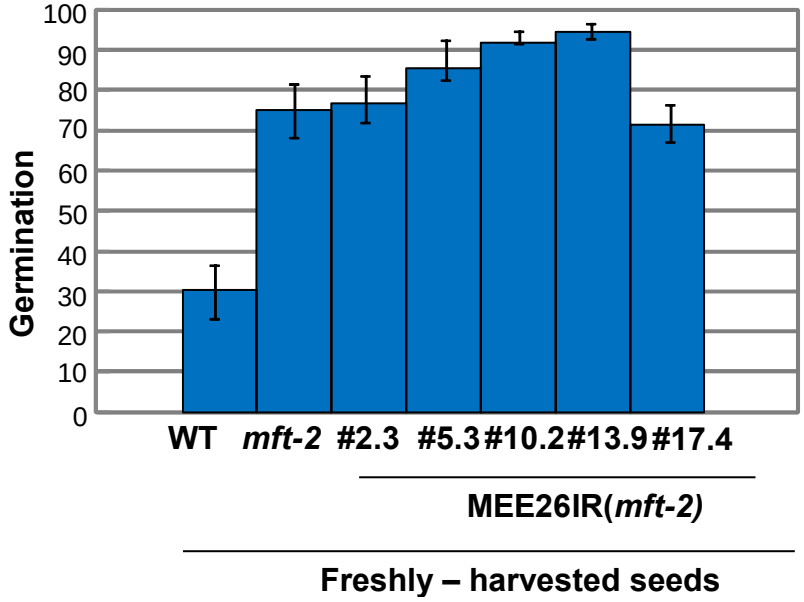


Figure 4.5: **Relative transcript levels of *MEE26*.** *MEE26* expression in the MEE26IR lines of the *mft-2* background. Gene expression is relative to fold change in *mft-2* values. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to the corresponding WT.

Germination phenotypes were assessed in the silenced lines to ascertain if the low dormancy phenotype of the *mft-2* seeds is due to the high expression of *MEE26*. Characterization of the phenotypes was carried out on freshly harvested seeds of the silencing lines along with WT and *mft-2*. All the lines analysed germinated at the same or slightly higher levels than *mft-2* seeds regardless of the degree of *MEE26* silencing in each of the transgenic lines (Figure 4.6a). Therefore, the low dormant phenotype of *mft-2* is not affected by the level of reduced expression of *MEE26*.

This phenotypic characterization was also extended to after-ripened seeds treated with light FR/R, FR, FR-FR as in chapter 3, figure 3.3a. All seeds treated with FR/R light had high germination frequencies. As in freshly harvested seeds, all the after-ripened seeds of MEE26IR lines germinated at levels similar to the *mft-2* mutant when treated with FR and FR-FR light (Figure 4.6b), thus the *mft-2* high germination rates are not compromised by the decreased transcript accumulation of *MEE26* in the silenced lines.

a



b

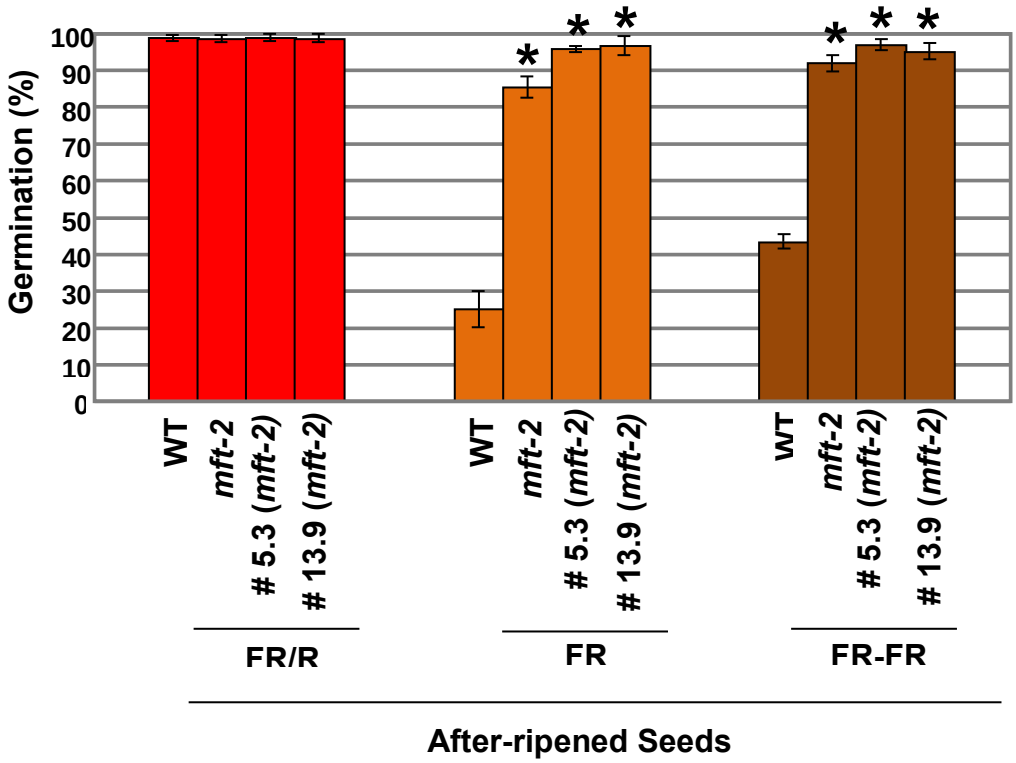


Figure 4.6: **Germination assays**: (a) Stratified freshly harvested seeds of WT, *mft-2* and MEE26IR lines in the *mft-2* background on water agar plates 7 days after imbibition (DAI) (b) after-ripened seeds of WT, *mft-2*, and 2 MEE26IR lines on water agar plates treated with light; 5 min pulse of FR followed 5min pulse of Red (FR/R), only 5 min pulse of FR (FR), 5 min pulse of FR followed by 2hrs pulse of FR 2 days later (FR-FR). Germination was scored 5 days after imbibition. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

In order to investigate a possible role of MEE26 independent of MFT in seed germination, line MEE26 13.9 which was the most silenced, was crossed to WT and homozygous plants were obtained (see

appendix 3 for description on how this was achieved) in order to remove the *mft-2* or bring the *MFT* to WT levels (see appendix 3 for description on how this line was crossed to WT). After crossing and further analysis, line 13.9(4) was identified as being homozygous for WT.

To confirm that *MEE26* has been silenced in MEE26IR 13.9, its expression was analysed in plants # 4.1 using RT-PCR and this was compared to WT. Figure 4.7a shows that there was approximately three-fold decreased expression in the transgenic line analysed. Expression of *MFT* was also assessed and surprisingly, there was a three-fold decrease compared to WT (Figure 4.7b). This indicates that MEE26 promoted *MFT* expression.

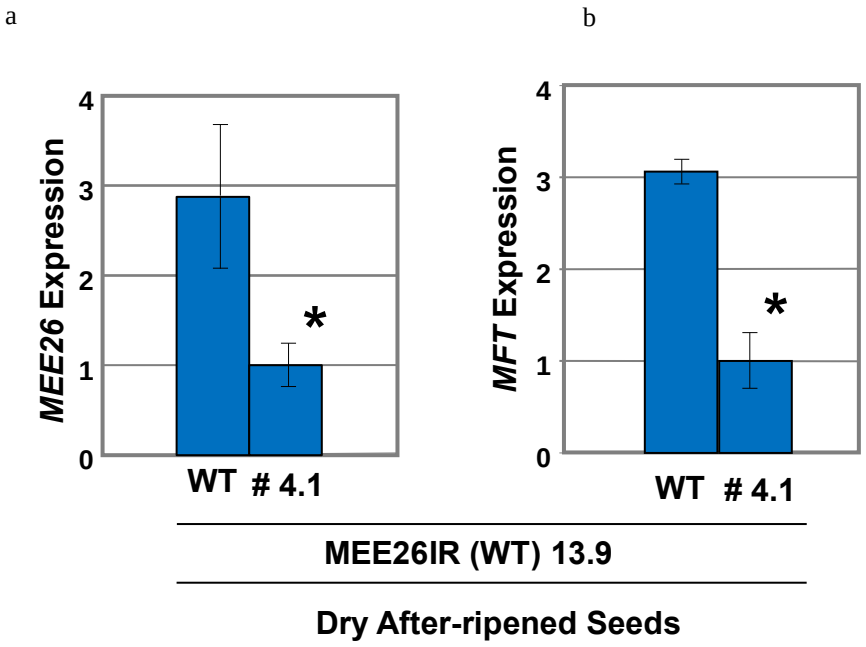


Figure 4.7: **Relative transcript levels of**: (a) *MEE26* in line 4.1 compared to WT (b)*MFT* in line 4.1 compared to WT. Gene expression is relative to fold change in # 4.1 values Germination was scored 5 days after imbibition. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

Germination rate was then assessed for MEE26IR (WT) 13.9. Freshly harvested T4 seeds of two sibling plants (# 4.1 and #4.4) were used for this experiment. Figure 4.8a shows that while WT seeds were extremely dormant, *MEE26* silenced seeds germinated 40 – 70%. In this experiment WT is extremely dormant and this could be due to the fact that these plants (WT and transgenic lines) were grown during the winter in the glasshouse. This is a season in which complete dormancy sets in to help plants overcome this harsh environmental condition, thus it is a survival mechanism. The same

experiment was repeated during the spring as conditions in the glasshouse may vary between winter and spring. In this experiment, WT seeds germinated at approximately 50%, whereas MEE26IR (WT) # 4.1 germinated 80% (Figure 4.8b). Taken together, this experiment shows that, contrary to our hypothesis, MEE26 is required to promote dormancy.

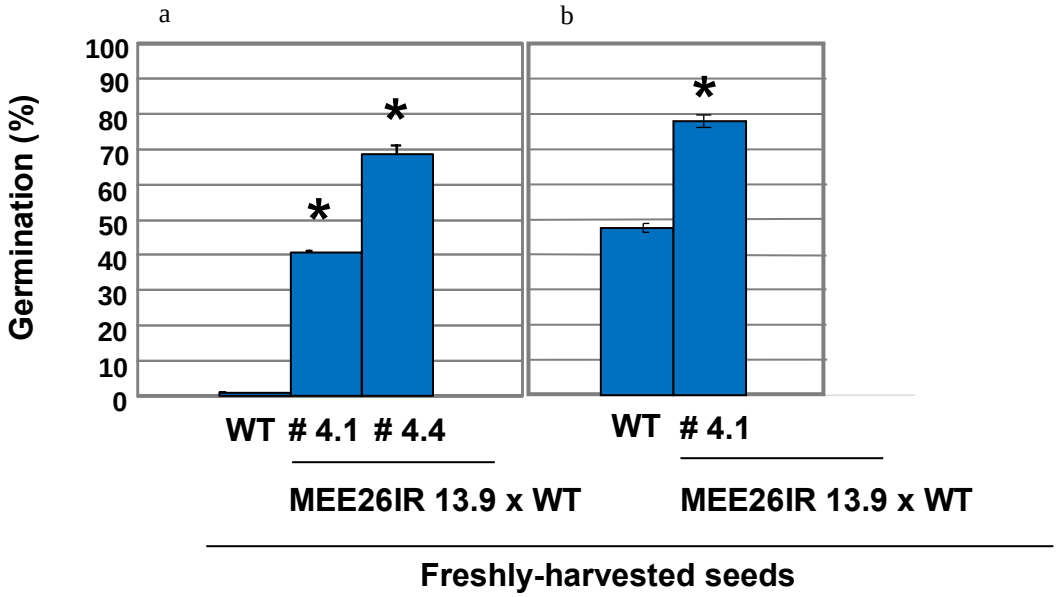


Figure 4.8: **Germination assays:** Stratified freshly harvested seeds of WT, *mft-2* and MEE26IR lines crossed to WT on water agar plates 7 DAI: (a) Seeds from plants grown in the winter (b) seeds from plants grown in spring. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

This phenotypic characterization was then extended to after-ripened seeds treated with light (figure 4.9). All seeds of WT, *mft-2,* and MEE26IR (WT) #4.1 treated with FR/R light have high levels of germination. As previously shown, seeds of the *mft-2* mutant treated with FR and FR- FR light all germinated more than WT controls. Seeds of MEE26IR (WT) #4.1 treated with FR germinated at even higher rates than *mft-2* seeds, whereas under FR-FR light conditions, MEE26IR (WT) seeds germinated at similar levels as WT control seeds. This shows that MEE26 is required to repress germination of after-ripened seeds under FR light conditions. However, under FR-FR light, which is a weaker germination repressing condition, MEE26 is not involved in regulating germination.

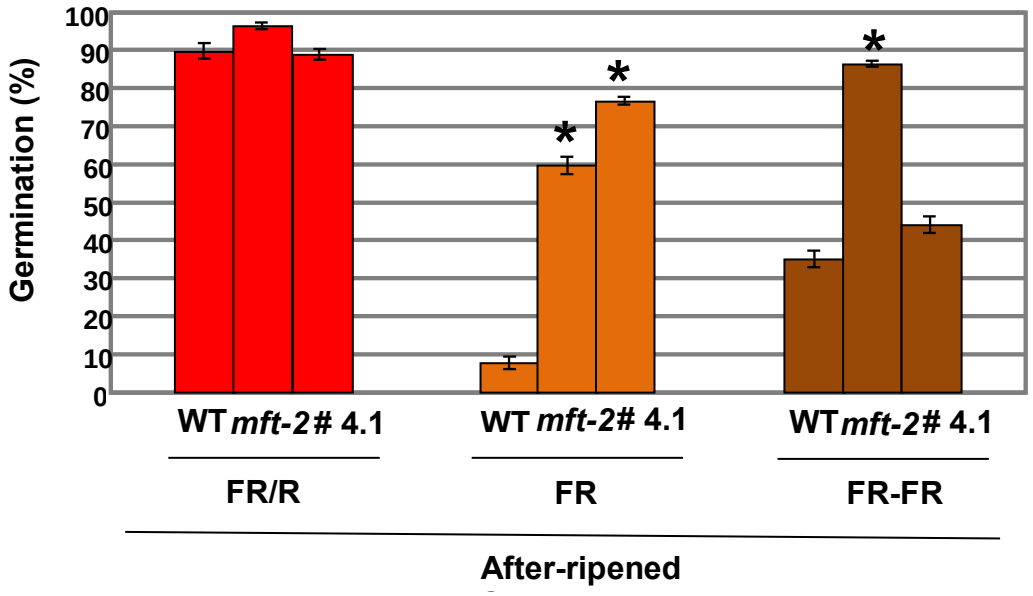


Figure 4.9: **Germination Assay**: After-ripened seeds of WT, *mft-2*, and MEE26IR 13.9 (4.1) X WT on water agar plates treated with light; 5 min pulse of FR followed 5min pulse of Red (FR/R), only 5 min pulse of FR (FR), 5 min pulse of FR followed by 2hrs pulse of FR 2 days later (FR-FR). Germination was scored 5 days after imbibition. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

Analysis of *MEE26* over-expressing lines

In order to further characterize the role of MEE26 in regulation of germination, *MEE26* over-expressing transgenic lines were also analysed. These lines were generated in the WT background (MEE26oe (WT)) before my arrival and had the *MEE26* coding sequence under the control of the 35S promoter (see appendix 2). These independent homozygous lines were first analysed for *MEE26* expression. Figure 4.10 shows that MEE26oe #2.5 and #9.6 had approximately 14-fold increase in expression compared to WT, whereas, MEE26oe #5.4 had only a 2-fold increased expression. Although the three over expressor lines did not show as high an expression of *MEE26* as in *mft*-2, they did express this gene at levels that are significantly higher than WT. As for the silenced lines, none of the over-expressing lines showed obvious growth and/or development phenotypes.

