ABSCISIC ACID AND GIBBERELLIN CONTROL SEED GERMINATION THROUGH NEGATIVE FEEDBACK REGULATION BY *MOTHER OF FT AND TFL1*

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SUMMARY

Seed germination is a critical stage in plant development, as it determines the time point when a plant starts its new life cycle. This process is under combinatorial control by endogenous and environmental cues. Abscisic acid (ABA) and gibberellin (GA) are two critical endogenous factors that integrate signals from biotic and abiotic environmental stresses. ABA and GA play antagonistic roles in the regulation of seed germination, with the former inhibiting while the latter promoting seed germination.

In this thesis, we demonstrate that *MOTHER OF FT AND TFL1 (MFT)*, which encodes a phosphatidylethanolamine-binding protein, acts as a novel regulator of seed germination via responding to both ABA and GA signaling pathways in *Arabidopsis*. *MFT* is specifically induced in the radicle-hypocotyl transition zone of the embryo in response to ABA and *mft* loss-of-function mutants show hypersensitivity to ABA in terms of seed germination. Genetic analyses revealed that in germinating seeds, *MFT* expression is directly regulated by ABA-INSENSITIVE3 (ABI3) and ABI5, two key transcription factors in ABA signaling pathway. On the other hand, *MFT* is also upregulated by DELLA proteins in the GA signaling pathway. *MFT* in turn provides negative feedback regulation of ABA signaling by directly repressing *ABI5*.

In summary, we conclude that during seed germination, *MFT* promotes the embryo growth potential by constituting a negative feedback loop in the ABA signaling pathway.

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LIST OF ABBREVIATIONS AND SYMBOLS

Chemicals and reagents

ABA	abscisic acid
BASTA	glufosinate ammonium
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DMSO	dimethyl sulfoxide
dNTP	deoxynucleoside triphosphate
EDTA	ethylene-diamine-tetra-acetate
GA_3	gibberellic acid
HCl	hydrochloric acid
H_2O	water
H_2SO_4	sulphuric acid
KCl	potassium chloride
$K_3Fe(CN)_6$	potassium ferricyanide
$K_4Fe(CN)_6$	potassium ferrocyanide
LiCl	lithium chloride
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
$MnCl_2$	magnesium chloride
NaAc	sodium acetate
NaCl	sodium chloride
NaOH	sodium hydroxide
$NaHCO_3$	sodium bicarbonate
Na_2CO_3	sodium carbonate
NBT/BCIP	nitro blue tetrazolium chloride/ 5-bromo-4-chloro-3-

	indolyl phosphate, toluidine salt
PBS	phosphate buffered saline
PIPES	piperazine-N,N′-bis(2-ethanesulfonic acid)
PMSF	phenylmethylsulfonylfluoride
PVA	polyvinyl alcohol
SDS	sodium dodecylsulphate
Tris	tris (hydroxymethyl)-aminomethane
X-Gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid
$ZnSO_4$	zinc sulfate

Units and measurements

°C	degree celsius
Ω	ohm
µF	microfarad(s)
µg	microgram(s)
µl	microlitre(s)
µm	micrometer(s)
µM	micromolar
bp	base pairs
Ct	threshold cycle
g	gram(s)
h	hour(s)
kb	kilo base-pairs
kDa	kilo Dalton(s)
kV	kilo volt(s)
M	molar
min	minute(s)
ml	milliliter(s)
mM	millimolar
ng	nanogram(s)

OD_{600}	optical density at wavelength 600 nm
rpm	revolution per minute
sec	second(s)
v/v	volume per volume
w/v	weight per volume

Others

BLAST	basic local alignment search tool
ChIP	chromatin immunoprecipitation
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
cDNA	complementary DNA
etc.	et cetera (and so forth)
et al.	et alii (and others)
i.e.	that is
RNA	ribonucleic acid
RNase	ribonuclease
mRNA	messenger RNA
tRNA	transfer RNA
PCR	polymerase chain reaction
RT-PCR	reverse transcription polymerase chain reaction
T-DNA	transfer DNA

CHAPTER 1

LITERATURE REVIEW

The first phase transition in the life cycle of a higher plant is seed germination. Seed germination *sensu stricto* (in a strict sense) can be defined as the reactivation of metabolism of seed embryo including the growth of embryonic root termed as radicle and embryonic leaf (leaves) termed as cotyledon(s). Seed germination is blocked by seed dormancy, which is sometimes considered as an adaptive trait that optimizes the distribution of germination over time (Bewley, 1997).

Whether a seed should remain dormant or proceed to germination under certain circumstances is important with respect to its survival as a species. When the environment is nonpermissive for germination, then the seed remains dormant. Such dormancy is advantageous for seed survival. For instance, dormancy prevents vivipary, a phenomenon of precocious germination prior to fruit harvest, which causes losses in fruit yield and is adverse to agricultural plants. However, when the environment becomes favorable for seed germination, dormancy must be released to allow germination to happen. This is important as seed germination marks the beginning of a new life and is prerequisite to agricultural sustainability.

Extensive work aiming to address the question of how seed dormancy and germination are regulated has been carried out in the model plant *Arabidopsis*. An *Arabidopsis* seed is comprised of three components: an embryo and two covering layers, that is endosperm and testa. The embryo is the new plant in miniature. The endosperm is a nutritive tissue with living cells surrounding and absorbed by the

embryo. The testa, or seed coat, usually consisting of dead cells, is mainly a protective tissue enclosing the embryo and endosperm. During seed germination, the endosperm and testa impose physical restraints on embryo growth. Such coat-imposed restraints must be overcome by the growth potential of the embryo for the successful transition of a seed from dormancy to germination under favorable conditions.

The phase transition from dormancy to germination is coordinated by both exogenous and endogenous cues (Koornneef et al., 2002). Exogenous cues, which include light, temperature, osmotic potential, pH and nutrients availability, in the control of seed dormancy and germination are well documented (Bewley and Black, 1994). For example, synergistic interaction of light and low temperature has been demonstrated to terminate seed dormancy and promote seed germination. Endogenous cues, especially phytohormones including abscisic acid (ABA), gibberellin (GA), brassinosteriods (BRs), ethylene, auxin, and cytokinin, interact with each other to form complicated signaling networks that regulate several processes in seed development. Among these phytohormones, ABA and GA are particularly well-known for their antagonistic roles in the regulation of seed dormancy and germination, with the former inhibiting while the latter promoting seed germination (Xie et al., 2006).

Basically, either exogenous or endogenous cues are ultimately processed by gene regulatory networks. In the past two decades, much effort has been devoted to identifying genes involved in the control of seed dormancy and germination. The classical technique is forward genetics, and this technique has led to the establishment of fundamental signaling networks. Nowadays, reverse genetics is widely adopted to discover the function of a specific gene of interest. Using this approach, some new genes regulating seed dormancy or germination process have come to light. However, considering that the number of genes is huge and lots of genes may have subtle or redundant phenotypes, there is a need to identify and characterize novel genes which can link those known genes together. Upon the combination of all the relevant genes, the genetic mechanism underlying seed dormancy and germination will be gradually uncovered.

The subsequent sections provide an overview of seed dormancy and germination, followed by the exogenous and endogenous factors influencing these biological events, as well as the major regulatory genes involved in these processes.

1.1 Seed Development, Germination and Dormancy

The creation of a seed in a higher plant happens when male and female sex cells meet and fuse together, and the plant comes through to nearly the end of its life

cycle. After the seed ripens, it can start the life cycle all over again from the very beginning step called seed germination.

Most fully ripened and dried seeds go through a quiescent period during which no active growth takes place. This property makes seed storage and transportation possible, most importantly, it enables the seeds to survive adverse environment until the favorable growth conditions occur. When such circumstances appear, the metabolism of a viable seed will be activated and the embryo inside starts to grow and penetrates the surrounding structures. A dormant seed may achieve virtually all of the metabolic steps required to complete germination, yet the embryonic axis fails to elongate. This may caused by either the constraints from the surrounding tissues or the embryo itself is dormant (Bewley, 1997).

1.1.1 Seed Structure

Before considering seed germination, it is appropriate first to briefly review the development of a seed. In angiosperms (flowering plants), seed development is initiated with the double fertilization event involving two sperm cells and two female gametes. The female gametes consist of a haploid egg cell and a diploid central cell, each is fertilized with one sperm cell to form the zygote (later differentiated into the embryo) and the endosperm, respectively. Surrounding the embryo and the endosperm is the testa, which is maternally derived in response to

fertilization. These three components undergo a series of cell division, differentiation, or death, and finally give rise to a mature seed. Taking model plant *Arabidopsis thaliana* (called *Arabidopsis* hereafter) as an example, a fully-developed embryo makes up most of the mass of a mature seed, while endosperm and testa are just two thin layers (Figure 1).

1.1.1.1 Embryo

Embryo development occurs in two distinct phases, morphogenesis and maturation. During the first morphogenesis phase, the basic body plan of the plant is progressively established. The zygote undergoes cell divisions to produce the embryo pattern from octant shape, through dermatogen shape, globular shape, heart shape, torpedo shape, to bent-cotyledon shape (Berleth and Chatfield, 2002). Thereafter, the embryo starts to accumulate storage macromolecules (lipids, starch, and proteins), and is converted to a state of metabolic quiescence as it desiccates (Harada, 1997). A mature *Arabidopsis* embryo consists of two cotyledons and an embryonic axis. The embryonic axis composed by a hypocotyl and a radicle (embryonic root) is a multilayer tissue (Figure 1), which is developed from a series of transverse and periclinal cell divisions (Benfey and Schiefelbein, 1994; Scheres et al., 1994). The epidermis is an outer layer of cells that take in water and nutrients as well as protect the underlying tissues of the root. Just inside the epidermis are two layers of cells known as the cortex, when the radicle protrudes through the

covering tissues and grows to postembryonic root, the double cortex layer develops into a single cortex layer (Dolan et al., 1993; Scheres et al., 1994). Root cortex has diverse functions in different plant species, it mainly serves as the path across which water and nutrients from the outside of the root pass and move easily through and around them. Root cortex cells also have active transport mechanisms in their membranes that keep water and nutrients moving deeper toward the center of the root. At the inner boundary of the cortex is a single layer of cells known as the endodermis, but it is argued that endodermis is actually a part of the cortex (Lux et al., 2004). The endodermis facilitates the movement of water from cortex to the center of the root, it also functions as a barrier to apoplastic ion movement and in preventing the backflow of ions from the inside of the root (Enstone et al., 2003). The endodermis wraps the provasculature, which develops into vascular cylinder or stele in the root of a growing plant. The vascular tissue is conducting tissue, which transport water and dissolved substances from the root to the aerial parts of the plant such as stems and leaves, it also receives organic substances from the leaves.

1.1.1.2 Endosperm

During the embryogenesis process, the nutrition for the embryo growth is persistently provided by the surrounding tissue called endosperm. The triploid endosperm is formed after double fertilization through the fusion of the diploid central cell with a sperm cell. There are three types of endosperm development in

flowering plants, termed nuclear, cellular, and helobial types based on their respective ontogenesis mode (Raghavan, 2006). In the nuclear mode, repeated free-nuclear divisions occur without cytokinesis for certain period of time before cell wall formation takes place to give rise to cellular structures. In the cellular type, nucleus divides with concomitant cytokinesis, i.e. cell plate formatiom, throughout the entire course of endosperm development. The helobial type is regarded as an intermediate type between the nuclear type and the cellular type, in which the first two daughter nuclei derived from the primary endosperm nucleus are first separated and then undergo distinct modes of development, one develops along the nuclear pattern and the other along the cellular pattern or remains undivided. The nuclear endosperm formation is the most common type and occurs in the model plant *Arabidopsis* (Olsen, 2004). During germination in angiosperm seeds, the endosperm has two known functions. In cereals, in which the endosperm makes up most of the mature seed, the endosperm serves as a source of starch and proteins, which provide nutrients to nourish the growing seedling. In *Arabidopsis*, the endosperm is largely absorbed during embryogenesis; therefore, it plays a nutritional role in nourishing the developing embryo rather than the seedling. During seed germination, this thin layer of endosperm imposes a constraint on radicle protrusion (Muller et al., 2006).

1.1.1.3 Testa

Enclosing both the embryo and endosperm and serving as a protective tissue is the testa. Unlike the embryo and endosperm, which are the products of fertilization, the testa is a maternal tissue derived from the differentiation of the integument cells, and it is initially developed with five layers in *Arabidopsis* (Beeckman et al., 2000; Windsor et al., 2000). By the time the seed is mature, the cells in all the layers of the testa are dead probably due to programmed cell death since those layers do not die simultaneously, but instead in a specific order (Nakaune et al., 2005). Mucilage is present in the testa of many plant species including *Arabidopsis* (not shown in Figure 1), which starts to accumulate at the torpedo stage and continues during maturation in the outermost layer of the testa until desiccates in a mature seed (Beeckman et al., 2000; Windsor et al., 2000). The mucilage of the testa provides assistance to seed dispersal, germination, and seedling establishment (Penfield et al., 2001; Huang et al., 2008). In addition to these aspects, the testa also plays important roles in the protection of the embryo from mechanical injury and pathogen attack, as well as the maintenance of seed dormancy.

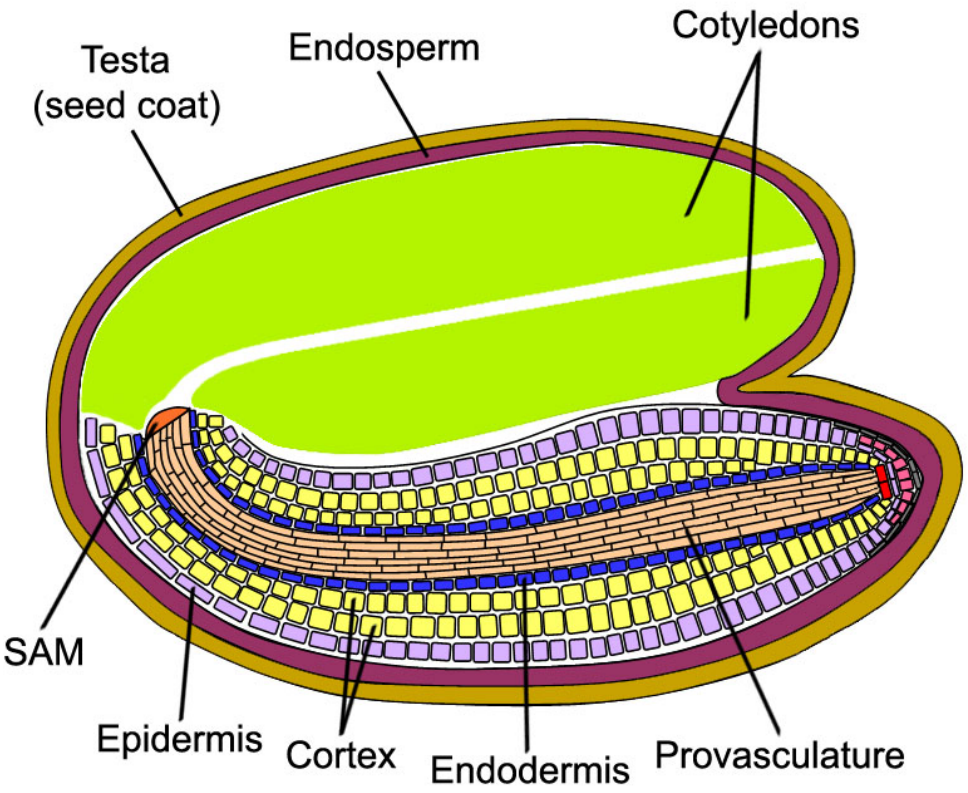

Figure 1. Schematic Drawing Showing the Anatomy of A Mature *Arabidopsis* Seed.

An embryo is enclosed in two layers, the inner one is endosperm (purple color), the outer one is testa or seed coat (yellow ochre color).The embryo consists of two cotyledons (green color) and an embryonic axis. The tissues of the embryonic axis are divided into four cell types, which are formed by highly organized cell arrays. These cell types are termed as epidermis, cortex, endodermis and provasculature shown in different colors from the outermost layer to the innermost layer. SAM, shoot apical meristem.

1.1.2 Three Phases of Imbibition Involved in Germination and Post-germinative Development

Generally, germination commences with water uptake, i.e. imbibition, by the dry seed, followed by a series of metabolic changes, and ends with the protrusion of the radicle of the embryo through all the surrounding tissues.

Under most circumstances, air-dried seeds must imbibe water to drive subsequent metabolic processes. This initial water uptake is a physical process which occurs in both living and dead seeds. For viable and nondormant seeds, there is a three-phase pattern of water uptake (Figure 2) (Bewley, 1997; Nonogaki et al., 2007).

1.1.2.1 Activation and Resumption of Metabolism

Phase I is primarily a physical process, during which seed water content increases sharply, to 5- to 10-fold higher than that in the dry seed. Initially, water absorption by the dry seed is dependent on the permeability of the testa, of which the microphylar region embracing the radicle is most permeable in many seeds (Mcdonald et al., 1994). Besides, it has been reported that the water channel proteins may also affect the seed imbibition (Maurel et al., 1997). With the increase of moisture content, the seed swells and some physiological activities, such as respiration, enzyme synthesis,

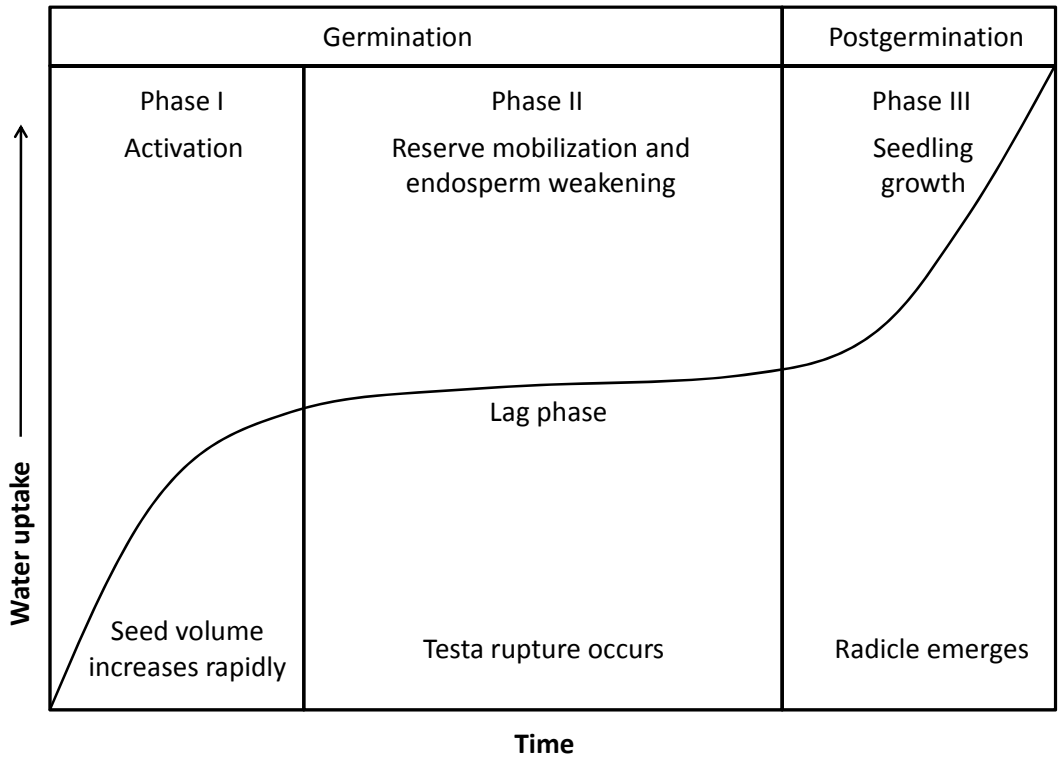

Figure 2. Three Phases of Seed Imbibition.

Phase I is characterized by rapid water uptake. During Phase I, seed volume increases rapidly and some physiological activities are activated. Phase II is a lag phase of imbibition. Physiological activities are speeded up, storage reserves are mobilized and endosperm constraint is weakened, and testa splits in this phase. Upon radicle penetrates through the covering layers, i.e. endosperm and testa, the seed enters Phase III. Radicle emergence indicates that the seed has just finished the transition from germination (cell elongation) to seedling growth (cell division).

and DNA repair, start to occur in the seed at this phase.

1.1.2.2 Reserve Mobilization and Endosperm Weakening

After Phase I, the rate of water intake slows down and seed water content is relatively constant or only slowly increases, which indicates the beginning of Phase II. At this plateau phase, the lipid and protein reserves accumulated during seed maturation are mobilized to fuel the rapid metabolism, and these storage reserves are mobilized first in the micropylar endosperm (Mansfield and Briarty, 1996). Meanwhile, new proteins essential for the support of normal cellular metabolism are synthesized as germination proceeds (Bewley and Marcus, 1990).

As an embryo is embedded in the endosperm that is further surrounded by the testa, the constraints imposed by these covering layers must be overcome by the growth potential of the embryo. The testa as a barrier for seed germination has been investigated by using testa mutants (Debeaujon et al., 2000). But testa rupture is not enough for radicle protrusion, weakening of endosperm is also required to allow radicle emergence, this process is associated with hydrolysis by cell wall-loosening proteins, such as expansin (Chen and Bradford, 2000), and endo-β-mannanase (Nonogaki et al., 2000). Testa rupture and endosperm rupture do not occur simultaneously, it has been demonstrated that testa rupture occurs firstly followed by endosperm rupture and finally radicle protrusion (Liu et al., 2005), and during

this process, ABA specifically inhibit endosperm rupture rather than testa rupture, the inhibitory effect of ABA can be counteracted by GA (Muller et al., 2006).

1.1.2.3 Radicle Emergence and Seedling Growth

Following the plateau phase (Phase II), there is a further increase in water uptake, with embryonic axis elongates and protrudes from the covering structures, which signals the end of germination and the beginning of post-germinative or seedling growth. Dead seeds can absorb water like viable seeds, but it cannot complete germination; therefore, it will never enter Phase III. Dormant seeds also cannot proceed to Phase III until dormancy is broken. Before Phase III, cell elongation is believed to be enough for the radicle protrusion since cell division, at most cases, takes place after radicle emergence (Barroco et al., 2005; Masubelele et al., 2005). Both cell elongation and division are essential for the subsequent seedling growth.

1.1.3 Seed Dormancy

Like many plant species, *Arabidopsis* possesses seed dormancy, which greatly contributes to the development of new species and dispersion of existing species (Baskin and Baskin, 1998). Besides, dormancy also helps reduce the risk of premature germination before seed harvesting. Thus, seed dormancy is to some extent considered as an advantageous trait of plant inherited during evolution.

Generally, dormancy can be classified into primary dormancy and secondary dormancy, the former occurs in an immature embryo during seed development, the latter occurs in a mature seed during seed imbibition (Amen, 1968).

1.1.3.1 Primary Dormancy

The induction of primary dormancy is correlated with the presence of ABA during seed maturation process. In many species, there are two peaks of ABA accumulation during seed development. Studies in *Arabidopsis* have showed that the first transient increase in ABA content is originated maternally and occurs prior to embryo maturation, thereby called maternal ABA; the second peak is present during maturation and is regulated by the genome of the embryo, thereby called embryonic ABA (Karssen et al., 1983). The accumulation of embryonic ABA, but not maternal ABA, is indispensable for the induction of primary dormancy (Karssen et al., 1983). Once the dormancy is established, endogenous ABA is not required and decreases significantly by seed maturity. In addition, disruption of ABA signal transduction also has a great impact on the induction of primary dormancy. For example, several ABA response loci like *ABA-INSENSITIVE 1* (*ABI1*), *ABI2*, and *ABI3*, upon loss-of-function, confer reduced dormancy phenotype (Koornneef et al., 1984); on the contrary, the ABA-hypersensitive mutants named *era*, short for *enhanced responsiveness to ABA*, exhibit enhanced primary dormancy (Cutler et al., 1996).

Primary dormancy ensures an embryo completes development, and prevents precocious germination in some species like maize. It has been shown that in the *Arabidopsis* accession Cvi, the seed loses its water content almost 5-fold from acquiring primary dormancy during late maturation phase until shed from the mother plant upon maturity (Baud et al., 2002). When the moisture content is further reduced to a certain level by dry storage, the seed loses primary dormancy. This process of breaking dormancy is called after-ripening and has many characteristics, including a decrease in ABA concentration and sensitivity, an increase in GA and light sensitivity, and a widening of temperature range for seed germination (Finch-Savage and Leubner-Metzger, 2006). Therefore, after-ripening releases the primary dormancy and determines the germination potential of seeds. Although the exact molecular mechanisms that regulate after-ripening process are unclear, initial attempt has shed some light on our understanding of such processes by a transcriptome profiling approach (Carrera et al., 2008). Another method to release dormancy is to subject seeds to moist chilling (cold stratification), or moist warming (warm stratification), depending on the species. In *Arabidopsis*, it has been recently found that cold treatment of imbibed seeds could increase endogenous GA level by inducing the GA biosynthesis genes (Yamauchi et al., 2004). Such cold-stimulated GA is more effective than exogenously-applied GA on dormancy breakage (Alonso-Blanco et al., 2003). Although as mentioned above that ABA is no longer required after the induction of primary dormancy, strong evidence suggests that *de novo*-synthesized ABA in dormant seeds during

imbibition maintain seed dormancy (Grappin et al., 2000; Ali-Rachedi et al., 2004) and such dormancy can be released with the decline of ABA content via ABA catabolism (Millar et al., 2006). Therefore, ABA-GA balance during imbibition is essential for the primary dormancy release.

1.1.3.2 Secondary Dormancy

After imbibed after-ripening seeds have lost primary dormancy, secondary dormancy might occur when improper conditions, such as unfavorable temperature, anoxia, inadequate light or nitrate, come into existence. Secondary dormancy is commonly observed in lots of species (Karssen, 1980; Hilhorst, 1998). In the soil seed bank, secondary dormancy enables cycling, through which different depths of dormancy are progressively gained or lost, until the environment is favorable for germination, and then seedling establishment (Baskin and Baskin, 1998; Hilhorst, 2007).

It is well-known that ABA plays a vital important role in the induction and maintenance of primary dormancy; however, whether it is also involved in the acquisition of secondary dormancy is largely unknown. Until recently, it has been reported that ABA is involved in the induction of secondary dormancy in Barley as exogenous ABA could significantly inhibit the germination of seeds which had lost primary dormancy (Leymarie et al., 2008). But the authors did not exclude the

possibility that the induction of secondary dormancy might due to reduced amount and sensibility of GA. Like its function in breaking primary dormancy, GA is also capable of releasing secondary dormancy as was discovered as early as 1970s (Bewley, 1979).

Since ABA and GA are involved in the control of both primary and secondary dormancy, a question was raised as to whether the molecular bases for the regulation of these two kinds of dormancy are different or not. Whole-genome microarrays have been developed as a powerful tool to address this question. Using this technology, people analyzed global transcript profiles of *Arabidopsis* seeds during dormancy and observed that significant differences exist in the transcriptomes of primary and secondary dormant seeds (Cadman et al., 2006; Finch-Savage et al., 2007).

1.2 Environmental Factors

Environment has a profound influence on seeds ranging from acquisition of dormancy to initiation of germination. Earlier research on the control of seed dormancy and germination was mainly focused on the environmental factors, including temperature, water, light, oxygen, etc. It was found that certain environmental condition may favor the germination of seeds in some species, but not other species. Therefore, no generalizations can be made as to which

environmental factor plays a constant role in the control of seed dormancy and germination over a variety of species. In view of this, the aim of this section is to review current knowledge concerning how environmental factors affect seed behavior in *Arabidopsis*.

1.2.1 Temperature

Temperature is a primary environmental cue affecting many aspects of plant development, including seed germination. In nature, the changes in seasonal temperature result in different germination timing of different plants. A well-known example is annual plants, which are principally classified into two categories, summer-annuals and winter-annuals. Summer-annuals overwinter as seeds and complete their life cycle during the same summer season; while winter-annuals germinate in the autumn, overwinter as seedlings before flowering in the spring. In other words, these two types of annual plants adopt different germination strategies in response to ambient temperature changes.

As an annual weed, *Arabidopsis* has both summer-annual accessions and winter-annual accessions in the field. In greenhouse conditions, winter-annual accessions of *Arabidopsis* are late flowering and summer-annual accessions flower early. The flowering time of winter-annuals can be greatly accelerated by an extended exposure to cold temperature, a process called vernalization. Genetic studies have

revealed that vernalization negatively regulates the expression of *FLOWERING LOCUS C* (*FLC*) to promote flowering time (Michaels and Amasino, 1999; Sheldon et al., 1999). Since then, *FLC* has long been regarded as a flowering repressor, but recently it has been reported that *FLC* also plays a critical role in the control of temperature-dependent seed germination (Chiang et al., 2009). It was found that high expression of *FLC* greatly increased the seed germination percentage under low temperature condition, and such enhancement of germination was substantially weakened when seeds were imbibed in a warm temperature (Chiang et al., 2009). Thus, in winter-annual *Arabidopsis*, highly expressed *FLC* responds to cool temperature to promote seed germination. On the contrary, *FLC* level is very low in summer-annual *Arabidopsis*, seeds remain dormant over winter and prepare for germination when ambient temperature reaches a proper level. Cold stratification is therefore a means to simulate overwintering to relieve seed dormancy and subsequently induces synchronized germination in *Arabidopsis*.

Opposite to cold treatment, which results in good germination performance of after-ripened *Arabidopsis* seeds, high temperature inhibits seed germination. Such suppression of seed germination at supraoptimal temperature is called thermoinhibition. The phenomenon of thermoinhibition was first found in lettuce seeds almost half a century ago (Berrie, 1966). In the case of winter-annual *Arabidopsis*, seed germination is inhibited by high temperature in summer, but the inhibition will be lost and germination occurs when the temperature falls into a

suitable range in autumn. Five genes have been identified to be involved in the germination response to supraoptimal temperature during imbibition through a screening in fully after-ripened seed pools in *Arabidopsis*. The mutants of these genes show resistance to thermoinhibition on seed germination, besides, the mutants exhibit reduced dormancy at harvest ripeness (Tamura et al., 2006). Four out of these five mutations have been mapped to their respective loci. One mutant is a new allele of *abi3*, an important ABA-insensitive mutant (Koornneef et al., 1984); a new mutant named *as thermoinhibition-resistant germination 2* (*trg2*) also exhibits ABA-insensitive germination phenotype (Tamura et al., 2006). These findings provide genetic evidence for the role of ABA in the thermoinhibition of seed germination.

1.2.2 Water

As described in section 1.1.2, a dry seed must first absorb water to initiate subsequent physiological and metabolic processes. Water can soften the seed coat and cause the endosperm to swell. Meanwhile, nutrients in the endosperm are dissolved for embryo growth. But a seed with hard seed coat which is impermeable to water remains quiescent until the seed coat is forced to open by weathering or scarification. Therefore, water penetration is essential in the Phase I of seed imbibition.

Besides, the ability of a seed to uptake water also determines the efficiency of seed germination. The mucilage on the seed coat plays an indispensable role in enhancing water uptake during germination. It has been reported that in *atsbt1.7* mutant seeds that are unable to release mucilage upon imbibition, the germination rate is strongly reduced under water-limiting conditions (Rautengarten et al., 2008). Water uptake during seed imbibition may also be controlled by aquaporins (a class of major intrinsic proteins), among which some members in the plasmamembrane intrinsic proteins (PIP) subgroup and the tonoplast intrinsic proteins (TIP) subgroup have been suggested to be involved in the regulation seed germination (Gao et al., 1999; Vander Willigen et al., 2006; Liu et al., 2007b). Transcript of *PIP1* in *Brassica napus* (*BnPIP1*) is expressed in seeds, and its abundance is correlated well with the germination rate of seeds primed with various stress treatment (Gao et al., 1999). Furthermore, functional study on one rice *PIP1* (*OsPIP1*) gene showed that overexpressing or knocking-down this *OsPIP1* results in elevated or reduced germination, respectively (Liu et al., 2007b). In addition to *PIP* genes, *TIP* genes may also contribute to seed germination. Certain *TIP* genes in *Arabidopsis* are exclusively expressed in seeds and may function in controlling the rate of water uptake during Phase II and therefore the onset of Phase III, thus regulating the speed of seed germination (Vander Willigen et al., 2006). Despite these attempts to initiate the study of the relationship between aquaporins and seed germination, much effort needs to be made towards a better understanding of the function of

aquaporins in seed germination and the underlying mechanism of aquaporin-regulated seed germination.

Water potential (ψ) is another important factor that controls seed water content. Reducing the ψ of the water supply exerts osmotic effects, creating a water stress for seeds. Polyethylene glycol (PEG) is commonly used to make a stress condition of low ψ without causing other side effects. Using solutions of PEG, people have established the correlation between ψ and germination rate (Bradford, 1990). If the ψ is sufficiently low, seed germination will be inhibited. This is because firstly, reduced ψ lowers seed water content, extending the time for seed hydration level to reach a certain threshold to allow germination to occur. Secondly and more importantly, reduced ψ alters the expression of a majority of genes associated with germination (Gallardo et al., 2001), which affects the embryo growth potential or testa/endosperm restraint.

1.2.3 Oxygen

Apart from suitable temperature and moisture status, the presence of oxygen is also of great importance to ensure the success of seed germination. Oxygen is an atmospheric gas, which means that it is deprived in deep soil or waterlogged environment. Therefore, seeds buried too deeply in the soil or immersed in water

can be oxygen starved, and most of them will eventually die although some can survive by going into dormancy.

Oxygen uptake occurs concurrently with water uptake during three phases of seed imbibition. Meanwhile, oxygen is consumed by seed respiration and energy (ATP) is produced in order to decompose the storage materials in the seed (Hourmant and Pradet, 1981). The correlation between a high ATP level and oxygen availability means that oxidative phosphorylation should occur during the beginning of seed germination, which is the case in lettuce seeds (Hourmant and Pradet, 1981). Although oxidative phosphorylation is good for ATP synthesis, it also has some detrimental effects like the production of reactive oxygen species (ROS). ROS is deleterious and will cause damage to seed; therefore, a natural antioxidant defense mechanism is adopted by aerobic organisms to provide repair and protection. The enzymes involved in such mechanism include superoxide dismutase (SOD), which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, and catalases and some peroxidases, which catalyze the decomposition of hydrogen peroxide. It has been shown that in soybean seeds, a SOD activity peak occurs during early imbibition (Phase I) and peroxidase activities significantly accumulate during later stage (Phase II and III) (Gidrol et al., 1994). Thus, the superoxide is gradually detoxified during seed imbibition and such fine tuning of oxidative stress seems to be beneficial for seed germination.

1.2.4 Light

Since plants are photosynthetic, their development from seed germination to flowering is tightly regulated by light. Light characteristics can be composed of intensity, wavelength, duration and direction, plants sense these different parameters of light to adapt themselves to the environment and to control various aspects of growth and development, such regulation is usually via the phytochrome family of photoreceptors (Quail et al., 1995).

Phytochrome is a pigmented protein encoded by five phytochrome genes called *PhyA* to *PhyE* in *Arabidopsis* (Mathews, 2006). It exists in two forms: a red-absorbing form (Pr) with maximum absorption at 660 nm and a far-red absorbing form (Pfr) with maximum absorption at 730 nm (Quail et al., 1995). Pr is considered as an inactive form, it is converted to the active form, i.e. Pfr, by red light (Seo et al., 2009). Thus, exposure of seeds to a high red light to far-red light ratio results in larger Pfr/P, which stimulates germination since the most dormancy-breaking wavelengths exist in the red region of the spectrum. It has been reported that in the dark, *phyB* mutant seeds do not germinate (Shinomura et al., 1994), but an increase in Pfr/P ratio induces PhyB activity. Active PhyB thereafter triggers the degradation of PIL5, a PhyB-interacting protein acting as a negative regulator of seed germination in *Arabidopsis*, to promote seed germination (Oh et al., 2004; Oh et al., 2006). In addition to PhyB, PhyA is also

involved in the control seed germination. It promotes seed germination in response to continuous far-red light in the absence of PhyB activity in the dark (Shinomura et al., 1994). Other phytochrome genes have little, if any, effect on seed germination. But once five phytochrome genes are simultaneously mutated, the resulted quintuple mutant does not germinate regardless the presence of light or not (Strasser et al., 2010).

Environmental factors are essential regulators in the process of seed germination, and their signalings can be tightly coupled at the molecular level. For example, it has been reported that two bHLH transcription factors SPATULA (SPT) and PHYTOCHROME-INTERACTING FACTOR3-LIKE5 (PIL5) are involved in a regulatory network and mediate the germination response to temperature and light (Penfield et al., 2005).

1.3 Hormone Signaling Pathways

Plant hormones or phytohormones, including abscisic acid (ABA), gibberellins (GA), brassinosteroids (BRs), ethylene, auxins, and cytokinins (CKs), are signalling molecules synthesized within the plant. They exert profound effects on many fundamental processes during plant growth and development even at extremely low concentrations. Among the phytohormones, ABA is a primary endogenous cue for the induction and maintenance of seed dormancy, resulting in the inhibition of seed

germination. On the contrary, GA acts antagonistically to ABA during seed development and germination. GA plays a vital role in releasing dormancy and promoting germination and thereby counteracts the inhibitory effect of ABA on seed germination. Other phytohormones are not as crucial as ABA and GA in the seed germination process, while they act synergistically with or antagonistically to ABA and/or GA in the control of seed dormancy maintenance and alleviation. Therefore, the regulatory roles of ABA and GA are in part achieved through interactions with other phytohormones. Such hormonal cross-talk forms a complex signaling web in which the interconnected processes that control dormancy release and germination initiation are well coordinated.

This section mainly outlines the roles of ABA and GA in regulating seed development and germination, followed by a brief discussion of the roles of other phytohormones and in controlling dormancy and germination and the interaction among the phytohormone signaling pathways.

1.3.1 Abscisic Acid

ABA is a sesquiterpene hormone that plays an integral part in mediating the adaptation of the plant to biotic and abiotic environmental stresses, and regulation of seed development and germination. It is ubiquitous in seed and fruit tissues. ABA levels decline with the maturation of seeds concomitantly with the decrease of

seed water content. Its levels increase dramatically during the first half period of seed development in response to environmental stresses, which plays a vital role in a series of physiological changes during embryogenesis, such as accumulation of storage reserves, inhibition of precocious germination, acquisition of desiccation tolerance, induction of seed dormancy for normal seed development (Finkelstein et al., 2002). The effect of ABA on seed dormancy can be efficiently alleviated by chilling (stratification treatment) so that endogenous ABA content drops precipitously with a concurrent increase in germination rate (Ali-Rachedi et al., 2004; Teaster et al., 2007). ABA can also enhance the tolerance of plants to adverse environmental conditions like drought, salinity, and temperature, and stimulate stomatal closure.

Although a direct relationship between ABA content and its physiological function in seed dormancy and germination is lacking, mechanisms about ABA-mediated inhibition of seed germination have been investigated to some extent. In *Brassica napus*, it has been suggested that one of the roles that ABA plays in seed germination is to inhibit water uptake by preventing loosening of the embryo cell wall, indicating that ABA is somehow capable of reducing embryo growth potential (Schopfer and Plachy, 1985). In addition, ABA has also been found to specifically inhibit endosperm rupture instead of testa rupture. This inhibitory effect can be partially counteracted by the antagonistic action of GA (White et al., 2000; Muller et al., 2006) .

Apart from the direct effect of ABA content on the inhibition of seed germination, sensitivity to ABA is also critical in determining whether a seed remains dormant or begins to germinate. Sensitivity is a reflection of the efficiency of both signal perception and transduction. During the past years, much effort has been devoted toward the identification of receptors for ABA perception and signaling factors involved in ABA transduction. Although a variety of proteins has been reported as ABA receptors (Razem et al., 2006; Shen et al., 2006; Liu et al., 2007e; Ma et al., 2009; Pandey et al., 2009; Park et al., 2009), so far no consensus has been reached as to the efficacy of these so-called receptors (Gao et al., 2007; Johnston et al., 2007; Liu et al., 2007d; Guo et al., 2008; McCourt and Creelman, 2008; Muller and Hansson, 2009; Risk et al., 2009). Therefore, the issue of ABA receptors has to be further clarified. With respect to signal transduction, genetic studies have greatly facilitated the elucidation of regulatory genes involved in ABA-regulated seed germination. The most well-known genes are probably the *ABA-INSENSITIVE (ABI)* genes, namely *ABI1* to *ABI5*. They were identified by selecting for mutant *Arabidopsis* seeds displaying ABA-resistant germination (Koornneef et al., 1984). Subsequent studies revealed that these *ABI* genes are primary signaling components responsible for ABA signal transduction.

Based on the insensitivity to ABA-mediated inhibition of germination, *abi1-1* and *abi2-1* mutants were identified. In addition, these mutants also exhibit reduced dormancy (Koornneef et al., 1984). Later, it was revealed that both *ABI1* and *ABI2*

encode protein phosphatases (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997), indicating that ABI1 and ABI2 may control phosphorylation state of cell signaling components in ABA signaling pathway. However, because the *abi1-1* and *abi2-1* mutations are both dominant, it was impossible to conclude whether *ABI1* and *ABI2* contribute to ABA signaling. To solve this problem, subsequent research using recessive mutants of *ABI1* and *ABI2* suggested that *ABI1* and *ABI2* played redundant roles in controlling ABA responsiveness, and these two genes negatively regulated ABA signaling (Gosti et al., 1999; Merlot et al., 2001). However, such conclusion was inferred from the observations on intragenic revertants of *abi1-1* or *abi2-1*, and it was questioned by other researchers whose finding did not agree with this conclusion (Wu et al., 2003). Thus, due to lack of null mutants of *ABI1* and *ABI2*, it remains unclear about the exact function of these two genes in ABA signaling.

Different from *ABI1* and *ABI2*, which encode enzymes, *ABI3*, *ABI4* and *ABI5* all encode transcription factors of the B3, APETALA2 (AP2) and basic leucine zipper (bZIP) domain, respectively (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000). Their mutants all show decreased sensitivity to the ABA inhibition of seed germination, but null mutations in *ABI3* are more severe than those in *ABI4* or *ABI5*. Besides, only *abi3* mutants have reduced dormancy (Koornneef et al., 1984; Finkelstein, 1994; Nambara et al., 1994; Parcy et al., 1994). During the past two decades, intensive research has been focused on the

functions of *ABI3*, *ABI4*, and *ABI5* in ABA signaling transduction. Since the embryos of *abi3* null mutant seeds remain green until maturation and immature seeds are completely non-dormant (Giraudat et al., 1992; Nambara et al., 1994), *ABI3* is thereby considered as a key regulator of the embryogenesis and seems to be involved in mediating the inhibitory role of endogenous ABA on seed germination. *ABI4* is a versatile gene involved in a variety of responses including ABA responses during seed germination (Finkelstein, 1994), salt responses during seed germination (Quesada et al., 2000), sugar responses in seedling growth (Arenas-Huertero et al., 2000), and chloroplast retrograde signaling (Koussevitzky et al., 2007). As *ABI4* is involved in regulating such a wide spectrum of developmental processes, whether it should be regarded as a key ABA signaling component is open to discussion. *ABI5* sets in after breakage of seed dormancy but prior to autotrophic growth, and it is essential to execute an ABA-dependent growth arrest (Lopez-Molina et al., 2001). Such growth arrest occurs through recruiting of *de novo* late embryogenesis programs, and confers the plant osmotic tolerance in harsh environment (Lopez-Molina et al., 2002).

With the identification of these transcription factors, intensive effort was also undertaken to study their interactions in ABA signaling. Being transcription factors, ABI3, ABI4, and ABI5 regulate downstream events via modulating the expression of their target genes. It has been shown that *ABI3* acts upstream of *ABI5* and mediates the induction of ABI5 activity by ABA (Lopez-Molina et al., 2002). On

the other hand, ectopic *ABI4* expression confers ABA inducible expression of *ABI3* and *ABI5* (Soderman et al., 2000). Considering these observations, it is not likely that these *ABI* genes act in a simple linear pathway, but rather in a combinatorial network. Apart from their cross-regulation, they also regulate many of the same downstream genes, in particular, those "seed-specific" genes, but in different manners during seed germination in response to ABA (Parcy et al., 1994; Finkelstein and Lynch, 2000; Soderman et al., 2000).

1.3.2 Gibberellins

Gibberellins are a group of tetracyclic diterpene acids that are well-known for their capability to promote plant growth. The gibberellins are named as GA_1 to GA_n according to the sequence of their discovery. GA_3, or gibberellic acid, is most frequently used in laboratory to trigger seed germination. In plants, GA biosynthesis and response are well coordinated during seed development and seed germination. In the process of seed development, GA levels are usually high during the embryo morphogenesis phase and decreased during the embryo maturation phase (Hedden and Kamiya, 1997). Active GAs may help promote the growth of embryo and later GAs are deactivated to avoid precocious germination (vivipary) (Hays et al., 2002). In fact, GA is likely to antagonize the ABA effect on seed development, particularly dormancy induction (White et al., 2000). It was found that in ABA-deficient or ABA-insensitive mutants of maize, when GA biosynthesis

is blocked during early seed development, vivipary is suppressed, indicating that GA can positively regulate vivipary (White and Rivin, 2000). In *Arabidopsis*, embryonic regulators *FUS3* and *LEC2* have been demonstrated to play vital roles in the control of GA and ABA biosynthesis during embryogenesis (Curaba et al., 2004; Gazzarrini et al., 2004). In immature seeds of *fus3* and *lec2*, GA levels are higher than that in wild-type and such misactivated GA biosynthesis results in some altered phenotypes (Curaba et al., 2004). Similarly, Gazzarrini et al. also showed that *FUS3* negatively regulates GA biosynthesis, moreover, it positively regulates ABA biosynthesis (Gazzarrini et al., 2004). *FUS3* on the one hand, modulates the GA/ABA ratio during seed development and, on the other hand, is negatively or positively regulated by GA and ABA, respectively. Such regulation loop helps regulate maturation events during seed development (Gazzarrini et al., 2004). Thus, GA/ABA ratio during seed development determines, to a large extent, whether seed dormancy and maturation can be induced and maintained. In addition, GA/ABA ratio is also of great importance in the control of seed germination. Accumulation of GA is accompanied with reduction of ABA during seed imbibition, suggesting that GA and ABA play antagonistic roles in germination process (Olszewski et al., 2002; Nambara and Marion-Poll, 2005). In *Arabidopsis*, ABA-deficient or ABA-insensitive mutants are able to rescue the germination of GA-deficient *ga1* mutant or seeds treated with GA biosynthetic inhibitor (Koornneef et al., 1982; Nambara et al., 1991; Leon-Kloosterziel et al., 1996).

GA counteracts the effect of ABA by promoting the embryo growth potential and the weakening of tissues covering the embryo (Bentsink and Koornneef, 2008; Holdsworth et al., 2008). It is known that GA upregulates the expression of two expansin genes in tomato, namely *LeEXPA8* and *LeEXPA10*, to promote the embryo growth potential (Chen et al., 2001). Expansins are so called because of their capability to cause expansion of cell walls. *LeEXPA8* is specifically expressed in the radicle cortex during and after germination, and *LeEXPA10* is expressed throughout the embryo during early stage of embryogenesis and germination. In GA-deficient *gib-1* mutant, the expression of both genes is suppressed and upon GA application, their expression is resumed to promote embryo growth (Chen et al., 2001). In *Arabidopsis*, microarray analysis revealed that many expansions are also rapidly upregulated by GA in GA-deficient *ga1-3* mutant upon imbibition. Besides, a large number of xyloglucan endotransglycosylase/hydrolase (XTH) genes, which function in loosening cell wall, and pectin methylesterase (PME) genes, which may be involved in cell elongation by modifying cell wall pectin, are upregulated by GA during seed germination (Ogawa et al., 2003). Expression of some XTH and PME genes are shown to be localized in the embryo. These data suggest that GA may induce some cell wall-modifying proteins in the embryo to promote the growth potential of the embryo during seed germination (Ogawa et al., 2003). On the other hand, weakening of covering tissues is also necessary for achieving germination. When GA biosynthesis is inhibited, both testa and endosperm rupture are blocked. Testa as a constraint to radicle protrusion has been shown by testa mutants that

have reduced seed dormancy (Debeaujon et al., 2000). On the contrary, GA-deficient mutants that have defects in GA biosynthesis cannot germinate in the absence of exogenous GA. But when some testa mutants are introduced into GA-deficient *ga1* mutant background, the resulting double mutants are able to fully germinate without exogenously-applied GA. Similarly, removal of the envelopes of *ga1* mutant seeds also makes them germinate successfully (Debeaujon and Koornneef, 2000). However, although ABA and GA have opposite effects during seed germination, ABA does not inhibit testa rupture. Instead, ABA specifically inhibits endosperm rupture, and such inhibitory effect of ABA can be counteracted by GA (Muller et al., 2006). In *Arabidopsis*, it is known that testa rupture and endosperm rupture are two sequential steps during germination (Liu et al., 2005). After testa rupture, endosperm must be further weakened prior to radicle protrusion. Extensive studies have been focused on cell wall-modifying proteins involved in endosperm weakening, including expansins, XTHs, PMEs, mannanase, cellulase, etc. (Nonogaki et al., 2007). Strong evidence show that de novo-synthesized GA in the micropylar endosperm after imbibition is required to induce the expression of the genes encoding the above-mentioned enzymes or cell wall proteins to weaken endosperm, thus relieving the inhibitory effect imposed by ABA (Ogawa et al., 2003; Muller et al., 2006).

GA signaling is regulated by a group of repressors collectively called DELLA proteins including REPRESSOR OF GA1-3 (RGA), GA-INSENSITIVE (GAI),

and RGA-LIKE 1-3 (RGL1-3) (Peng and Harberd, 1997; Dill and Sun, 2001; Lee et al., 2002; Wen and Chang, 2002; Tyler et al., 2004). DELLA proteins have been found to function in mediating cotyledon expansion to overcome seed dormancy imposed by the seed coat in *Arabidopsis* (Penfield et al., 2006). Although all DELLA proteins seem to participate in the regulation of seed germination, RGL2 appears as the major DELLA factor involved in repressing seed germination (Lee et al., 2002; Tyler et al., 2004). Among all the *della* single mutants except *rgl3*, it was found that only the germination of *rgl2* mutants is strongly resistant to the effects of GA biosynthesis imhibitor, paclobutrazol (PAC). Moreover, Loss of *RGL2* function suppresses the nongerminating phenotype of GA-deficient *ga1-3* mutant (Lee et al., 2002). Recent studies have shown that RGL2 stimulates ABA biosynthesis and ABI5 activity, while ABA enhances the *RGL2* expression (Ko et al., 2006; Zentella et al., 2007; Piskurewicz et al., 2008), indicating that RGL2 plays a role in mediating the interaction of GA and ABA during seed germination.

1.3.3 Brassinosteroids

BRs are a class of polyhydroxysteroids that are found in a wide variety of plant species. They can be detected in almost every plant tissue, while the most abundant BRs are present in the pollen and seeds (Schmidt et al., 1997). The most active component in the family of BRs is 24-epibrassinolide (BL), it is detected in dry seeds and capable of activating BR signaling (Schmidt et al., 1997). Although BRs

production is plentiful in seeds, they are generally not considered as major regulators of seed dormancy and germination, but they are involved in these processes through the communication with other hormones.

In *Arabidopsis*, BL is able to rescue the nongerminating phenotypes of GA-deficient mutants *ga1-1*, *ga2-1*, and *ga3-1* and greatly increase the germination of GA-insensitive *sly1* mutants. In addition, it was found that BR biosynthetic *det2-1* mutant and BR responsive *bri1-1* mutant are more sensitive to ABA on seed germination than wild-type (Steber and McCourt, 2001). In *Nicotiana*, both BL and GA have the ability to promote seed germination and their actions are suggested to be independent to each other, and BL counteracts the inhibitory effect of ABA on endosperm rupture (Leubner-Metzger, 2001). An inference is that BR and GA act in parallel to promote germination, and they antagonize ABA during seed germination. Recently, it was further found that exogenous ABA rapidly and directly inhibits BR signaling outputs through modulating the phosphorylation status of BES1, a BR signaling marker, and the expression of many BR-responsive genes. Such regulation is through ABI1 and ABI2 in the ABA signaling pathway, and through BIN2 in the BR signaling pathway (Zhang et al., 2009). Thus, the molecular mechanism of the interaction between BR and ABA signaling pathways is partially unveiled.

1.3.4 Ethylene

Ethylene is an organic compound with the molecular formula C_2H_4. It has been reviewed that ethylene might play a role in the release of dormancy and the promotion of germination of non-dormant seeds of many species (Kepczynski and Kepczynska, 1997; Matilla, 2000). Ethylene production is higher in non-dormant seeds than dormant seeds, and is induced during seed imbibition before radicle emergence. Since the ethylene production is correlated well with the progress of seed germination, it has been proposed that the primary action of ethylene in germinating seeds may be in the promotion of radial cell expansion in the embryonic axis, increasing seed respiration, or increasing water potential (Kucera et al., 2005).

Studies on ethylene-insensitive *etr1* and *ein2* mutants show that they display enhanced dormancy and low germination rate. Both mutants are ABA-hypersensitive, and *ein2* is able to suppress the high germination rate of *abi1-1*. (Beaudoin et al., 2000). In addition to disrupting ABA signaling, *ein2* and *ert1* mutants also accumulate ABA to higher levels (Ghassemian et al., 2000; Chiwocha et al., 2005). On the contrary, another ethylene-hypersensitive mutant *ctr* that exhibits slightly reduced dormancy is ABA-insensitive, and can enhance the germination rate of *abi1-1* (Beaudoin et al., 2000). Therefore, ethylene counteracts ABA during the control of seed dormancy and germination.

Ethylene also interacts with GA to synergistically promote the transition from seed dormancy to germination. It was found that ethephon, a compound that releases ethylene in solution, has similar effect like GA on suppressing the expression of genes encoding GA 20-oxidase in *Fagus sylvatica* L. seeds, thus releasing dormancy (Calvo et al., 2004). Indeed, it has already been found in earlier research that ethylene can restore the germination of *ga1* mutant (Karssen et al., 1989). On the other hand, GA can also promote the germination of *etr1* mutant (Bleecker et al., 1988). Thus, ethylene acts in concert with GA to promote seed germination.

1.3.5 Auxins

Auxins are the first plant hormones discovered. In auxin family, the most important member produced by plants is indole-3-acetic acid (IAA). Auxins play a vital role in embryogenesis, providing positional information for the establishment of the basic body plan of the embryo (Fischer-Iglesias et al., 2001; Friml et al., 2003; Weijers and Jurgens, 2005). It has been reported that IAA might be involved in the control of dormancy and preharvest sprouting in wheat seeds (Ramaih et al., 2003).

Although present in the seed during imbibition, auxin itself seems not to directly regulate germination since auxin-related mutants do not reportedly have obvious defects in seed germination. However, the involvement of auxin in the control of seed germination is reflected through the crosstalk with other hormones.

Germination of auxin response mutants *axr1* and *ibr5* is slightly more insensitive to low ABA concentrations compared with the response of wild-type seeds (Tiryaki and Staswick, 2002; Monroe-Augustus et al., 2003). Another study also show the interaction between auxin and ABA by a detailed investigation of an *AUXIN RESPONSE FACTOR* called *ARF10* in the process of seed germination (Liu et al., 2007c). When expression of *ARF10* is repressed by overexpression of microRNA160 (miR160), the seeds exhibits reduced sensitivity to ABA. Conversely, in transgenic seeds expressing an miR160-resistant form of *ARF10*, germination is more sensitive to ABA.

The interaction between auxin and GA during seed germination is largely inferred by an microarray analysis of GA-regulated genes during *Arabidopsis* seed germination. It was found that the expression of several auxin transporter genes, including *AUX1*, *PIN2*, and *PIN7*, and some CYP genes that are necessary for the biosynthesis of auxin, is highly upregulated in *ga1-3* mutant seeds treated with exogenous GA (Ogawa et al., 2003). Therefore, GA activity may affect auxin biosynthesis and transport to regulate seed germination.

1.3.6 Cytokinins

CKs are compounds with an adenine-like structure that promote cell division. Kinetin is the first CK discovered, but since it is not synthesized in plants, it is

regarded as a "synthetic" CK. Zeatin is named because it was originally isolated from *Zea mays*, it is the most common form of naturally occurring CKs. CKs are present in developing seeds and accumulate mainly in the endosperm (Emery et al., 2000; Mok and Mok, 2001). Like auxin, CKs also participate in embryo pattern formation; besides, they control endosperm growth and grain filling (Bewley and Black, 1994; Mok and Mok, 2001).

It has been found that CKs alone are able to break dormancy of seeds of various species, but whether they play a role in promoting seed germination was largely unknown. Until recently, an analysis of a triple mutant in which three *Arabidopsis* cytokinin receptors lose their activities reveals a possible function of CKs in germination control (Riefler et al., 2006). The triple cytokinin receptor mutant exhibits faster germination rates in white light or red light and increased germination percentage in the dark (Riefler et al., 2006). Thus, CKs may act as a negative regulator of seed germination.

The interactions between CKs and other hormones in the control of seed dormancy and germination have not been well established. However, there are some studies suggest the existence of such interactions. For example, some cytokinin-resistant mutants in *Nicotiana* show reduced seed dormancy and lower endogenous ABA levels compared with wild-type (Rousselin et al., 1992). Furthermore, a connection between CKs and ethylene is suggested in a research that in an *Arabidopsis*

ethylene-insensitive *etr1* mutant, the profiles of CKs are considerably different from that in wild-type during seed germination (Chiwocha et al., 2005).

1.3.7 Summary

In the past, our knowledge about dormancy and germination was almost zero; nowadays, significant progress has been made towards our understanding of the factors and processes involved in controlling the transition from dormancy to germination. In addition to environmental cues, hormones are also indispensable for the balance between dormancy and germination, recent years extensive hormonal interactions have been found to modulate downstream physiological events, thus control seed germination (Figure 3). Despite the progress made toward elucidating hormone signaling, the complexities of "integrated signaling" are still far beyond our comprehension and understanding. To gain an in-depth understanding of this biological event, much effort can be done, such as searching for the substrates of those known kinases and phosphatases, establishing direct or indirect gene-gene interaction and protein-protein interaction, and identifying novel signaling components linking the known elements need to be further identified and incorporated into the hormonal web.

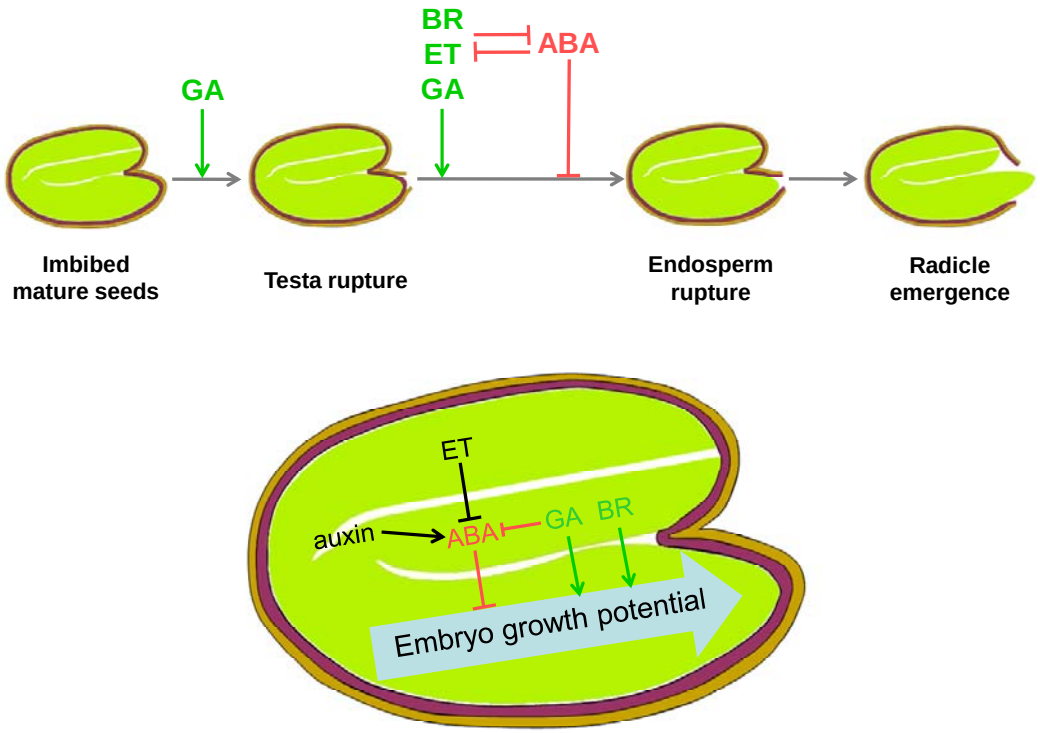

Figure 3. Hormonal Control of Seed Germination in *Arabidopsis*.

Under favorable environmental conditions, seed germination is also under combinatorial control by phytohormones. ABA is a dominant repressor of seed germination, while GA is a major promoter of seed germination. Other hormones also play a role in the control of seed germination. During *Arabidopsis* seed germination process, testa rupture and endosperm rupture are two sequential steps regulated by different hormones. Besides, embryo growth potential is also under hormonal regulation. ET, ethylene.

1.4 PEBP Family

In this study, a gene called *MFT* is characterized and its role in the control of seed germination has been established. *MFT* was named after its sequence similarity with two important flowering time regulators, *FLOWERING LOCUS T (FT)* and *TERMINAL FLOWER 1 (TFL1)*. *FT*, one of the floral pathway integrators (Simpson and Dean, 2002), is expressed in the leaves, but its protein moves to the shoot meristems where it interacts with the bZIP transcription factor FD to induce flowering (Abe et al., 2005; Corbesier et al., 2007). Although *TFL1* shares high sequence similarity with *FT*, it exerts an opposite action to potently repress flowering (Shannon and Meeks-Wagner, 1991). Its expression is restricted to the inner cells of mature shoot meristems, while its protein moves to outer cells to control shoot meristem identity throughout the plant life cycle (Conti and Bradley, 2007).

Both *FT* and *TFL1* encode small proteins similar with phosphatidylethanolamine - binding proteins (PEBPs) or Raf kinase inhibitor proteins (RKIPs) (Kardailsky et al., 1999; Kobayashi et al., 1999). Initially a basic cytosolic protein with a molecular weight of 23 kDa was isolated, and binding studies indicated that it has a preference for binding to phosphatidylethanolamine compared to other phospholipids (Bernier et al., 1986). Based on sequence homology, all other similar

proteins isolated were assigned to this PEBP family. The first structural information was derived from human PEBP, from which a novel fold topology was found, and the regions that likely execute biological function were suggested (Banfield et al., 1998). Members of the PEBP family have diverse roles in animals, yeast and bacteria, and they are small peptide precursors, inhibitors of various enzymes and modulators of signaling complexes (Tohdoh et al., 1995; Bruun et al., 1998; Hengst et al., 2001; Chautard et al., 2004). Among the PEBP family, RKIP is the most extensively studied protein that is widely expressed in mammals. It can bind to Raf-1 kinase and interfere with Raf activity, thus affecting relevant signaling pathways to control numerous biological processes including growth and differentiation (Park et al., 2006). It can also facilitate membrane associated heterotrimeric G protein coupled signaling to trigger subsequent cellular responses (Kroslak et al., 2001). Structural analysis on PEPBs from both human and bovine identified a highly conserved phosphate-binding site, which may be the crucial domain for the inhibitory activity of PEPBs on Raf-1 (Banfield and Brady, 2000). Crystal structure of a plant PEBP family member, *Antirrhinum* CENTRORADIALIS (CEN), confirmed the structural similarity of CEN with other known PEBP members (Banfield and Brady, 2000). Although the plant CEN homologues, including MFT, form a distinctive cluster, the striking conservation of a ligand binding site and accessory features in PEBP members strongly supports the hypothesis that the CEN members share a common biological function with their mammalian PEBP

counterparts as regulators of cell differentiation mediated by their interaction with kinase cascades (Banfield and Brady, 2000).

While FT and TFL1 proteins share high sequence similarity (~59% identity), their function is surprisingly different. Domain swapping strategy has been applied to reveal the molecular basis for their opposite function. Hanzawa et al. (2005) showed that a single amino acid exchange can convert FT or TFL1 protein into a new form with TFL1-like or FT-like function, respectively. Ahn et al. (2006) further expanded our knowledge on this antagonistic activity by defining an external loop along with an adjacent peptide that is responsible for the opposite function of FT and TFL1. The key residue identified by Hanzawa et al. (2005) can interact with the external loop. The proposed model that FT and TFL1 compete for common interacting partner(s) that may have some intermediate levels of activity in the absence of FT or TFL1 (Ahn et al., 2006) still awaits evidence.

In addition to *FT* and *TFL1*, *MFT* also has several other homologs, including *BROTHER OF FT AND TFL1 (BFT)*, *TWIN SISTER OF FT (TSF)*, *and ARABIDOPSIS THALIANA CENTRORADIALIS HOMOLOGUE (ATC)* (Kobayashi et al., 1999; Mimida et al., 2001; Yamaguchi et al., 2005). Among these homologs, *TSF* has been suggested as another floral pathway integrator with redundant function with *FT* (Yamaguchi et al., 2005). *BFT* has recently been found to act redundantly with *TFL1* in inflorescence meristem development (Yoo et al., 2010).

Apart from the well characterized *FT* and *TFL1*, the function of other members of this small gene family is yet to be further explored.

Although *MFT* was ascribed as a weak floral inducer (Yoo et al., 2004), the characteristics of this gene, such as spatial expression of its mRNA and protein localization, are largely unknown.

1.5 Objectives and Significance of the Study

Bioinformatics analyses revealed that there are thousands of ABA-responsive genes in *Arabidopsis* imbibed seeds. But till now less than 100 genes have been well investigated. This means that studies regarding the overwhelming majority of genes remain untouched hitherto. Among these yet-to-be characterized genes, *MFT*, short for *MOTHER OF FT AND TFL1*, appeared as one of the ABA-upregulated genes. *MFT* was named after its high sequence similarity with two important flowering time regulators, *FLOWERING LOCUS T (FT)* and *TERMINAL FLOWER 1 (TFL1)*. However, it was found that *MFT* had little, if any, effect on the flowering time. Thus, this molecule is intriguing and is worthy of further investigation.

To gain a comprehensive understanding of how *MFT* participates in the control of seed germination in the ABA signaling pathway, we sought to characterize *MFT* by

genetic, molecular, biochemical, and physiological approaches. Our specific objectives were listed as follows:

- Generation of loss- and gain-of-function mutants of *MFT*
- Examination of the temporal and spatial expression of *MFT* by RT-PCR and in situ hybridization methods
- Determination of the protein localization of MFT by GFP-tagging and confocal microscopy
- Identification of upstream regulatory factors of *MFT* by screening assay
- Identification of downstream target genes of *MFT* by microarray and chromatin immunoprecipitation (ChIP) techniques

Since our study focused on the interaction of *MFT* with ABA signaling factors during seed germination, particular attention was paid to the identified genes or proteins that are in the ABA pathway. Thereafter, more detailed analyses between *MFT* and these genes/proteins were further performed.

The achievement of the above-mentioned objectives will disclose the detailed characteristics of *MFT* that are largely unknown so far. This information would be useful for other relevant research, such as studies on *MFT* homologs in *Arabidopsis*, or *MFT* orthologs in other plant species. More importantly, the results of how *MFT* affects seed germination in response to ABA should provide valuable information for expanding our current understanding of the genetic regulation of

seed germination, and make a nice contribution to further research in the underlying mechanism of seed germination.

This thesis focuses on the function of *MFT* during seed germination in response to ABA. But it is known that a gene may respond to multiple signals to execute its function in certain biological process, as is the case for *MFT*. In fact, bioinformatics analyses showed that besides ABA, *MFT* is upregulated by salt and osmotic stresses as well in imbibed *Arbidopsis* seeds. However, it is impossible for us to perform detailed investigation of all the factors in a single research study. From this thesis, we should be able to understand how *MFT* affects seed germination in response to ABA. Such information could shed light on further studies of how *MFT* is regulated by other factors during seed germination.