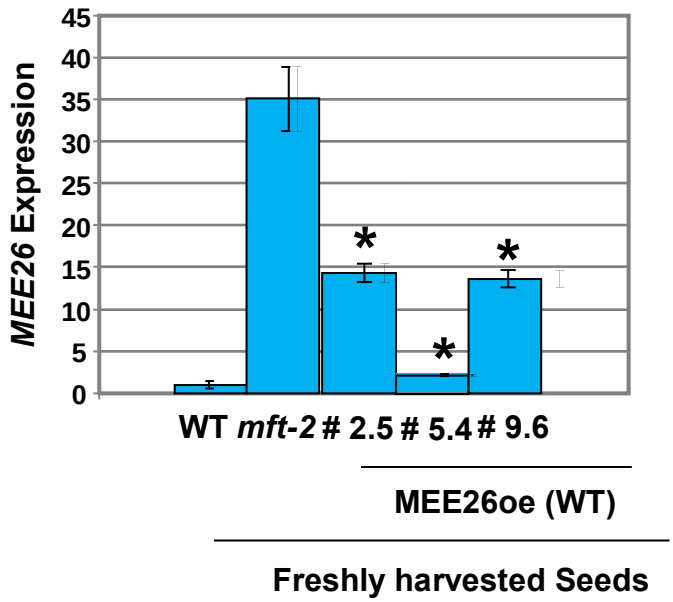


Figure 4.10: **Relative expression of *MEE26*.** Expression of *MEE26* in three over-expressing freshly harvested transgenic lines. Gene expression is relative to fold change in WT. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$).

To phenotypically characterize the MEE26oe lines, germination assays were performed on freshly harvested seeds of these lines along with WT and *mft-2* as controls. Figure 4.11 shows that WT seeds germinated at 50%, *mft-2* seeds germinated at higher rates (almost 100%), as expected. Regarding MEE26oe seeds of the independent transgenic lines, they all germinated at lower rates (approximately 10-25%) than WT control. This indicates that overexpressing *MEE26* promotes dormancy in freshly harvested seeds.

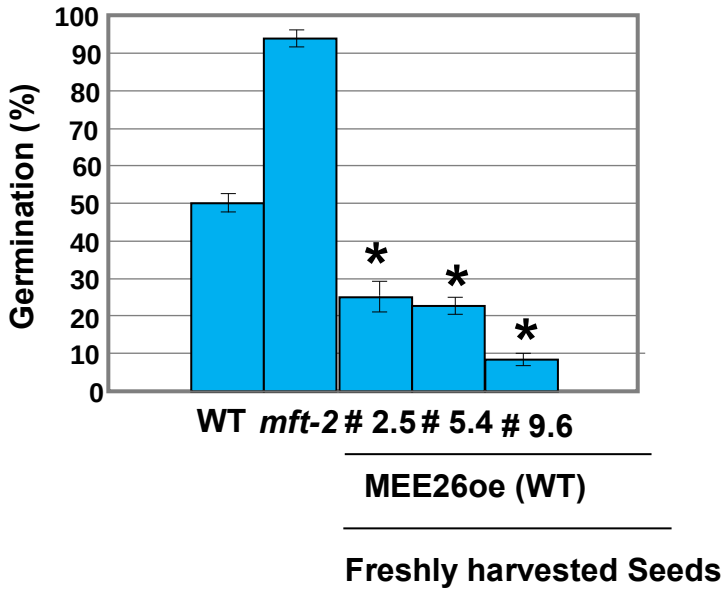


Figure 4.11: **Germination assay**: Stratified freshly harvested seeds of WT, *mft-2* and MEE26oe lines on water agar plates 7 DAI. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

This phenotypic analysis was extended to after-ripened seeds treated with light alongside WT and *mft-2* as controls. To determine if the repression of germination by MEE26 is mediated by FR light, after-ripened seeds were treated as in chapter 3, figure 3.3A. Figure 4.12 shows that all seeds treated with FR/R light had high germination frequencies as expected. Seeds of *mft-2* mutant germinated more than WT under FR and FR-FR light conditions, as shown previously. MEE26oe seeds germinated less than WT under FR and FR-FR light conditions. Taken together, this result indicates that overexpressing *MEE26* represses germination in the presence of FR and FR-FR light.

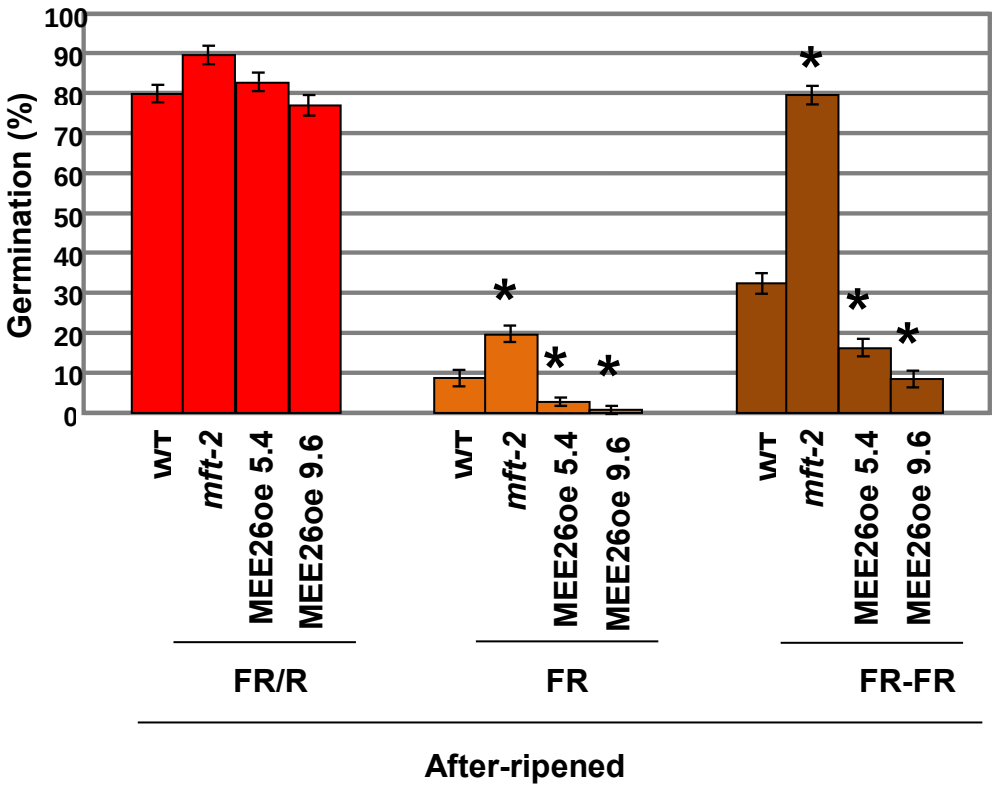


Figure 4.12: **Germination Assay**: After-ripened seeds of WT, *mft-2*, and 2 MEE26oe lines on water agar plates treated with light; 5 min pulse of FR followed 5min pulse of Red (FR/R), only 5 min pulse of FR (FR), 5 min pulse of FR followed by 2hrs pulse of FR 2 days later (FR-FR). Germination was scored 5 days after imbibition. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

Overexpression of *MEE26* Promotes Hypocotyl Elongation

The fact that MEE26 repression of germination in after-ripened seeds is mediated by light prompted the decision to test whether *MEE26* is also important in light signalling in seedlings. *MEE26* is involved in the FR-light signalling pathway and is promoted by PIF1. PIF1 is also known to promote hypocotyl elongation in dark grown seedlings. Thus, it was hypothesized that MEE26 will function in promoting

hypocotyl elongation. In this experiment, seeds were grown in the dark because PIF1 also functions in the dark (its ubiquitination by phyB is cancelled) (Leivar *et al,* 2008*)* similar to FR light conditions. To test this hypothesis, seeds of WT and two MEE26oe lines were treated as in figure 4.13a. Seeds of *35S:SPT* had been included in this experiment to be used as a tool for comparison as it had an elongated hypocotyl when grown in the dark and is hypersensitive to FR light (Penfield *et al,* 2005). Figure 4.13b shows that there was approximately 1.5-fold increase in hypocotyl length of MEE26oe 5.4 and 9.6 compared to WT seedlings. The hypocotyl lengths of these two overexpressor lines were also similar to the SPToe line, which usually had an elongated hypocotyl in the dark.

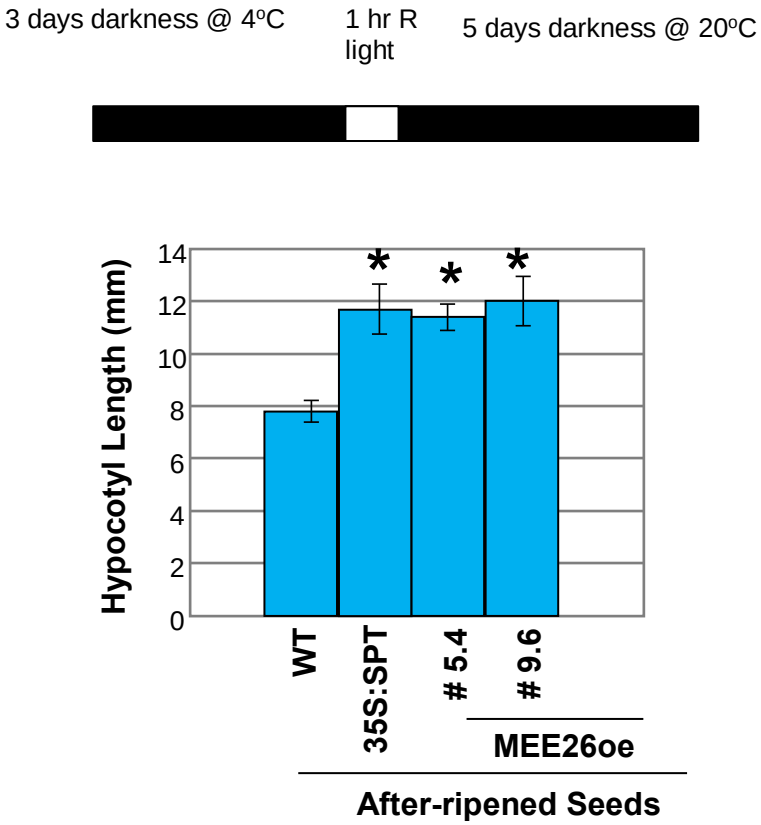


Figure 4.13: **Hypocotyl Lengths of Dark Grown Seedlings**: (a) schematic illustration of the plan of experiment (b) seedling morphology of dark-grown seeds of WT, *mft-2,* and *35S:SPTmyc* and the two MEE26oe lines. Error bars represent SD of at least ten determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

4.3 Discussion

MEE26 was chosen for further studies and investigation because results of the transcriptomic analysis showedthat it was first in the list of genes that were up-regulated in the *mft-2* mutant, therefore, it is repressed by MFT. MFT also represses germination, thus it was hypothesized that MEE26 will promote

germination. However, when *MEE26* is silenced, there is an increase in germination levels compared to WT. The opposite was observed in the overexpressor lines. This indicates that MEE26 functions to repress germination, which invalidates the hypothesis. Thus, it cannot explain the phenotype of *mft-2*, but it does provide more insight into the role that *MEE26* plays in the bigger picture of seed dormancy and germination.

MFT is known to promote dormancy. Thus, the promotion of dormancy by MEE26 could be explained by the fact that MEE26 promotes the expression of *MFT*. *MEE26* expression is promoted by FR light, which is a negative regulator of germination. Figure 4.3 shows that there was a massive increase in expression of *MEE26* in *mft-2* seeds treated with FR light compared to those treated with FR/R light. Seeds of *mft-2* treated with FR light had higher *MEE26* expression compared to WT seeds treated with FR light. This validates the findings of the transcriptomic analysis. Initially, it was surprising to observe that WT seeds treated with FR had fifteen times more expression of *MEE26* compared to the corresponding WT seeds treated with FR/R light. However, this was clarified by the fact that MEE26 promotes the expression of *MFT*, thus, in FR light conditions, expression of *MEE26* was induced, which in turn promotes *MFT*.

The promotion of *MEE26* by FR light is in a PIF1 dependent manner. This is consistent with the fact that PIF1 is a negative regulator of germination and so is *MEE26*. PIF1 also promotes *MFT*, but the results indicate that the FR induced PIF1-dependent expression of *MFT* might have been mediated at least partially by *MEE26*. As expected, ABI5 and the DELLAs, which are all components of the FR light signalling pathway, also promote *MEE26* expression. FR light deactivates PhyB and this cancels the ubiquitination of PIF1 (Leivar *et al*, 2008), which has been reported to stimulate the expression of genes that are involved in the positive signalling components of ABA (e.g., ABI3 and ABI5), and promotes the expression of the negative signalling component genes (the DELLAs) of Gibberellic acid (Oh *et al*, 2009). Therefore, PIF1 promotes ABI5 and the DELLAs, which in turn promotes *MEE26* and this causes germination to be suppressed (Figure 4.14). The results show that *SOM* suppressed *MEE26*. This differed from what was expected because SOM and MEE26 are both negative regulators of seed germination.

PIF1 also actively controls genes that sustain etiolated type of growth and mutants lacking PIF1 exhibit a constitutive photomorphogenic development in the dark (Leivar *et al,* 2008). A study carried out by Soy *et al,* (2014) provides evidence that PIF1 also participates in stimulating hypocotyl elongation in the dark. Similarly, when *MEE26* is overexpressed, it led to elongated hypocotyls of seedlings grown in the dark. This is consistent with the fact that PIF1 promotes *MEE26* and this led to elongated hypocotyls of dark grown seedlings. PIF1, *MFT* and *MEE26* have similar phenotypes, i.e., they are involved in the responses to FR light, which are repression of germination and elongation of hypocotyl. Thus, mutants in these factors showed increased germination and decreased hypocotyl elongation in the dark.

Pagnussat *et al* (2005) identified *MEE26* as one of the genes whose mutant had an arrested endosperm development phenotype. *MEE50* encodes an Armadillo repeat (Arm) protein (Ngo *et al,* 2012) and in its mutant (*mee50*), growth is delayed and prevented at the initial stages of embryo and endosperm development. The Arabidopsis *capulet1* (*cap1*) and *capulet2 (cap2),* which are maternal gametophytic mutants that have the embryo and endosperm developmentally arrested only in the case whereby the female gametophyte has the mutant alleles (Grini *et al,* 2002). *Embryo Defective (EMB) 1611,* which is also known as *MEE22,* is an important gene in Arabidopsis that plays a vital role in the maintenance of shoot and apical meristem, roots, and developing rosette leaves. Leasure *et al* (2009) showed that *emb1611-2* is a partial loss of function mutant that formed various phenotypes indicating pleiotropic development such as a continuing loss of apical meristem function, which can result in early prevention of meristem growth. In the MEE26IR lines, there were no growth/developmental abnormalities observed, although histological analysis was not performed on seeds of these lines and overexpressing lines in order to identify development phenotypes in embryo or endosperm. Nevertheless, it is important to note that perhaps silencing *MEE26* does not cause a developmental arrest of the embryo and/or endosperm, which might have been the case if they were mutant. For this reason, germination phenotypes were observed in the silencing lines. Recommendations for future work would be to perform histological analysis on seeds of MEE26IR andMEE26oe lines in order to check for any abnormalities or defects in the embryo or endosperm.

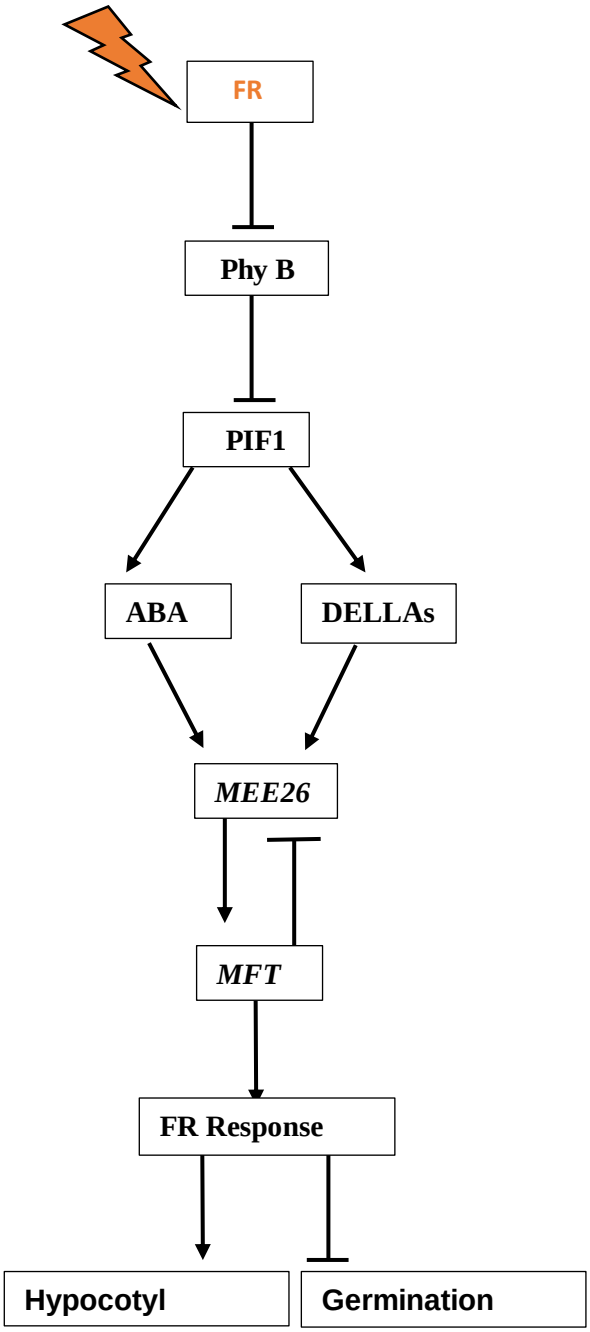


Fig 4.14: **A Proposed model of *MEE26* in the FR light signalling pathway**. FR light represses phyB which in turn represses PIF1. PIF1 promotes the ABA response transcription factors and the DELLAS which also promote *MEE26* expression. MEE26 induces *MFT* expression which in turn represses germination and promotes hypocotyl elongation.

CHAPTER 5: Overexpression of *CYP89A2* Promotes Germination

5.1 Introduction

Transcriptomic analysis was performed on three pairs of WT vs *mft-2* seeds (as described in chapter 4) in order to get an insight into the genes that are deregulated in the *mft-2* mutant. Results of the transcriptomic analysis revealed that *Cytochrome P450 89A2(CYP89A2)* was at the top of the list of genes that were down-regulated in *mft-2* (see table 2 in Appendix 1). In the first comparison, in which freshly matured dry seeds were developed at 15^{o}C, there was an approximately 52-fold decreased expression of *CYP89A2* in *mft-2* compared to WT seeds. In the second comparison, freshly matured (developed at 15^{o}C) seeds were imbibed in the dark for 7 days at 4^{o}C and in the third one, seeds were imbibed in the dark for 7 days at 4^{o}C, and both these comparisons had a seven-fold decrease in the expression of *CYP89A2* in *mft-2* compared to WT. Thus, *CYP89A2* was chosen for further research.

The Cytochromes (CYPs) are an ancient superfamily that has been discovered in all domains of organisms like plants, animals, fungi, protists, archaea, bacteria and viruses (Lamb *et al*, 2009). CYPs greatly absorb 450 nm light and were initially identified by the group of Martin Klingenberg (1958) during their study on the spectrophotometric properties of pigments in a microsomal fraction made from livers of a rat (Klingenberg 1958). Analysis carried out showed that CYPs are not pigments but are enzymes that take part in multiple catalytic pathways (Jun *et al*, 2015). CYPs contain heme cofactors which function essentially in the catalysis pathway and give P450s the ability to bind carbon monoxide. Even though P450s control a range of metabolic pathways, they share the same oxidative and reductive process (Jun *et al*, 2015). A general cytochrome P450 oxygenase reaction involves the transfer of two electrons by cytochrome P450 reductase from NADPH to their substrate. The initial oxygen atom joins (hydroxylates) with the substrate (SH) and the next one forms water: S-H+O2 +NADPH, H+ → NADP++H2 O+S-OH (Munroe *et al*, 2018). Sequence homology and phylogenetic criteria have been used to group CYPs into various families and subfamilies. Presently, there are 135 families of plant cytochrome P450s (CYPs) (Hori *et al*, 2018). In individual plant genomes, CYPs represent the third biggest family of plant genes (Arabidopsis contains of 272) (Mao *et al*, 2013).

Ehlting *et al*, (2008) analysed Affymetrix ATH1 microarray data from experiments that were available publicly in order to produce a complete gene expression matrix for all P450s in *Arabidopsis thaliana*. The study showed that there are some flower, root, and leaf specific CYPs and there were others that were highly expressed in seedlings, siliques and seeds. Studies on subcellular localization of CYPs have mostly been biochemical and its identification in microsomal fractions is common. Different techniques have also identified CYPs in the Endoplasmic Reticulum (ER) (Durst, 1991).

Cytochrome P450 controls a large number of significant cell processes that have an effect on plant growth and development. Some P450 genes play a role in the biosynthesis of compounds in a variety of metabolic pathways. Biosynthesis of a wide range of secondary metabolites including terpenoids, flavonoids, steroids, alkaloids, involve reactions performed by CYPs. Metabolites like terpenes have a vital role to play in biotic stress response. For example, some CYPs are associated with the synthesis of homoterpene volatiles, which are involved in attracting pollinators and act as defence compounds that are harmful to herbivores (Lee *et al*, 2010).

CYPs also function in the control of plant hormone metabolism. Hormones are necessary for the production of flowers, stems, leaves, the process of shedding of leaves, as well as fruit development and ripening. In Arabidopsis, some CYPs inactivate the brassinosteroid class of phytohormones and are involved in modulating photomorphogenesis and the signal transduction of plant steroid (Turk *et al*, 2003; Thornton *et al*, 2010). CYPs control the production of auxin in Arabidopsis (Vadassery *et al*, 2008), and they also take part in the metabolic pathways of gibberellin (Helliwell *et al*, 2001; Zhang *et al*, 2011). They are also involved in the modulation of abscisic acid contents in both Arabidopsis and barley (Millar *et al*, 2006).

Numerous pigments, which are necessary for the physiological processes in plants, are strongly associated with the functions of P450s as well. Carotenoids are pigments that are widely distributed and derived from the ubiquitous isoprenoid biosynthetic pathway. They are involved in various roles in plant primary and secondary metabolism (Tian *et al*, 2004; Kim *et al*, 2009). Some CYPs are essential for the hydroxylation of carotenoids. Anthocyanins are other important pigments, which contribute to

colour of petals and fruits. CYPs control the expression of flavonoid 3´-hydroxylase and flavonoid 3´, 5´-hydroxylase, which function in the synthesis of many anthocyanins (Castellarin *et al*, 2006).

P450s also function in plant defence through their involvement in the biosynthesis of phytoalexin, the metabolism of hormones and the biosynthesis of a few other secondary metabolites. Phytoalexins are known as antimicrobial compounds that have low molecular weight and are synthesized in plants in response to a diverse range of plant pathogens (Glawischnig 2007).

A large number of P450s take part in the biosynthesis of components of the cell wall. The cell wall functions as a barrier to shield plants from a variety of abiotic and biotic stresses. For example, CYP86A2 acts as an important producer of cutin needed for cuticle biosynthesis (Xiao *et al,* 2004). De Giogi *et al,* (2015) identified *CYP86A8* (*LACERATA –LCR*) as a gene involved in cuticle biosynthesis. To investigate the role of cutin in mature seed germination, they carried out germination assays on *lcr* mutant seeds along with other mutants of cutin biosynthesis genes. The cutin mutant seeds showed a less dormant phenotype compared to WT Col-0. This shows that the accelerated loss in seed dormancy is due to the deficiency in the deposition of cutin in dry mature seeds. Analyses of publicly available data (Belmonte *et al*, 2013; and Vseed website) shows that in newly developed seeds *CYP89A2* is mainly expressed in the seed coat with high expression in the chalazal seed coat and there is low expression in the endosperm and embryo (Figure 5.1).

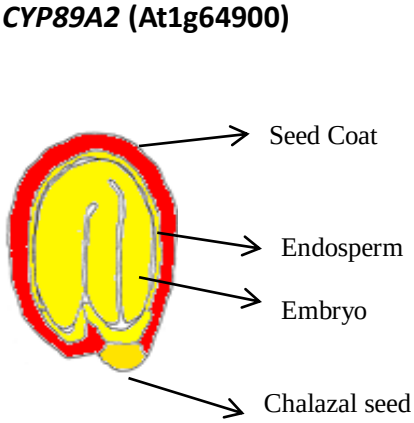


Figure 5.1: **Expression of *CYP89A2* in seed tissues**. Red colour indicates the maximum absolute expression level of this gene (main expression), orange corresponds to high expression i.e expression levels very close to the maximum absolute expression, and yellow indicates no expression or low expression i.e. absolute expression levels close to zero (Belmonte et al. 2013; eFP browser)

CYP89A2 has been cited in a study carried out by Hudson *et al,* (2003). This study investigated the *FAR1* and *FHY3* genes and the role(s) that these signalling components play in the phyA signalling

pathway. The phyA receptor is vital for etiolated seedlings to respond to far red light (Quail, 1998). Some etiolated plants that are deficient of phyA are unresponsive to light of this wavelength. Several mutations have been identified that interfere with the transfer of signals from phyA to the process of photomorphogenic development. Included in a class of mutants that are hyposensitive to FR are *fhy3* and *far1* (Ballesteros *et al*, 2001), which are part of the FAR1 gene family and are closely related. In order to identify the genes that are downstream of the signalling pathways or branches outlined by *far1* and *fhy3*, Hudson *et al*, (2013) conducted a microarray analysis and detected a downstream group of genes, which *CYP89A2* was a part of. *CYP89A2* expression in seedlings was promoted by far-red light in wild-type Arabidopsis (Tepperman *et al*, 2001), but not in the phyA photoreceptor-null mutant (*fhy3*). This is suggestive of the fact that the promotion of expression of this gene can be linked to a signal from the phyA photoreceptor.

The transcriptomic analysis indicates that MFT promotes *CYP89A2*. Thus, it is hypothesized that CYP89A2 could function in repressing germination, thereby explaining the *mft*-2 phenotype.

5.2 Results

FR Promotes Expression of CYP89A2

To validate the results of the transcriptomic analysis, *CYP89A2* expression was analysed in WT and *mft-2* freshly harvested seeds. Figure 5.2 shows that there was 90 times more expression of *CYP89A2* in WT compared to *mft-2*. This is in agreement with the results of the transcriptomic analysis and it shows that MFT promotes *CYP8989A2.*

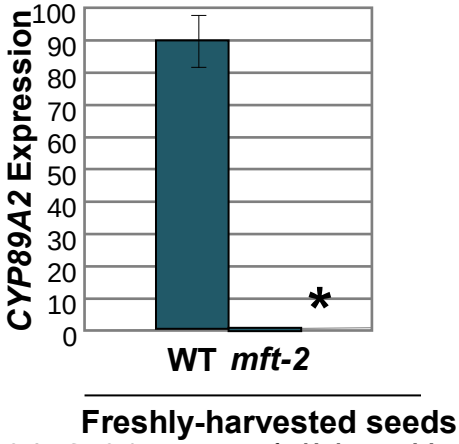


Figure 5.2: **Relative Expression of *CYP89A2*.** *CYP89A2* expression in freshly harvested dry seeds of WT (Col) and *mft-*. Gene expression is relative to fold-change in *mft-2* values. Error bars represent SD of at least three determinations. Asterisks over the bar specify statistically significant differences ($P < 0.05$) compared to its corresponding WT.

The fact that *CYP89A2* expression was induced by FR light in seedlings (Hudson *et al,* 2003) prompted the decision to check if a similar occurrence would be observed in seeds. In chapter 3, figure 3.2, it is shown that FR light promotes the expression of *MFT*. Results of the transcriptomic analysis suggested that MFT promotes *CYP89A2*. It is thus hypothesized that the expression of *CYP89A2* will be induced by FR light. To test this hypothesis, *CYP89A2* expression was analysed in WT and *mft-2* mutant after-ripened seeds treated with FR/R and FR light as described in chapter 3 (Figure 3. 5). Figure 5.3 shows that there was approximately 5 times more expression of *CYP89A2* in WT seeds treated with FR light compared to those treated with FR/R light. This indicates that FR promotes *CYP89A2*. In *mft-2* seeds treated with FR light, there is a 7-fold decreased expression compared to the WT seeds treated with FR, which indicates that the induced expression of *CYP89A2* by FR light is dependent on MFT. In the *mft-2* mutant seeds, *CYP89A2* expression is induced by FR light, which indicates that other factors are involved in this induction in addition to MFT.