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Materials

Arabidopsis thaliana ecotype Columbia (Col-0) was used in the generation of all transgenic plants. *abi1-1*, *abi2-1*, and *abi3-1* are in Landsberg *erecta* (L*er*) background; *abi4-1*, *mft-2*, *mft-3*, *aba1-5*, *cyp707a1-1*, and *cyp707a2-1* are in Col background; and *abi5-1* is in Wassilewskija (Ws) background. *mft-2* introgressed into L*er* was obtained by three backcrosses of *mft-2* into L*er*. Different combinations of *DELLA* mutants in *ga1-3* background (L*er*) have been described previously (Yu et al., 2004b).

2.2 Plant Growth Conditions, Seed Germination Assay and Stress Treatment

Plants were grown at 22°C under long days (16 h of light/8 h of dark). Dry seeds were collected and stored at a dehumidifier cabinet for at least 2 months before seed germination test was carried out. After-ripened seeds were washed with 70% (v/v) ethanol for 30 seconds, sterilized with 10% (v/v) commercial Clorox bleach for 15 min, and washed three times with sterile water. Sterilized seeds were subsequently plated on MS medium (Sigma) containing 0.8% (w/v) Bacto Agar (Difco/BD) supplemented with ABA, NaCl or GA_3. Stock solution of ABA (mixed isomers, Sigma) was in methanol, while GA_3 (Sigma) was in ethanol. Control plates

contained equal amount of the corresponding solvents. Plates were kept at 4°C in darkness for 3 d for stratification and then transferred to a tissue culture room set at 22°C with a 16 h light/8 h dark photoperiod. For the germination assay, at least 100 seeds for each genotype were sterilized and sown on MS medium supplemented with or without phytohormones or chemicals. Germination was defined as the first sign of radicle tip emergence and scored daily until the 7th day of the incubation, and the germination results were calculated based on at least three independent experiments with seeds from independent batches grown under same conditions. The standard deviation was calculated based on the results from three independent experiments. Drought treatment and measurement of transpiration rate were performed as previously described (Kang et al., 2002).

2.3 Plasmid Construction

2.3.1 Fragment Amplification and Cloning

To construct *35S:MFT*, the *MFT* coding region was amplified using primers MFT_P1_PstI (5'-CCCTGCAGATATATATCTCCCTCCCCGC-3') and MFT_P2_SpeI (5'-CCACTAGTTTTTTGTACTAGCGTCTGCG-3'). The PCR products were digested with *Pst*I and *Spe*I and inserted into the corresponding sites of the pGreen 0229-35S binary vector (Yu et al., 2004a).

To construct *MFT(P2)-GUS*, the 1.8 kb *MFT* 5' upstream sequence was amplified with MFTGUS_P1_HindIII (5'-CCAAGCTTCTACGCGATTGGACGTTGC-3') and MFTGUS-P5-XmaI (5'-ACCCGGGCGATCAGCGGGGAGGGAGAT-3') primer pairs. To construct *MFT(P6)-GUS*, the 900 bp *MFT* 5' upstream sequence was amplified with the primers MFTGUS-P4-HindIII (5'-CCAAGCTTCGATGAA TATGCGACCGACC-3') and MFTGUS-P5-XmaI. The digested PCR products were cloned into pHY107 (Liu et al., 2007). These constructs were mutagenized to produce the mutated ABA response elements (Figure 22) using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene). Two *MFT* genomic fragments (*gMFT-P2* and *gMFT-P6*) were used for the complementation test. *gMFT-P2* was comprised of the 1.8 kb upstream sequence and the 2.1 kb coding sequence plus introns, and amplified using the primers MFTGUS_P1_HindIII and MFT_P2'_XmaI (5'-ACCCGGGCTAGCGTCTGCGTGAAGCAGGTTCC-3'). *gMFT-P6* contained the 900 bp upstream sequence and the 2.1 kb coding sequence plus introns, and was amplified by MFTGUS-P4-HindIII and MFT_P2'_XmaI. These fragments were digested and inserted into pHY105 (Liu et al., 2007a).

To construct *gMFT-HA*, a single HA tag was incorporated into pHY105-*gMFT-P2* construct using the primers MFT_inverse_RC (5'-CAGCGAATTATCTAG AACTAGCTAGCGTCTGCGTGAAGCAGGTTCC-3') and gMFT-HA_R (5'-AGCGTAATCTGGAACGTCATATGGATAGCGTCTGCGTGAAGCAGGTTCC -3') by the mutagenesis PCR method.

To construct *35S:ABI3-6HA*, the *ABI3* cDNA was amplified with the primers ABI3-6HA_P1_XhoI (5'-CCCTCGAGCCACTTCAACGATGAAAAGCTTGCATGTGG-3') and ABI3-6HA_P2_SpeI (5'-GGACTAGTTTTAACAGTTTGAGAAGTTGGTGAAGCGACCAC-3'). The resulting products were digested by *Spe*I and *Xho*I, and cloned into pGreen-35S-6HA to obtain an in-frame fusion of *ABI3-6HA* under the control of 35S promoter (Liu et al., 2008). Similarly, *35S:ABI5-6HA* and *35S:RGL2-6HA* were constructed using the primers ABI5-6HA_P1_XhoI (5'-CCCTCGAGGCAGTTGTTAAATGGTAACTAGAG-3') and ABI5-6HA_P2_SpeI (5'-GGACTAGTGAGTGGACAACTCGGGTTCCTCATC-3'), and the primers RGL2_EcoRV_F (5'-GGGATATCAACAAGAAAGATGAAGAGAGGATACGGAG-3') and RGL2_XmaI_R2 (5'-CCCCCCGGGGGCGAGTTTCCACGCCGAGG-3'), respectively.

2.3.2 Preparation and Transformation of *E. coli* Competent Cells

The method for the preparation of competent cells of *Escherichia coli* for heat-shock transformation was modified based on a previous published protocol (Inoue et al., 1990). The XL1-Blue strain of *E. coli* (Stratagene, USA) that is both endonuclease (*endA*) and recombination (*recA*) deficient is known to significantly improve the quality of miniprep DNA and insert stability, thus it is used in our lab.

The procedures are briefly summarized as follows: Single colony of XL1-Blue cells is inoculated into 1.5 ml of SOB medium (yeast extract 5 g/l, Tryptone 20 g/l, NaCl 0.58 g/l, KCl 0.19 g/l, $MgCl_2 \cdot 6H_2O$ 2.03 g/l, $MgSO_4 \cdot 7H_2O$ 2.46 g/l) and incubated for 12 h by vigorously shaking at 37°C. An aliquot of 500 µl of the overnight culture was transferred to 100 ml fresh SOB medium and cultured at 20°C until an OD_{600} value reached 0.6. The culture was then cooled on ice for 10 min before centrifuged at 3000 rpm for 5 min at 4°C. The pellet was gently re-suspended in 20 ml of freshly-prepared TB medium (10 mM PIPES, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7), placed on ice for 10 min, and centrifuged for 5 min at 4°C. The cell pellet was gently re-suspended again in 4 ml of fresh ice-cold TB medium. DMSO was added (to a final concentration of 7%) with gentle swirling to the cell suspension as a stabilizer. Subsequently, 100 µl of cell suspension was aliquoted into a pre-chilled 1.5 ml tube. The tubes were then quick-frozen by liquid nitrogen, and kept at -80°C for long term storage up to several months without dramatic decrease of competency.

A tube of frozen XL1-Blue competent cells was thawed and placed on ice. 5 µl of ligation reaction was pipetted to the tube and mixed sufficiently by gentle tapping of the tube. After incubation on ice for 15 min, the mixture was then placed in a water bath preheated to 42°C to do heat-shock for 90 sec. The tube was then placed on ice for an incubation of another 2 min before added 1 ml LB medium (yeast extract 5 g/l, Tryptone 10 g/l, NaCl 10 g/l). The bacteria were cultured at 37°C for 1

h with shaking, and centrifuged for 3 min at 3000 rpm. The cell pellet was re-suspended in 100 µl LB and spread evenly onto an LB agar plate containing a specific antibiotic for selection. The LB agar plate was then incubated overnight at 37°C.

2.3.3 PCR Verification and Sequence Analysis

The verification of pGreen binary vector based constructs was taken as an example here. For clones with the inserted DNA in *E. coli*, single colony was suspended in 5 µl H_2O. 1 µl of bacterial suspension was added to a buffered PCR reaction mix containing 0.2 mM dNTP, 1 unit of Taq DNA Polymerase, 0.2 mM of each primer (PG-P1: 5'-CGACGGCCAGTGAATTGTAATACG-3' and PG-P2: 5'-CCTTATCGGGAAACTACTCACAC-3'). Polymerase chain reaction (PCR) was performed as follows: denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 15 sec, annealing at 52.5°C for 15 sec, and elongation at 72°C for 1 min, and final elongation at 72°C for 5 min. PCR products were viewed on a 1% agarose gel by electrophoresis. Colonies with PCR products of expected size were grown for plasmid extraction and DNA sequencing.

Plasmid DNA was extracted with the High-Speed Plasmid Minikit (Geneaid, Taiwan) according to manufacturer's instruction. DNA sequences were determined by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). About

100-200 ng of purified plasmid was added into a reaction mix with 3.2 pmols of PG-P1 or PG-P2 primer. PCR program for sequencing was performed as follows: 25 cycle of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and extension at 60°C for 30 sec. The resulting PCR products were then precipitated with 80 µl of 75% isopropanol, pelleted by centrifugation, washed with 500 µl of 70% ethanol, and finally air-dried. The pelleted DNA was sequenced by a sequencing machine. The sequence returned was subject to BLAST search at the web site of National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov).

2.4 Plant Transformation

2.4.1 Preparation of *A. tumefaciens* Competent Cells

The *Agrobacteria tumefaciens* GV3101 strain was used in our lab for making electroporation-competent cells. The procedures are briefly summarized as follows. as follows: Single colony of *A. tumefaciens* cells is inoculated into 1.5 ml of LB medium and incubated for 12 h by vigorously shaking at 28°C. An aliquot of 500 µl of the overnight culture was transferred to 100 ml fresh LB medium and cultured at 28°C until an OD_{600} value reached 0.6. The culture was then cooled on ice for 10 min before centrifuged at 3000 rpm for 10 min at 4°C. The pellet was gently re-suspended in 32 ml of ice-cold sterile H_2O, placed on ice for 10 min, and

centrifuged for 10 min at 4°C. The cell pellet was gently re-suspended again in 8 ml of fresh ice-cold sterile H_2O and placed on ice for 10 min. 50 µl of cell suspension was aliquoted into a pre-chilled 1.5 ml tube. The tubes were then quick-frozen by liquid nitrogen, and kept at -80°C for long term storage up to several months without dramatic decrease of competency.

2.4.2 Plasmid Transformation of *A. tumefaciens* Competent Cells

A tube of frozen GV3101 competent cells was thawed and topped up to 100 µl with sterile H_2O. 1-2 µl of purified plasmid was pipetted to the tube and mixed sufficiently by gentle tapping of the tube. The mixture was transferred into a 1 mm Gene Pulser® cuvette (Bio-Rad) and subject to electroporation at 25 µF, 2.5 kV, 200Ω. The electroporated bacteria were then cultured in 1 ml of LB medium for 3 h with shaking at 28°C and precipitated at 3000 rpm for 10 min. The cell pellet was re-suspended in 100 µl LB and spread evenly onto an LB agar plate containing 25µg/ml gentamycin, 10 µg/ml tetracycline and 25 µg/ml rifampicin for the selection of the GV3101 strain and a specific antibiotic for the selection of the plasmid. For pGreen-based plasmids, 50 µg/ml kanamycin was used. The plate was incubated for 2-3 days at 28°C. The colonies were verified by PCR and the confirmed colonies with the transgene were grown for subsequent floral dip.

2.4.3 Floral Dip and Selection of Transgenic Plants

Agrobacterium-mediated floral dip method was established previously (Clough and Bent, 1998). GV3101 cells transformed by a desired construct were cultured at 28°C until an OD_{600} value reached 0.8 and then pelleted at 4000 rpm for 10 min. The cell pellet was re-suspended completely in a solution containing 5% sucrose and 0.015% surfactant Silwet L-77. Flower buds of desired plants that were ready for transformation were dipped and submerged in *Agrobacterium* cell suspension for several seconds. The dipping can be repeated once more. The inoculated plants were then covered in a black plastic bag overnight to improve the transformation efficiency. The dipped plants were then grown under normal growth conditions and their seeds were later collected as T1 generation, which was grown and screened by 3% BASTA (for the selection of pGreen-based constructs) after the emergence of the first rosette leaf.

2.5 Expression Analysis

2.5.1 RNA Extraction and Reverse Transcription for cDNA Synthesis

Total RNAs from seeds were extracted using the RNAqueous® Small Scale Phenol-Free Total RNA Isolation Kit (Ambion) according to the manufacturer's

instructions. RNase-free DNase (Roche) was used to eliminate genomic DNA contamination. The RNAs were reverse-transcribed with SuperScript RT-PCR System (Invitrogen) according to the manufacturers' instructions. For future expression analysis, if *TUBULIN* (*TUB2*) was included as an internal control, Oligo$(dT)_{20}$ was used to synthesize first-strand cDNA; if 18S rRNA was included as an internal control, random hexamers were used to synthesize first-strand cDNA.

2.5.2 Semi-quantitative RT-PCR

For checking the expression of *MFT* in two T-DNA insertion lines designated as *mft-2* and *mft-3*, semi-quantitative RT-PCR analysis was employed. PCR amplification of cDNA templates using using a pair of primers flanking the T-DNA insertion sites in *mft-2* and *mft-3* was performed. The amplified PCR products were viewed on a 1% agarose gel by electrophoresis, the expression of *TUB2* was adjusted to give approximately equal intensities in different cDNA samples.

2.5.3 Quantitative Real-time RT-PCR

For all the other expression analyses, quantitative real-time RT-PCR was performed. Samples were run in triplicate on 7900HT Fast Real-Time PCR system (Applied Biosystems) using the Power SYBR® Green PCR Master mix (Applied

Biosystems). Either *TUB2* or 18S rRNA was used as an internal control. Taking *TUB2* as an example, the difference between the cycle threshold (Ct) of the target gene and the Ct of *TUB2* ($\Delta Ct = Ct_{target\ gene} - Ct_{tubulin}$) was used to obtain the normalized expression of target genes, which correspond to $2^{-\Delta Ct}$. The specificity of real-time primers was evaluated by examining the plot of dissociation curve for any abnormal amplification or bimodal dissociation curve, while the efficiency were determined by plotting a standard curve base on a series of 10-fold dilutions of DNA templates for each pair of primers. All real-time RT-PCR primers used for detecting gene expression levels are listed in Table 1.

2.6 Non-radioactive In Situ Hybridization

2.6.1 Preparation of RNA Probes

A fragment of DNA sequence that is unique to *MFT* was amplified using forward primer 5'-CCTCTCTGTTTCTCTCTCTCTC-3' and reverse primer 5'-AAGTATCTCTTTTCCTCTTGAG-3'. Full-length *ABI5* coding sequence was amplified to synthesize *ABI5* RNA probe. The PCR products were cloned into pGEM-T Easy vector (Promega) for subsequent synthesis of RNA probes. A Digoxigenin (DIG) RNA Labeling Kit (Roche) was used for synthesizing RNA probes. Transcription and labeling reaction was set up by mixing 2 µl of transcription buffer, 1µl of 10× DIG labelling Mix, 0.5 µl of RNase inhibitor, 1 µl

RNA polymerase, 1 μg of linerized plasmid DNA and RNase H_2O to a total volume of 10 μl. After incubation at 37°C for 2 h, 1 μl of RNase free DNase was added to the reaction mixture for another 30 min at 37°C to degrade the template DNA. For the verification of the RNA production, 0.5 μl of the reaction product was analyzed by 1% agarose gel. The synthesized DIG-labeled probe was hydrolysed into 75-100 bp fragments using carbonate hydrolysis as follows: the RNA products were mixed with 50 μl of 2× carbonate buffer (80 mM $NaHCO_3$ and 120 mM Na_2CO_3) and topped up to 50 μl with 0.1% diethylpyrocarbonate (DEPC)-treated H_2O. The mixture was then incubated at 60°C for a period of time calculated using the following formula.

$$\text{Incubation time (min)} = \frac{\text{Initial length of probe (in kb)} - 0.15}{0.11 \text{ x initial length of probe (in kb)} \text{ x } 0.15}$$

After RNA hydrolysis, 5 μl of 10% acetic acid was added to neutralize the solution. Subsequently, neutralized solution was mixed with 10 μl of 3 M NaAc (pH 5.2), 250 μl of ethanol and 1 μl of 10 mg/ml tRNA, followed by incubation at -80°C overnight. The RNA was precipitated at 13k rpm for 20 min at 4°C. The RNA pellet was washed with 80% ethanol and spun down for 5 min at 4°C. The pellet was finally resuspended in 40 μl of 50% formamide and kept in -80°C fridge.

2.6.2 Tissue Preparation

The fixative solution for imbibed seeds was prepared as follows: 1× PBS buffer was adjusted to pH 11 using saturated NaOH and heated to 60°C. Paraformaldehyde was added to a final concentration of 4% (w/v). After paraformaldehyde was completely dissolved, the solution was cooled on ice and adjusted to pH 7.0 with H_2SO_4. Freshly collected imbibed seeds were immersed in the ice-cold fixative in a glass bottle and under vacuum 1 h. The fixative was replaced and the seeds were incubated overnight at 4°C on a shaker.

After fixation, dehydration was performed at 4°C on a shaker as follows: 2×30 min 1×PBS, 60 min 30% ethanol, 60 min 40% ethanol, 60 min 50% ethanol, 60 min 60% ethanol, 60 min 70% ethanol, 60 min 85% ethanol, and finally 95% ethanol with 0.05 g/ml of eosin for overnight.

After dehydration, staining and clearing steps were performed at room temperature on a shaker as follows: 2×30 min 100% ethanol with 0.05 g/ml of eosin, 2×60 min 100% ethanol with 0.05 g/ml of eosin twice, 60 min 25% Histoclear in ethanol, 60 min 50% Histoclear in ethanol, 60 min 75% Histoclear in ethanol, 2×60 min 100% Histoclear. At last, the seeds were immersed in 100% Histoclear with 1/4 volume of paraplast chips for overnight without shaking.

After staining, the seeds in the glass bottle was transferred to an incubator set at 42°C until paraplast chips were completely melted, another 1/4 volume of paraplast

chips were added and the temperature was increased to 55°C. Subsequently, the mixture of Histoclear and paraplast was removed and replaced with freshly melted paraplast for overnight at 55°C. In the following three days, the paraplast was replaced by newly-melted paraplast twice a day. The well embedded-seeds in paraplast were transferred to a plastic container and hardened at room temperature.

2.6.3 Sectioning

The paraplast blocks were secured on sectioning molds using melted paraplast. Sectioning was performed on a Leica RM2165 microtome with a section thickness set at 8 μm. The tissue ribbons were arranged on a ProbeOn Plus glass slide (Fisher Biotechnology) and a few drops of DEPC-treated H_2O were added to float the ribbon. The slide was then placed on the top of a 42°C heated slide warmer to allow evaporation of H_2O and allow the ribbon to flatten out on the slide. Excessive H_2O was then removed and the slide was kept on the slide warmer at 42°C overnight to achieve complete drying and tight adherence of tissues on the slide.

2.6.4 Section Pre-treatment

All solutions in this step were made RNase-free, tips and a glass slide container was autoclaved at 121°C for 1 h, a plastic container was treated with 0.1 M NaOH

overnight and rinsed with sterile H_2O before use. All washing steps were done on a platform rocker.

The slides were placed in the glass slide holder. The sections were deparaffinised by warm Histoclear for 10 min twice. Rehydration of slides were carried out at room temperature with the following washes: 2×1 min 100% ethanol, 1 min 95% ethanol, 1 min 90% ethanol, 1 min 80% ethanol, 1 min 60% ethanol, 1 min 30% ethanol and 1 min RNase-free H_2O. After rehydration, slides were incubated in 2× SSC buffer (150 mM NaCl, 15 mM Sodium Citrate, pH 7.0) for 15-20 min at room temperature followed by proteinase K solution (1 μg/ml proteinase K in 100 mM Tris pH 8, 50 mM EDTA) for 30 min at 37°C. After proteinase K treatment, the slide holder was returned to room temperature for the following steps. The solution was replaced by 2 mg/ml glycine in 1× PBS buffer for 2 min to quench the remaining proteinase K, followed by 1× PBS washing for 2 min twice. Next, slides were fixed with freshly-made 4% (w/v) paraformaldehyde in 1× PBS solution (pH 7) for 10 min and washed with 1×PBS for 5 min twice. Slides were incubated for 10 min in triethanolamine solution (freshly-prepared by adding 2.68 ml of triethanolamine into 200 ml RNase-free H_2O containing 0.8 ml of 37% HCl and 1 ml of acetic anhydride, mix vigorously) and followed by 2× 5 min washes with 1× PBS. Dehydration of slides was done with the following washes: 30 sec 30% ethanol, 30 sec 60% ethanol, 30 sec 80% ethanol, 30 sec 90% ethanol, 30 sec 95% ethanol and 2×30 sec 100% ethanol.

2.6.5 Hybridization

Hybridization buffer contains 100 µl 10× in situ salts, 400 µl deionised formamide, 200 µl 50% dextran sulphate, 20 µl 50× Denhardt's solution (warm to 50°C before pipetting), 10 µl tRNA (10 mg/ml) and 70 µl H_2O (DEPC-treated). The total volume of 800 µl hybridization buffer was enough for 3 pairs of slides. The probe solution was prepared as follows: 1-2 µl of RNA probe from stock at -80°C was topped up to 60 µl with 50% formamide, followed by heating at 80°C for 2 min and immediate cooling with ice. For each pair of slides, 240 µl of hybridization buffer was mixed with 60 µl of probe solution to make the hybridization solution.

Slides were completely dried in a clean laminar flow cabinet before application of hybridization solution. 300 µl of hybridization solution was added to one slide and another slide was slowly placed onto the previous slide so that the probe can spread throughout the slides interface. Slides were elevated on a rack in a sealed plastic container containing sterile H_2O and incubated between 50-55°C in a hybridization oven overnight.

2.6.6 Post-hybridization

Each pair of slides was separated by dipping in 55°C pre-warmed 0.2× SSC buffer before placing in the glass slide holder. The slides were washed three times with

0.2× SSC for 60 min at 55°C in the hybridization oven equipped with a shaker. Thereafter, the slides were washed with 1× PBS buffer at room temperature for 5 min. Subsequently, the slides were blocked for 45min with 1% Boehringer blocking reagent dissolved in 100 mM Tris pH 7.5, 150 mM NaCl, followed by another 45 min incubation with a second blocking solution (BSA/Tris/NaCl/Triton) containing 1.0% Bovine Serum Albumin (BSA) dissolved in 100 mM Tris pH 7.5, 150 mM NaCl, 0.3% Triton X-100. All incubation steps were performed at room temperature on a platform rocker.

After blocking, anti-DIG antibody (Roche) was diluted 1:500 in 4 ml of new BSA/Tris/NaCl/Triton solution described in previous washing step, and the antibody solution was poured in a plastic weighing dish. Slides were sandwiched together and dipped into the antibody solution, which would be drawn by capillary action. Solution was drained on Kimwipes and the dipping process was repeated. Great care was taken to avoid bubbles between two slides. Slides were elevated on a rack in a sealed plastic container containing sterile H_2O and allowed to sit at room temperature for 2 h. After antibody incubation, slides were drained on Kimwipes and separated in the glass slide holder containing BSA/Tris/NaCl/Triton solution. This washing step was repeated 4 times for 15 min each at room temperature on a platform rocker, followed by washing with 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ solution (Tris pH 9.5/NaCl/$MgCl_2$) for 10 min to remove detergent. The substrate solution Tris-NaCl-PVA for color development was prepared as follows:

10% (w/v) 40kD polyvinyl alcohol (PVA) (Sigma) was dissolved in Tris pH 9.5/NaCl/$MgCl_2$ solution, which was then heated to 50°C, mixed vigorously, and cooled down to room temperature. 60 µl of NBT/BCIP stock solution (Roche) was mixed with 3 ml of Tris-NaCl-PVA stock solution and the solution was spun down briefly to remove the bubbles. 270 µl of prepared substrate solution was added to each pair of slides which were then sandwiched with face-to-face manner. Slides were elevated on a rack in the plastic container containing sterile H_2O in total darkness overnight at room temperature.

On the next day, slides were separated and placed in the glass slide holder. The slides were rinsed with tap water for three times to stop the reaction. The slides were dehydrated by washing with 70% ethanol for 5 sec and 2 times 100% ethanol for 2 sec each. Slides were air dried before being mounted with 50% glycerol for maintenance of signals for at least three months and observation under microscopes.

2.7 GUS Activity Analysis

For each MFT-GUS reporter construct, we checked at least 15 independent transgenic lines at the T3 generation. A representative line for each MFT-GUS construct was selected for further analysis. GUS (β-glucuronidase) staining was performed according to the method described previously (Sieburth and Meyerowitz,

1997). The GUS substrate X-Gluc (cyclohexylammonium salt) (Gold Biotechnology) was dissolved in N-N-Dimethylformamide to make a 100 mM stock solution, which was kept at -20°C in darkness. The solution went bad if the color turned to light red. The staining procedures are briefly summarized as follows: plant tissues were fixed in 90% acetone for 15-20 min on ice, followed by three times washes with rinse buffer (50 mM sodium phosphate pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$). The staining solution is the rinse buffer supplemented with 2mM X-Gluc, the tissues in the staining solution were under vacuum for 30 min before incubated in a 37°C oven overnight. After staining, the tissues were cleared in an ethanol series.

2.8 ChIP Assay

2.8.1 Fixation

About 300 mg germinating seeds were fixed for 45 min at 4°C with 1% formaldehyde in MC buffer (10 mM potassium phosphate, pH 7.0, 50 mM NaCl, 0.1 M sucrose) under vacuum. After adding glycine to a concentration of 150 mM, the fixed seeds were shaken for 20 min at 4°C, washed three times with MC buffer. The fixed seeds were kept at -80°C or used directly for ChIP assay.

2.8.2 Homogenization and Sonication

The fixed seeds were homogenized in 500 µl reaction buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS) supplemented with PMSF and Protease Inhibitor Cocktail Tablets (Roche). The mixture was sonicated to produce DNA fragments between 200 bp and 1 kb. After sonication, the mixture was spun down at 14k rpm for 5 min at 4°C and the supernatant was transferred to a new 1.5 ml tubes. The pellet was washed by another 500 µl reaction buffer and the supernatant was combined with the previous one. 10% solubilized chromatin was saved as an input control.

2.8.3 Immunoprecipitation and DNA Recovery

The solubilized chromatin was incubated with anti-HA or anti-myc agarose beads (Sigma) for 1.5 h at 4°C. Beads were washed twice with IP buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 5 mM $MgCl_2$, 10 µM $ZnSO_4$, 1% Triton X-100, 0.05% SDS), twice with high salt IP buffer with the concentration of KCl increased to 500 mM, once with LNDET buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.0), and once with IP buffer again. Beads were then incubated with the elution buffer (50 mM Tris, pH 8.0, 1% SDS, 10 mM EDTA) for 30 min at 65°C. The eluted supernatant and the input control that was topped up

with the elution buffer to a final volume equal to that of the eluted supernatant were added with NaCl to a final concentration of 200 mM. They were incubated at 65°C for 6 h for reverse crosslinking followed by 40 mM proteinase K treatment for 1 h at 45°C. DNA was then recovered using the QIAquick PCR Purification Kit (Qiagen). For each ChIP assay, three independent experiments were performed using seeds collected separately.

2.8.4 Calculation of Fold Enrichment

DNA enrichment was examined by quantitative real-time PCR in triplicates as previously described (Liu et al., 2008). The enrichment of a *TUB* genomic fragment was used as negative control. Primer pairs used for ChIP enrichment test are listed in Table 2.

2.9 Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *MFT* (At1g18100), *ABI3* (At3g24650), *ABI4* (At2g40220), *ABI5* (At2g36270), *RGL2* (At3g03450), *RD29A* (At5g52310), *RD29B* (At5g52300), *AtEm6* (At2g40170), *CRC* (At4g28520), *At2S3* (At4g27160), *OsMFT1* (Os06g0498800), *OsMFT2* (Os01g0111600), *ZCN9* (Eu241925), *ZCN10* (Eu241926), and *ZCN11* (Eu241927).

Table 1. Primers for real-time RT-PCR.

Primers pairs used for real-time RT-PCR (Sequence 5'→3')	
MFT	CGAGCCGAACATGAGAGAAT AAGTATCTCTTTTCCTCTTGAGGG
ABI3	CGGGAGGGACCTGGATGTATT CCATCACTGGCGGTAATTGAG
ABI4	GCTCACTGATGTTCCGGTAACTAA TTACACCCACTTCCTCCTTGTTC
ABI5	CAGCTGCAGGTTCACATTCTG CACCCTCGCCTCCATTGTTAT
RGL2	GCCCTTACCTGAAGTTCGCTC TGCATTAAAGCAGGCCATTGC
RD29A	CCTGAAGTGATCGATGCACCAG TGGTGTAATCGGAAGACACGAC
RD29B	GTGAAGATGACTATCTCGGTGG CACCACTGAGATAATCCGATCC
AtEm6	AGGATATCAGCAGATGGGACGC CGTCTATCTCGACTCCTTCCTC
CRC	CGTGTTAAGGGACCTTTCCAGG ATAGTCTCCTCAAGGCCGTTGC
At2S3	GCACGATGATCATGAGTGTTGC AGTGCTAATTACATTAGCCTC
18S rRNA	CCTCTGTGCTGGCGACGCAT CCAGACTTGCCCTCCAATGGA

Table 2. Primers for ChIP assays.

Primers pairs used for ChIP assays (Sequence 5'→3')	
MFT-1	GTTCCTCCTGATTATTCTACG GAAACCGTGAATTTCATGCCA
MFT-2	ATGATCCTCCTAACCGACGGC AACCTATAGGCCATGCATAGG
MFT-3	TAACGACAAAATAACGCACAT TCACGTGTTGCATGATTAGCC
MFT-4	AGAGGAAATTATCGCCAACGT ATGGGCATCTATGTCATGGTT
ABI5-1	TTGTCCCTGTGACTCTGCAAC ACCATACCTGGAGCAAACGG
ABI5-2	CTCCCAATGGAAGTTCGGAA AGAATTACGTTTAGTGAGACA
ABI5-3	TTGACCTTCACGCCTCTCTTC GCTTCTTGTTATGGAGGTTC
ABI5-4	ATCTTGTGTTGATAAGTTCGC AAGAACGGTCCACATCGTGAC
ABI3-1	GTTCGTGCATGATGGTAATGG AACACTTGTACGTCGAGATGG
ABI3-2	CTGCTGAGGTAATTGAATGCTGC ATAAGAGAGCCCATGTGTTCC
ABI3-3	TTCGATGATAGCCAAGTTGGG TTTTGTACGGACAGATAGGG
TUB2	ATCCGTGAAGAGTACCCAGAT AAGAACCATGCACTCATCAGC
AtEm6	AAGAAGTACGGCCACTACCAC TGGTGACTCAGGGACAATCG
ACTIN	CGTTTCGCTTTCCTTAGTGTTAGCT AGCGAACGGATCTAGAGACTCACCTTG

CHAPTER 3

RESULTS

3.1 Phenotypic Characterization of *mft* Mutants in *Arabidopsis*

To examine the spatial expression pattern of *MFT* in *Arabidopsis*, quantitative real-time RT-PCR analysis was carried out with total RNA extracted from various plant tissues. It was found that *MFT* was expressed highly in developing siliques, moderately in roots and rosette leaves, but weakly in other tissues (Figure 4A). To better define the expression of *MFT* in siliques, we further dissected siliques into developing seeds and pods & placentas. The result showed that *MFT* was preferentially expressed in the developing seeds (Figure 4B), implying a possible role of *MFT* in seed development.

To investigate the biological function of *MFT*, two T-DNA insertion alleles of *MFT* in Col-0 background were isolated (Figure 5A). Since mft-1 has been adopted in a previous publication (Yoo et al., 2004), thus these two new alleles were designated as *mft-2* and *mft-3*, respectively. Semi-quantitative RT-PCR analysis showed that there was no detectable expression of *MFT* in these two homozygous mutant lines, suggesting that *mft-2* and *mft-3* are null alleles (Figure 5B). Next, we compared the phenotypes between *mft* mutants and wild-type plants under normal growth conditions, it turned out that the *mft* homozygous mutants did not show obvious defects. Considering the high expression of *MFT* in the developing seeds and the well-known role of ABA in the control of seed development and seed germination,

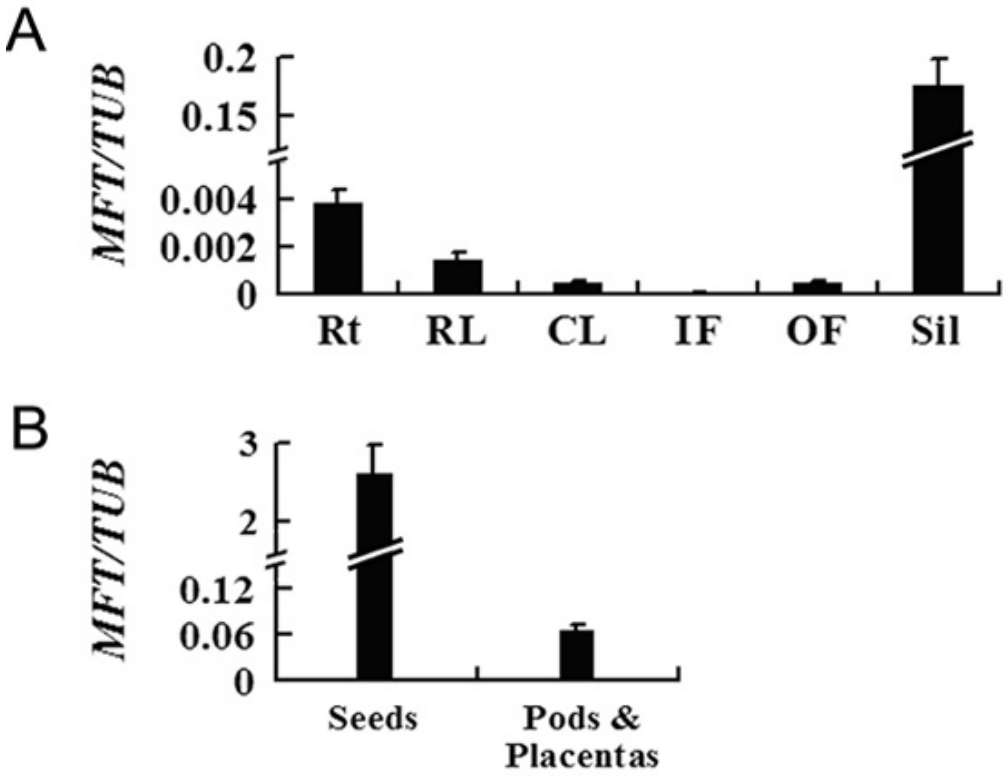

Figure 4. Spatial Expression of *MFT*.

(A) Quantitative real-time PCR analysis of *MFT* expression in various tissues. Results were normalized against the expression of *TUB2*. Rt, roots; RL, rosette leaves; CL, cauline leaves; IF, inflorescences without open flowers; OF, open flowers; Sil, siliques.

(B) *MFT* expression in developing siliques dissected into seeds and pods plus placentas.

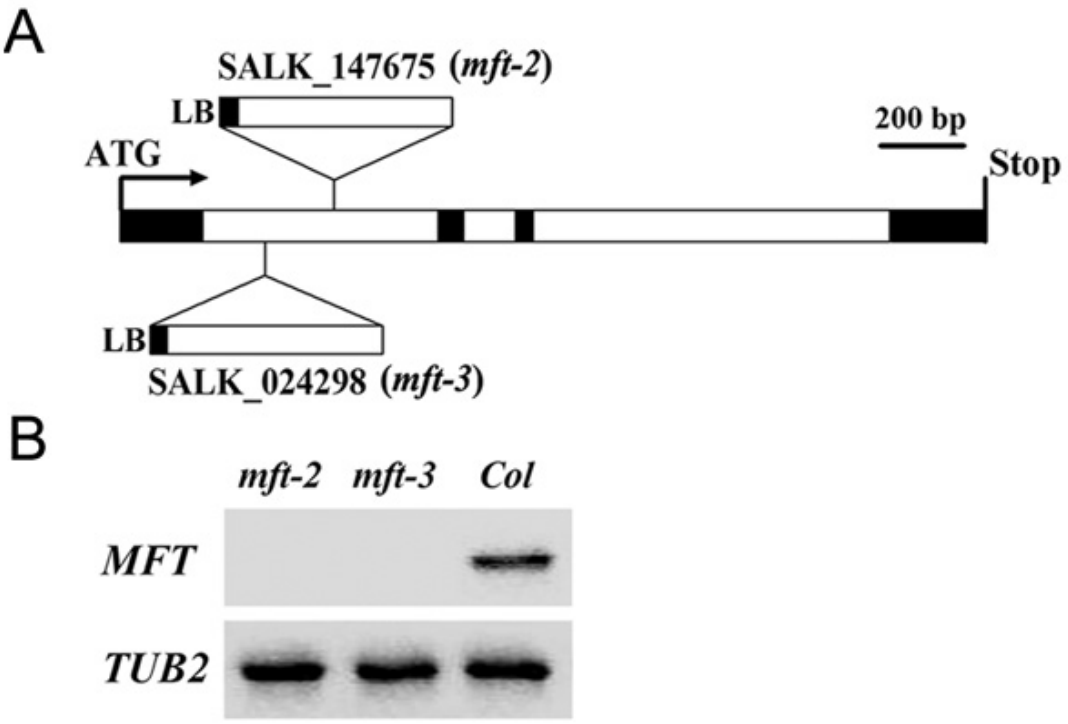

Figure 5. T-DNA Insertion Alleles of *MFT*.

(A) Schematic diagram indicating the T-DNA insertions in two *mft* loss-of-function mutants, *mft-2* (SALK_147675) and *mft-3* (SALK_024298). Black and white boxes indicate exons and introns of *MFT*, respectively.
(B) Semi-quantitative RT-PCR analysis using a pair of primers flanking the T-DNA insertion sites did not detect *MFT* expression in *mft-2* and *mft-3*, indicating that both of them are null alleles.

we thereafter carefully observed the response of *mft* mutants to exogenous ABA. We found that in the presence of exogenous ABA, the germination rate of both *mft-2* and *mft-3* seeds was much lower than that of wild-type seeds (Figure 6), indicating that *MFT* is involved in seed germination in response to ABA. To further assess the role of *MFT* in seed germination, we generated *35S:MFT* transgenic plants. Among 23 lines generated, 16 lines showed higher germination rates to different extents particularly at early stages of seed germination when exogenous ABA was applied (Figure 7). These results suggest that *MFT* regulates seed germination in response to exogenous ABA. As endogenous ABA levels in *mft-2* seeds after imbibition were comparable to those in wild-type (Figure 8), *MFT* may not be directly involved in regulating ABA levels during seed germination.

As ABA induces growth retardation and stomata closure, we further studied whether *MFT* is also involved in regulating other ABA-related physiological responses. After seed germination, both *mft-2* and wild-type seedlings showed similar degree of growth retardation when exogenous ABA was applied (Figure 9). In addition, there was no significant difference in ABA-induced drought tolerance among wild-type plants and the plants with altered *MFT* expression (Figure 10), implying that *MFT* might have a specific function in seed germination.

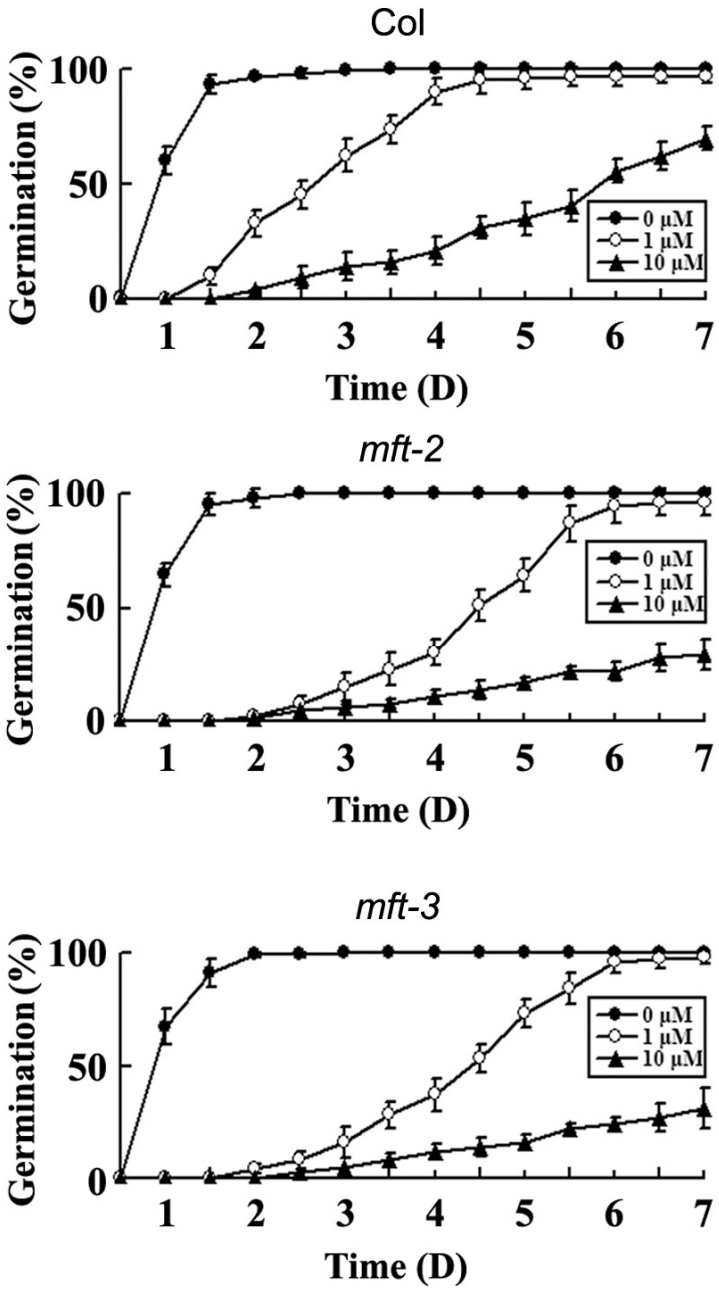

Figure 6. Germination Rate of *mft* Mutants in Response to ABA.

Germination phenotype of wild-type, *mft-2*, and *mft-3* treated with different concentrations of ABA (0, 1, and 10 μM).

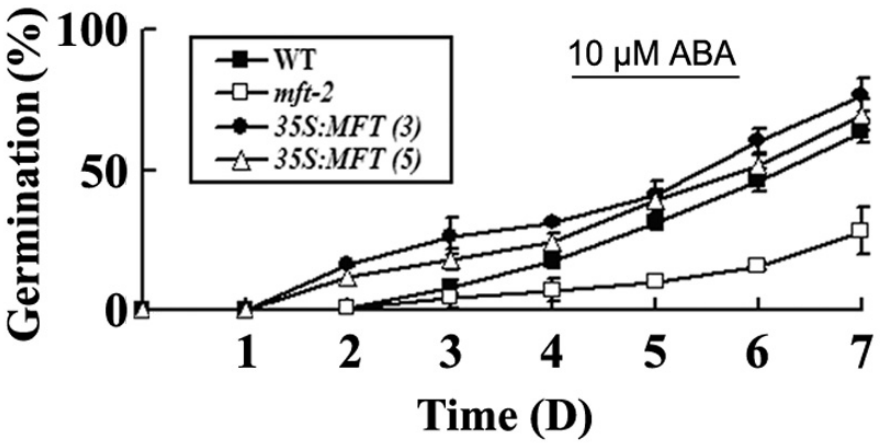

Figure 7. Germination Rate of Seeds Overexpressing *MFT*.

Germination phenotype of two representative *35S:MFT* lines (3 and 5) in response to 10 μM ABA.

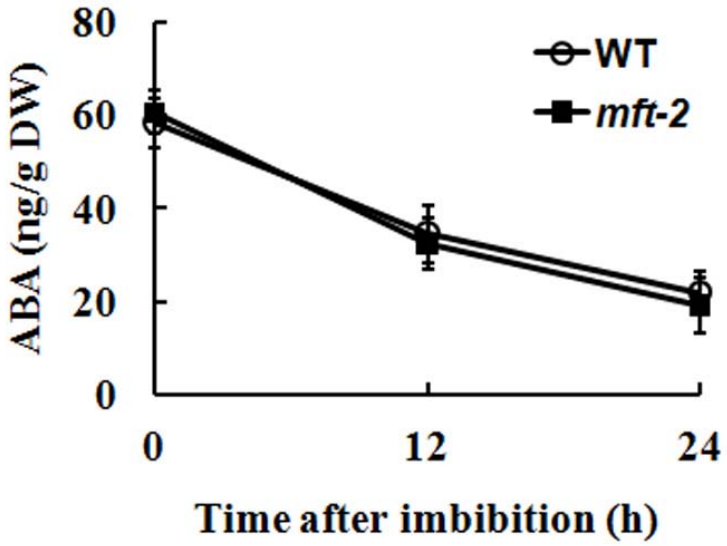

Figure 8. Quantification of Endogenous ABA Levels in Wild-type and *mft-2* Seeds after Imbibition.

Endogenous ABA levels were measured using a Plant Hormone ABA ELISA kit (Cusabio biotech). The values were calculated based on three independent batches of seed samples. DW, dry weight.

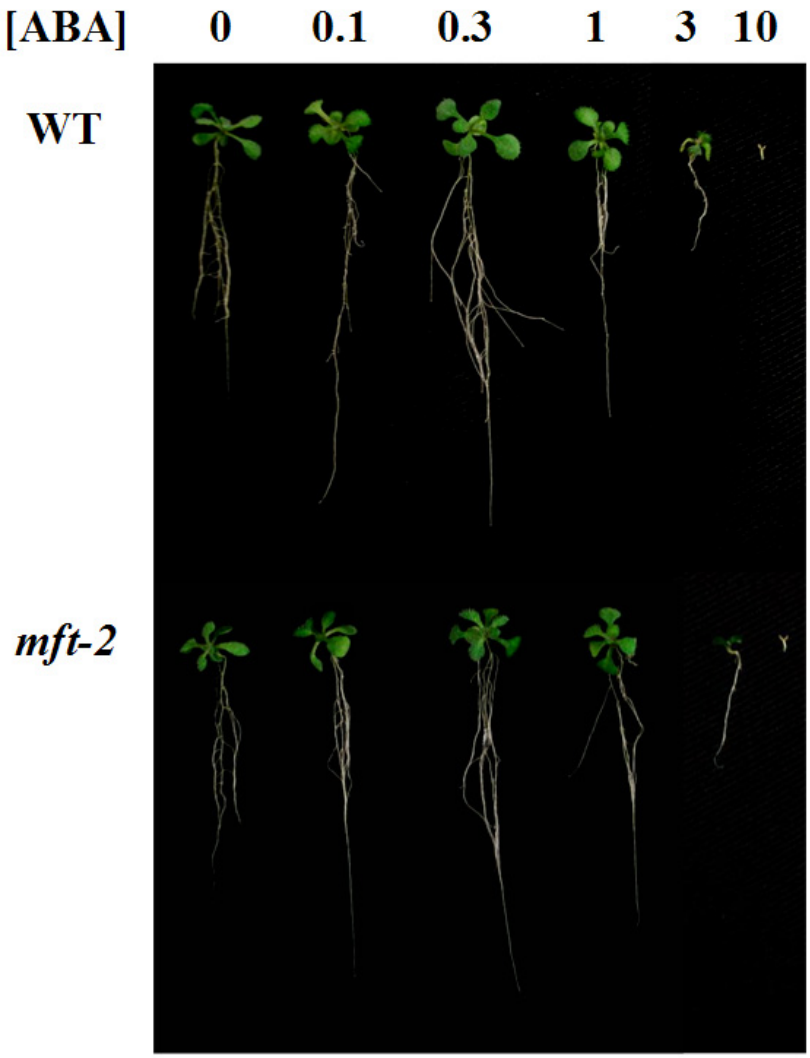

Figure 9. Post-germination Growth of *mft* Is Not Hypersensitive to ABA Treatment.

Seeds were germinated firstly on common MS medium. Two days after germination, they were transferred to MS medium supplemented with different concentrations of ABA (0, 0.1, 0.3, 1, 3, and 10 μM). Phenotypes of seedling growth were compared 10 days after the transfer.

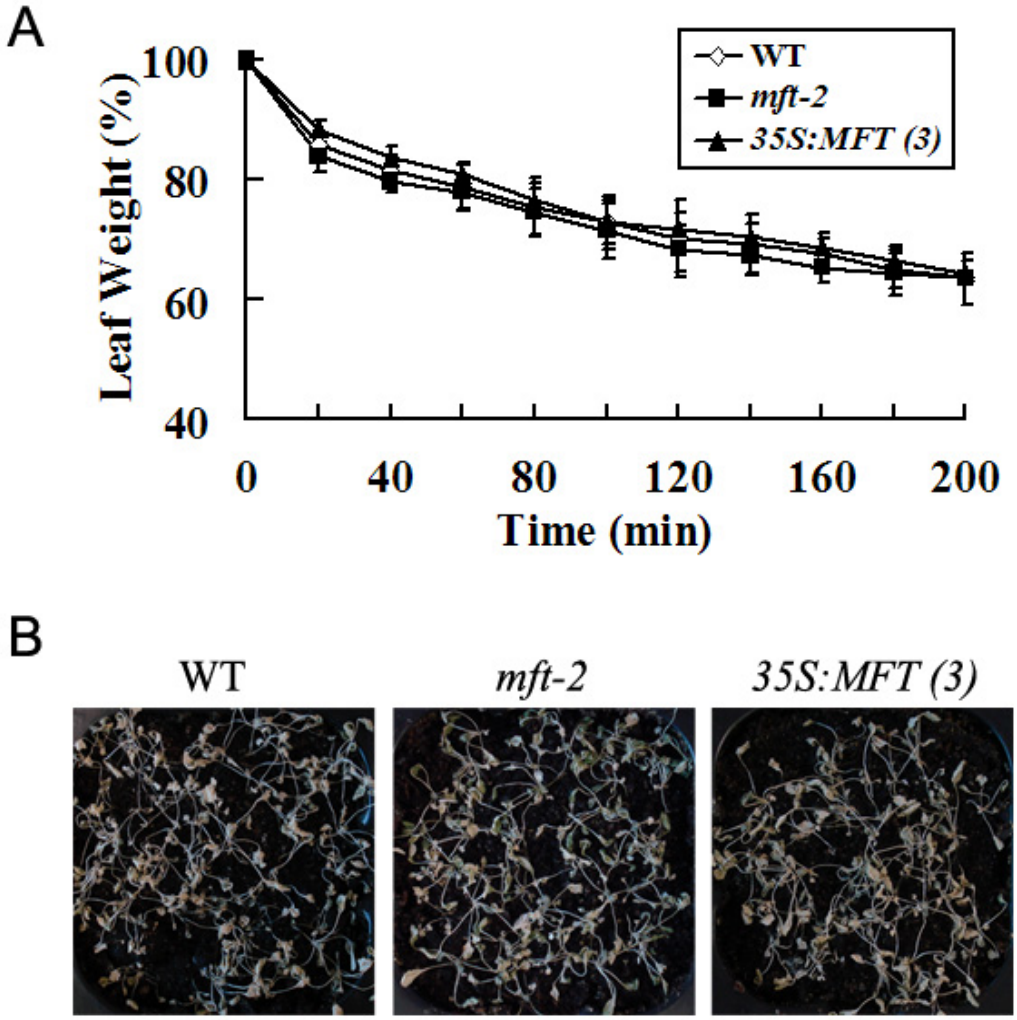

Figure 10. *mft* Is Not Hypersensitive to Drought Stress.

(A) Transpiration rates of wild-type, *mft-2*, and *35S:MFT (No.3)* plants. Young rosette leaves of similar developmental stages (2-week-old; n = 4 each) were excised and weighed at different time points as indicated.

(B) Drought tolerance of wild-type, *mft-2*, and *35S:MFT (No.3)* plants. 2-week-old plants (n = 50 each) grown in soil under the same growth conditions were withheld from water for 11 days, and re-watered. The photos were taken 3 days after re-watering.

3.2 *MFT* Expression Is Upregulated in Response to ABA

Because the germination of *mft* mutant seeds was hypersensitive to exogenously applied ABA, we further examined *MFT* expression levels in response to ABA. Information retrieved from public *Arabidopsis* microarray database (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) showed that *MFT* was greatly upregulated by ABA (Figure 11A), which is in agreement with our observation that *MFT* expression levels in germinating seeds were gradually elevated along with increased concentrations of ABA (Figure 11B). To reveal the exact location where *MFT* is upregulated by ABA, in situ hybridization assay was further performed. The results showed that *MFT* was not detectable in both the embryo and endosperm of seeds collected 6 h post-stratification, which is an early stage of germinating seeds (Figure 12), whereas its upregulation by ABA occurred strongly in the epidermis and cortex and weakly in the provasculature of the radicle-hypocotyl transition zone of seeds collected at a later germination stage (Figure 13). Furthermore, we checked the time-course expression of *MFT* during seed imbibition in two *cyp707a* mutants with high levels of endogenous ABA (Okamoto et al., 2006). Our results demonstrated that the expression of *MFT* in both *cyp707a1-1* and *cyp707a2-1* mutants was much higher than that in wild-type, especially at 24 h after imbibition (Figure 14). Thus, *MFT* is also upregulated by endogenous ABA. These findings indicate that *MFT* is highly upregulated at late

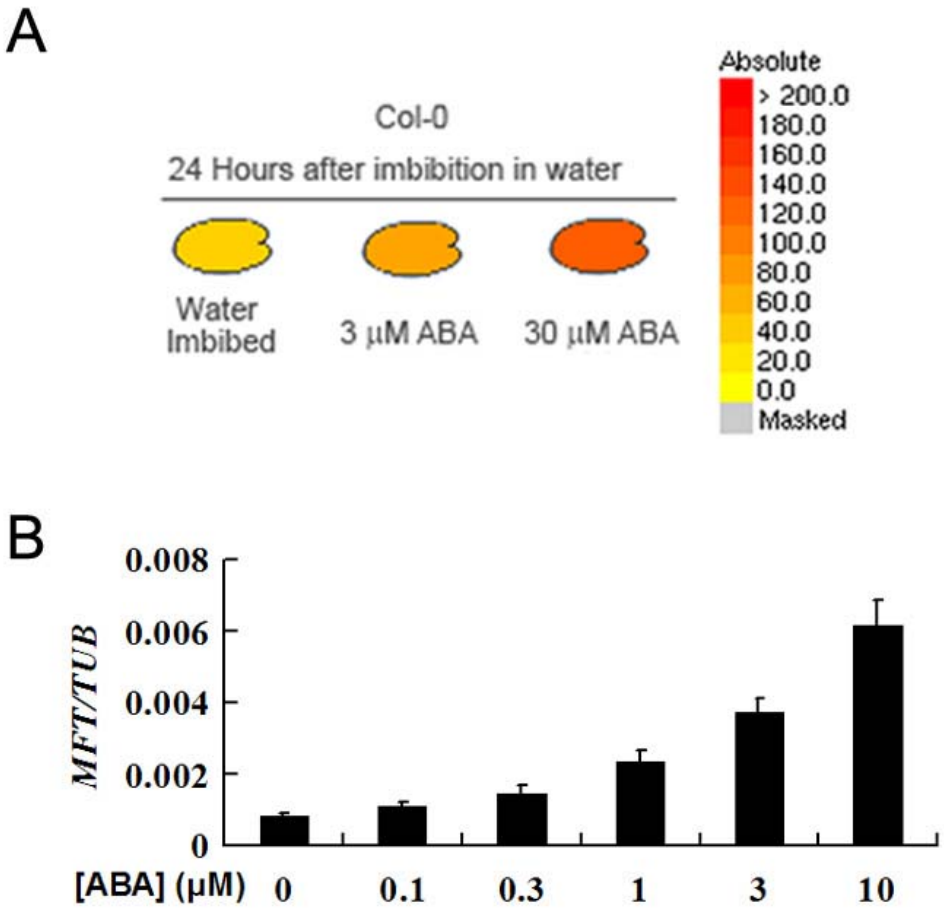

Figure 11. *MFT* Is Upregulated by ABA.

(A) Public microarray data showing upregulation of *MFT* by ABA in germinating seeds (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). The colors from yellow to red indicate the increased absolute signal values of *MFT* expression retrieved from microarray data.

(B) *MFT* expression in germinating seeds treated with different concentrations of ABA. All seeds were collected 16 h post-stratification.

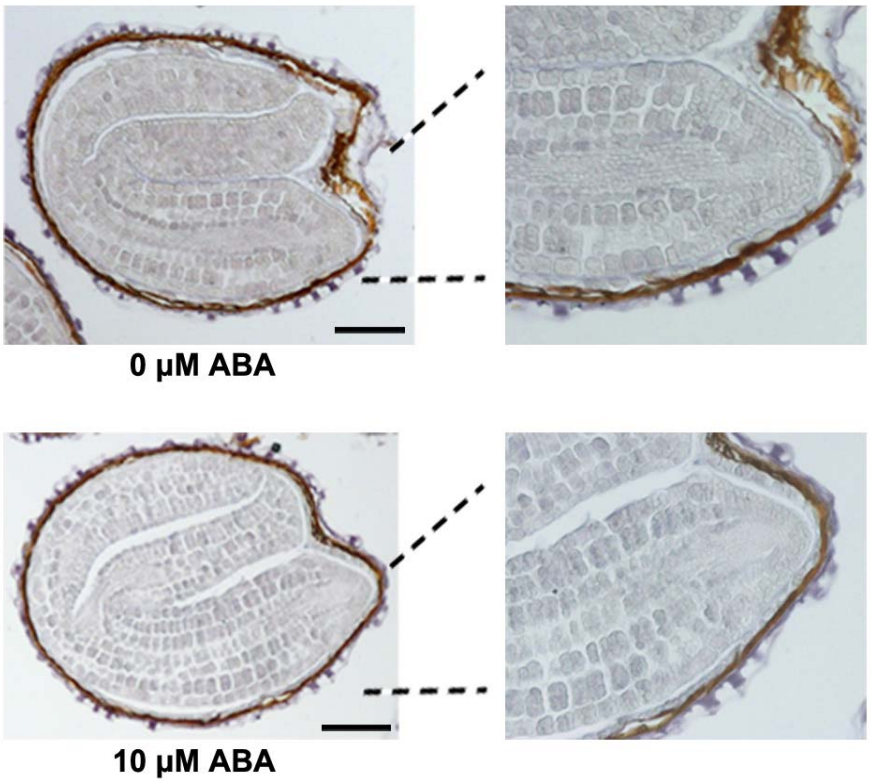

Figure 12. In Situ Localization of *MFT* in Germinating Seeds at An Early Stage.

Seeds were collected 6 h post-stratification treated without or with 10 µM ABA. The right panels show the magnified views of the micropylar endosperm surrounding the radicle. Bars = 100 µm.

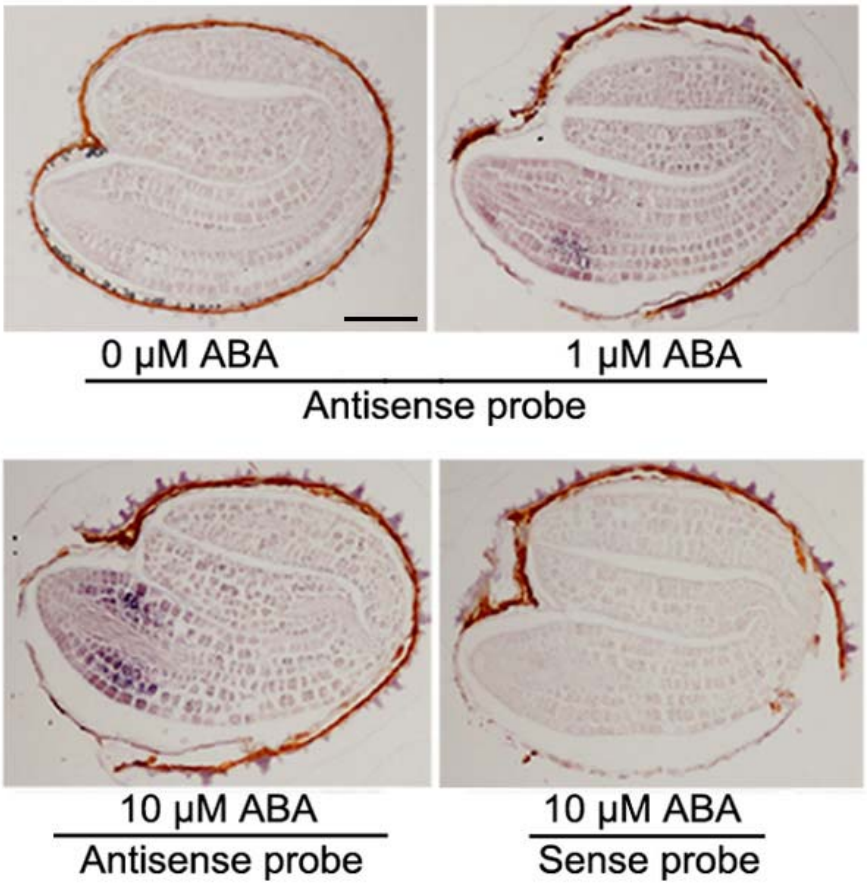

Figure 13. In Situ Localization of *MFT* in Germinating Seeds at Later Stages.

Seeds without ABA treatment were collected 12 h post-stratification, while other seeds treated with 1 and 10 μM ABA were collected 24 h post-stratification. These seeds at the same developmental stage were hybridized with the antisense or sense *MFT* probe as indicated. Bar = 100 μm.

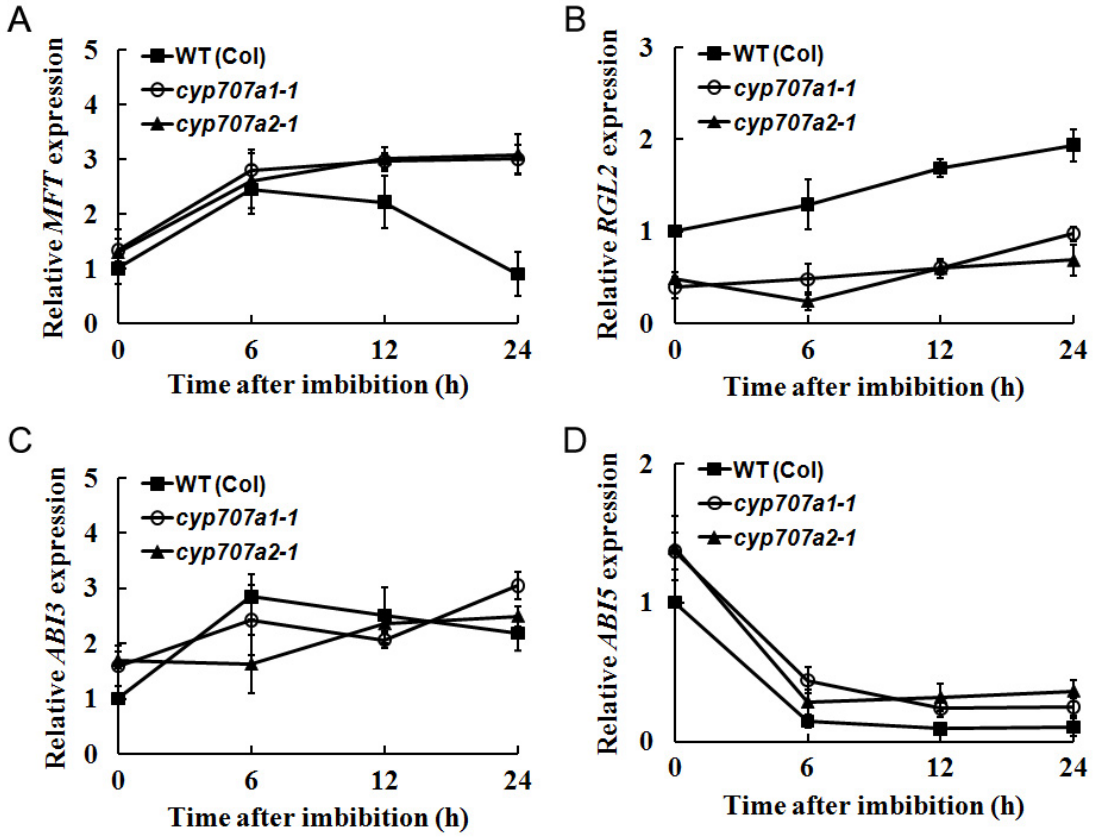

Figure 14. Expression of *MFT*, *RGL2*, *ABI3*, and *ABI5* in Wild-type, *cyp707a1-1*, and *cyp707a2-1* Seeds after Imbibition.

Expression levels of *MFT* **(A)**, *RGL2* **(B)**, *ABI3* **(C)**, and *ABI5* **(D)** were compared in wild-type, *cyp707a1-1*, and *cyp707a2-1* seeds sown on MS medium. The expression levels were normalized against the expression of *18S rRNA*. In each panel, the relative expression in wild-type dry seeds (0 h) is set as 1.

stages of germinating seeds to promote germination.

As high salinity prevents seed germination and stimulates the biosynthesis and accumulation of ABA by activating genes encoding ABA biosynthetic enzymes (Xiong et al., 2002; Xiong and Zhu, 2003), we next investigated whether *MFT* could affect seed germination in response to high salinity. The germination of *mft* seeds was also hypersensitive to NaCl treatment (Figure 15). In addition, high salt concentrations caused dramatic upregulation of *MFT* expression, which is similar to the ABA effect (Figure 16A). To test if upregulation of *MFT* by high salinity was mediated via the ABA pathway, we treated seeds of *aba1-5* mutants, in which the ABA biosynthesis was severely impaired (Leon-Kloosterziel et al., 1996), with high salinity. Upregulation of *MFT* by high salinity was significantly attenuated in *aba1-5* seeds as compared with wild-type (Figure 16A), suggesting that high salinity induces *MFT* expression mainly through the ABA signaling pathway. Furthermore, *MFT* was markedly upregulated in just germinated seeds compared with geminating seeds treated with high concentrations of ABA (e.g. 10 μM), whereas high salinity upregulated *MFT* in just germinated seeds to the same level as in geminating seeds (Figure 16B). This demonstrates a cumulative effect of ABA on promoting *MFT* expression during seed germination. Taken together, these results show that *MFT* is upregulated by ABA during seed germination, but loss of *MFT* function results in ABA hypersensitivity, suggesting an antagonistic function of *MFT* against the inhibitory effect of ABA on seed germination.