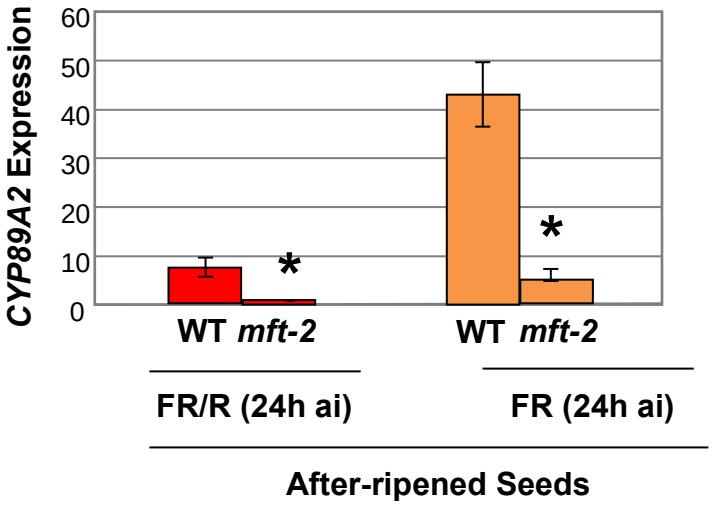


Figure 5.3: **Relative *CYP89A2* expression:** in after-ripened seeds WT and *mft-2* seeds treated with FR/R and FR light. Gene expression is relative to fold-change in the sample with the lowest expression (*mft-2* (*FR/R*)). Samples from RNA extractions were collected 24h after imbibition (ai). Error bars represent SD of at least three determinations. Error bars represent SD of at least ten determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

The fact that *CYP89A2* is promoted by FR light led to the decision to investigate if its expression is induced by other FR-pathway factors like PIF1, SOM, ABI5 and the DELLAs. Figure 5.4a shows that there was an 18 and 17 times reduced expression of *CYP89A2* in *pif1-1* and *della4* respectively

compared to WT, and this indicates that they both promote *CYP89A2* expression. There is a 3.5-fold decreased expression of *CYP89A2* in *abi5-7*, which indicates that it promotes *CYP89A2* (Figure 5.4b and c). However, the induced expression of *CYP89A2* is stronger in the *pif1-1* and the *della4* compared to *abi5-7*. In the *som-3* mutant, there was no statistically significant decrease in expression of *CYP89A2* compared to the corresponding WT (Figure 5.4d). Taken together, the FR-pathway factors, with the exception of *SOM*, all promote the expression of *CYP89A2*. This indicates that the induced expression of this gene by FR light is mediated by these factors.

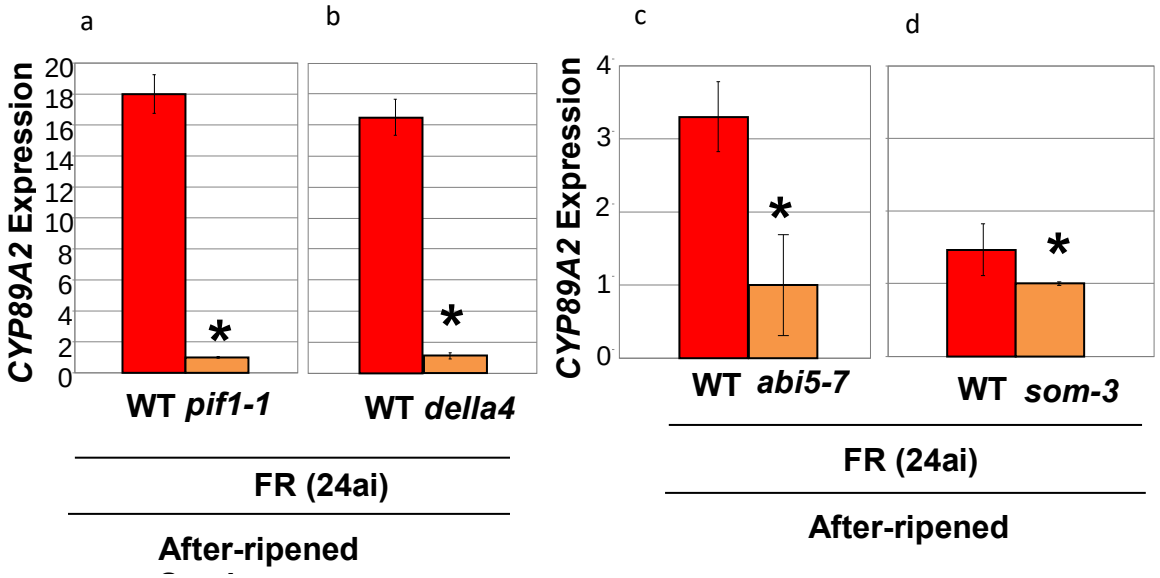


Figure 5.4: **Relative *CYP89A2* expression:** in after-ripened seeds (a) WT and *pif1-1*, (b) WT and *della4*, (c) WT and *abi5-7*, and (d) WT and *som-3* mutants treated with 5' pulse of FR light. Gene expression is relative to fold-change in the sample with lowest expression. Samples from RNA extractions were collected 24h after imbibition (ai). Error bars represent SD of at least three determinations. Error bars represent SD of at least ten determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$).

Characterization of CYP89A2 T-DNA Insertion Lines

In order to characterize the gene function of CYP89A2, two T-DNA insertion lines, GABI-169E08 and Salk_082987 (*cyp89a2-1)* were obtained from Nottingham Arabidopsis Stock Centre (NASC), and thus it was unnecessary to make mutant or transgenic lines to silence this gene. PCR was used to select for the homozygous T-DNA insertions for the Gabi line. PCR using the Forward(For) and Reverse(Rev) primers flanking the T-DNA insertion (Figure 5.5a) and genomic DNA from WT and sibling GABI plants amplified the predicted 900 bp fragments corresponding to the amplicon of the *CYP89A2* gene without the insertion. This is indicative that none of the siblings were homozygous for the insertion.

PCR using 'For' and T-DNA specific primer (Figure 5.5a) failed to amplify any WT (as expected) and siblings of the Gabi line. The GABI T-DNA insertion is linked to resistance to sulfadiazine. None of the seedlings of the GABI line were resistant to sulfadiazine (data not shown). Hence, taken together the PCR and resistant assays, these results indicate that the GABI line either did not contain the annotated T-DNA insertion, or it was not what NASC claimed it to be.

For the SALK line, PCR was carried out using the Left Primer (LP) and Right Primer (RP) flanking the T-DNA insertion (Figure 5.5a) and genomic DNA from WT and sibling SALK plants. WT amplified the predicted 900 bp fragments (as expected) corresponding to the amplicon of the *CYP89A2* gene without the insertion, however, the sibling SALK plants did not show any amplification (Figure 5.5c). PCR using RP and T-DNA specific primer, Left Border (LB) (see Figure 5.5a) failed to amplify any WT (as expected), but SALK lines amplified the 300 bp fragments. This is indicative that these lines were homozygous for the insertion. Thus, further studies continued with this line.

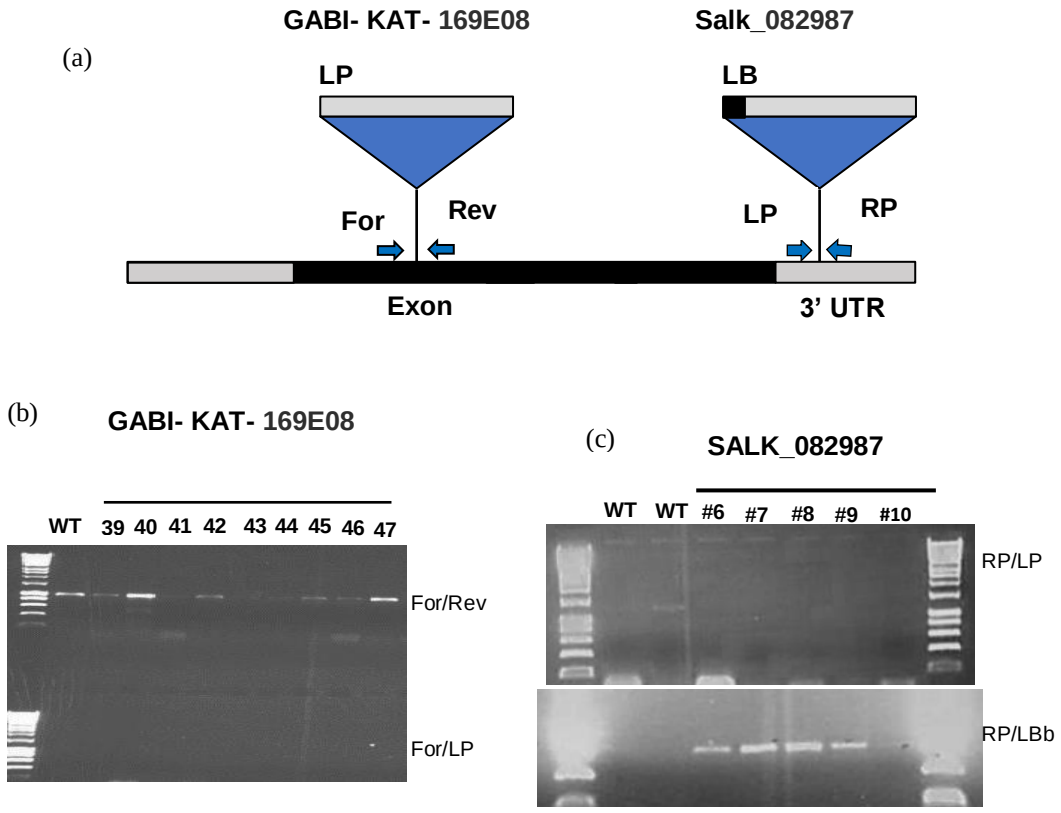


Figure 5.5: **PCR Genotyping for GABI and SALK lines**. (a) Schematic representation of the gene with the believed insertions (b) PCR using For/Rev primers identifies the existence of bands at the 900bp lane for all the Gabi lines and WT, no bands are identified when For/LP are used. (c) PCR reaction uses primes RP/LP and a band was amplified in the WT control, but none in the sibling SALK plants. Bands were amplified at the 300bp lane for all SALK sibling plants but not for WT. This indicates that lines are not homozygous for the insertion.

To characterize the *cyp89a2-1* T-DNA insertion line, germination assays were carried out on freshly harvested seeds of this line along with WT and *mft-2*. As expected *mft-2* had higher germination frequency than WT, however, there was no significant difference in the level of germination between the T-DNA insertion line and WT (Figure 5.6).

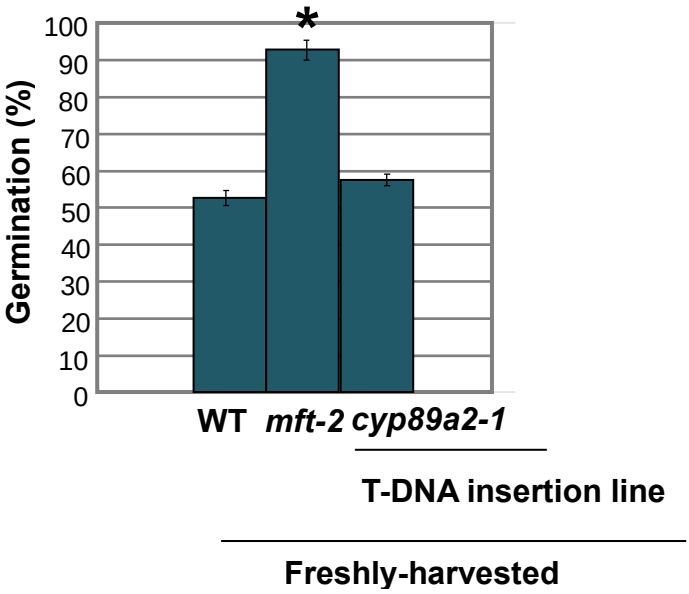


Figure 5.6: **Germination assay**: Stratified freshly harvested seeds of WT, *mft-2* and *cyp89a2-1* on water agar plates 7 DAI. Error bars represent SD of at least four determinations. Asterisks over the bar specify statistically significant differences ($P < 0.05$) compared to its corresponding WT.

The expression of *CYP89A2* was then analysed in the *cyp89a2-1* compared to WT. Figure 5.7 shows that there was no significant difference between the expression of *CYPP89A2* in the t-DNA insertion line *cyp89A2*-1 and WT. This could be due to the fact that the T-DNA insertion of *cyp89A2*-1 was in the 3' UTR. For this reason, no further analysis was pursued for this line.

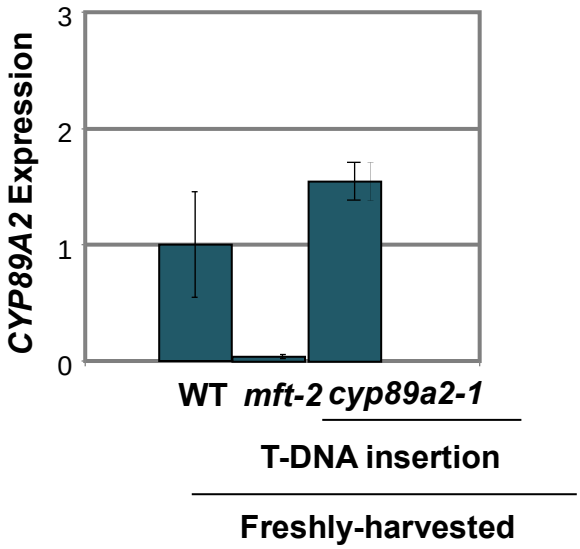


Figure 5.7: **Relative *CYP89A2* expression.** *CYP89A2* expression in WT, *mft-2* and T-DNA insertion. Gene expression is expressed relative expression in *mft-2*. Error bars represent SD of at least three determinations.

CYP89A2 Overexpression Promotes Germination

Prior to the commencement of the PhD programme, transgenic lines were designed to over-express *CYP89A2* in the WT background in order to further characterize its gene function. The over-expression of *CYP89A2* was carried out by linking it to the 35S promoter sequence (Appendix 2). This was done due to the fact that *CYP89A2* is down-regulated in the *mft-2* mutant. This is a gain-of-function approach that will increase the expression and eventually assist in establishing the role that CYP89A2 plays in Arabidopsis. This approach generated multiple independent CYP89A2-overexpressor (oe) transgenic lines in the WT background, but, this research concentrated on only three, CYP89A2oe #2.3, CYP89A2oe #3.5 and CYP89A2oe #4.2 (see appendix 2 on how the transformations are made).

To confirm that *CYP89A2* was indeed over-expressed in these lines, real-time PCR was used to analyse its expression in their seedlings along with WT. Figure 5.8 shows that *CYP89A2* expression in seedlings of the three transgenic lines ranged from 2-fold to 26-fold increase compared to WT.

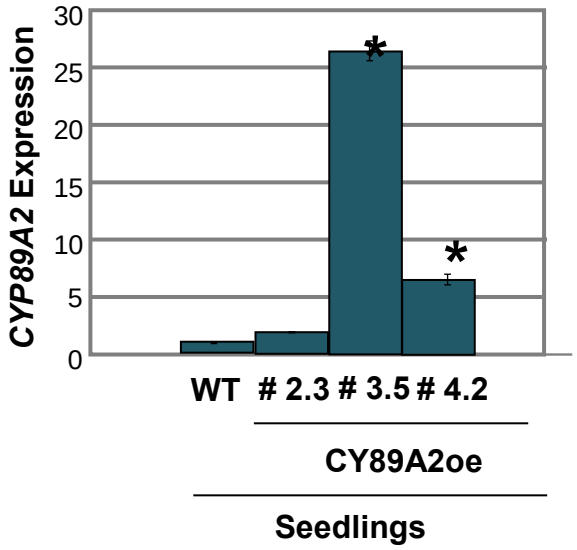


Figure 5.8: **Relative *CYP89A2* expression.** *CYP89A2* expression in seedlings of WT and CYP89A2oe lines. Expression is standardized relative to WT. Error bars represent SD of at least three determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

CYP89A2oe lines were phenotypically characterized by carrying out germination assays on freshly harvested seeds. Seeds of WT and *mft-2* were used as controls. Figure 5.9 shows that the over-expressor lines achieved higher germination percentages (75%-85%), whilst WT had 50% germination. Apparently, only a two-fold increase in the expression of *CYP89A2* in line #2.3 resulted to a high germination frequency similar to the other transgenic lines. The result indicates that overexpressing *CYP89A2* promotes germination in freshly harvested seeds.