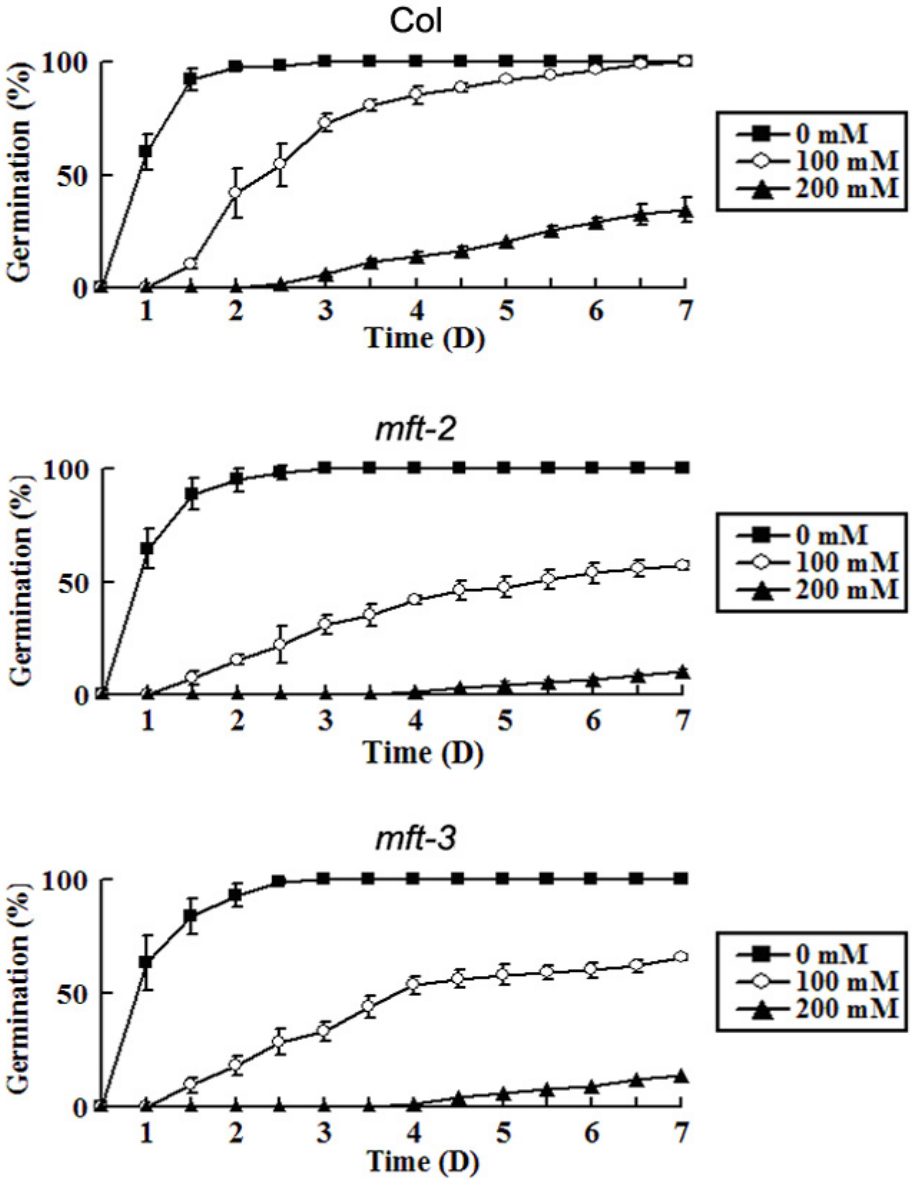

Figure 15. Germination Rate of *mft* Mutants in Response to NaCl.

Germination phenotype of wild-type, *mft-2*, and *mft-3* treated with different concentrations of NaCl.

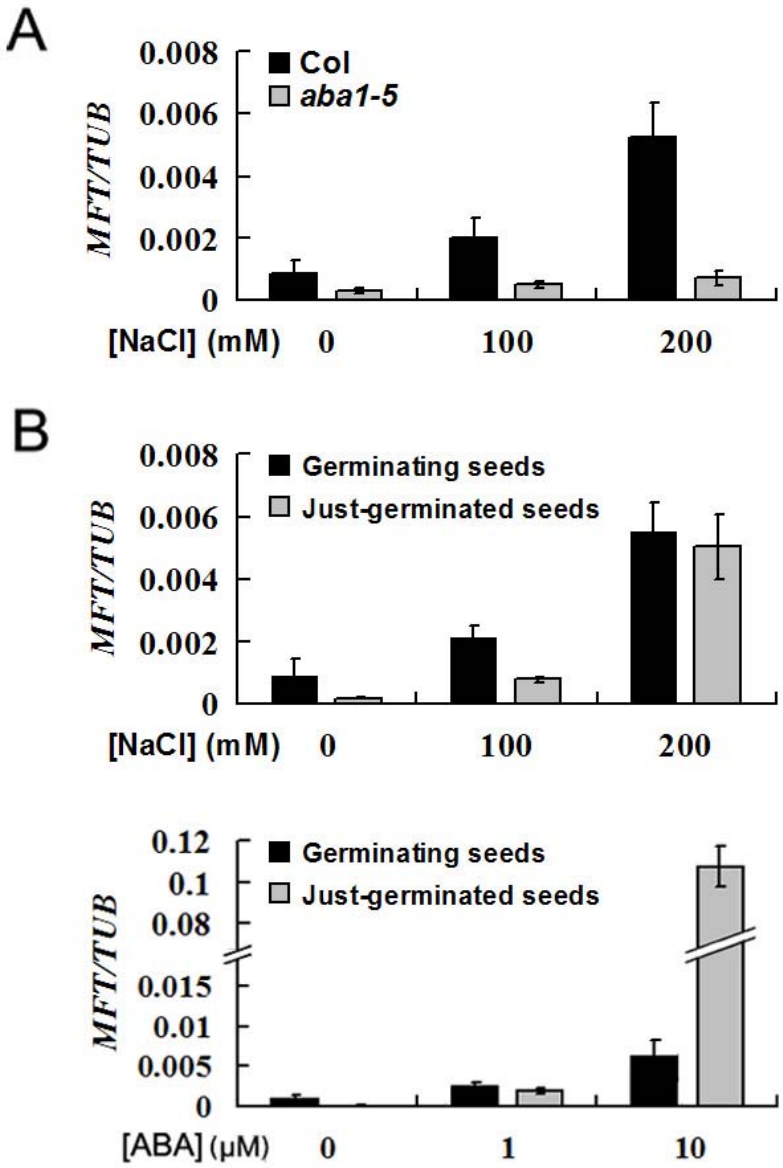

Figure 16. Expression of *MFT* in Response to NaCl and ABA.

(A) *MFT* expression in wild-type and *aba1-5* germinating seeds treated with different concentrations of NaCl. All seeds were collected 16 h post-stratification.
(B) *MFT* expression in germinating and just-germinated seeds treated with NaCl and ABA. All germinating seeds were collected 16 h post-stratification. Seeds with visible protrusion of the radicle tip through all the covering layers were collected as just-germinated seeds.

3.3 The Response of *MFT* to ABA Is Directly Mediated by ABI3 and ABI5

Next we sought to understand the molecular mechanism by which ABA regulates *MFT* expression. *ABI* loci (*ABI1* to *ABI5*) have been identified as essential regulators in the ABA signaling network (Koornneef et al., 1984; Finkelstein, 1994). The expression of *ABI3-5* had a similar trend as that of *MFT* in wild-type seeds during imbibition under normal growth conditions (Figure 17). We further tested whether these *ABI* genes are involved in the regulation of *MFT* by ABA by examining *MFT* transcript levels in various *abi* mutant seeds in the absence or presence of exogenous ABA. Upregulation of *MFT* by ABA was completely abolished in *abi3-1* and attenuated in *abi5-1* (Figure 18), suggesting that *ABI3* and *ABI5* mediate the expression of *MFT* in response to ABA. It is noteworthy that with or without ABA treatment, *MFT* expression in *abi3-1* mutant was strikingly higher than that in wild-type (Figure 18), indicating that *ABI3* negatively regulates *MFT*. On the contrary, *MFT* expression in *abi5-1* was the lowest among all the mutants treated with high concentrations of ABA (Figure 18), indicating that *ABI5* promotes *MFT* expression in response to ABA. As the expression levels of *MFT* in *abi3-1 abi5-1* were similar to those in *abi3-1* (Figure 18), *abi3-1* is epistatic to *abi5-1* in terms of regulating *MFT* expression.

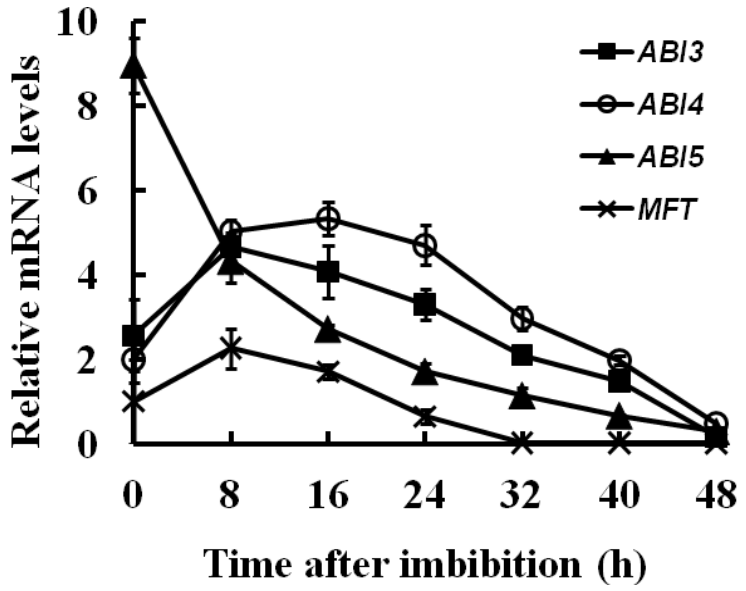

Figure 17. Expression of *MFT*, *ABI3*, *ABI4*, and *ABI5* in Wild-type Seeds after Imbibition.

Expression levels of *MFT*, *ABI3*, *ABI4*, and *ABI5* were compared in wild-type seeds sown on MS medium. The expression levels were normalized against the expression of *18S rRNA*. The relative expression of *MFT* in dry seeds (0 h) is set as 1.

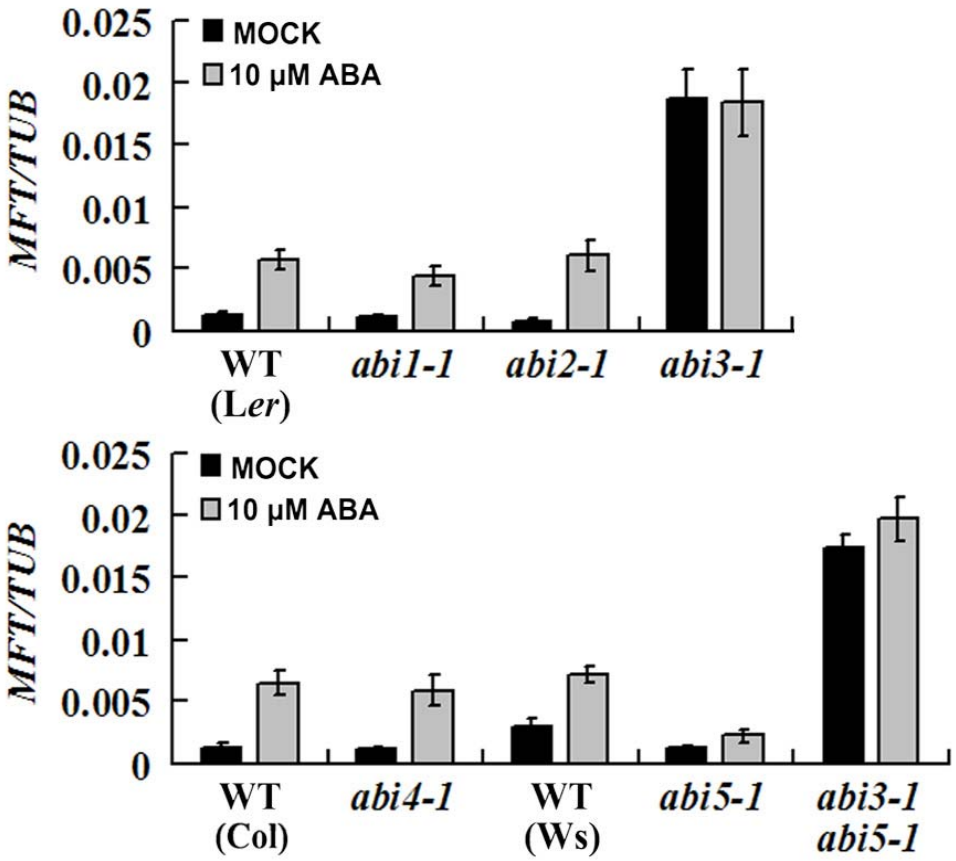

Figure 18. Expression of *MFT* in Wild-type and *abi* Mutant Seeds.

Expression levels of *MFT* were analyzed in wild-type and various *abi* mutants mock-treated or treated with 10 μM ABA. Because *abi3-1* seeds germinated around 14 h post-stratification, we collected all germinating seeds 12 h post-stratification for comparing *MFT* expression.

To elucidate the relationship between *ABI3*, *ABI5*, and *MFT*, we created *35S:ABI3-6HA* and *35S:ABI5-6HA* transgenic lines. To test the functionality of these two transgenic lines, germination assay was performed. The results showed that when low concentrations (1 μM or 5 μM) of ABA was applied, the germination rates of these two transgenic plants were significantly lowered, which is in agreement with previous reports on *35S:ABI3* and *35S:ABI5* (Lopez-Molina et al., 2001; Zhang et al., 2005), suggesting that both ABI3-6HA and ABI5-6HA fusion proteins are biologically functional (Figure 19).

We further checked the expression of *ABI3*, *ABI5*, and *MFT* in the germinating seeds of these two functional transgenic plants in the absence or presence of exogenous ABA. It was found that when *ABI3* was overexpressed, *ABI5* expression was upregulated, whereas *MFT* expression was downregulated in the presence of ABA (Figure 20). Overexpression of *ABI5* did not affect *ABI3* expression, but did lead to upregulated *MFT* expression in the presence of ABA (Figure 20). As it has been reported that ABA prevents the degradation of ABI3 and ABI5 proteins (Zhang, 2005; Lopez-Molina, 2001), these results, together with the observation on *MFT* expression in *abi3-1* and *abi5-1*, suggest that ABI3 activity inhibits *MFT* expression, whereas ABI5 has an opposite effect.

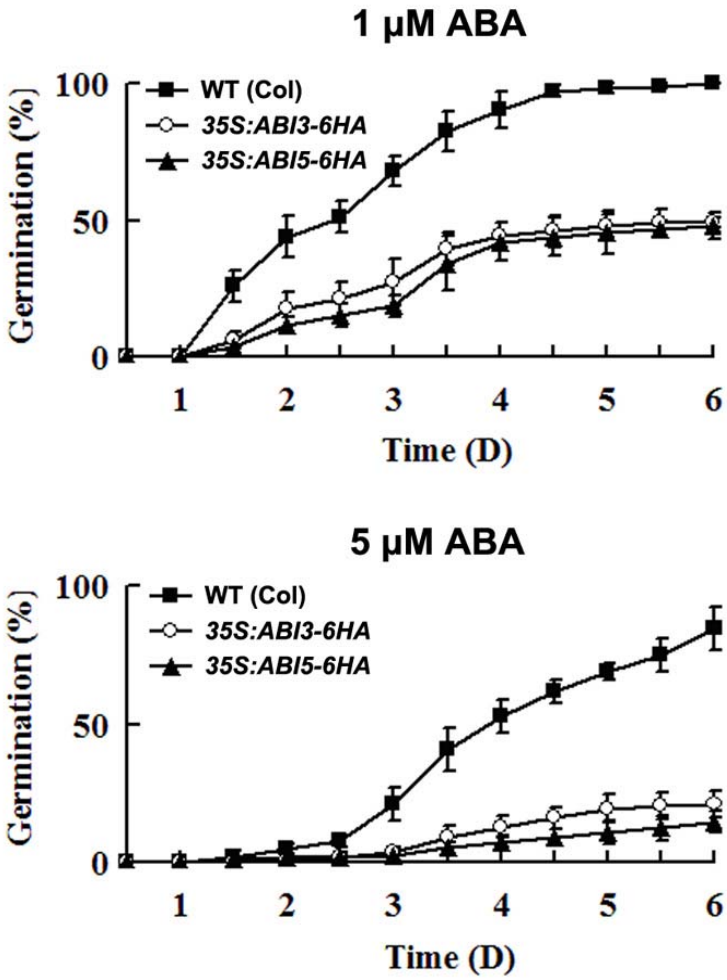

Figure 19. Biological Functional Lines of *35S:ABI3-6HA* and *35S:ABI3-6HA*.

Germination phenotype of wild-type, *35S:ABI3-6HA* and *35S:ABI5-6HA* treated with 1 or 5 µM ABA.

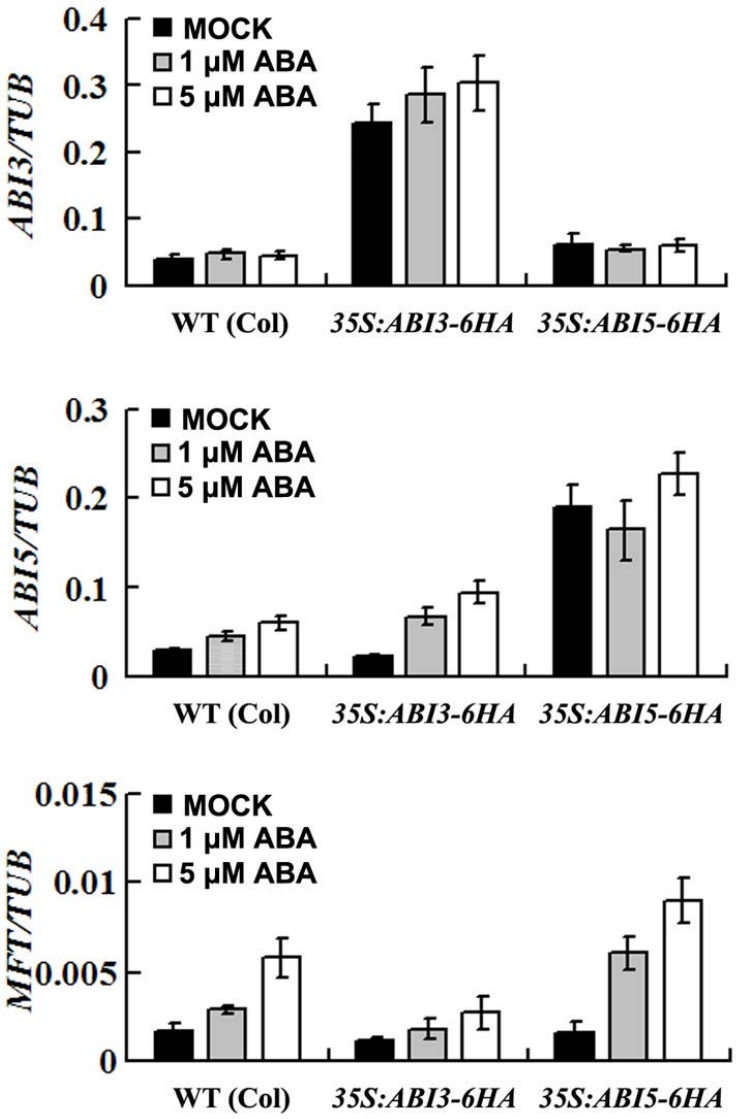

Figure 20. Expression of *ABI3*, *ABI5*, and *MFT* in Germinating Seeds of *35S:ABI3-6HA* and *35S:ABI3-6HA*.

Expression of *ABI3*, *ABI5* and *MFT* in germinating seeds of wild-type, *35S:ABI3-6HA* and *35S:ABI5-6HA* mock-treated or treated with 1 or 5 μM ABA. All germinating seeds were collected 16 h post-stratification.

To further investigate whether ABI3 and ABI5 directly regulate *MFT*, chromatin immunoprecipitation (ChIP) assays were carried out. We first did promoter analysis and identified several putative ABA-responsive elements (ABREs) in the *MFT* promoter. There is a single ABRE and a separate cluster of 5 ABREs located about 700 bp and 1.7 kb upstream of the initiation codon, respectively (Figure 21, upper panel). The presence of several ABREs in the *MFT* promoter implies that ABI3 and ABI5 may directly regulate *MFT* expression, since both ABI3 and ABI5 have been shown to regulate ABRE-containing genes (Kim et al., 1997; Ezcurra et al., 1999; Ezcurra et al., 2000; Finkelstein and Lynch, 2000; Kim et al., 2002; Lopez-Molina et al., 2002). Hence, *35S:ABI3-6HA* and *35S:ABI5-6HA* tagging lines were applied for ChIP assays using four pairs of the primers designed in the *MFT* promoter (MFT-1 to MFT-4). ChIP enrichment test revealed that ABI3-6HA was mainly associated with the genomic region near MFT-2, while ABI5-6HA bound to the regions near both MFT-2 and MFT-3 (Figure 21, middle and lower panels), suggesting that both ABI3 and ABI5 directly bind to the *MFT* promoter in vivo.

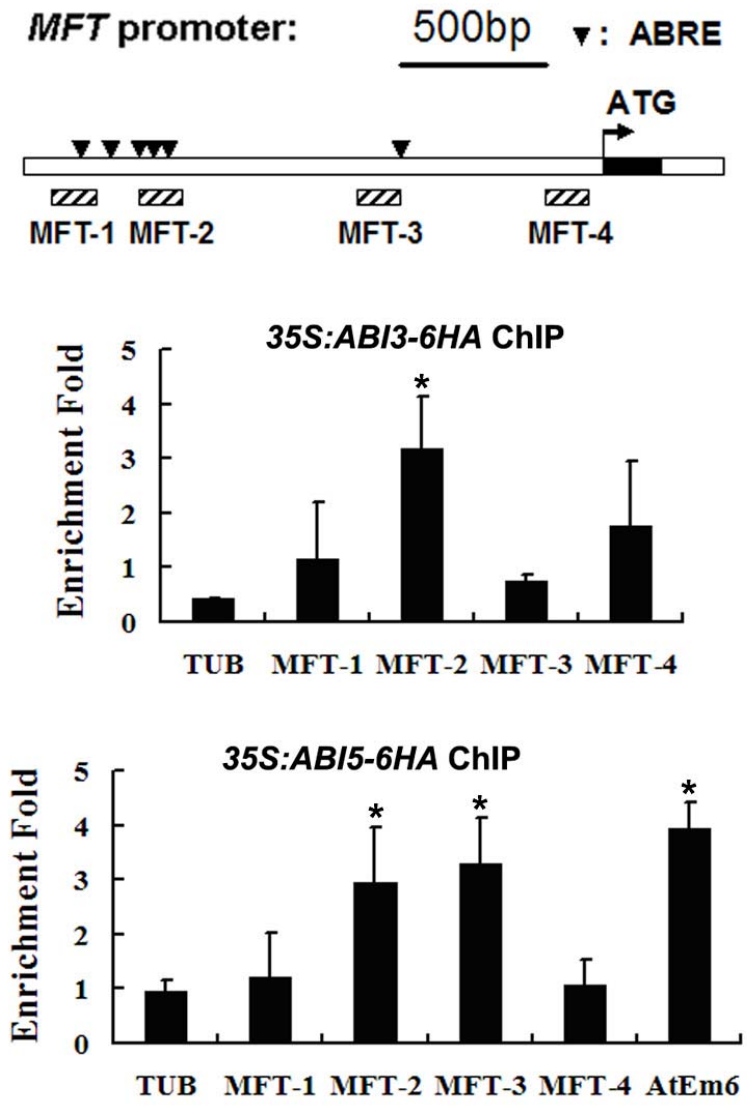

Figure 21. ChIP Enrichment Test Showing the Binding of ABI3-6HA and ABI5-6HA to the *MFT* Promoter.

The upstream region and the first intron of *MFT* are represented by white boxes, while the first exon is represented by a black box. The arrowheads in the upper panel indicate the sites containing putative ABREs on the *MFT* promoter. Hatched boxes represent the DNA fragments amplified in ChIP assays. ChIP assay results of *35S:ABI3-6HA* and *35S:ABI5-6HA* are shown in the lower panels. Seeds were sown on MS medium supplemented with 10 μM ABA and harvested 16 h post-stratification for ChIP assays. *AtEm6*, which has been identified as a direct target of ABI5 (Lopez-Molina et al., 2001; Lopez-Molina et al., 2002), is used as a positive control for ABI5-6HA ChIP assay. Significant differences in comparison with the enrichment of a *TUB2* fragment are indicated with asterisks ($P < 0.05$, Student's *t* test).

3.4 A G-box Motif Mediates Spatial Regulation of *MFT* in Response to ABA

To further understand how ABA regulates *MFT* through *ABI3* and *ABI5* during seed germination, we generated two versions of *MFT*:*GUS* constructs, *MFT(P2)-GUS* and *MFT(P6)-GUS*, in which 1.8 kb and 900 bp promoter sequences with 6 and 1 ABREs upstream of the translational start site, respectively, were fused with the *GUS* reporter gene (Figure 22, upper panel). The *MFT* coding sequence driven by the 1.8 kb promoter was able to rescue the low germination phenotype of *mft*-2 in response to ABA (Figure 23A), implying that this promoter region contains essential *cis*-elements required for the regulation of *MFT* expression by ABA. The *MFT* coding sequence driven by the 900 bp promoter could partially rescue the low germination phenotype of *mft*-2 in response to ABA (Figure 23B), suggesting that this promoter region also functions to certain extent. For both *MFT(P2)-GUS* and *MFT(P6)-GUS*, moderate GUS signals were detected in embryos without ABA treatment (Figure 24). In agreement with *MFT* gene expression profiles (Figures 11 and 13), when exogenous ABA was applied, GUS staining in these two reporter lines was enhanced throughout the embryos, especially in the radicle-hypocotyl transition zone (Figure 24). Since the sensitivity of GUS staining is higher than that of in situ hybridization, thus, ABA upregulates *MFT* in the whole embryo particularily in the radicle-hypocotyl transition zone. While GUS staining was also

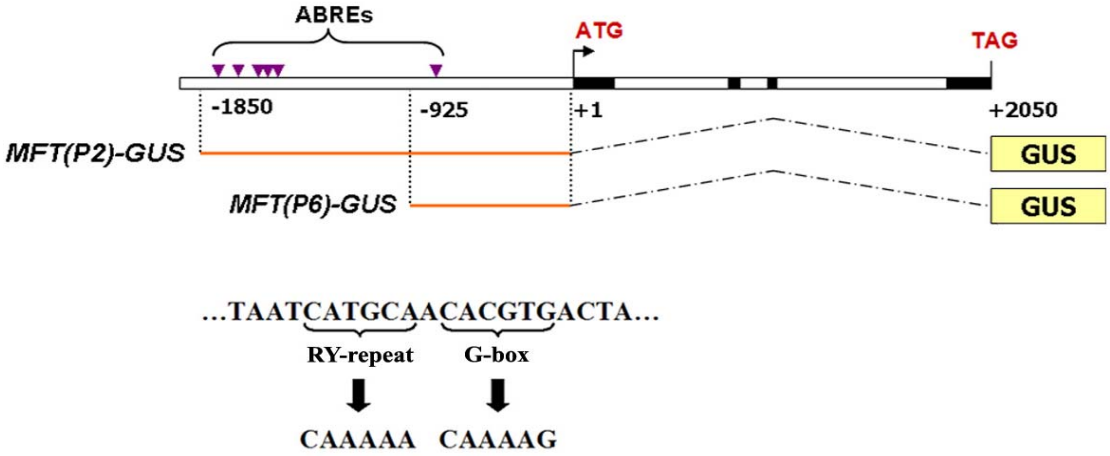

Figure 22. Schematic Diagram of *MFT(P2)-GUS* and *MFT(P6)-GUS* Constructs.

The upper panel shows the constructs in which 5' upstream sequences of *MFT* containing ABREs were transcriptionally fused with the *GUS* gene (left panel). The lower panel shows the mutagenesis of the RY-repeat and the G-box in the ABRE that is located 700 bp upstream of the ATG start codon.

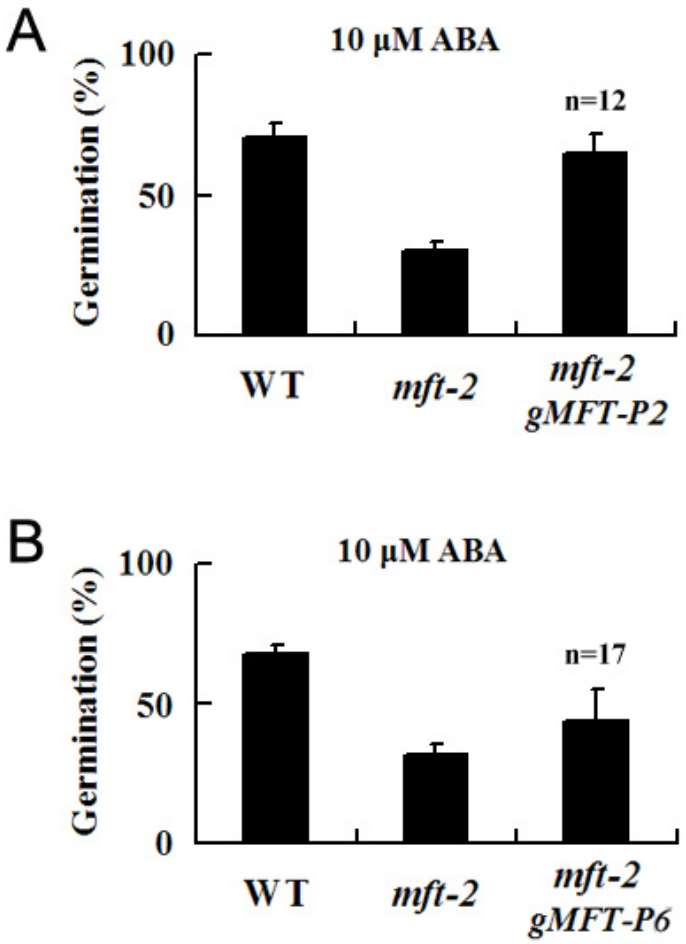

Figure 23. Complementation of *mft-2* by Two *MFT* Genomic Fragments *gMFT-P2* and *gMFT-P6*.

(A) *gMFT-P2* comprises of the 1.8 kb upstream sequence with 6 ABREs (Fig. 4A) and the 2.1 kb coding sequence plus introns rescues the low germination phenotype of *mft*-2 in response to 10 µM ABA.
(B) *gMFT-P6* comprises of the 900 bp upstream sequence with 1 ABRE (Fig. 4A) and the 2.1 kb coding sequence plus introns only partially rescues the low germination phenotype of *mft*-2 in response to 10 µM ABA.
The germination percentage was scored 7 day post-stratification. The number of independent transgenic lines tested for statistical analysis was indicated.

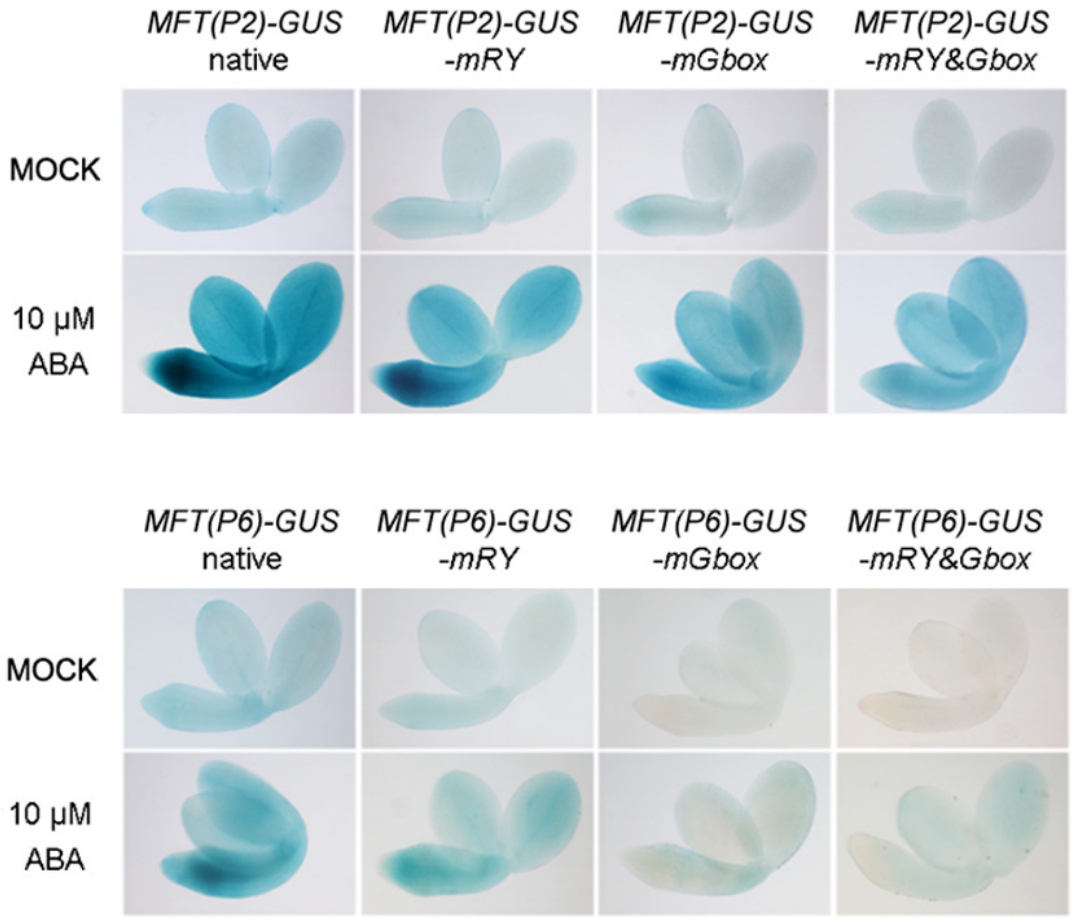

Figure 24. **GUS Staining in Germinating Seeds of *MFT-GUS* Transgenic Plants.**

GUS staining in germinating seeds of the transformants containing *MFT(P2)-GUS*, *MFT(P6)-GUS* and their derived constructs with the mutated RY-repeat (mRY) and G-box motif (mGbox). Seeds from T3 homozygous plants with a single insertion of the transgene for each construct were analyzed and representative images are shown. Germinating seeds mock-treated or treated with 10 µM ABA, which were at the same developmental stage, were stained 12 h or 24 h post-stratification, respectively.

observed in the seed coat with the endosperm, it was not affected by ABA.

Although *MFT(P6)-GUS* showed weaker staining in whole embryos than *MFT(P2)-GUS* in response to ABA, increased GUS staining was specifically observable in the radicle-hypocotyl transition zone of *MFT(P6)-GUS* (Figure 24). This implies that the 5 clustered ABREs at about 1.7 kb upstream of the start codon may modulate the extent of *MFT* upregulation throughout the embryo, while the single ABRE close to the start codon may confer the spatial upregulation of *MFT* in the radicle-hypocotyl transition zone in response to ABA. To test this hypothesis, we further mutagenized the single ABRE in both *MFT(P2)-GUS* and *MFT(P6)-GUS* to evaluate the function of this single ABRE in response to ABA (Figure 22, lower panel). Sequence analysis revealed that this ABRE contains one RY repeat that can be recognized by ABI3 and one G-box that can be recognized by both ABI3 and ABI5 (Figure 22, lower panel) (Kim et al., 1997; Ezcurra et al., 2000). Our results demonstrated that mutation of the RY motif had no obvious effect on the GUS staining of either *MFT(P2)-GUS* or *MFT(P6)-GUS* in response to ABA, whereas mutation of the G-box motif notably attenuated the responses to ABA, for both *MFT(P2)-GUS* and *MFT(P6)-GUS* (Figure 24). In particular, upregulation of GUS staining in the radicle-hypocotyl transition zone was significantly abolished when the G-box motif was mutated (Figure 24). Therefore, the G-box in this single ABRE is essential for upregulating *MFT* expression particularly in the radicle-hypocotyl transition zone in response to ABA.

3.5 *MFT* Is Promoted by ABI5 but Suppressed by ABI3

As ChIP assays demonstrated that ABI3 and ABI5 physically bound to the *MFT* promoter, we crossed *MFT:GUS* lines into the plants with overexpression or loss of function of *ABI3* and *ABI5* to monitor how they control *MFT* expression in response to ABA during seed germination. Since the germination of *35S:ABI3* and *35S:ABI5* seeds was hypersensitive to low ABA concentrations (Figure 19) (Lopez-Molina et al., 2001; Zhang et al., 2005), GUS staining of *MFT:GUS* lines in these backgrounds was examined under the treatment of 1 or 3 μM ABA.

The staining results clearly demonstrated that *MFT(P2)-GUS* exhibited enhanced staining specifically in the radicle-hypocotyl transition zone in the background of *35S:ABI5-6HA* in response to ABA, while mutation of the G-box in *MFT(P2)-GUS* did not show any change of GUS staining in the background of *35S:ABI5-6HA* in response to ABA (Figure 25A). This finding, together with the previous ChIP result showing the binding of ABI5 to the promoter region near MFT-3 (Figure 21), strongly suggests that ABI5 directly and specifically regulates *MFT* expression in the radicle-hypocotyl transition zone through the G-box motif in the single ABRE near the start codon of *MFT*. To further confirm this notion, *abi5-1* mutation was introduced into *MFT(P2)-GUS*, and it was observed that the resulting GUS staining was decreased in the radicle elongation zone compared with that in wild-type background in response to ABA (Figure 25B).

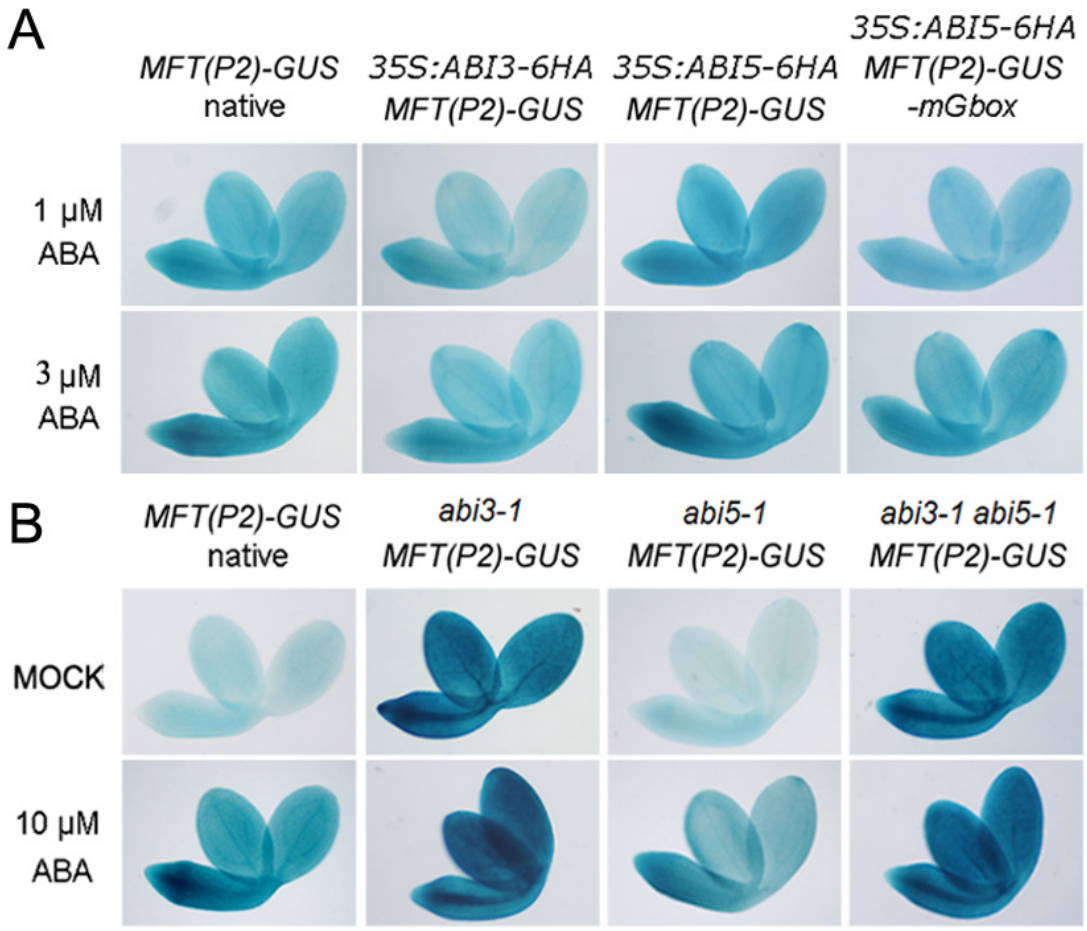

Figure 25. GUS Staining in Germinating Seeds of *MFT(P2)-GUS* in Different Genetic Background.

(A) GUS staining in germinating seeds of *MFT(P2)-GUS*, *35S:ABI3-6HA MFT(P2)-GUS*, *35S:ABI5-6HA MFT(P2)-GUS* and *35S:ABI5-6HA MFT(P2)-GUS-mGbox*. Germinating seeds treated with 1 and 3 µM ABA, which were at the same developmental stage, were stained 24 h post-stratification.
(B) GUS staining in germinating seeds of *MFT(P2)-GUS*, *abi3-1 MFT(P2)-GUS*, *abi5-1 MFT(P2)-GUS* and *abi3-1 abi5-1 MFT(P2)-GUS*. To examine GUS expression in seeds at the same developmental stage, *MFT(P2)-GUS* and *abi5-1 MFT(P2)-GUS* treated with 10 µM ABA were stained 24 h post-stratification, while other germinating seeds mock-treated or treated with 10 µM ABA were stained 12 h post-stratification.

On the other hand, *MFT(P2)-GUS* showed slightly reduced GUS staining in the *35S:ABI3-6HA* background in response to ABA (Figure 25A), but exhibited an extraordinarily strong GUS staining in the whole embryo of *abi3-1* independently of ABA treatment (Figure 25B). Further loss of *ABI5* in *abi3-1* had little effect on the intensity of GUS staining in *MFT(P2)-GUS* (Figure 25B). These GUS staining patterns are in agreement with the *MFT* expression in the corresponding transgenic plants or mutants (Figures 18 and 20), suggesting that ABI3 plays a dominant role in suppressing *MFT* expression in the whole embryo, while ABI5 recognizes the G-box motif to promote *MFT* expression particularly in the radicle-hypocotyl transition zone.

3.6 *MFT* Is Regulated by DELLA Proteins

Since GA functions as a major counteracting hormone against ABA to promote both embryo growth potential and endosperm weakening during seed germination (Ogawa et al., 2003; Muller et al., 2006), we asked whether *MFT* is involved in the crosstalk of ABA and GA signaling. We firstly examined if GA could rescue the low germination rate of *mft-2* caused by ABA. In the presence of 10 μM ABA, increasing the concentration of exogenously applied GA from 1 to 10 μM clearly elevated the germination rate of wild-type seeds, but had little effect on the germination of *mft-2* (Figure 26), implying that *MFT* plays a role in mediating the

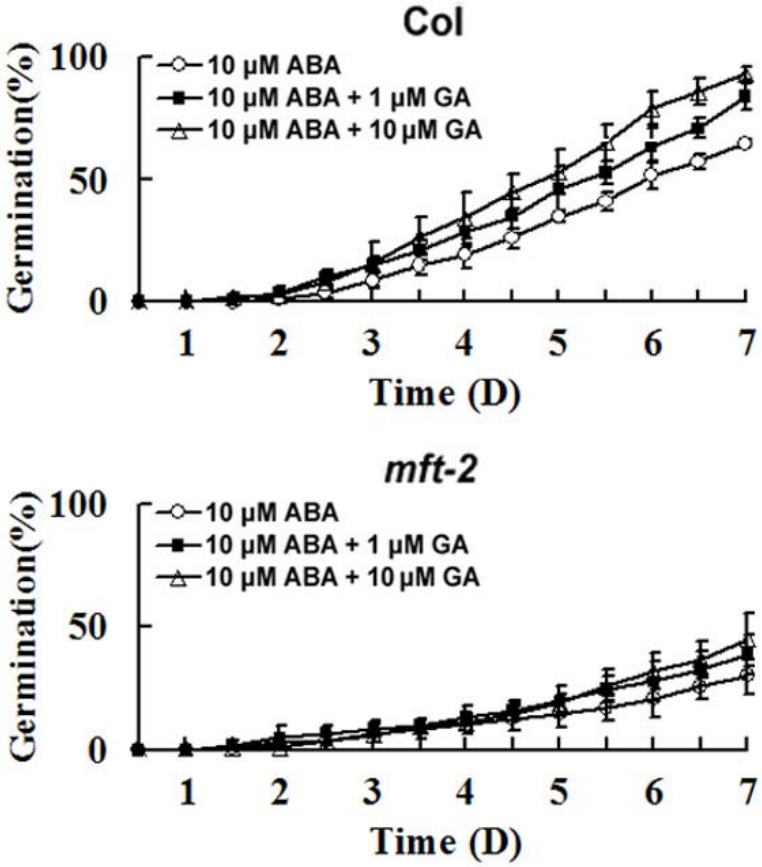

Figure 26. Germination Rate of *mft-2* in Response to ABA and GA.

Germination phenotype of wild-type and *mft-2* treated with 10 μM ABA plus different concentrations (1 μM and 10 μM) of GA.

interaction between ABA and GA signals during seed germination. We next examined *MFT* expression in the GA-deficient *ga1-3* mutant, in which GA biosynthesis is blocked (Wilson et al., 1992). It was shown that *MFT* was highly expressed in *ga1-3* seeds, and exogenously applied GA downregulated *MFT* (Figure 27A), suggesting that GA represses *MFT* expression in seeds. As it is known that DELLA proteins are a major family of growth-restricting nuclear proteins mediating the GA effect on growth, and RGA and RGL2 are predominant DELLA proteins involved in the control of seed germination (Lee et al., 2002; Tyler et al., 2004), we thereafter tested if DELLA proteins mediate GA regulation of *MFT* by checking *MFT* expression in various *DELLA* mutants in the *ga1-3* background. The results showed that further loss of RGL2 or RGA activity noticeably reduced *MFT* expression in *ga1-3* seeds with a stronger effect exerted by RGL2 (Figure 27B). In *ga1-3 rga-t2 rgl2-1* triple mutants, *MFT* expression was much reduced (Figure 27B). Further loss of the activity of other two DELLA proteins, GAI and RGL1, reduced *MFT* expression to a level comparable to that in wild-type (Figure 27B). GA treatment that resulted in the degradation of DELLA proteins considerably downregulated *MFT* expression in almost all the mutants tested except the penta mutant *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* (Figure 27B). Taken together, these results suggest that all DELLA proteins tested contribute to the upregulation of *MFT* in *ga1-3* in which GA is absent, and that RGL2 and RGA are two major regulators controlling *MFT* expression with the former as the most important regulator.

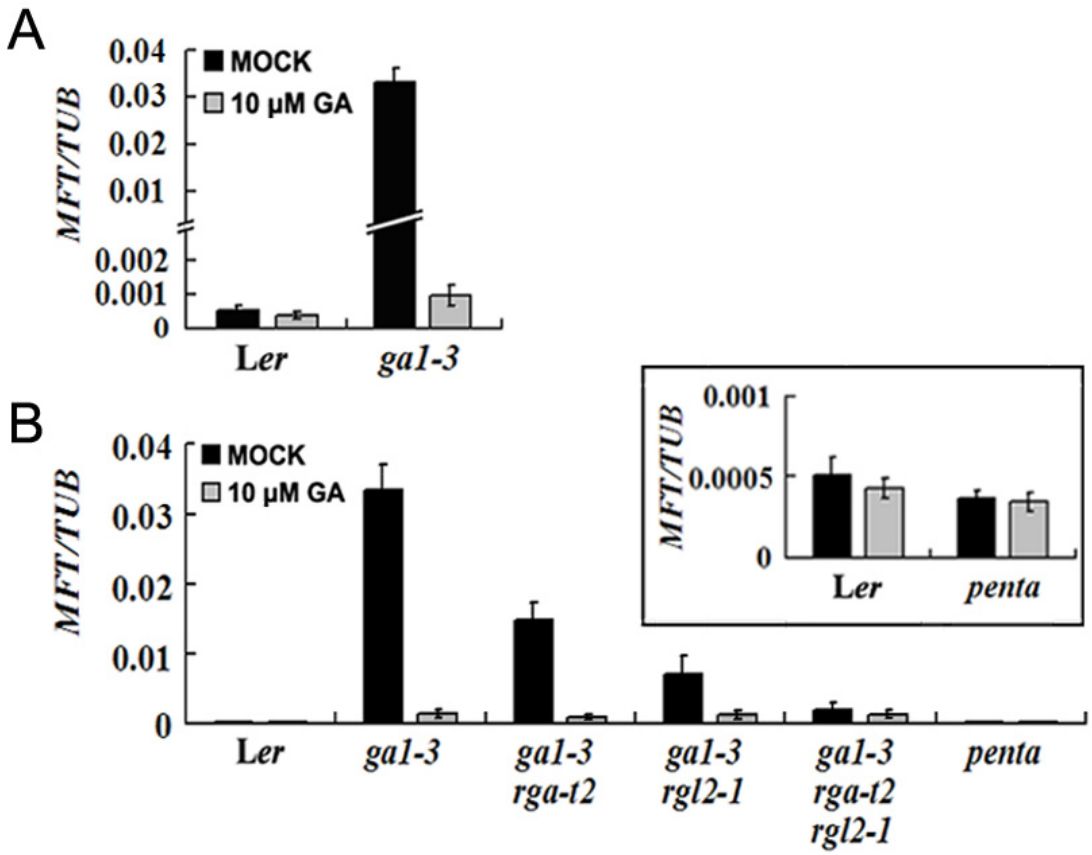

Figure 27. Expression of *MFT* in Various *DELLA* Mutants.

(A) *MFT* expression in wild-type and *ga1-3* seeds mock-treated or treated with 10 µM GA. All germinating seeds were collected 16 h post-stratification.
(B) *MFT* expression in wild-type and various *DELLA* mutant seeds in *ga1-3* background mock-treated or treated with 10 µM GA. All germinating seeds were collected 16 h post-stratification. *penta* indicates the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant. Inset shows the comparison of *MFT* expression between wild-type and *penta* mutants.

To examine if DELLA proteins directly modulate *MFT* transcription, we created *ga1-3 rgl2-1 rga-t2 35S:RGL2-GR* inducible lines, in which glucocorticoid receptor (GR) fusion protein could be activated by its ligand dexamethasone (DEX). Application of DEX delayed the germination rate of *ga1-3 rgl2-1 rga-t2 35S:RGL2-GR* (Figure 28A), suggesting that RGL2-GR is biologically functional in inhibiting seed germination. Using this system, we checked *MFT* expression by inducing RGL2 activity. Combined treatment of *ga1-3 rgl2-1 rga-t2 35S:RGL2-GR* by DEX and cycloheximide (CYC), an inhibitor of protein synthesis, resulted in an increase in *MFT* expression. This demonstrates that RGL2 modulates *MFT* expression independently of protein synthesis, indicating that *MFT* may be an immediate target of RGL2 (Figure 28B).

Since it was shown that RGL2 may directly regulate *MFT* expression, we further tested whether RGL2 could be associated with the *MFT* promoter. To this end, we created *35S:RGL2-6HA* tagging lines for ChIP analysis. *35S:RGL2-6HA* was introduced into *ga1-3 rgl2-1* background, and the functional lines that mimicked *ga1-3* phenotypes were chosen for further ChIP assays. ChIP enrichment test showed that RGL2-6HA was associated with the region near the MFT-2 fragment (Figure 29), indicating that RGL2 is directly involved in the regulation of *MFT* expression.

In addition, we found that both *ABI3* and *ABI5* expression was elevated in *ga1-3*

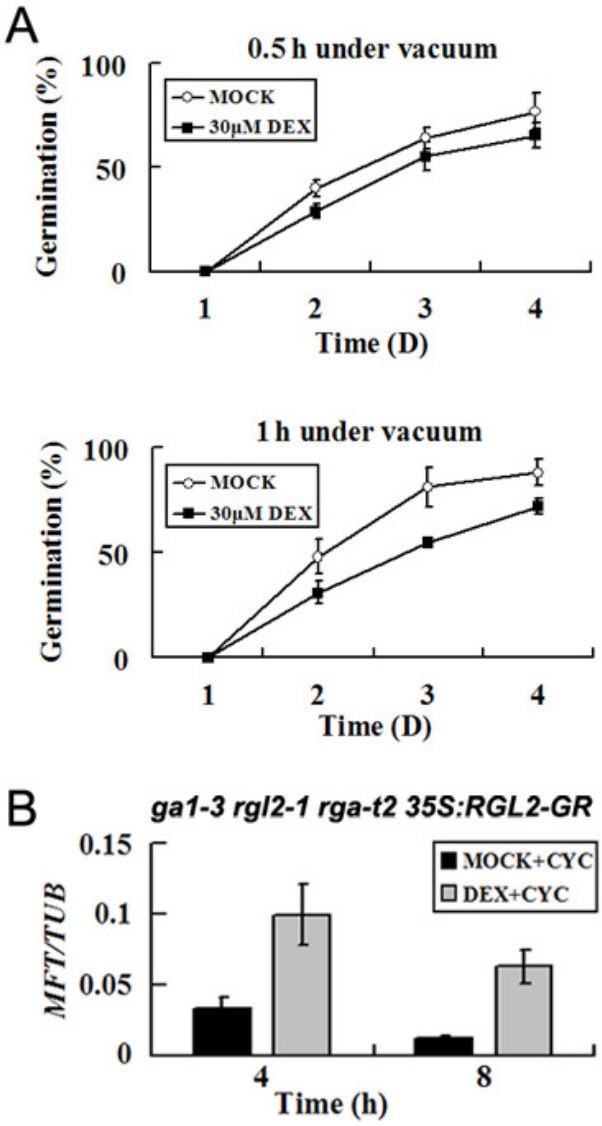

Figure 28. A Biologically Active RGL2-GR Fusion.

(A) Surface-sterilized *ga1-3 rgl2-1 rga-t2 35S:RGL2-GR* seeds were subject to vacuum infiltration in 30 μM dexamethasone (DEX) solution or MOCK solution (0.09% ethanol) for 0.5 h or 1 h. Seeds were subsequently washed three times and sown on common MS medium. The plates were transferred to a tissue culture room and germination percentage was scored over time. Mock-treated seeds show higher germination rate than DEX-treated seeds, suggesting that RGL2-GR is biologically functional in inhibiting seed germination.
(B) *MFT* expression in *ga1-3 rgl2-1 rga-t2 35S:RGL2-GR* seeds. Seeds were treated with 30 μM dexamethasone (DEX) plus 30 μM cycloheximide (CYC) or MOCK (0.09% ethanol) plus CYC under vacuum for 1 h. They were subsequently washed three times, and collected 4 h and 8 h after sowing on MS medium.

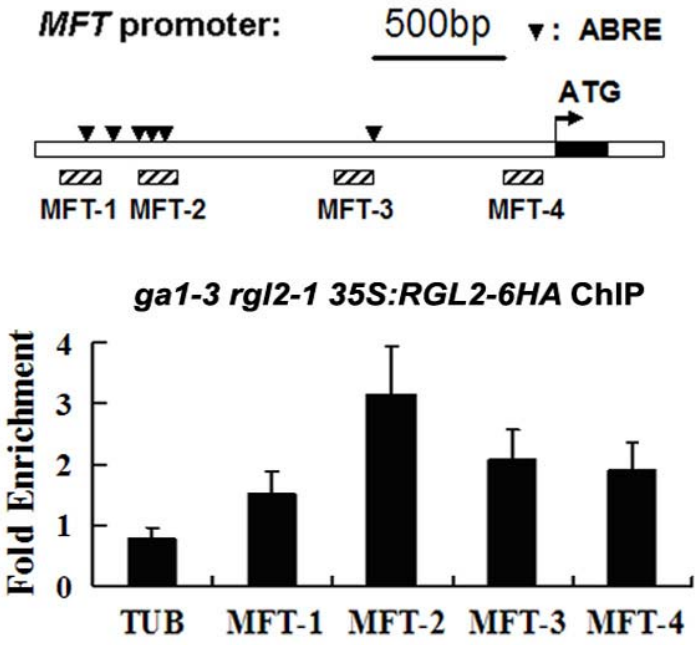

Figure 29. ChIP Enrichment Test Showing the Binding of RGL2-6HA to the *MFT* Promoter.

Seeds were sown on MS medium and harvested 16 h post-stratification for ChIP assays. A significant difference in comparison with the enrichment of a *TUB2* fragment is indicated with an asterisk ($P < 0.05$, Student's *t* test).

seeds, and that their expression levels in *ga1-3* lacking DELLA proteins (particularly RGL2 and RGA) decreased to the same levels as in wild-type seeds (Figure 30). GA treatment of these mutant seeds downregulated *ABI3* and *ABI5* expression to the levels comparable to those of wild-type seeds (Figure 30). These results suggest that DELLA proteins promote ABA signaling, which is consistent with the previous findings showing that DELLA proteins stimulate endogenous ABA synthesis (Ko et al., 2006; Zentella et al., 2007; Piskurewicz et al., 2008). Thus, it is likely that in addition to direct regulation of *MFT* expression, RGL2 also indirectly affects *MFT* expression through the ABA signaling pathway.

To understand the biological significance of the upregulation of *MFT* by DELLA proteins, we crossed *mft-2* in L*er* background with *ga1-3* to remove *MFT* activity. As *ga1-3* germinates only upon GA treatment, seed germination was examined in the presence of 1 μM GA. Under the same growth conditions, *ga1-3 mft-2* germinated at a lower rate than *ga1-3* in response to GA treatment, especially within 5 days after stratification (Figure 31A). When GA levels are low, DELLA proteins accumulate, thus concomitantly promoting *MFT* expression and ABA signaling. The lower germination rate in *ga1-3 mft-2* than in *ga1-3* indicates that when GA biosynthesis is impaired and ABA signaling is stimulated, *MFT* is required to maintain the seed germination potential. In agreement with this, *ga1-3 rgl2-1 mft-2* exhibited a lower germination rate than *ga1-3 rgl2-1* in the absence of exogenous GA (Figure 31B).

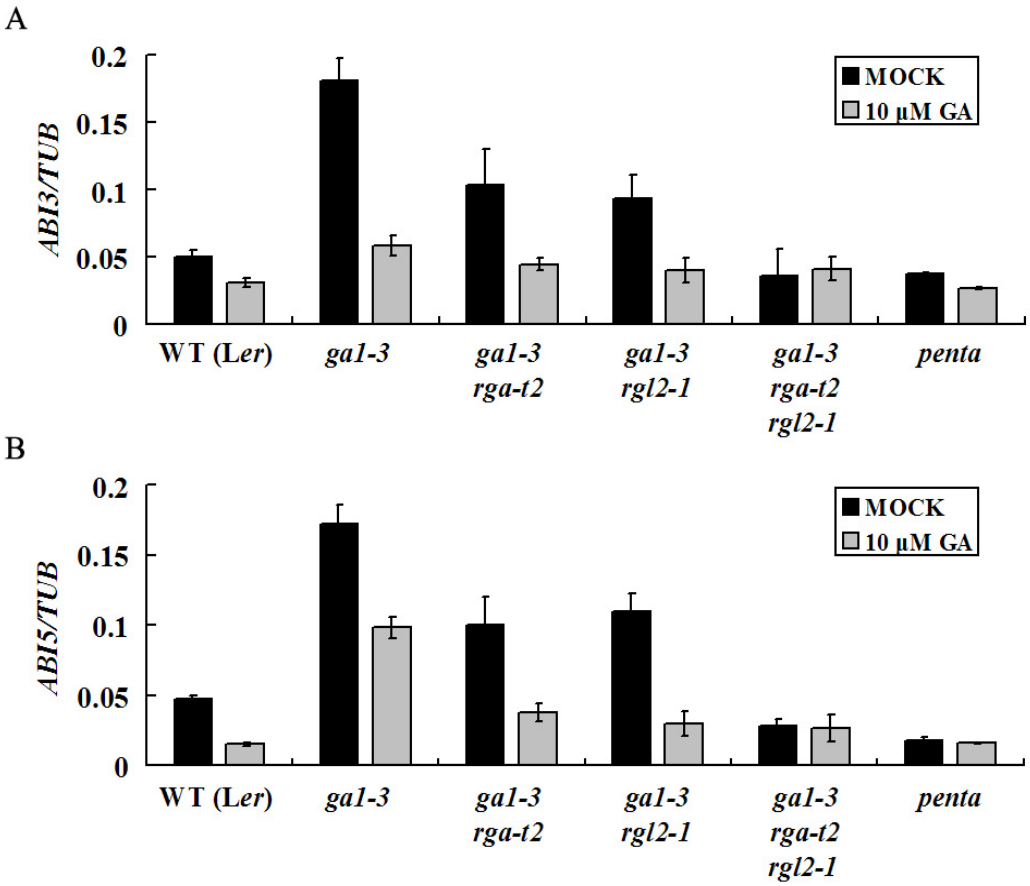

Figure 30. Expression of *ABI3* and *ABI5* in Various *DELLA* Mutants.

(A) *ABI3* expression in seeds of wild-type and *ga1-3* lacking DELLA proteins that were mock-treated or treated with 10 µM GA.
(B) *ABI5* expression in seeds of wild-type and *ga1-3* lacking DELLA proteins that were mock-treated or treated with 10 µM GA.
All germinating seeds were collected 16 h post-stratification. *penta* indicates the *ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1* mutant.

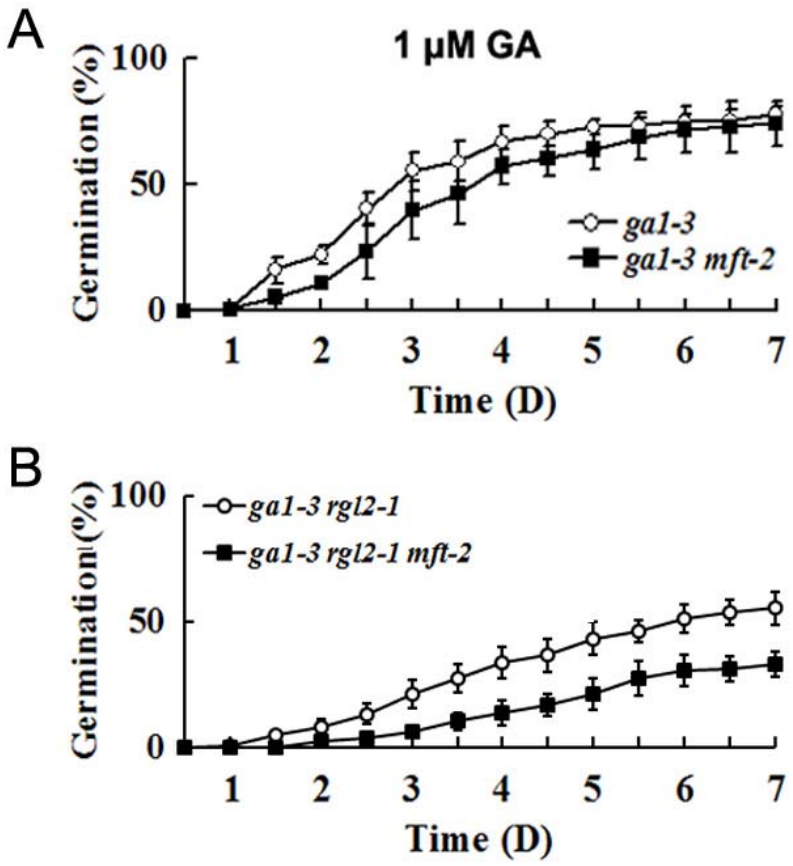

Figure 31. *MFT* Maintains the Germination Potential when GA Levels Are Low.

(A) Germination phenotype of *ga1-3* and *ga1-3 mft-2* treated with 1 μM GA.

(B) Germination phenotype of *ga1-3 rgl2-1* and *ga1-3 rgl2-1 mft-2* in the absence of exogenous GA.

3.7 MFT Represses *ABI5* Expression during Seed Germination

To understand the mechanism by which *MFT* regulates seed germination, we first checked MFT intracellular localization by examining the localization of gMFT-GFP, in which the *MFT* coding sequence driven by the 1.8 kb promoter was fused with the GFP reporter gene. Among 16 *mft-2 gMFT-GFP* transgenic lines obtained, 10 lines could rescue the ABA hypersensitivity phenotype of *mft-2*, indicating that the MFT-GFP fusion protein is biologically functional (Figure 32A). Using the functional *gMFT-GFP* transgenic plants, we were able to visualize the localization of MFT by confocal microscopy. Although according to the sequence analysis, MFT is not designated as a transcription factor, we found that MFT-GFP signals in the cells of the radicle-hypocotyl transition zone were located in the nucleus, implying that MFT may function as a transcription co-regulator that modulates the expression of downstream genes (Figure 32B). The similar nuclear localization has also been observed in another MFT homolog, FT, which functions in the nucleus to regulate the expression of other flowering genes (Abe et al., 2005; Wigge et al., 2005).

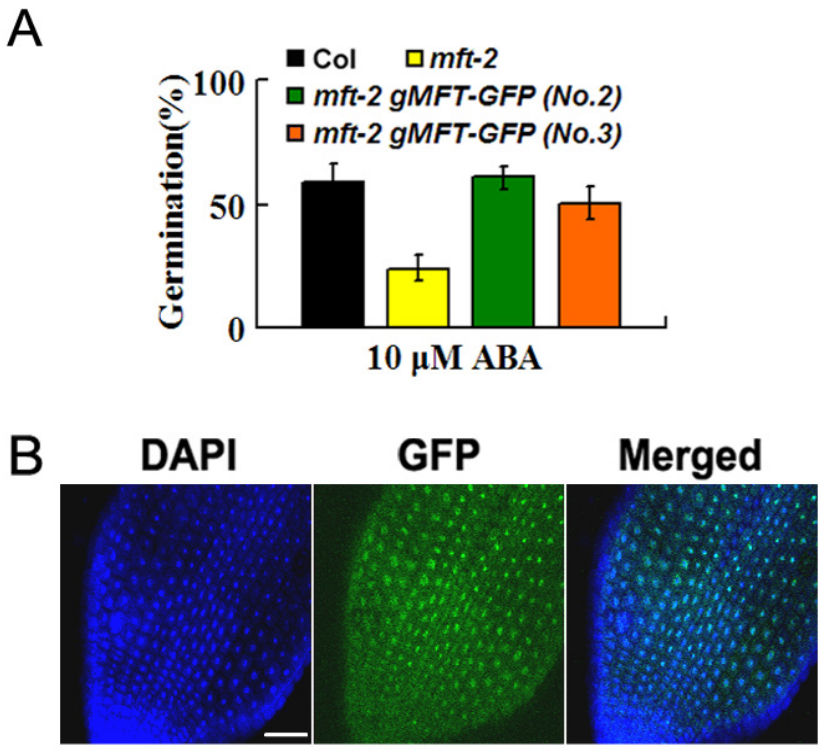

Figure 32. MFT Is Localized in the Nucleus.

(A) Two representative *mft-2 gMFT-GFP* transgenic lines showing rescued germination phenotype in response to 10 µM ABA. The percentage of germination was scored 7 day post-stratification.

(B) MFT-GFP localization in the cells of the radicle-hypocotyl transition zone. DAPI, fluorescence of 4',6-diamino-2-phenylindol; Merged, merge of DAPI and GFP. Bar = 50 µm.

To evaluate how *MFT* affects the expression of other genes during seed germination, we tested a group of ABA marker genes and found that the expression of *RD29A, RD29B*, and *AtEm6* was higher in *mft-2* than in wild-type in response to ABA (Figure 33). Since *AtEm6* is a known direct downstream target of *ABI5* (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2002) and *ABI5* expression in the radicle overlaps with *MFT* expression in response to ABA (Piskurewicz et al., 2008) (Figure 13), we asked if *MFT* regulates *ABI5*, thus affecting *AtEm6* expression. We found that in germinating seeds, *ABI5* expression in *mft-2* was higher than in wild-type in response to exogenous ABA (Figure 34A). Furthermore, in situ hybridization revealed higher *ABI5* expression levels in the radicle of *mft-2* than in wild-type (Figure 34B). These suggest that *MFT* negatively regulates *ABI5* in the radicle of germinating seeds in response to ABA.