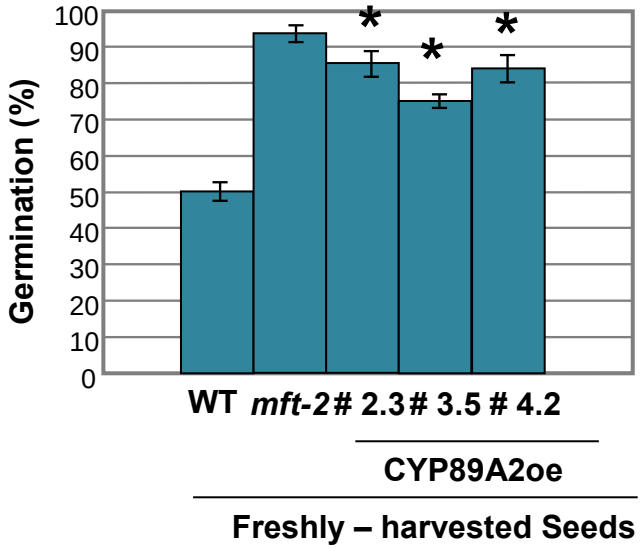


Figure 5.9: **Germination assay**: Stratified freshly harvested dry seeds of WT, *mft-2* and CYP89A2oe lines on water agar plates 7 days after imbibition (DAI). Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

To gain more insight into the mode of action of CYP89A2, exogenously applied ABA was used in germination assays. All seeds plated on water agar only had above 90% germination and this is because they were after-ripened (dormancy loss has occurred) and had also been stratified. Seeds of *mft-2* mutant were hypersensitive to ABA (Xi *et al,* 2010) and thus germinated less than WT when treated with 1uM and 10 µM ABA (figure 5.10). The CYP89A2oe lines germinated slightly higher than WT when treated with 1µM ABA. When treated with 10uM exogenous ABA, WT seeds germinated at 42% while all the CYP89A2oe lines germinated at levels ranging from 60 – 65%. In conclusion, CYP89A2oe lines were hyposensitive to ABA when compared to WT. This is in agreement with the fact that the freshly harvested seeds of the over-expressor lines were less dormant.

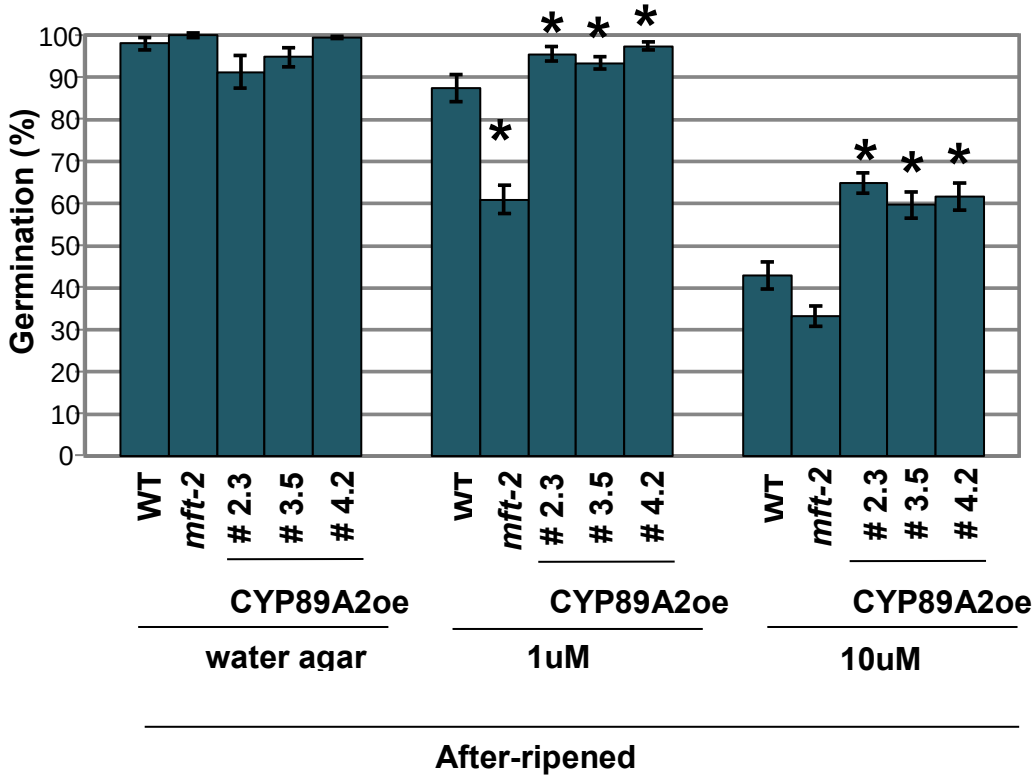


Figure 5.10. **ABA Sensitivity Test**: Stratified seeds of WT, *mft-2*, and CYP89A2oe lines on water agar, water agar supplemented with 1uM ABA, and, water agar supplemented with 10uM ABA. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

The fact that the expression of *CYP89A2* is induced by FR light prompted the decision to investigate the germination response of CYP89A2oe seeds treated with light. After-ripened seeds were given similar treatments as shown in chapter 3, figure 3.3A. Figure 5.11 shows that all seeds treated with

FR/R light had high germination frequencies. Seeds of *mft-2* treated with FR and FR-FR germinated more than their corresponding WT. All CYP89A2oe lines treated with FR light had germination percentages that ranged from 53-67%, whilst WT germinated at 20%. After FR-FR light treatment, WT germinated at 38%, whilst CYP89A2oe #2.3 germinated at 5.4%. Both CYP89A2oe #3.5 and #4.2 display germination frequencies close to 90%. Taken together, these results indicate that overexpressing *CYP89A2* increased germination in the presence of FR and FR-FR. This experiment was repeated several times and in some of the cases line #2.3 had higher germination frequencies (up to 80%), similar to the other two CYP89A2oe lines.

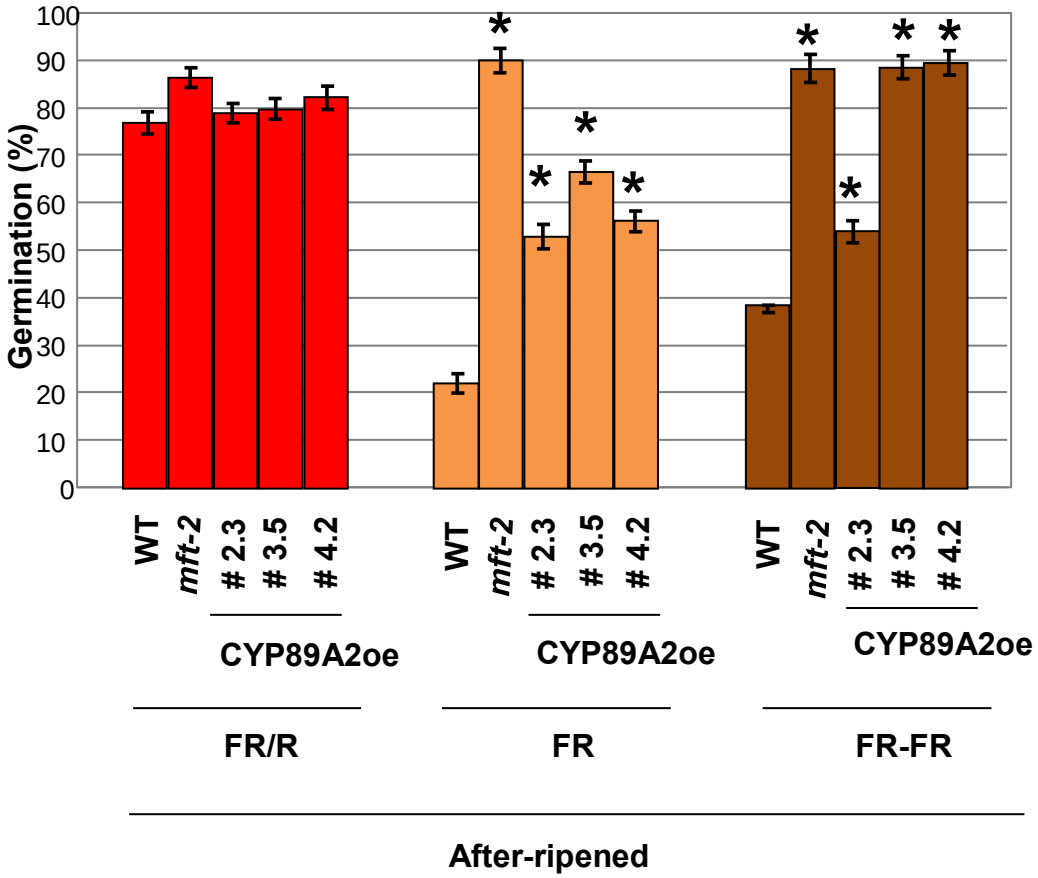


Figure 5.11: **Germination Assay**: After-ripened seeds of WT, *mft-2*, and CYP89A2oe lines on water agar plates treated with light; 5 min pulse of FR followed 5min pulse of Red (FR/R), only 5 min pulse of FR (FR), 5 min pulse of FR followed by 2hrs pulse of FR 2 days later (FR-FR). Germination was scored 5 days after imbibition. Error bars represent SD of at least four determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

CYP89A2 Overexpression Promotes Seeds Coat Permeability

Publicly available data indicate that *CYP89A2* is strongly expressed in the seed coat during seed development (Figure 5.1). This led to the investigation into the effect of CYP89A2oe on seed permeability. The seed coat mostly restricts germination either by remaining impermeable to water and/or oxygen or exerting mechanical resistance to the protrusion of the radicle (Debeaujon *et al,* 2000). It is difficult to assess water uptake in Arabidopsis because of the capacity of the mucilage to hold water. Therefore, to assess the permeability of the testa, the absorption of 2, 3, 5 triphenyltetrazolium chloride (tetrazolium salts) by the embryo was used (Macgregor *et al,* 2014). After-ripened seeds were stained with this for 48 hours. The seed coat of CYP89A2oe lines stain red upon entry of the tetrazolium solution into the seed, which indicates that they are more permeable. This is in agreement with the fact that seeds with permeable seed coats are less dormant (Debeaujon *et al,* 2000). WT and *mft-2* seeds had not shown any observable staining, and are therefore, less permeable (Figure 5.12).

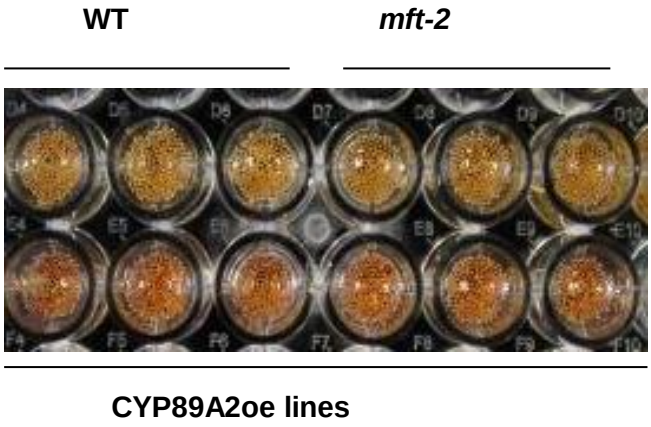


Figure 5.12: **Seeds Permeability (Tetrazolium Salts) Assay**: Characterization of seed coats of WT, *mft-2,* and CYP89A2oe by assessing their permeability to tetrazolium salts 48h after incubation at 37ºC

CYP89A2 Overexpression Promotes Hypocotyl Elongation

Hudson *et al,* 2003 showed that expression of *CYP89A2* is induced by FR light in seedlings. Thus, it was decided to assess hypocotyl length of seedlings grown in the dark. After ripened seeds were given similar treatment as shown in chapter 4, figure 4.12. Both CYP89A2oe lines had seedlings with longer hypocotyls compared to WT seedlings, and almost similar in length to the 35S:SPT (Figure 5.13). Seeds of *35S:SPT* had been included in this experiment to be used as a tool for comparison as it had an elongated hypocotyl.

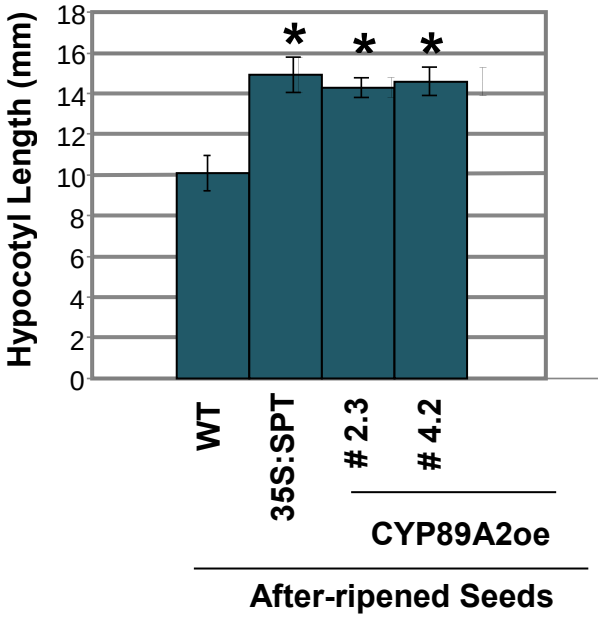


Figure 5.13: **Hypocotyl length of dark-grown seedlings**: Seeds of WT, *mft-2*, and *35S:SPTmyc* and the two CYP89A2oe lines were plated on ½ MS and grown in the dark. Hypocotyl length was assessed 8 DAI. Error bars represent SD of at least ten determinations. Asterisks over the bars specify statistically significant differences ($P < 0.05$) compared to their corresponding WT.

5.3 Discussion

In this chapter the role of CYP89A2 in the process of seed dormancy and germination was studied. The original hypothesis was that, since MFT promotes *CYP89A2*, CYP89A2 would in turn suppress germination. But, the data presented shows that overexpression of *CYP89A2* promoted germination in both freshly harvested and after-ripened seeds of CYP89A2oe lines. Therefore, the *mft-2* phenotype cannot be explained simply by the increase in *CYP89A2* gene expression. To better understand the role of CYP89A2, a T-DNA insertion, *cyp89A2-1*, a SALK line that was homozygous for the insertion, was used. However, after several analyses, it was observed that there was no difference in level of germination between it and WT. Expression analysis was carried out which showed that the transcript level of *CYP89A2* in *cyp89a2-1* was not significantly different from WT, and this is most likely due to the fact that the insertion is in the 3'UTR. For this reason, the lack of effect on germination was dismissed and this SALK line was discarded.

Because of the availability of the T-DNA insertion from NASC, it was initially thought it was unnecessary to create mutants by CRISPR Cas9 or transgenic lines made to silence *CYP89A2*. At the point in this PhD programme, when it was discovered that the T-DNA insertion line was not altered in gene expression, there was insufficient time left to take another approach to obtain knock-outs or knock-downs in the *CYP89A2* gene.

The CYP89A2oe displayed hyposensitivity to ABA compared to WT when water agar was supplemented with 10 µM ABA. This is not surprising because seeds that have higher germination frequencies are known to demonstrate a hyposensitive phenotype in the presence of ABA (Finkelstein *et al,* 2002). However, the opposite was observed in *mft-2* phenotype, which germinateed higher than WT but was more hypersensitive to ABA compared to WT (Xi *et al*, 2010*)*.

FR promotes *MFT* expression. Thus, it was hypothesized that since MFT promotes *CYP89A2*, then FR will also induce expression of *CYP89A2* and thereby lead to repression of germination. Though FR did induce *CYP89A2* expression, overexpression of *CYP89A2* promoted germination. This, therefore, also

invalidates the hypothesis. *CYP89A2* expression was analysed in relation to other FR light-pathway factors. The results show that it was promoted by PIF1, DELLAs and the ABI5, but with the promoting effect of the latter being weaker than the other two. The promotion of *CYP89A2* by SOM was not statistically significant. These components of the FR light signalling pathway are all negative regulators of germination which is in contradiction with the fact that overexpression of *CYP89A2* promoted seed germination. FR light deactivates PhyB and this cancels the ubiquitination of PIF1, which has been reported to stimulate the expression of genes that are involved in the positive signalling components of ABA (e.g., ABI3 and ABI5), and promotes the expression of the negative signalling component genes (the DELLAs) of Gibberellic acid (Oh *et al*, 2009). Therefore, PIF1 promotes ABI5, the DELLAs, and *SOM,* which all promote *MFT*. MFT in turn promotes *CYP89A2* (Figure 5.14), but SOM does not promote *CYP89A2*. This indicates that in the FR light signalling pathway, the promotion of *CYP89A2* by MFT is not through SOM.

A study carried out by Soy *et al,* (2014) provides evidence that PIF1 also participates in stimulating hypocotyl elongation in the dark. Similarly, when *CYP89A2* was overexpressed, it led to elongated hypocotyls of seedlings grown in the dark. This is consistent with the fact that PIF1 promotes *CYP89A2* and this led to elongated hypocotyls of dark grown seedlings. Usually mutants like *mft-2* and *phyB* (Devlin *et al,* 2003) germinate more under FR light and have shorter hypocotyls. The over-expressors of *CYP89A2* germinated more under FR light and have longer hypocotyls. This hypocotyl phenotype is in agreement with the results of Soy *et al,* 2014 and the role of MFT in promoting *CYP89A2*. The contradictory part here is the CYP89A2oe germination phenotype. The expression of *CYP89A2* is induced by FR-light and the FR-light pathway factors as well as MFT, which are all negative regulators of germination. Therefore, it is rather surprising that CYP89A2oe lines have higher germination frequencies.

Tetrazolium salt staining showed that CYP89A2oe lines are more permeable than WT. Perhaps the overexpression of *CYP89A2* has an effect on the seed coat and makes it more permeable. Also, the CYP89A2oe lines germinated more than WT. This is consistent with the fact that the seed coat is known to reduce germination by controlling permeability to water (Wyatt, 1977) or oxygen (Corbineau &

Come, 1993). Reduced dormancy can be related to the increased permeability and a decrease in testa thickness (Debeaujon *et al,* 2000). De Giorgi *et al,* 2015 indicate that in seed coats of many species, including *Arabidopsis,* are cutin-containing layers that regulate permeability to external compounds. They identified a *bodygaurd1* mutant that is deficient in cuticle and is linked with changes in endospermic permeability. This mutant also had a low seed dormancy phenotype. Although the CYP89A2oe are not mutants, perhaps the overexpression of *CYP89A2* caused a defect or deficiency in the cuticular layer. However, a mutant line could also have had a defect in the cuticle, in which case it may be more permeable and germinate more. If this was the case, then the contradictory phenotypes of the over-expressor lines and the hypocotyl elongation may be explained.

Babineau *et al,* 2017 performed a comprehensive reference transcriptome for *Apera spica venti* (a weed that is highly resistant to herbicides) in various tissues and growth stages. Genes that play a role in herbicide resistance mechanisms were identified and one of these was a CYP, which was the second most abundant P450 in seeds and leaf tissues of *A. spica venti*. After obtaining the sequence of this gene, I carried out a BLAST search on TAIR website and the result indicated that it had sequence similarity with *CYP89A2* in *Arabidopsis*. This suggests that CYP89A2 has a role to play in cuticle biosynthesis as a thick cuticle renders the plant resistant to herbicides. Nawrath, (2006) states that 'Typical phenotypes of plants having a permeable cuticle include an increased loss of water, leading to desiccation sensitivity, and a facilitated uptake of molecules, leading to, for example, herbicide sensitivity. Under most conditions, increased permeability of the cuticle is detrimental to the plant'.

Recommendations for further work would be to make mutant or knockout lines of *CYP89A2* in order to gain a proper understanding of this gene. Furthermore, metabolite profiling of seed tissues could be carried out on CYP89A2oe lines so that their metabolite composition can be compared to WT. This could provide an answer to the involvement of CYP89A2 in cuticle and cell wall biosynthesis.

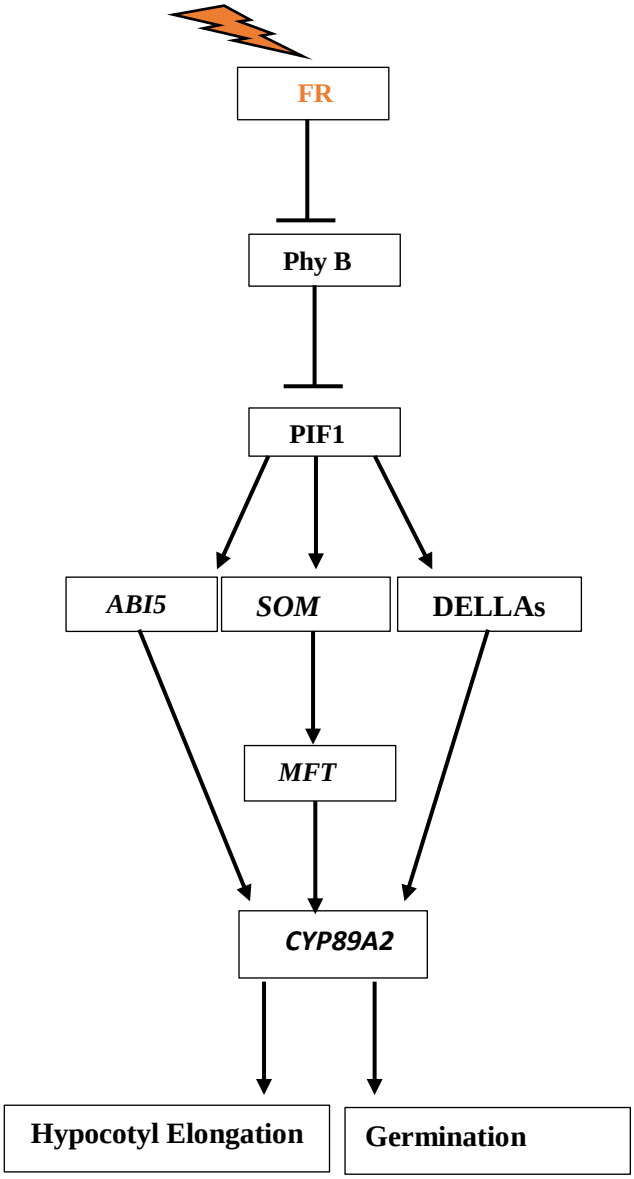


Fig 5.14: **A Proposed model of *CYP89A2* in the FR light signalling pathway**. FR light represses phyB which in turn represses PIF1. PIF1 promotes the ABI5 and the DELLAS and *MFT* which all promote *CYP89A2* expression. However, overexpression of *CYP89A2* promotes germination and hypocotyl elongation.

CHAPTER 6: CONCLUSION

The findings indicate that MFT does supresses germination in after-ripened seeds treated with FR light. This is also consistent with the fact that *MFT* is promoted by FR light as well as other FR signalling pathway factors like PIF1, SOM and ABI5, which are all negative regulators of germination. The findings also showed that MFT acts downstream of ABA and OPDA, thus, alterations in these phytohormones could not explain the phenotype of *mft-2* (high germination rate compared to WT). MFT does not regulate germination through ABA and OPDA. However, Xi *et al* (2010) showed that ABA does induce *MFT* expression and Vaistij *et al* (2018) indicated that MFT played a role in transducing the signals of ABA thereby leading to suppression of germination, even though this repression on germination by ABA was not entirely on its effect on MFT.

The germination phenotype of the after ripened seeds treated with FR light contradicts the findings of Xi *et al* (2010), which showed that MFT promotes germination in after-ripened seeds treated with ABA. Mach (2014) showed that under FR light conditions, phyA causes ABA levels to increase. Thus, *mft-2* seeds treated with ABA should have similar germination pattern with those treated with FR light (they should germinate more than WT). The phenotype of *mft-2* treated with ABA is being investigated further by the Graham laboratory.

Figure 3.1 shows that the *MFT* is mainly expressed in the endosperm which is also known to suppress germination of nondormant seeds subjected to FR light treatment (Lee *et al*, 2012). Further studies will have to be carried out to investigate if MFT is also involved in the repression of germination by the endosperm.

MEE26 was used as a candidate gene that could help in understanding the phenotype of *mft-2*. It was initially hypothesized that because it is up-regulated in the *mft-2* mutant (MFT suppresses it), it will be a promoter of germination. Surprisingly, the results indicated that it suppresses germination in freshly harvested seeds and promotes dormancy in after-ripened seeds treated with FR light. This is consistent with the fact that *MEE26* expression is up-regulated by FR light, PIF1, ABI5, and the DELLAs, which are all negative regulators of germination. The seedlings of *mft-2* and *pif1*-1have shorter hypocotyls in

the dark, thus in the future, it will be worthwhile to investigate if the MEE26IR lines will have the same phenotype. Although the role of MEE26 in the wider context of seed dormancy and germination has been unravelled, it cannot explain the phenotype of *mft-2*.

MEE26 is part of a group of genes involved in gametophyte development and also plays a role in the development of embryo and endosperm. Some *mee* mutants, like *mee22,* show phenotypes such as a decreased growth of roots and shoots (Leasure, *et al,* 2008). Pagnussat *et al* (2004) identified *mee26* mutants amongst a group of mutants having an arrested endosperm development. Although there were no observable growth or developmental problems observed on MEE26oe and MEE26IR lines, future work could be to carry out histological analyses on the seeds of MEE26IR lines to check for any developmental issues. Perhaps, silencing *MEE26* does not lead to developmental and growth abnormalities as seen in some *mee* mutants.

CYP89A2 was another candidate gene that was identified for further studies in order to understand *mft-2*. Because *CYP89A2* is down-regulated in *mft-2* mutant (MFT promotes it), our hypothesis was that it is a repressor of germination. Surprisingly, though, the results indicated that it promoted germination in freshly harvested and after-ripened seeds treated with FR light. Therefore, *CYP89A2* cannot explain the *mft-2* phenotype. The contradictory aspect of these results is that expression analyses indicated that *CYP89A2* is induced by FR light as well as other FR-light pathway factors such as PIF1, ABI5 and the DELLAs which are all negative regulators of germination. Therefore, it is rather surprising that CYP89A2oe lines had higher germination frequencies. However, this could be explained by the fact that CYP89A2oe lines were more permeable than WT after staining with tetrazolium salts. Since it is highly expressed in the seed coat, perhaps its overexpression had an effect on the seed coat and made it more permeable. When there is a reduction in the thickness of the testa and an increase in its permeability, the dormancy level of the seed is reduced (Debeaujon *et al,* 2000). It is unfortunate that there were no silencing lines for *CYP89A2*, and the only T-DNA insertion line available (a Salk line) germinated similarly to WT. Thus, future work would be to make silencing lines or mutants as this could provide a better understanding of the role of CYP89A2 in regulating seed germination. However, it could be possible that mutation of *CYP89A2* could also have had an effect on the seed coat, which

could alter the germination phenotype. For example, the *bodyguard1* mutant has a permeable endosperm and a low seed dormancy phenotype (De Giorgi *et al*, 2015).

Furthermore, *CYP89A2* is highly expressed in the seed coat, however, this tissue is dead at maturity, thus it be useful to extract RNA from endosperm and seed coat and compare expression of *CYP89A2* on both tissues. It could be that it is highly expressed in the endosperm.

It would be worthwhile to note that the strong overexpressor lines of *CYP89A2* had stunted growth or grew very weakly. This could signify that overexpressing this gene could cause some cytotoxic effects in the plant. Thus, it will be helpful to perform metabolite profiling in the future which could be used to investigate the metabolic composition of the seed coats of these transgenic lines compared to WT. This could also help in investigating if CYP89A2 is involved in seed coat/endosperm biosynthesis.

Taken together, the results of *MEE26* and *CYP89A2* indicate that the *mft-2* phenotype cannot be explained by one gene. There could be a cross-talk between a combination of different genes and/or multiple other factors. This shows that the *mft-2* phenotype could be the result of a complex network of genetic interactions.