To test whether MFT is directly associated with the *ABI5* promoter, we created *mft-2 gMFT-HA* transgenic plants that fully rescued the *mft-2* phenotype (data not shown). ChIP assays showed the enrichment of MFT-HA at the promoter region near the ABI5-2 fragment (Figure 35), whereas no binding was observed on the *ABI3* promoter (Figure 36), suggesting that *MFT* antagonizes ABA signaling by directly repressing *ABI5*. This was supported by the expression analysis showing that *ABI3* upregulation of *ABI5* in *35S:ABI3-6HA* was enhanced in *mft-2* in response to ABA (Figure 37). Furthermore, *abi5-1 mft-2* double mutants could fully rescue the low germination defects of *mft-2* in response to ABA (Figure 38).

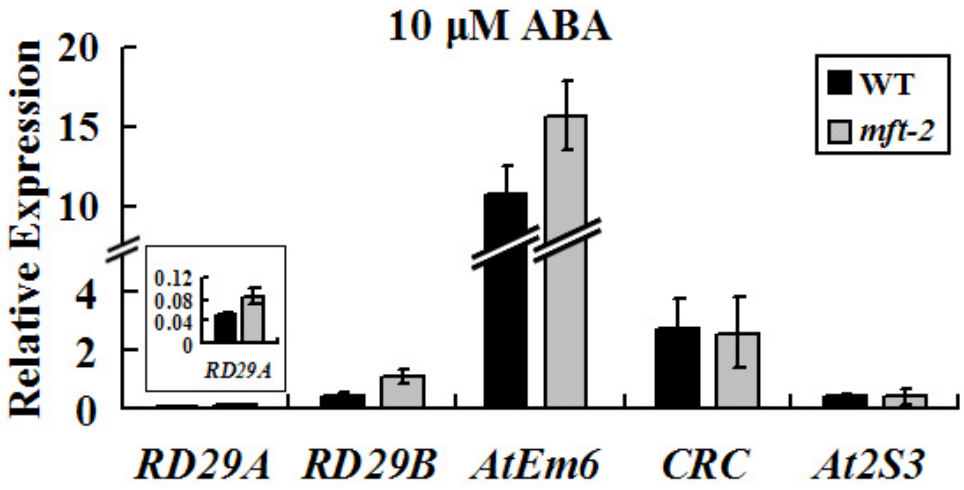

Figure 33. Expression of Several ABA Marker Genes in Wild-type and *mft-2* in Response to ABA.

Expression levels of *RD29A*, *RD29B*, *AtEm6*, *CRC*, and *At2S3* were compared in wild-type and *mft-2* germinating seeds treated with 10 µM ABA. Inset at left corner shows the *RD29A* expression. The expression levels were normalized to *TUB2*.

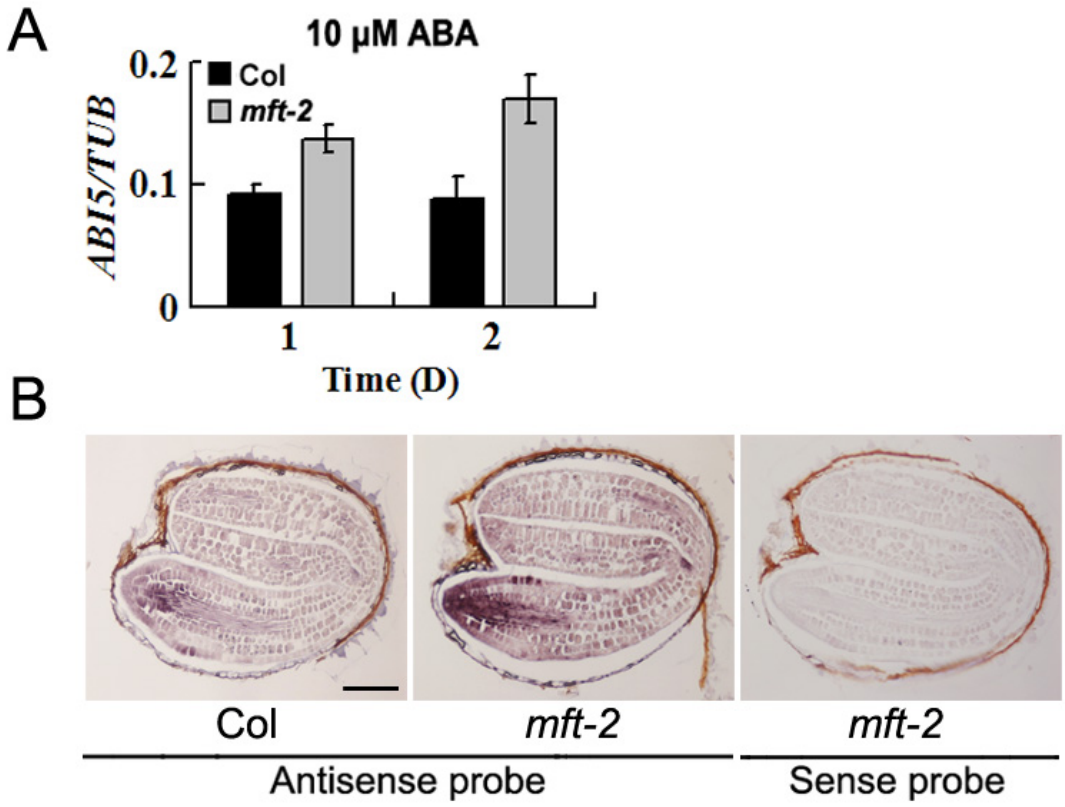

Figure 34. MFT Suppresses *ABI5* Expression in Response to ABA.

(A) Expression of *ABI5* in wild-type and *mft-2* seeds treated with 10 µM ABA at 1 day or 2 day post-stratification.
(B) In situ localization of *ABI5* in wild-type and *mft-2* seeds treated with 10 µM ABA. Seeds were collected 24 h post-stratification. Bar = 100 µm.

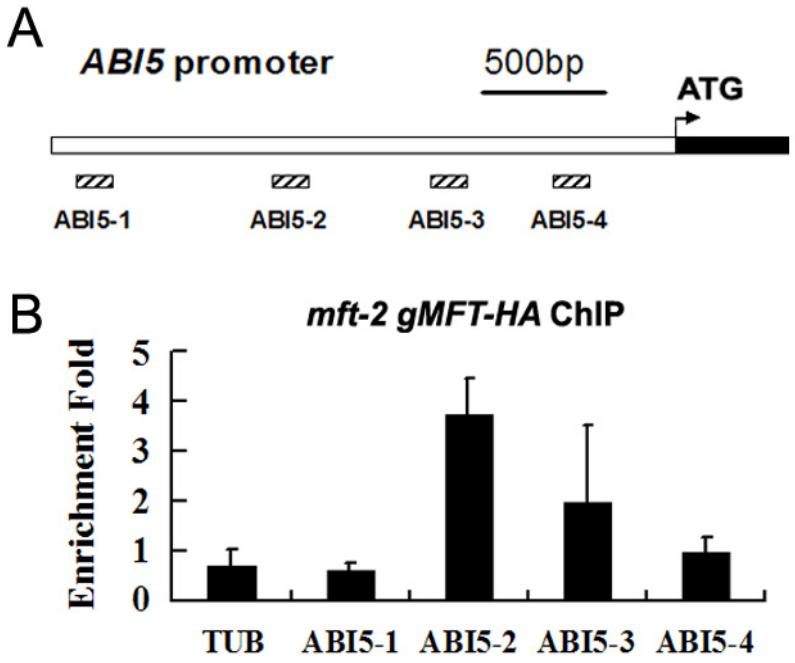

Figure 35. ChIP Enrichment Test Showing the Binding of MFT-HA to the *ABI5* Promoter.

(A) Schematic diagram of the *ABI5* promoter region. White and black boxes represent the upstream region and part of the first exon, respectively. Hatched boxes represent the DNA fragments amplified in ChIP assays
(B) ChIP enrichment test showing the binding of MFT-HA to the *ABI5* promoter. Seeds were sown on MS medium supplemented with 10 µM ABA and harvested 24 h post-stratification for ChIP assays. A significant difference in comparison with the enrichment of a *TUB2* fragment is indicated with an asterisk ($P < 0.05$, Student's *t* test).

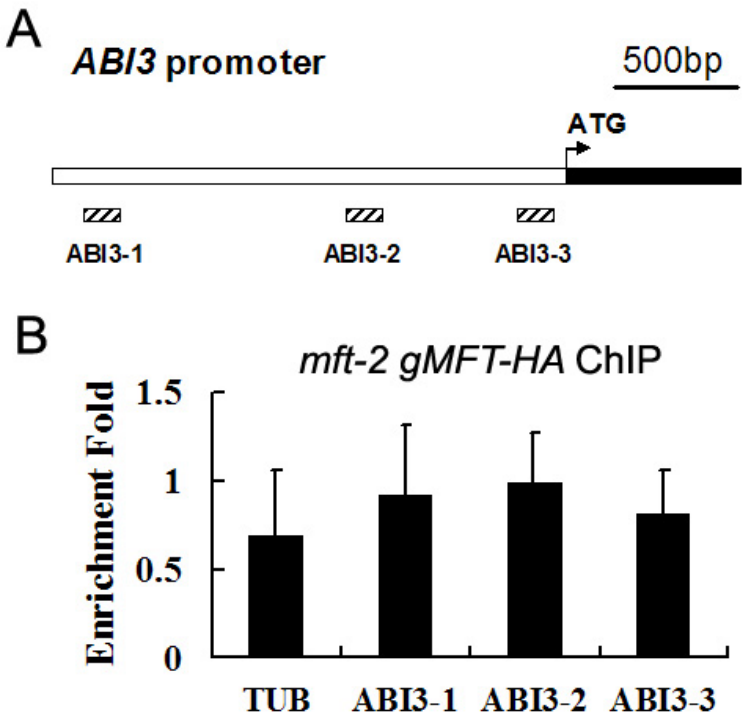

Figure 36. *ABI3* Promoter Is Not Directly Bound by MFT-HA.

(A) Schematic diagram of the *ABI3* promoter region. White and black boxes represent the upstream region and part of the first exon, respectively. The hatched boxes represent the DNA fragments amplified in the ChIP assay.
(B) ChIP assay shows no binding of MFT-HA on the *ABI3* promoter.

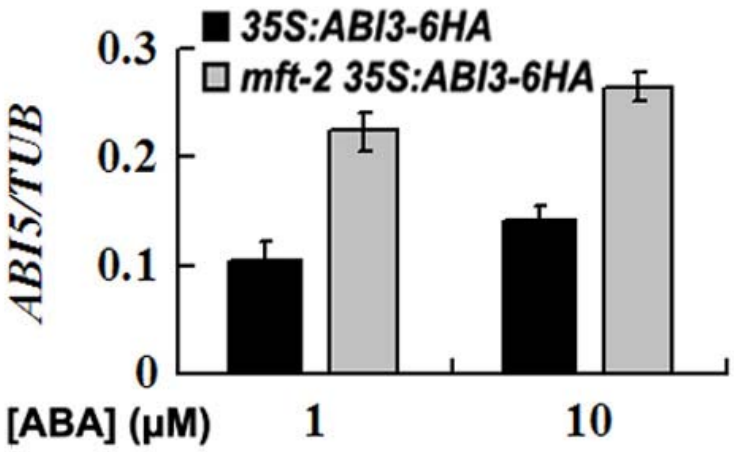

Figure 37. *ABI5* Expression in Germinating Seeds of *35S:ABI3-6HA* and *mft-2 35S:ABI3-6HA*.

Germinating seeds were treated with ABA (1 and 10 μM) and collected 24 h post-stratification.

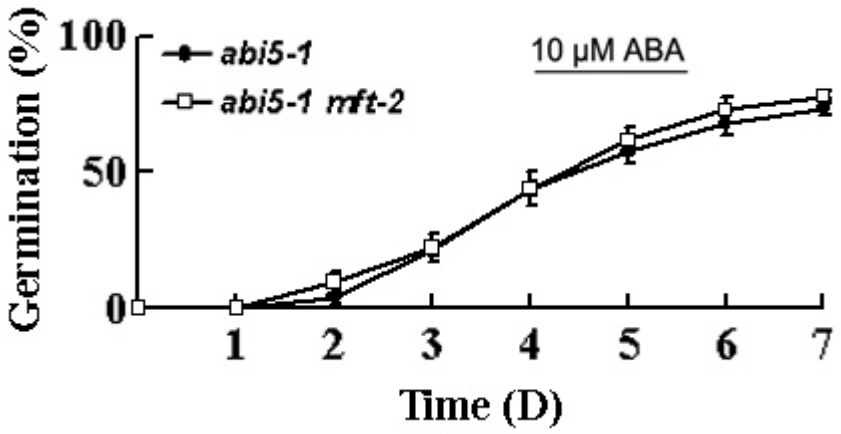

Figure 38. Germination Rate of *mft-2* and *abi5-1 mft-2* Mutants in Response to ABA.

Germination phenotype of *mft-2* and *abi5-1 mft-2* in response to 10 μM ABA.

CHAPTER 4

DISCUSSION

The primary objective of this research study was to characterize the role of *MFT* during plant growth and development in *Arabidopsis*. *MOTHER OF FT AND TFL1 (MFT)* is named after its sequence homology with two important flowering time genes, *FT* and *TFL1* (Bradley et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999). But loss of *MFT* function does not result in observable defects in flowering time (Yoo et al., 2004), indicating that *MFT* might be involved in other biological processes. Dedicated to investigating the exact function of *MFT*, our study is the first to report that *MFT* regulates seed germination in response to ABA.

4.1 *MFT* Expression Is Mediated by ABA and GA Signaling Pathways

Seed germination starts with the uptake of water by the dry seed and terminates with the visible penetration of the structures surrounding the embryo by the tip of the embryonic axis, i.e. radicle (Bewley, 1997). This process is governed by two major counteracting phytohormones, ABA and GA, in response to various environmental factors. As these two hormones act through a complex crosstalk rather than through independent pathways (Kucera et al., 2005), integration of their mutual interaction is critical for a plant to make a decision on whether it should initiate the vegetative growth of its life cycle. The DELLA protein RGL2 and ABA biosynthesis are two known factors involved in such integration, in which they promote each other via a positive feedback regulatory loop (Piskurewicz et al.,

2008). In this study, we show that *MFT* responds to ABA and GA signaling to modulate *ABI5* expression in the radicle of the embryo, thus specifically regulating the timing of seed germination in response to environmental factors (Figure 39).

Several pieces of evidence suggest that *MFT* serves as a convergence point of ABA and GA signaling pathways during seed germination. First, ABI5, which represses seed germination in response to ABA and GA (Piskurewicz et al., 2008), directly upregulates *MFT* in the radicle-hypocotyl transition zone of the embryo. In the absence of *ABI5*, upregulation of *MFT* in response to ABA is abolished, while overexpression of *ABI5* increased *MFT* expression. ABI5 binds to the *MFT* promoter region close to the start codon, where there is a single ABRE. Mutation of the G-box in this ABRE abolishes the upregulation of *MFT* expression in the radicle-hypocotyl transition zone by increased ABI5 activity. These results strongly suggest that ABI5 specifically regulates *MFT* expression in the radicle-hypocotyl transition zone through the G-box in the ABRE near the start codon. It is noteworthy that in *abi5-1*, GUS staining of *MFT(P2)-GUS* in the whole embryo still increased in response to ABA (Figure 25B). This is consistent with the expression analysis showing slightly upregulated *MFT* expression in *abi5-1* in response to ABA (Figure 18), implying that some other factor(s) act concomitantly with ABI5 to upregulate *MFT* in the ABA pathway. In addition to ABI5, there are other four bZIP ABRE-Binding Factors (ABFs) that recognize the G-box motif (Choi et al., 2000). Among them, ABF3 has

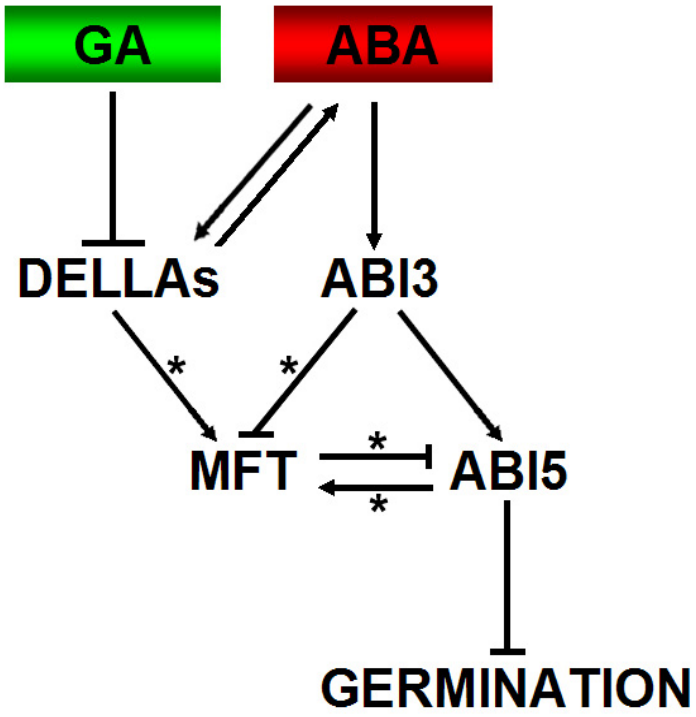

Figure 39. A Proposed Model of Seed Germination Mediated by *MFT*.

ABA regulates *MFT* expression via *ABI3* and *ABI5* with the former acting as a repressor and the latter as a promoter. *MFT* confers a negative feedback regulation of the ABA signaling pathway through directly repressing *ABI5*. On the other hand, GA downregulates *MFT* expression and inhibits ABA synthesis via DELLA proteins (i.e. RGL2). Therefore, *MFT* serves as a mediator in response to ABA and GA signals to promote seed germination through constituting a negative feedback regulation of ABA signaling. Asterisks represent direct transcriptional regulation.

been shown to function redundantly with ABI5 during seed germination (Finkelstein et al., 2005), and thus it might be another potential upstream regulator of *MFT*.

Second, ABI3, another key transcription factor in the ABA pathway, also directly represses *MFT*. *MFT* is downregulated in *35S:ABI3* germinating seeds treated with ABA which has been revealed by both expression analysis and GUS staining assay. Furthermore, ChIP results show that the genomic region near the MFT-2 fragment, where several ABREs are located, is associated with ABI3-6HA (Figure 21). Therefore, these data suggest that *ABI3* plays a role in directly repressing *MFT* in response to ABA during seed germination. Interestingly, *MFT* was found to be dramatically upregulated in the germinating seeds of *abi3-1* mutant even without the treatment of exogenous ABA (Figures 18 and 25B). It is known that ABI3 is an essential embryogenesis factor, and when the activity of ABI3 is impaired, it will cause global changes in transcription profiling during embryo development (McCourt, 1999). Thus, we speculate that such extraordinarily high expression of *MFT* in *abi3-1* may partly result from a failure in establishing seed maturation in *abi3-1*. In agreement with this idea, GUS staining demonstrates that *MFT* expression is particularly strong in the whole embryo of the germinating seeds of *abi3-1* (Figure 25B), which resembles its expression pattern in wild-type immature seeds (Figure 40F). Thus, the significantly elevated *MFT* expression in *abi3-1* may reflect ABI3 regulation of *MFT* expression in the underdeveloped embryo.

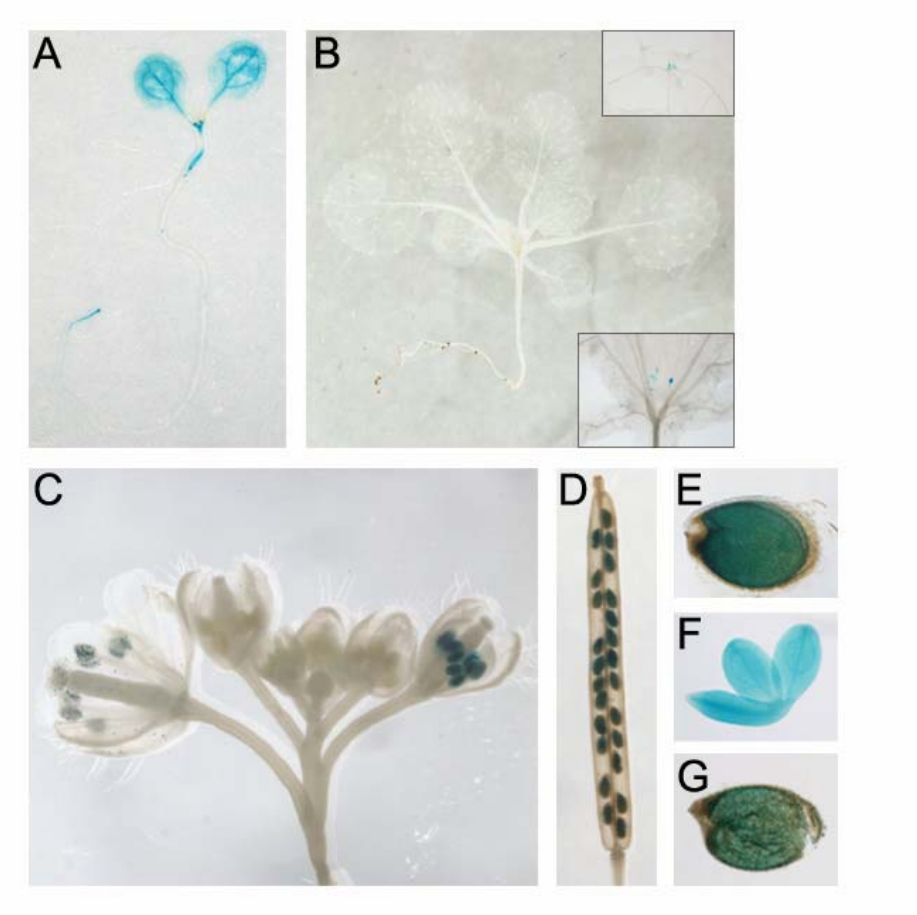

Figure 40. GUS Staining Pattern of *MFT(P2)-GUS* in Different Tissues.

(A) A 5-day-old seedling.
(B) A 2-week-old seedling. Insets show tiny GUS staining signals in the tip of a rosette leaf (upper) and the basal region of the seedling (lower).
(C) An inflorescence apex.
(D) A developing silique.
(E) A developing seed from an immature silique.
(F) and **(G)** An immature embryo (F) and a seed coat with the endosperm (G) dissected from a developing seed.

Third, our data suggest that among all the DELLA proteins involved in the regulation of *MFT*, RGL2 is the main one that directly modulates *MFT* expression. When GA levels are low, RGL2 promotes ABA biosynthesis through stimulating the expression of *XERICO*, which encodes a RING-H2 zinc finger factor (Ko et al., 2006; Zentella et al., 2007; Piskurewicz et al., 2008). In turn, an elevation of endogenous ABA levels promotes the mRNA expression of *RGL2* and *ABI5* and the protein expression and activity of ABI5 (Piskurewicz et al., 2008). Thus, direct upregulation of *MFT* by RGL2 allows *MFT* to respond to both GA and ABA pathways when they generate a signal output that does not permit germination under unfavorable environmental conditions.

4.2 Negative Feedback Regulation of *ABI5*

ABI5 has been identified as the final and common downstream repressor of seed germination in response to ABA and GA (Piskurewicz et al., 2008). The function of ABI5 is modulated by phosphorylation mediated by SnRK2-type kinases, and sumoylation mediated by SUMO E3 ligase (Lopez-Molina et al., 2001; Fujii et al., 2007; Miura et al., 2009). ABI5 prevents seed germination partly by activating a group of *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) genes, including *AtEm1* and *AtEm6* (Finkelstein and Lynch, 2000; Lopez-Molina et al., 2002), which encode hydrophilic proteins possibly required for desiccation tolerance (Vicient et al., 2000). So far several upstream regulators of *ABI5* have been reported. *ABI3*

function as an upstream promoter of *ABI5* to regulate the growth arrest of germinating seed (Lopez-Molina et al., 2002). Because *35S:ABI5* could rescue ABA insensitivity of *abi3* during germination, *ABI5* has been suggested as an essential factor acting downstream of *ABI3* and executing an ABA-dependent growth arrest (Lopez-Molina et al., 2002). Genetic analysis has also suggested that *ABF1* and *ABF3* participate in antagonizing *ABI5* expression (Finkelstein et al., 2005). In addition, HY5, a light-signaling mediator, directly activates *ABI5* to integrate light and ABA signaling (Chen et al., 2008).

Here we show that *MFT* responds to the signals from ABA and GA pathways and generates a negative feedback loop in the ABA pathway by directly repressing *ABI5* (Figure 39). When ABA levels are high, ABI5 directly promotes the expression of *MFT*, which in turn directly represses *ABI5* expression in the radicle of the embryo, thus maintaining the embryo growth potential even under high levels of ABA. Upon ABA treatment, *mft* mutants exhibit enhanced upregulation of *ABI5* in the radicle of the embryo, thus resulting in the ABA hypersensitive phenotype. As MFT protein does not contain any DNA-binding domain, there are likely to be other transcription factor(s) involved in guiding the MFT protein to the *ABI5* promoter. A bZIP transcription factor FD has been shown to interact with the MFT homolog, FT, and such protein complex participates in activating a floral homeotic gene *APETALA1* (*AP1*) (Abe et al., 2005; Wigge et al., 2005). However, our preliminary test did not show an interaction between FD and MFT (data not

shown), and the MFT-interacting partner(s) remain to be identified. To this end, yeast two-hybridization screening, in vitro pull-down assay can be carried out in the future. Among all the putative MFT protein partners identified, particular attention should be paid to those transcription factors which play a role in the process of seed dormancy and seed germination. To verify the interaction between MFT and those transcription factors of interest, bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (Co-IP) assays can be further utilized.

4.3 *MFT*-like Genes May Have Conserved Function in Plants

The PEBP family is evolutionarily conserved in a wide range of multi-cellular land plants, and phylogenetic studies of PEBP-like genes in angiosperms have divided them into three main subfamilies, *FT*-like, *TFL1*-like and *MFT*-like clades (Kobayashi et al., 1999; Carmel-Goren et al., 2003; Chardon and Damerval, 2005; Carmona et al., 2007; Hedman et al., 2009). A recent study on *MFT*-like genes in a basal plant lineage *Physcomitrella patens* (bryophyte) has suggested that the MFT-like clade is ancestral to the other two clades (Hedman et al., 2009). In *Arabidopsis*, three PEBP members, *FT*, *TFL1*, and *TWIN SISTER OF FT* (*TSF*), have been shown to regulate flowering time and shoot meristem identity (Bradley et al., 1997; Kardailsky et al., 1999; Kobayashi et al., 1999; Yamaguchi et al., 2005). Although *MFT* shares sequence similarity with *FT* and *TFL1*, its loss-of-function mutant in Ws-2 ecotype background does not exhibit relevant defects in flowering and

meristem development (Yoo et al., 2004), which is consistent with what we have observed in *mft-2* and *mft-3* in Col. A comparison of protein sequences has revealed a critical amino acid residue (tryptophan) in MFT that differs from tyrosine or histidine in FT or TFL1, respectively, in which this residue is located in a potential ligand binding pocket and determines the protein function as a flowering inducer or repressor (Hanzawa et al., 2005). Notably, this residue is well conserved in most *MFT*-like genes (Hedman et al., 2009). Furthermore, almost all MFT-like proteins share another conserved proline residue near the C-terminus, which is absent in FT-like or TFL1-like proteins (Hedman et al., 2009). These observations imply that MFT may have different roles in plant development as compared with FT and TFL1.

In this study, we show that *MFT* functions as an antagonistic factor of ABA signaling during seed germination. *MFT* expression is boosted by ABA and inhibited by GA, and promotes seed germination particularly when ABA levels are elevated under unfavorable environmental stresses. Like *MFT* expression in *Arabidopsis* (Figures 4A, 4B, and 40), most of the identified *MFT*-like genes in various plant species show preferential expression in seeds (Chardon and Damerval, 2005; Danilevskaya et al., 2008), implying a highly conserved function of *MFT*-like genes in seed development across the plant kingdom. Analysis of the genomic sequences of *MFT*-like genes in maize and rice have revealed that they all contain several ABREs near their coding regions, which is comparable to the ABREs

identified in *MFT* in *Arabidopsis* (Figure 41). The presence of many ABREs in those *MFT* orthologs in rice and maize indicates that they may have similar function like *Arabidopsis MFT* in the control of seed germination in response to ABA. Here we only performed promoter analysis on *MFT*-like genes in two monocots, in order to gain more information, promoter studies could also be extended to those species which are more closely related to *Arabidopsis* such as papaya, *Populus*, and grapevine. Thus, it will be interesting and of practical importance to study further whether *MFT*-like genes in other plants have a conserved function in tuning seed sensitivity to ABA, thus modulating the growth potential of the embryo in response to environmental cues. To achieve this goal, we can first check whether the expression of *MFT*-like genes in other plants is responsive to ABA treatment, upon the confirmation of ABA regulation on the expression of certain *MFT*-like genes, further focus can be put on the detailed analysis of the ABREs in those genes by mutagenesis method and phenotypic analysis of those *mft*-like mutants, especially in the process of seed development and seed germination. Our findings in *Arabidopsis* should shed light on the studies of *MFT*-like genes in other plant species and a broader picture of *MFT* evolution is expected to be built.

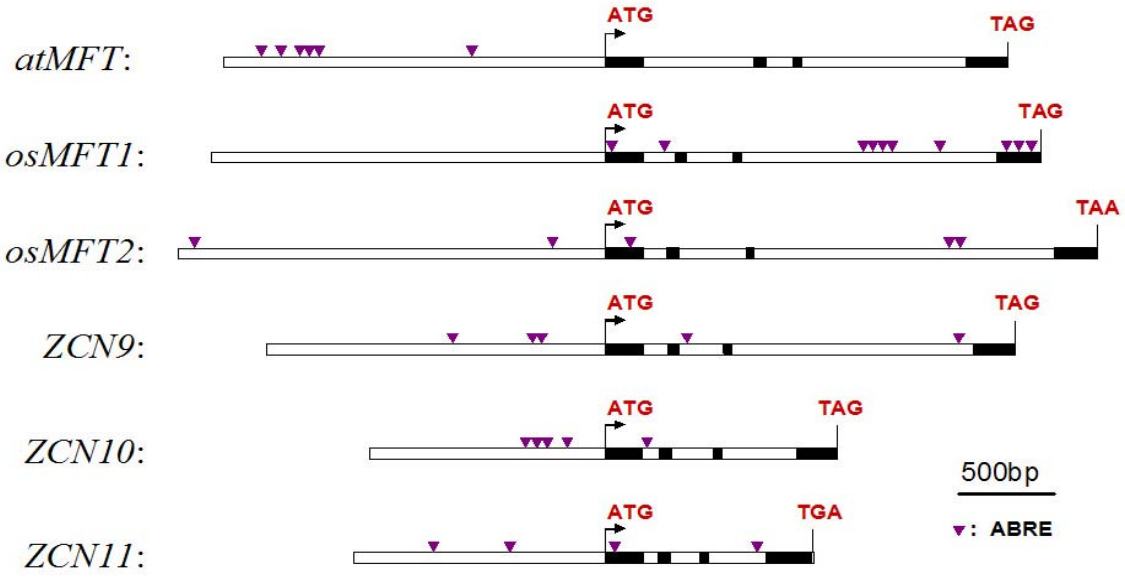

Figure 41. Promoter Analysis of *MFT*-like Subfamily Genes in *Arabidopsis*, Rice and Maize.

The sequences for *osMFT1* and *osMFT2* were derived from *Oryza sativa ssp.japonica*, and those of *ZCN9*, *ZCN10*, and *ZCN11* were identified from bacterial artificial chromosome (BAC) clones in *Zea mays*. Putative ABREs were identified using online MatInspector software (http://www.genomatix.de/) and marked by inverted triangles. Upstream regions and introns are represented by white boxes, while exons are represented by black boxes.

REFERENCES

Abe, M., Kobayashi, Y., Yamamoto, S., Daimon, Y., Yamaguchi, A., Ikeda, Y., Ichinoki, H., Notaguchi, M., Goto, K., and Araki, T. (2005). FD, a bZIP protein mediating signals from the floral pathway integrator FT at the shoot apex. Science **309,** 1052-1056.

Ahn, J.H., Miller, D., Winter, V.J., Banfield, M.J., Lee, J.H., Yoo, S.Y., Henz, S.R., Brady, R.L., and Weigel, D. (2006). A divergent external loop confers antagonistic activity on floral regulators FT and TFL1. Embo J **25,** 605-614.

Ali-Rachedi, S., Bouinot, D., Wagner, M.H., Bonnet, M., Sotta, B., Grappin, P., and Jullien, M. (2004). Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of Arabidopsis thaliana. Planta **219,** 479-488.

Alonso-Blanco, C., Bentsink, L., Hanhart, C.J., Blankestijn-de Vries, H., and Koornneef, M. (2003). Analysis of natural allelic variation at seed dormancy loci of Arabidopsis thaliana. Genetics **164,** 711-729.

Amen, R.D. (1968). A model of seed dormancy. Bot Rev **34,** 1-31.

Arenas-Huertero, F., Arroyo, A., Zhou, L., Sheen, J., and Leon, P. (2000). Analysis of Arabidopsis glucose insensitive mutants, gin5 and gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev **14,** 2085-2096.

Banfield, M.J., and Brady, R.L. (2000). The structure of Antirrhinum centroradialis protein (CEN) suggests a role as a kinase regulator. J Mol Biol **297,** 1159-1170.

Banfield, M.J., Barker, J.J., Perry, A.C., and Brady, R.L. (1998). Function from structure? The crystal structure of human phosphatidylethanolamine-binding protein suggests a role in membrane signal transduction. Structure **6,** 1245-1254.

Barroco, R.M., Van Poucke, K., Bergervoet, J.H., De Veylder, L., Groot, S.P., Inze, D., and Engler, G. (2005). The role of the cell cycle machinery in resumption of postembryonic development. Plant Physiol **137,** 127-140.

Baskin, C.C., and Baskin, J.M. (1998). Seeds: ecology, biogeography, and evolution of dormancy and germination. (Acadmic Press, New York).