APPENDICES

Appendix 1: **Results of the Transcriptomic Analysis**

		Gene number	DS (15oC mat)		7dai @ 4oC (15oC mat)		7dai @4oC (20oC mat)		Gene symbol	Gene description
			WT	mft-2	WT	mft-2	WT	mft-2		
Genes UP-regulated in mft-2 background	#1	AT2G34870.1	0.07 ± 0.12	26.80 ± 1.82	0.33 ± 0.31	17.73 ± 0.78	0.10 ± 0.17	6.07 ± 2.18	MEE26	hydroxyproline-rich glycoprotein family protein
	#2	AT4G24480.1	0.33 ± 0.15	9.60 ± 1.54	0.93 ± 0.12	11.00 ± 1.64	3.33 ± 0.75	18.43 ± 1.01		Protein kinase superfamily protein
	#3	AT5G05965.1	11.97 ± 0.81	183.43 ± 8.27	2.37 ± 0.75	59.77 ± 5.34	10.03 ± 2.91	55.00 ± 6.16		unknown protein
	#4	AT3G63210.1	0.23 ± 0.15	4.57 ± 0.60	1.33 ± 0.29	6.23 ± 0.60	5.63 ± 0.40	15.77 ± 1.03	MARD1	Protein of unknown function (DUF581)
	#5	AT4G01525.1	1.40 ± 0.70	19.33 ± 0.86	1.77 ± 0.65	57.07 ± 3.20	1.40 ± 0.40	24.50 ± 4.48	SADHU5-1	transposable element gene
	#6	AT5G62850.1	0.93 ± 0.23	12.30 ± 0.10	0.83 ± 0.15	31.00 ± 1.60	0.63 ± 0.21	6.60 ± 1.97	AtVEX1, SWEET5	Nodulin MtN3 family protein
	#7	AT3G55800.1	1.07 ± 0.21	11.43 ± 1.04	1.30 ± 0.53	13.60 ± 2.33	3.83 ± 1.42	14.03 ± 0.55	SBPASE	sedoheptulose-bisphosphatase
	#8	AT2G34700.1	2.30 ± 1.15	22.67 ± 3.61	2.47 ± 0.71	89.37 ± 14.10	0.80 ± 0.26	18.63 ± 4.12		Pollen Ole e 1 allergen and extensin family protein
	#9	AT1G80440.1	1.43 ± 0.40	14.30 ± 0.36	5.87 ± 0.61	18.63 ± 0.65	18.03 ± 1.02	39.30 ± 5.36		Galactose oxidase/kelch repeat superfamily protein
	#10	AT2G13547.1	0.60 ± 0.79	6.47 ± 1.43	2.37 ± 0.55	31.27 ± 4.06	2.30 ± 2.00	12.37 ± 1.27		unknown protein
	#11	AT5G62350.1	1.40 ± 0.44	12.40 ± 0.87	7.50 ± 1.61	17.57 ± 3.55	108.23 ± 8.38	317.17 ± 38.65		Plant invertase/pectin methylesterase inhibitor superfamily prot
	#12	AT1G71695.1	16.37 ± 2.60	132.63 ± 5.35	7.43 ± 0.15	50.07 ± 3.42	4.70 ± 1.22	24.97 ± 2.18		Peroxidase superfamily protein
	#13	AT3G21800.1	4.60 ± 1.01	34.83 ± 0.97	11.17 ± 0.38	51.53 ± 5.64	18.67 ± 1.27	52.33 ± 1.80	UGT71B8	UDP-glucosyl transferase 71B8
	#14	AT5G08260.1	1.03 ± 0.38	7.30 ± 0.44	11.23 ± 0.71	42.03 ± 7.24	139.07 ± 12.63	296.20 ± 27.50	scpl35	serine carboxypeptidase-like 35
	#15	AT4G08290.1	17.80 ± 2.50	107.20 ± 7.49	3.47 ± 0.49	47.43 ± 2.81	19.23 ± 2.50	101.37 ± 4.66		nodulin MtN21 /EamA-like transporter family protein
	#16	AT2G15970.1	5.40 ± 1.25	29.63 ± 0.50	61.97 ± 6.21	197.53 ± 6.41	83.30 ± 7.35	174.93 ± 11.22	COR413, FL3-5A3, ATCYP19	cold regulated 413 plasma membrane 1
	#17	AT4G15910.1	14.37 ± 2.40	76.00 ± 3.14	74.67 ± 5.40	199.90 ± 13.09	186.97 ± 30.83	301.80 ± 30.88	ATDI21, DI21	drought-induced 21
	#18	AT3G28740.1	6.30 ± 0.46	33.17 ± 1.53	26.87 ± 1.15	196.20 ± 2.54	29.30 ± 1.56	95.87 ± 16.31	CYP81D1	Cytochrome P450 superfamily protein
	#19	AT1G47540.2	680.03 ± 60.09	3107.40 ± 232.93	425.97 ± 10.89	2543.97 ± 267.66	436.50 ± 139.14	1012.73 ± 290.69		Scorpion toxin-like knottin superfamily protein
	#20	AT1G43650.1	12.63 ± 3.69	57.00 ± 4.81	3.17 ± 0.55	62.77 ± 7.84	6.73 ± 1.42	20.70 ± 3.12		nodulin MtN21 /EamA-like transporter family protein
	#21	AT2G41070.1	47.07 ± 3.42	199.97 ± 2.37	29.10 ± 0.79	130.17 ± 8.50	21.63 ± 4.85	51.57 ± 9.85	EEL, ATBZIP12, DPBF4	bZIP transcription factor family protein
	#22	AT1G04660.1	30.70 ± 1.57	124.43 ± 3.19	28.97 ± 5.33	132.50 ± 6.61	29.40 ± 0.44	98.47 ± 2.83		glycine-rich protein
	#23	AT3G63040.1	55.47 ± 9.78	215.03 ± 10.99	44.73 ± 1.99	274.30 ± 27.18	36.00 ± 3.93	76.83 ± 17.74		unknown protein
	#24	AT5G04470.1	6.83 ± 2.25	26.03 ± 4.06	7.57 ± 0.85	15.87 ± 1.36	18.03 ± 3.46	41.87 ± 2.56	SIM	cyclin-dependent protein kinase inhibitors
	#25	AT1G33790.2	11.97 ± 0.71	37.97 ± 2.95	40.03 ± 4.48	80.33 ± 3.47	154.70 ± 15.71	437.37 ± 43.59		jacalin lectin family protein
	#26	AT1G15010.1	8.27 ± 1.19	26.33 ± 0.85	1.77 ± 0.96	10.77 ± 1.76	2.87 ± 0.78	15.23 ± 2.81		unknown protein
	#27	AT1G65970.1	38.03 ± 0.91	106.83 ± 18.99	48.93 ± 7.64	320.63 ± 5.84	95.07 ± 30.65	198.13 ± 39.45	TPX2	thioredoxin-dependent peroxidase 2
	#28	AT1G62290.1	56.20 ± 4.45	151.63 ± 11.20	42.03 ± 1.70	199.63 ± 10.84	46.50 ± 2.75	177.83 ± 6.18		Saposin-like aspartyl protease family protein
	#29	AT2G26010.1	18.00 ± 5.00	44.90 ± 3.05	25.17 ± 1.50	70.63 ± 10.67	2.93 ± 0.67	10.70 ± 0.61	PDF1.3	plant defensin 1.3

Table 1: **Genes Up-regulated in *mft-2* Compared to WT Subjected to Three Different Treatments.** 1) DS (15 ºC mat) = seeds matured 15 ºC and are therefore highly dormant. 2) 7dai @4 ºC (15 ºC mat) = seeds matured at 15 ºC and left to imbibe in the dark for 7 days at 4 ºC. 3) 7dai@4 ºC (20 ºC mat) = *seeds* matured at 20 ºC and left to imbibe in the dark for 7 days at 4 ºC. Due to these different treatments, the level of dormancy will differ. *MEE26* was at the top of the list of genes that were up-regulated in the *mft-2* background. The units represents Reads Per Kilobase of transcript per Million (RPKM) mapped reads.

		Gene number	DS (15oC mat)		7dai @ 4oC (15oC mat)		7dai @4oC (20oC mat)		Gene symbol	Gene description
			WT	mft-2	WT	mft-2	WT	mft-2		
Genes DOWN-regulated in mft-2 background	#1	AT1G64900.1	29.57 ± 3.69	0.57 ± 0.23	31.80 ± 2.46	4.53 ± 0.84	36.63 ± 1.72	4.90 ± 0.53	CYP89A2	cytochrome P450, family 89
	#2	AT1G17430.1	12.13 ± 1.97	0.17 ± 0.15	17.53 ± 1.06	1.73 ± 0.70	8.93 ± 0.15	1.47 ± 0.06		alpha/beta-Hydrolases superfamily protein
	#3	AT1G23800.1	6.20 ± 0.35	0.33 ± 0.25	7.97 ± 0.15	0.20 ± 0.10	4.83 ± 0.57	0.37 ± 0.25	ALDH2B7	aldehyde dehydrogenase 2B7
	#4	AT1G19440.1	1002.27 ± 257.67	99.03 ± 4.92	831.50 ± 55.09	146.13 ± 4.53	1042.10 ± 223.96	74.37 ± 12.98		Polyketide cyclase/dehydrase and lipid transport superfamily prot
	#5	AT1G72570.1	18.40 ± 1.39	1.90 ± 0.40	32.83 ± 2.29	3.83 ± 0.75	33.80 ± 2.88	9.17 ± 0.87		Integrase-type DNA-binding superfamily protein
	#6	AT2G18570.1	116.60 ± 1.06	12.93 ± 1.11	145.47 ± 5.40	42.57 ± 2.20	123.73 ± 6.73	45.43 ± 2.90		UDP-Glycosyltransferase superfamily protein
	#7	AT1G14960.1	73.90 ± 15.16	10.67 ± 3.74	18.10 ± 1.80	2.47 ± 0.42	29.80 ± 3.25	5.27 ± 1.85		Polyketide cyclase/dehydrase and lipid transport superfamily prot
	#8	AT5G57550.1	7377.83 ± 246.26	1083.30 ± 10.25	3620.33 ± 361.32	901.60 ± 74.95	572.53 ± 38.15	125.93 ± 21.39	XTR3, XTH25	xyloglucan endotransglucosylase/hydrolase 25
	#9	AT4G28520.1	24174.57 ± 2313.45	3592.73 ± 94.51	27659.37 ± 1540.36	5226.60 ± 174.07	14431.60 ± 693.46	2052.93 ± 267.89	CRU3, CRC	cruciferin 3
	#10	AT1G52920.1	32.17 ± 0.51	4.97 ± 0.55	11.53 ± 1.42	1.13 ± 0.06	5.73 ± 1.33	0.70 ± 0.26	GCR2, GPCR	G protein coupled receptor
	#11	AT1G18100.1	26.07 ± 4.04	4.07 ± 0.55	50.97 ± 1.47	2.07 ± 0.49	90.37 ± 2.89	2.80 ± 0.10	MFT	PEBP (phosphatidylethanolamine-binding protein) family protein
	#12	AT3G25780.1	7.60 ± 1.05	1.17 ± 0.25	5.53 ± 0.21	0.60 ± 0.44	3.67 ± 0.35	0.33 ± 0.21	AOC3	allene oxide cyclase 3
	#13	AT4G19170.1	156.20 ± 13.34	32.03 ± 0.65	66.57 ± 5.60	4.77 ± 0.76	6.07 ± 2.31	1.33 ± 0.15	NCED4, CCD4	nine-cis-epoxycarotenoid dioxygenase 4
	#14	AT4G11310.1	3453.53 ± 208.08	768.87 ± 28.82	3140.63 ± 144.54	452.10 ± 66.98	1611.47 ± 55.75	152.27 ± 20.43		Papain family cysteine protease
	#15	AT3G21360.1	741.57 ± 227.45	175.47 ± 5.56	427.80 ± 44.09	176.07 ± 13.80	275.60 ± 37.42	62.57 ± 9.55		2-oxoglutarate and Fe(II)-dependent oxygenase superfamily
	#16	AT5G54740.1	20010.17 ± 1337.98	4902.97 ± 220.75	23396.80 ± 549.82	6910.53 ± 355.64	17874.77 ± 1737.73	1793.10 ± 369.37	SESA5	seed storage albumin 5
	#17	AT3G21380.1	1391.63 ± 183.54	341.73 ± 17.34	64.37 ± 5.16	31.40 ± 1.93	64.97 ± 6.07	9.87 ± 1.93		Mannose-binding lectin superfamily protein
	#18	AT1G14930.1	1136.30 ± 282.19	304.77 ± 10.79	1103.67 ± 69.09	575.60 ± 24.97	1119.67 ± 212.76	742.00 ± 109.66		Polyketide cyclase/dehydrase and lipid transport superfamily prot
	#19	AT3G22500.1	3318.80 ± 347.57	892.57 ± 27.70	2377.43 ± 115.43	450.07 ± 46.08	920.33 ± 63.85	62.23 ± 8.64	ATECP31	Seed maturation protein
	#20	AT5G54770.1	506.83 ± 91.89	150.80 ± 13.65	622.30 ± 46.06	199.93 ± 9.52	388.13 ± 27.49	175.93 ± 7.16	THI1, TZ, THI4	thiazole biosynthetic enzyme, chloroplast (ARA6) (THI1) (THI4)
	#21	AT1G03890.1	778.00 ± 49.41	231.10 ± 15.62	74.70 ± 3.34	12.67 ± 1.90	12.87 ± 2.63	4.23 ± 1.72		RmlC-like cupins superfamily protein
	#22	AT2G43510.1	18.13 ± 4.12	5.30 ± 0.66	82.90 ± 9.92	51.87 ± 1.78	84.33 ± 19.49	48.80 ± 10.13	ATTI1, TI1	trypsin inhibitor protein 1
	#23	AT3G51810.1	1668.87 ± 266.90	501.80 ± 23.53	1338.80 ± 65.48	611.77 ± 28.80	776.07 ± 52.65	176.90 ± 36.92	ATEM1, GEA1, AT3, EM1	Stress induced protein
	#24	AT3G21370.1	5098.30 ± 857.55	1542.40 ± 74.07	3404.87 ± 180.54	1519.43 ± 95.73	2009.87 ± 182.51	469.73 ± 31.27	BGLU19	beta glucosidase 19
	#25	AT3G48660.1	260.33 ± 9.35	82.20 ± 4.16	104.77 ± 21.76	35.23 ± 1.19	49.03 ± 4.08	24.20 ± 3.73		Protein of unknown function (DUF 3339)
	#26	AT1G70840.1	1815.20 ± 367.86	578.67 ± 24.27	2260.77 ± 197.76	1389.87 ± 77.75	1937.20 ± 303.30	402.03 ± 63.83	MLP31	MLP-like protein 31
	#27	AT4G15630.1	159.40 ± 12.87	51.13 ± 3.01	222.20 ± 6.27	133.90 ± 19.58	131.27 ± 10.03	54.00 ± 7.84		Uncharacterised protein family (UPF0497)
	#28	AT3G26770.1	96.20 ± 3.42	33.20 ± 1.30	80.40 ± 5.20	41.00 ± 0.72	60.87 ± 7.35	21.13 ± 3.46		NAD(P)-binding Rossmann-fold superfamily protein
	#29	AT5G44380.1	9.53 ± 1.62	3.27 ± 0.67	36.77 ± 3.23	5.57 ± 0.49	23.30 ± 1.70	5.53 ± 0.06		FAD-binding Berberine family protein
	#30	AT4G20820.1	14.67 ± 0.87	5.33 ± 0.67	28.90 ± 1.87	12.43 ± 0.23	7.97 ± 0.86	2.63 ± 1.31		FAD-binding Berberine family protein
	#31	AT2G29340.1	30.43 ± 1.90	11.27 ± 0.58	54.97 ± 6.18	13.60 ± 0.89	27.90 ± 6.19	2.47 ± 0.38		NAD-dependent epimerase/dehydratase family protein
	#32	AT4G15620.1	82.60 ± 2.57	30.90 ± 1.74	108.13 ± 11.46	53.13 ± 1.04	51.73 ± 4.29	9.47 ± 3.41		Uncharacterised protein family (UPF0497)
	#33	AT5G07190.1	175.40 ± 12.28	68.77 ± 8.10	82.10 ± 11.27	17.47 ± 1.36	38.77 ± 1.66	7.40 ± 0.35	ATS3	seed gene 3
	#34	AT5G01520.1	74.97 ± 5.32	29.60 ± 1.61	45.07 ± 4.72	23.27 ± 1.53	27.00 ± 2.21	8.20 ± 1.76		RING/U-box superfamily protein
	#35	AT1G19570.1	81.70 ± 2.38	34.17 ± 0.87	82.37 ± 1.88	40.00 ± 2.52	83.33 ± 7.61	52.67 ± 2.61	DHAR1, DHAR5	dehydroascorbate reductase
	#36	AT3G23570.1	80.53 ± 6.61	37.73 ± 0.21	92.40 ± 6.50	44.67 ± 7.10	101.27 ± 3.36	40.77 ± 7.17		alpha/beta-Hydrolases superfamily protein
	#37	AT5G44120.3	20313.13 ± 1940.63	9599.33 ± 482.00	25782.70 ± 669.85	15138.80 ± 613.02	19876.87 ± 1035.59	5879.13 ± 1135.23	CRA1, CRU1	RmlC-like cupins superfamily protein
	#38	AT3G22490.1	963.23 ± 136.06	482.03 ± 19.11	337.73 ± 16.86	152.10 ± 11.59	85.23 ± 14.26	18.13 ± 2.86		Seed maturation protein

Table 2: Results of the transcriptomic analysis showing genes up-regulated in *mft-2*. This ranking was based on the expression of the genes in dry seeds. 1) DS (15 ºC mat) = seeds matured 15 ºC and are therefore highly dormant. 2) 7dai @4 ºC (15 ºC mat) = seeds matured at 15 ºC and left to imbibe in the dark for 7 days at 4 ºC. 3) 7dai@4 ºC (20 ºC mat) = seeds matured at 20 ºC and left to imbibe in the dark for 7 days at 4 ºC. Due to these different treatments, the level of dormancy will differ. *CYP89A2* was at the top of the list of genes that were down-regulated in the *mft-2* background. The units represent Reads Per Kilobase of transcript per Million (RPKM) mapped reads.

Appendix 2: **Designing of Transgenic Lines**

The vectors of MEE26-OE, MEE26-IR and CYP89A2-OE were utilised in transforming Arabidopsis Col plants by Agrobacterium floral dipping procedure (Bent, 2006; Mara *et al*, 2010). MEE26 was overexpressed in WT, MEE26 silenced in *mft*-2 and CYP89A2 overexpressed in WT and *mft-2*.

The BAR gene which is resistant to the BASTA herbicide was used to select for transformed plants. This gene is in the pFGC5941 found adjacent to the 35S promoter in the LB and RB of the T-DNA. This gene selects for plants that have been transformed which are called the 'T1'. Different T1 plants come from various independent transformation procedures. Plants from T1 generation will later produce T2 seeds.

The vector for the **MEE26-OE** was created by PCR amplifying MEE26 coding sequence (CDS) from Col genomic DNA. To PCR-engineer MEE26 CDS to carrying the XhoL and BamHI, which are restriction enzymes sites, Xhol and BamHI were used to clone it down-stream of the CaMV-35S promoter of the binary vector pFGC5941 (figure 2.1).

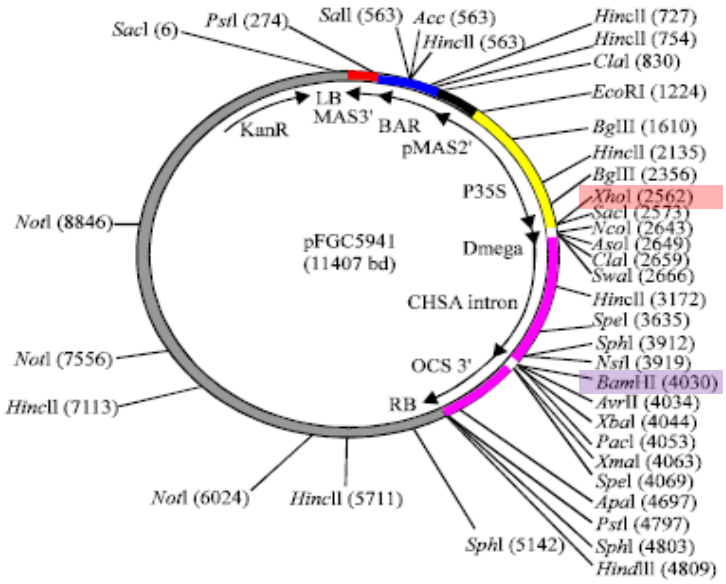


Figure 2.1: Vector design for transformation of MEE26oe lines

The vector for MEE26IR was engineered by cloning the MEE26 CDS in the sense direction in the the XhoL and NcoL sites found in the pFGC5941 and then in the anti-sense direction in the BamHI and

XmaL sites (figure 3.2). This vector will drive transcription of an IR. This causes an IR-RNA which has a hair-pin structure that promotes silencing or RNA interference of endogenous transcripts of MEE26.

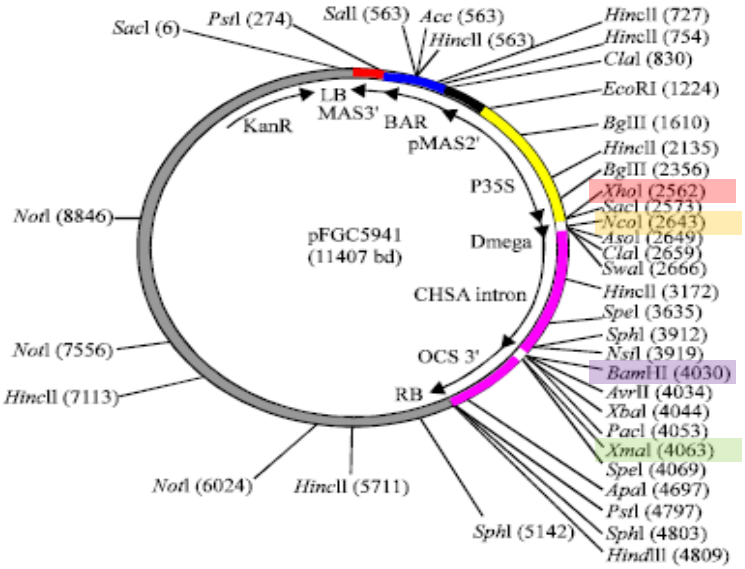


Figure 2.2: Vector design for transformation of MEE26IR lines

The vector for the **CYP89A2-OE** lines were made by using the CYP89A2 coding sequences (CDS) amplified from Col genomic DNA. PCR was used to engineer the CDS to carrying the XhoL and XmaL restriction enzymes sites that used to clone it into pFGC5941. Because T-DNA insertion lines were available in NASC, CYP89A2-IR lines were not made.

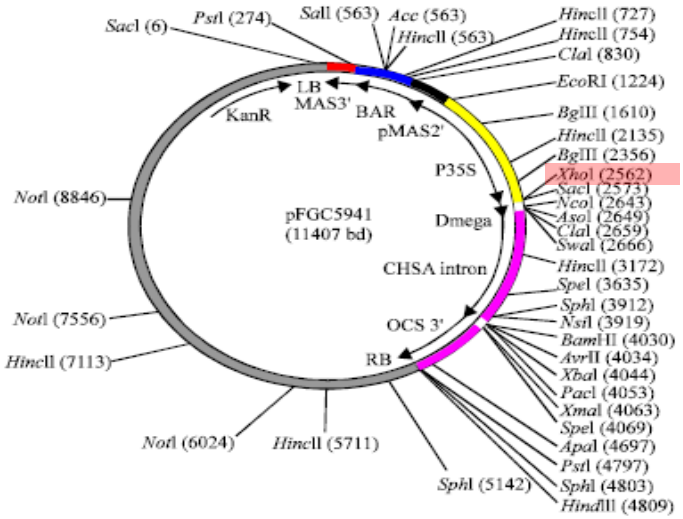


Figure 2.3: Vector design for transformation of CYP89A2oe lines

Appendix 3: **Selection of MEE26IR(WT) Crosses that are Homozygous for WT**

In order to get rid of the *mft-2* or bring *MFT* back to WT levels, MEE26IR lines in *mft-2* were crossed to WT. The F1 seeds from these crosses were allowed to self-pollinate and produce F2 seeds, which were then assessed for basta resistance so as to select for single insertion lines. The seedlings were then left to seed and form the F3 generation. Isolation of homozygous lines occurred through selection for basta resistance and those that were 100% basta resistant were selected and sown in soil. Figure 2.1 shows that line # 4.1 and # 4.5 was resistant to basta.

PCR was used to select for crosses that were homozygous for WT. PCR was carried out using the LP and RP primers flanking the insertion (figure 2.2a) and genomic DNA from WT, *mft-2* and sibling of MEE26IR (WT) 13.9 crosses. WT and sibling of MEE26IR (WT) 13.9 crosses were amplified in the predicted 900bp lane, but *mft-2* was not amplified (figure 2.2b). PCR using RP and LB (see figure 2.2a) failed to amplify any WT (as expected) and siblings of MEE26IR (WT) 13.9 crosses (figure 2.2b), but amplified *mft-2*. This indicates that sibling of MEE26IR (WT) 13.9 crosses are homozygous for WT.

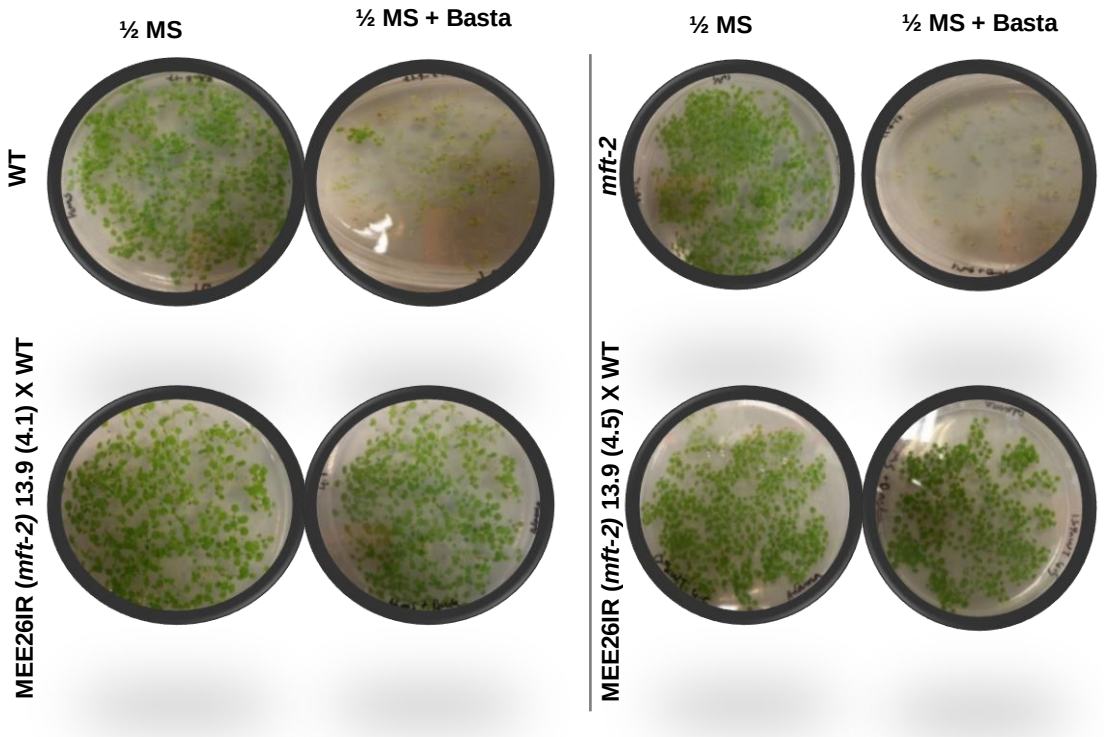


Figure 3.1: MEE26IR X WT lines grown in ½ ms and ½ ms +basta water agar plates along with WT and *mft-2* as controls. MEE26IR lines are 100% resistant.

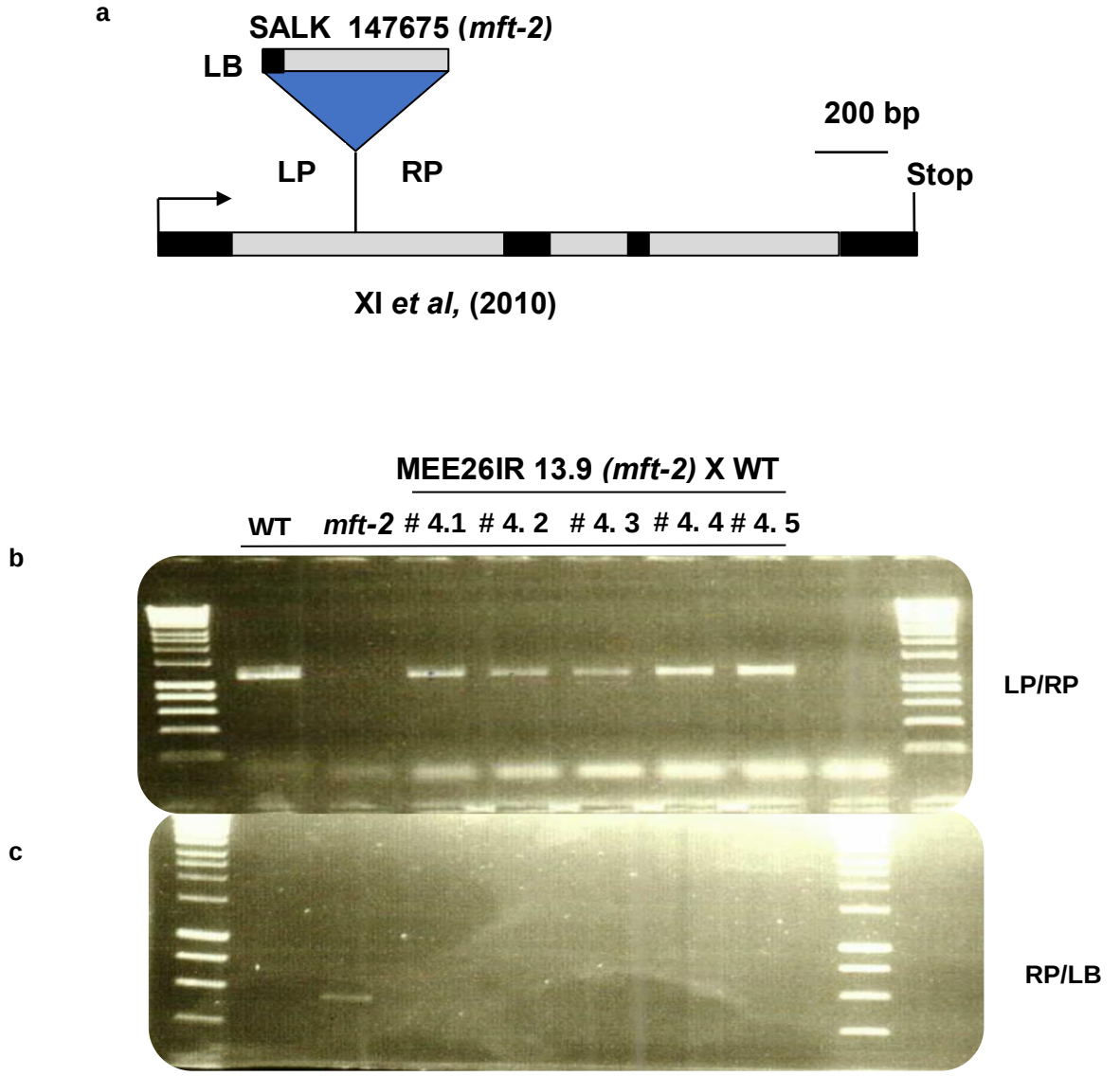


Figure 3.2: **PCR Genotyping of MEE26IR (WT)** (a)Schematic representation of the *MEE26* gene showing location of the SALK T-DNA insertion and the DNA primers (RP and LP) used to assay for the presence/absence of the insertion. (b) when RP/LP are used, a band will be amplified in the WT control as well as the homozygous lines. (c) PCR reaction using RP/LB is carried out to verify that the homozygous line is free of *mft-2*. This PCR reaction will not amplify WT control and lines homozygous for WT in the 900bp lane. However, *mft-2* control is amplified in the 300bp lane. This indicates that lines 4.1 to 4.5 are homozygous for the WT

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