Baud, S., Boutin, J.-P., Miquel, M., Lepiniec, L., and Rochat, C. (2002). An integrated overview of seed development in Arabidopsis thaliana ecotype WS. Plant Physiol Biochem **40,** 151-160.

Beaudoin, N., Serizet, C., Gosti, F., and Giraudat, J. (2000). Interactions between abscisic acid and ethylene signaling cascades. Plant Cell **12,** 1103-1115.

Beeckman, T., Rycke, R.D., Viane, R., and Inze, D. (2000). Histological study of seed coat development in Arabidopsis thaliana. J Plant Res **113,** 139-148.

Benfey, P.N., and Schiefelbein, J.W. (1994). Getting to the root of plant development: the genetics of Arabidopsis root formation. Trends Genet **10,** 84-88.

Bentsink, L., and Koornneef, M. (2008). Seed dormancy and germination. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), doi/10.1199/tab.0050, http://www.aspb.org/publications/arabidopsis/.

Berleth, T., and Chatfield, S. (2002). Embryogenesis: pattern formation from a single cell. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD.: American Society of Plant Biologists).

Bernier, I., Tresca, J.P., and Jolles, P. (1986). Ligand-binding studies with a 23 kDa protein purified from bovine brain cytosol. Biochim Biophys Acta **871,** 19-23.

Berrie, A.M.M. (1966). The effect of temperature and light on the germination of lettuce seeds. Physiol Plant **19,** 429-436.

Bewley, J.D. (1979). Secondary dormancy (skotodormancy) in seeds of lettuce (Lactuca sativa cv. Grand Rapids) and its release by light, gibberellic acid and benzyladenine. Physiol Plant **49,** 277-280.

Bewley, J.D. (1997). Seed germination and dormancy. Plant Cell **9,** 1055-1066.

Bewley, J.D., and Marcus, A. (1990). Gene expression in seed development and germination. Prog Nucleic Acid Res Mol Biol **38,** 165-193.

Bewley, J.D., and Black, M. (1994). Seeds: physiology of development and germination. (New York: Plenum Press).

Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H. (1988). Insensitivity to Ethylene Conferred by a Dominant Mutation in Arabidopsis thaliana. Science **241,** 1086-1089.

Bradford, K.J. (1990). A Water Relations Analysis of Seed Germination Rates. Plant Physiol **94,** 840-849.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997). Inflorescence commitment and architecture in Arabidopsis. Science **275,** 80-83.

Bruun, A.W., Svendsen, I., Sorensen, S.O., Kielland-Brandt, M.C., and Winther, J.R. (1998). A high-affinity inhibitor of yeast carboxypeptidase Y is encoded by TFS1 and shows homology to a family of lipid binding proteins. Biochemistry **37,** 3351-3357.

Cadman, C.S., Toorop, P.E., Hilhorst, H.W., and Finch-Savage, W.E. (2006). Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. Plant J **46,** 805-822.

Calvo, A.P., Nicolas, C., Nicolas, G., and Rodriguez, D. (2004). Evidence of a cross-talk regulation of a GA 20-oxidase (FsGA20ox1) by gibberellins and ethylene during the breaking of dormancy in Fagus sylvatica seeds. Physiol Plant **120,** 623-630.

Carmel-Goren, L., Liu, Y.S., Lifschitz, E., and Zamir, D. (2003). The SELF-PRUNING gene family in tomato. Plant Mol Biol **52,** 1215-1222.

Carmona, M.J., Calonje, M., and Martinez-Zapater, J.M. (2007). The FT/TFL1 gene family in grapevine. Plant Mol Biol **63,** 637-650.

Carrera, E., Holman, T., Medhurst, A., Dietrich, D., Footitt, S., Theodoulou, F.L., and Holdsworth, M.J. (2008). Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. Plant J **53,** 214-224.

Chardon, F., and Damerval, C. (2005). Phylogenomic analysis of the PEBP gene family in cereals. J Mol Evol **61,** 579-590.

Chautard, H., Jacquet, M., Schoentgen, F., Bureaud, N., and Benedetti, H. (2004). Tfs1p, a member of the PEBP family, inhibits the Ira2p but not the Ira1p Ras GTPase-activating protein in Saccharomyces cerevisiae. Eukaryot Cell **3,** 459-470.

Chen, F., and Bradford, K.J. (2000). Expression of an expansin is associated with endosperm weakening during tomato seed germination. Plant Physiol **124,** 1265-1274.

Chen, F., Dahal, P., and Bradford, K.J. (2001). Two tomato expansin genes show divergent expression and localization in embryos during seed development and germination. Plant Physiol **127,** 928-936.

Chen, H., Zhang, J., Neff, M.M., Hong, S.W., Zhang, H., Deng, X.W., and Xiong, L. (2008). Integration of light and abscisic acid signaling during seed germination and early seedling development. Proc Natl Acad Sci U S A **105,** 4495-4500.

Chiang, G.C., Barua, D., Kramer, E.M., Amasino, R.M., and Donohue, K. (2009). Major flowering time gene, flowering locus C, regulates seed germination in Arabidopsis thaliana. Proc Natl Acad Sci U S A **106,** 11661-11666.

Chiwocha, S.D., Cutler, A.J., Abrams, S.R., Ambrose, S.J., Yang, J., Ross, A.R., and Kermode, A.R. (2005). The etr1-2 mutation in Arabidopsis thaliana affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. Plant J **42,** 35-48.

Choi, H., Hong, J., Ha, J., Kang, J., and Kim, S.Y. (2000). ABFs, a family of ABA-responsive element binding factors. J Biol Chem **275,** 1723-1730.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. **16,** 735-743.

Conti, L., and Bradley, D. (2007). TERMINAL FLOWER1 is a mobile signal controlling Arabidopsis architecture. Plant Cell **19,** 767-778.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., and Coupland, G. (2007). FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. Science **316,** 1030-1033.

Curaba, J., Moritz, T., Blervaque, R., Parcy, F., Raz, V., Herzog, M., and Vachon, G. (2004). AtGA3ox2, a key gene responsible for bioactive gibberellin biosynthesis, is regulated during embryogenesis by LEAFY COTYLEDON2 and FUSCA3 in Arabidopsis. Plant Physiol **136,** 3660-3669.

Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. (1996). A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. Science **273,** 1239-1241.

Danilevskaya, O.N., Meng, X., Hou, Z., Ananiev, E.V., and Simmons, C.R. (2008). A genomic and expression compendium of the expanded PEBP gene family from maize. Plant Physiol **146,** 250-264.

Debeaujon, I., and Koornneef, M. (2000). Gibberellin requirement for Arabidopsis seed germination is determined both by testa characteristics and embryonic abscisic acid. Plant Physiol **122,** 415-424.

Debeaujon, I., Leon-Kloosterziel, K.M., and Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. Plant Physiol **122,** 403-414.

Dill, A., and Sun, T. (2001). Synergistic derepression of gibberellin signaling by removing RGA and GAI function in Arabidopsis thaliana. Genetics **159,** 777-785.

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., and Scheres, B. (1993). Cellular organisation of the Arabidopsis thaliana root. Development **119,** 71-84.

Emery, R.J., Ma, Q., and Atkins, C.A. (2000). The forms and sources of cytokinins in developing white lupine seeds and fruits. Plant Physiol **123,** 1593-1604.

Enstone, D.E., Peterson, C.A., and Ma, F. (2003). Root endodermis and exodermis: structure, function, and responses to the environment. J Plant Growth Regul **21,** 335-351.

Ezcurra, I., Ellerstrom, M., Wycliffe, P., Stalberg, K., and Rask, L. (1999). Interaction between composite elements in the napA promoter: both the B-box ABA-responsive complex and the RY/G complex are necessary for seed-specific expression. Plant Mol Biol **40,** 699-709.

Ezcurra, I., Wycliffe, P., Nehlin, L., Ellerstrom, M., and Rask, L. (2000). Transactivation of the Brassica napus napin promoter by ABI3 requires interaction of the conserved B2 and B3 domains of ABI3 with different cis-elements: B2 mediates activation through an ABRE, whereas B3 interacts with an RY/G-box. Plant J **24,** 57-66.

Finch-Savage, W.E., and Leubner-Metzger, G. (2006). Seed dormancy and the control of germination. New Phytol **171,** 501-523.

Finch-Savage, W.E., Cadman, C.S., Toorop, P.E., Lynn, J.R., and Hilhorst, H.W. (2007). Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directed by environmentally specific sensing. Plant J **51,** 60-78.

Finkelstein, R.R. (1994). Mutations at two new Arabidopsis ABA response loci are similar to the abi3 mutations. plant J **5,** 765-771.

Finkelstein, R.R., and Lynch, T.J. (2000). The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell **12,** 599-609.

Finkelstein, R.R., Gampala, S.S., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14 Suppl,** S15-45.

Finkelstein, R.R., Wang, M.L., Lynch, T.J., Rao, S., and Goodman, H.M. (1998). The Arabidopsis abscisic acid response locus ABI4 encodes an APETALA 2 domain protein. Plant Cell **10,** 1043-1054.

Finkelstein, R.R., Gampala, S.S., Lynch, T.J., Thomas, T.L., and Rock, C.D. (2005). Redundant and distinct functions of the ABA response loci ABAINSENSITIVE(ABI)5 and ABRE-BINDING FACTOR (ABF)3. Plant Mol Biol **59,** 253-267.

Fischer-Iglesias, C., Sundberg, B., Neuhaus, G., and Jones, A.M. (2001). Auxin distribution and transport during embryonic pattern formation in wheat. Plant J **26,** 115-129.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jurgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature **426,** 147-153.

Fujii, H., Verslues, P.E., and Zhu, J.K. (2007). Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. Plant Cell **19,** 485-494.

Gallardo, K., Job, C., Groot, S.P., Puype, M., Demol, H., Vandekerckhove, J., and Job, D. (2001). Proteomic analysis of arabidopsis seed germination and priming. Plant Physiol **126,** 835-848.

Gao, Y., Zeng, Q., Guo, J., Cheng, J., Ellis, B.E., and Chen, J.G. (2007). Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination and early seedling development in Arabidopsis. Plant J **52,** 1001-1013.

Gao, Y.P., Young, L., Bonham-Smith, P., and Gusta, L.V. (1999). Characterization and expression of plasma and tonoplast membrane aquaporins in primed seed of Brassica napus during germination under stress conditions. Plant Mol Biol **40,** 635-644.

Gazzarrini, S., Tsuchiya, Y., Lumba, S., Okamoto, M., and McCourt, P. (2004). The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. Dev Cell **7,** 373-385.

Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., and McCourt, P. (2000). Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. Plant Cell **12,** 1117-1126.

Gidrol, X., Lin, W.S., Degousee, N., Yip, S.F., and Kush, A. (1994). Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur J Biochem **224,** 21-28.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F., and Goodman, H.M. (1992). Isolation of the Arabidopsis ABI3 gene by positional cloning. Plant Cell **4,** 1251-1261.

Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A., Vartanian, N., and Giraudat, J. (1999). ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. Plant Cell **11,** 1897-1910.

Grappin, P., Bouinot, D., Sotta, B., Miginiac, E., and Jullien, M. (2000). Control of seed dormancy in Nicotiana plumbaginifolia: post-imbibition abscisic acid synthesis imposes dormancy maintenance. Planta **210,** 279-285.

Guo, J., Zeng, Q., Emami, M., Ellis, B.E., and Chen, J. (2008). The GCR2 gene family is not required for ABA control of seed germination and early seedling development in Arabidopsis. PloS One **3,** e2982.

Hanzawa, Y., Money, T., and Bradley, D. (2005). A single amino acid converts a repressor to an activator of flowering. Proc Natl Acad Sci U S A **102,** 7748-7753.

Harada, J.J. (1997). Seed maturation and control of germination. In Advances in cellular and molecular biology of plants, B.A. Larkins and I.K. Vasil, eds (dordrecht: Kluwer Academic Publishers), pp. 545-592.

Hays, D.B., Yeung, E.C., and Pharis, R.P. (2002). The role of gibberellins in embryo axis development. J Exp Bot **53,** 1747-1751.

Hedden, P., and Kamiya, Y. (1997). GIBBERELLIN BIOSYNTHESIS: Enzymes, Genes and Their Regulation. Annu Rev Plant Physiol Plant Mol Biol **48,** 431-460.

Hedman, H., Kallman, T., and Lagercrantz, U. (2009). Early evolution of the MFT-like gene family in plants. Plant Mol Biol **70,** 359-369.

Hengst, U., Albrecht, H., Hess, D., and Monard, D. (2001). The phosphatidylethanolamine-binding protein is the prototype of a novel family of serine protease inhibitors. J Biol Chem **276,** 535-540.

Hilhorst, H.W.M. (1998). The regulation of secondary dormancy. The membrane hypothesis revisited. . Seed Sci Res **8,** 77-90.

Hilhorst, H.W.M. (2007). Definition and hypotheses of seed dormancy. In Seed Development, Dormancy and Germination., K.J. Bradford and H. Nonogaki, eds (Blackwell Publishing, Oxford), pp. 50-71.

Holdsworth, M.J., Bentsink, L., and Soppe, W.J. (2008). Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. New Phytol **179,** 33-54.

Hourmant, A., and Pradet, A. (1981). Oxidative Phosphorylation in Germinating Lettuce Seeds (Lactuca sativa) during the First Hours of Imbibition. Plant Physiol **68,** 631-635.

Huang, Z., Boubriak, I., Osborne, D.J., Dong, M., and Gutterman, Y. (2008). Possible role of pectin-containing mucilage and dew in repairing embryo DNA of seeds adapted to desert conditions. Ann Bot **101,** 277-283.

Inoue, H., Nojima, H., and Okayama, H. (1990). High-efficiency transformation of *Escherichia coli* with plasmids. Gene **96,** 23-28.

Johnston, C.A., Temple, B.R., Chen, J.G., Gao, Y., Moriyama, E.N., Jones, A.M., Siderovski, D.P., and Willard, F.S. (2007). Comment on "A G protein coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid". Science **318,** 914; author reply 914.

Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y. (2002). Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14,** 343-357.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J., and Weigel, D. (1999). Activation tagging of the floral inducer FT. Science **286,** 1962-1965.

Karssen, C., Brinkhorst-van der Swan, D., Breekland, A., and Koornneef, M. (1983). Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of Arabidopsis thaliana (L.) Heynh. . Planta **157,** 158-165.

Karssen, C.M. (1980). Environmental conditions and endogenous mechanisms involved in secondary dormancy of seeds. Jsr J Bot **29,** 45-64.

Karssen, C.M., Zagorski, S., Kepczynski, J., and Groot, S.P.C. (1989). Key role for endogenous gibberellins in the control of seed germination. Ann Bot **63,** 71-80.

Kepczynski, J., and Kepczynska, E. (1997). Ethylene in seed dormancy and germination. Physiol Plant **101,** 720-726.

Kim, S.Y., Chung, H.J., and Thomas, T.L. (1997). Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant J **11,** 1237-1251.

Kim, S.Y., Ma, J., Perret, P., Li, Z., and Thomas, T.L. (2002). Arabidopsis ABI5 subfamily members have distinct DNA-binding and transcriptional activities. Plant Physiol **130,** 688-697.

Ko, J.H., Yang, S.H., and Han, K.H. (2006). Upregulation of an Arabidopsis RING-H2 gene, XERICO, confers drought tolerance through increased abscisic acid biosynthesis. Plant J **47,** 343-355.

Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M., and Araki, T. (1999). A pair of related genes with antagonistic roles in mediating flowering signals. Science **286,** 1960-1962.

Koornneef, M., Reuling, G., and Karssen, C.M. (1984). The isolation and characterization of abscisic acid-insensitive mutants of Arabidopsis thaliana. Physiol Plant **61,** 377-383.

Koornneef, M., Bentsink, L., and Hilhorst, H. (2002). Seed dormancy and germination. Curr Opin Plant Biol **5,** 33-36.

Koornneef, M., Jorna, M.L., Brinkhorst-van der Swan, D.L.C., and Karssen, C.M. (1982). The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) heynh. . Theor Appl Genet **61,** 385-393.

Koussevitzky, S., Nott, A., Mockler, T.C., Hong, F., Sachetto-Martins, G., Surpin, M., Lim, J., Mittler, R., and Chory, J. (2007). Signals from chloroplasts converge to regulate nuclear gene expression. Science **316,** 715-719.

Kroslak, T., Koch, T., Kahl, E., and Hollt, V. (2001). Human phosphatidylethanolamine-binding protein facilitates heterotrimeric G protein-dependent signaling. J Biol Chem **276,** 39772-39778.

Kucera, B., Cohn, M.A., and Leubner-Metzger, G. (2005). Plant hormone interactions during seed dormancy release and germination. Seed Sci Res **15,** 281-307.

Lee, S., Cheng, H., King, K.E., Wang, W., He, Y., Hussain, A., Lo, J., Harberd, N.P., and Peng, J. (2002). Gibberellin regulates Arabidopsis seed germination via RGL2, a GAI/RGA-like gene whose expression is up-regulated following imbibition. Genes Dev **16,** 646-658.

Leon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A., and Koornneef, M. (1996). Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. Plant J **10,** 655-661.

Leubner-Metzger, G. (2001). Brassinosteroids and gibberellins promote tobacco seed germination by distinct pathways. Planta **213,** 758-763.

Leung, J., Merlot, S., and Giraudat, J. (1997). The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell **9,** 759-771.

Leung, J., Bouvier-Durand, M., Morris, P.C., Guerrier, D., Chefdor, F., and Giraudat, J. (1994). Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science **264,** 1448-1452.

Leymarie, J., Robayo-Romero, M.E., Gendreau, E., Benech-Arnold, R.L., and Corbineau, F. (2008). Involvement of ABA in induction of secondary dormancy in barley (Hordeum vulgare L.) seeds. Plant Cell Physiol **49,** 1830-1838.

Liu, C., Zhou, J., Bracha-Drori, K., Yalovsky, S., Ito, T., and Yu, H. (2007a). Specification of Arabidopsis floral meristem identity by repression of flowering time genes. Development **134,** 1901-1910.

Liu, C., Chen, H., Er, H.L., Soo, H.M., Kumar, P.P., Han, J.H., Liou, Y.C., and Yu, H. (2008). Direct interaction of AGL24 and SOC1 integrates flowering signals in Arabidopsis. Development **135,** 1481-1491.

Liu, H.Y., Yu, X., Cui, D.Y., Sun, M.H., Sun, W.N., Tang, Z.C., Kwak, S.S., and Su, W.A. (2007b). The role of water channel proteins and nitric oxide signaling in rice seed germination. Cell Res **17,** 638-649.

Liu, P.P., Koizuka, N., Homrichhausen, T.M., Hewitt, J.R., Martin, R.C., and Nonogaki, H. (2005). Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes. Plant J **41,** 936-944.

Liu, P.P., Montgomery, T.A., Fahlgren, N., Kasschau, K.D., Nonogaki, H., and Carrington, J.C. (2007c). Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. Plant J **52,** 133-146.

Liu, X., Yue, Y., Li, W., and Ma, L. (2007d). Response to comment on "A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid". Science **318,** 914.

Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W.H., and Ma, L. (2007e). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science **315,** 1712-1716.

Lopez-Molina, L., Mongrand, S., and Chua, N.H. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci U S A **98,** 4782-4787.

Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T., and Chua, N.H. (2002). ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J **32,** 317-328.

Lux, A., Luxova, M., Abe, J., and Morita, S. (2004). Root cortex, structural and functional variability and responses to environmental stress. Root Res **13,** 117-131.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science **324,** 1064-1068.

Mansfield, S.G., and Briarty, L.G. (1996). The dynamics of seedling and cotyledon cell development in arabidopsis thaliana during reserve mobilization. Int J Plant Sci **157,** 280-295.

Masubelele, N.H., Dewitte, W., Menges, M., Maughan, S., Collins, C., Huntley, R., Nieuwland, J., Scofield, S., and Murray, J.A. (2005). D-type cyclins activate division in the root apex to promote seed germination in Arabidopsis. Proc Natl Acad Sci U S A **102,** 15694-15699.

Mathews, S. (2006). Phytochrome-mediated development in land plants: red light sensing evolves to meet the challenges of changing light environments. Mol Ecol **15,** 3483-3503.

Matilla, A.J. (2000). Ethylene in seed formation and germination. Seed Sci Res **10,** 111-126.

Maurel, C., Chrispeels, M., Lurin, C., Tacnet, F., Geelen, D., Ripoche, P., and Guern, J. (1997). Function and regulation of seed aquaporins. J Exp Bot **48,** 421-430.

McCourt, P. (1999). Genetic Analysis of Hormone Signaling. Annu Rev Plant Physiol Plant Mol Biol **50,** 219-243.

McCourt, P., and Creelman, R. (2008). The ABA receptors -- we report you decide. Curr Opin Plant Biol **11,** 474-478.

Mcdonald, M.B., Sullivan, J., and Lauer, M.J. (1994). The pathway of water-uptake in maize seeds. Seed Sci Tech **22,** 79-90.

Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., and Giraudat, J. (2001). The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. Plant J **25,** 295-303.

Meyer, K., Leube, M.P., and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. Science **264,** 1452-1455.

Michaels, S.D., and Amasino, R.M. (1999). FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. Plant Cell **11,** 949-956.

Millar, A.A., Jacobsen, J.V., Ross, J.J., Helliwell, C.A., Poole, A.T., Scofield, G., Reid, J.B., and Gubler, F. (2006). Seed dormancy and ABA metabolism in Arabidopsis and barley: the role of ABA 8'-hydroxylase. Plant J **45,** 942-954.

Miura, K., Lee, J., Jin, J.B., Yoo, C.Y., Miura, T., and Hasegawa, P.M. (2009). Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. Proc Natl Acad Sci U S A **106,** 5418-5423.

Mok, D.W., and Mok, M.C. (2001). Cytokinin Metabolism and Action. Annu Rev Plant Physiol Plant Mol Biol **52,** 89-118.

Monroe-Augustus, M., Zolman, B.K., and Bartel, B. (2003). IBR5, a dual-specificity phosphatase-like protein modulating auxin and abscisic acid responsiveness in Arabidopsis. Plant Cell **15,** 2979-2991.

Muller, A.H., and Hansson, M. (2009). The barley magnesium chelatase 150-kd subunit is not an abscisic acid receptor. Plant Physiol **150,** 157-166.

Muller, K., Tintelnot, S., and Leubner-Metzger, G. (2006). Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of Lepidium sativum (cress) and endosperm rupture of cress and Arabidopsis thaliana. Plant Cell Physiol **47,** 864-877.

Nakaune, S., Yamada, K., Kondo, M., Kato, T., Tabata, S., Nishimura, M., and Hara-Nishimura, I. (2005). A vacuolar processing enzyme, deltaVPE, is involved in seed coat formation at the early stage of seed development. Plant Cell **17,** 876-887.

Nambara, E., and Marion-Poll, A. (2005). Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol **56,** 165-185.

Nambara, E., Akazawa, T., and McCourt, P. (1991). Effects of the Gibberellin Biosynthetic Inhibitor Uniconazol on Mutants of Arabidopsis. Plant Physiol **97,** 736-738.

Nambara, E., Keith, K., McCourt, P., and Naito, S. (1994). Isolation of an internal deletion mutant of the Arabidopsis thaliana ABI3 gene. Plant Cell Physiol **35,** 509-513.

Nonogaki, H., Gee, O.H., and Bradford, K.J. (2000). A germination-specific endo-beta-mannanase gene is expressed in the micropylar endosperm cap of tomato seeds. Plant Physiol **123,** 1235-1246.

Nonogaki, H., Chen, F., and Bradford, K.J. (2007). Mechanisms and genes involved in germination sensu stricto. . In Seed Development, Dormancy and Germination, K.J. Bradford and H. Nonogaki, eds (Blackwell Publishing, Oxford), pp. 264-304.

Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A., Kamiya, Y., and Yamaguchi, S. (2003). Gibberellin biosynthesis and response during Arabidopsis seed germination. Plant Cell **15,** 1591-1604.

Oh, E., Kim, J., Park, E., Kim, J.I., Kang, C., and Choi, G. (2004). PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key negative regulator of seed germination in Arabidopsis thaliana. Plant Cell **16,** 3045-3058.

Oh, E., Yamaguchi, S., Kamiya, Y., Bae, G., Chung, W.I., and Choi, G. (2006). Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in Arabidopsis. Plant J **47,** 124-139.

Okamoto, M., Kuwahara, A., Seo, M., Kushiro, T., Asami, T., Hirai, N., Kamiya, Y., Koshiba, T., and Nambara, E. (2006). CYP707A1 and CYP707A2, which encode abscisic acid 8'-hydroxylases, are indispensable for proper control of seed dormancy and germination in Arabidopsis. Plant Physiol **141,** 97-107.

Olsen, O.A. (2004). Nuclear endosperm development in cereals and Arabidopsis thaliana. Plant Cell **16 Suppl,** S214-227.

Olszewski, N., Sun, T.P., and Gubler, F. (2002). Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell **14 Suppl,** S61-80.

Pandey, S., Nelson, D.C., and Assmann, S.M. (2009). Two novel GPCR-type G proteins are abscisic acid receptors in Arabidopsis. Cell **136,** 136-148.

Parcy, F., Valon, C., Raynal, M., Gaubier-Comella, P., Delseny, M., and Giraudat, J. (1994). Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. Plant Cell **6,** 1567-1582.

Park, S., Rath, O., Beach, S., Xiang, X., Kelly, S.M., Luo, Z., Kolch, W., and Yeung, K.C. (2006). Regulation of RKIP binding to the N-region of the Raf-1 kinase. FEBS Lett **580,** 6405-6412.

Park, S.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.F., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Desveaux, D., Rodriguez, P.L., McCourt, P., Zhu, J.K., Schroeder, J.I., Volkman, B.F., and Cutler, S.R. (2009).

Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science **324,** 1068-1071.

Penfield, S., Meissner, R.C., Shoue, D.A., Carpita, N.C., and Bevan, M.W. (2001). MYB61 is required for mucilage deposition and extrusion in the Arabidopsis seed coat. Plant Cell **13,** 2777-2791.

Penfield, S., Josse, E. M., Kannangara, R., Gilday, A. D., Halliday, K. J., and Graham, I. A. (2005). Cold and light control seed germination through the bHLH transcription factor SPATULA. Curr Biol **15**, 1998-2006.

Penfield, S., Gilday, A. D., Halliday, K. J., and Graham, I. A. (2006). DELLA-mediated cotyledon expansion breaks coat-imposed seed dormancy. Curr Biol **16**, 2366-2370.

Peng, J., and Harberd, N.P. (1997). Gibberellin deficiency and response mutations suppress the stem elongation phenotype of phytochrome-deficient mutants of Arabidopsis. Plant Physiol **113,** 1051-1058.

Piskurewicz, U., Jikumaru, Y., Kinoshita, N., Nambara, E., Kamiya, Y., and Lopez-Molina, L. (2008). The gibberellic acid signaling repressor RGL2 inhibits Arabidopsis seed germination by stimulating abscisic acid synthesis and ABI5 activity. Plant Cell **20,** 2729-2745.

Quail, P.H., Boylan, M.T., Parks, B.M., Short, T.W., Xu, Y., and Wagner, D. (1995). Phytochromes: photosensory perception and signal transduction. Science **268,** 675-680.

Quesada, V., Ponce, M.R., and Micol, J.L. (2000). Genetic analysis of salt-tolerant mutants in Arabidopsis thaliana. Genetics **154,** 421-436.

Raghavan, V. (2006). Double fertilization - embryo and endosperm development in flowering plants. (Berlin: Springer-Verlag).

Ramaih, S., Guedira, M., and Paulsen, G.M. (2003). Relationship of indoleacetic acid and tryptophan to dormancy and preharvest sprouting of wheat. Funct Plant Biol **30,** 939-945.

Rautengarten, C., Usadel, B., Neumetzler, L., Hartmann, J., Bussis, D., and Altmann, T. (2008). A subtilisin-like serine protease essential for mucilage release from Arabidopsis seed coats. Plant J **54,** 466-480.

Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D. (2006). The RNA-binding protein FCA is an abscisic acid receptor. Nature **439,** 290-294.

Riefler, M., Novak, O., Strnad, M., and Schmulling, T. (2006). Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. Plant Cell **18,** 40-54.

Risk, J.M., Day, C.L., and Macknight, R.C. (2009). Reevaluation of abscisic acid-binding assays shows that G-Protein-Coupled Receptor2 does not bind abscisic Acid. Plant Physiol **150,** 6-11.

Rousselin, P., Kraepiel, Y., Maldiney, R., Miginiac, E., and Caboche, M. (1992). Characterization of three hormone mutants of *Nicotiana plumbaginifolia:* evidence for a common ABA deficiency Theor Appl Genet **85,** 213-221.

Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C., and Weisbeek, P. (1994). Embryonic origin of the Arabidopsis primary root and root meristem initials. Development **120,** 2475-2487.

Schmidt, J., Altmann, T., and Adam, G. (1997). Brassinosteroids from seeds of Arabidopsis thaliana. Phytochemistry **45,** 1325-1327.

Schopfer, P., and Plachy, C. (1985). Control of Seed Germination by Abscisic Acid : III. Effect on Embryo Growth Potential (Minimum Turgor Pressure) and Growth Coefficient (Cell Wall Extensibility) in Brassica napus L. Plant Physiol **77,** 676-686.

Seo, M., Nambara, E., Choi, G., and Yamaguchi, S. (2009). Interaction of light and hormone signals in germinating seeds. Plant Mol Biol **69,** 463-472.

Shannon, S., and Meeks-Wagner, D.R. (1991). A Mutation in the Arabidopsis TFL1 Gene Affects Inflorescence Meristem Development. Plant Cell **3,** 877-892.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J., and Dennis, E.S. (1999). The FLF MADS box gene: a repressor of flowering in Arabidopsis regulated by vernalization and methylation. Plant Cell **11,** 445-458.

Shen, Y.Y., Wang, X.F., Wu, F.Q., Du, S.Y., Cao, Z., Shang, Y., Wang, X.L., Peng, C.C., Yu, X.C., Zhu, S.Y., Fan, R.C., Xu, Y.H., and Zhang, D.P. (2006). The Mg-chelatase H subunit is an abscisic acid receptor. Nature **443,** 823-826.

Shinomura, T., Nagatani, A., Chory, J., and Furuya, M. (1994). The Induction of Seed Germination in Arabidopsis thaliana Is Regulated Principally by Phytochrome B and Secondarily by Phytochrome A. Plant Physiol **104,** 363-371.

Sieburth, L.E., and Meyerowitz, E.M. (1997). Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. Plant Cell **9,** 355-365.

Simpson, G.G., and Dean, C. (2002). Arabidopsis, the Rosetta stone of flowering time? Science **296,** 285-289.

Soderman, E.M., Brocard, I.M., Lynch, T.J., and Finkelstein, R.R. (2000). Regulation and function of the Arabidopsis ABA-insensitive4 gene in seed and abscisic acid response signaling networks. Plant Physiol **124,** 1752-1765.

Steber, C.M., and McCourt, P. (2001). A role for brassinosteroids in germination in Arabidopsis. Plant Physiol **125,** 763-769.

Strasser, B., Sanchez-Lamas, M., Yanovsky, M.J., Casal, J.J., and Cerdan, P.D. (2010). Arabidopsis thaliana life without phytochromes. Proc Natl Acad Sci U S A **107,** 4776-4781.

Tamura, N., Yoshida, T., Tanaka, A., Sasaki, R., Bando, A., Toh, S., Lepiniec, L., and Kawakami, N. (2006). Isolation and characterization of high temperature-resistant germination mutants of Arabidopsis thaliana. Plant Cell Physiol **47,** 1081-1094.

Teaster, N.D., Motes, C.M., Tang, Y., Wiant, W.C., Cotter, M.Q., Wang, Y.S., Kilaru, A., Venables, B.J., Hasenstein, K.H., Gonzalez, G., Blancaflor, E.B., and Chapman, K.D. (2007). N-Acylethanolamine Metabolism Interacts with Abscisic Acid Signaling in Arabidopsis thaliana Seedlings. Plant Cell.

Tiryaki, I., and Staswick, P.E. (2002). An Arabidopsis mutant defective in jasmonate response is allelic to the auxin-signaling mutant axr1. Plant Physiol **130,** 887-894.

Tohdoh, N., Tojo, S., Agui, H., and Ojika, K. (1995). Sequence homology of rat and human HCNP precursor proteins, bovine phosphatidylethanolamine-binding protein and rat 23-kDa protein associated with the opioid-binding protein. Brain Res Mol Brain Res **30,** 381-384.

Tyler, L., Thomas, S.G., Hu, J., Dill, A., Alonso, J.M., Ecker, J.R., and Sun, T.P. (2004). Della proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. Plant Physiol **135,** 1008-1019.

Vander Willigen, C., Postaire, O., Tournaire-Roux, C., Boursiac, Y., and Maurel, C. (2006). Expression and inhibition of aquaporins in germinating Arabidopsis seeds. Plant Cell Physiol **47,** 1241-1250.

Vicient, C.M., Hull, G., Guilleminot, J., Devic, M., and Delseny, M. (2000). Differential expression of the Arabidopsis genes coding for Em-like proteins. J Exp Bot **51,** 1211-1220.

Weijers, D., and Jurgens, G. (2005). Auxin and embryo axis formation: the ends in sight? Curr Opin Plant Biol **8,** 32-37.

Wen, C.K., and Chang, C. (2002). Arabidopsis RGL1 encodes a negative regulator of gibberellin responses. Plant Cell **14,** 87-100.

White, C.N., and Rivin, C.J. (2000). Gibberellins and seed development in maize. II. Gibberellin synthesis inhibition enhances abscisic acid signaling in cultured embryos. Plant Physiol **122,** 1089-1097.

White, C.N., Proebsting, W.M., Hedden, P., and Rivin, C.J. (2000). Gibberellins and seed development in maize. I. Evidence that gibberellin/abscisic acid balance governs germination versus maturation pathways. Plant Physiol **122,** 1081-1088.

Wigge, P.A., Kim, M.C., Jaeger, K.E., Busch, W., Schmid, M., Lohmann, J.U., and Weigel, D. (2005). Integration of spatial and temporal information during floral induction in Arabidopsis. Science **309,** 1056-1059.

Wilson, R.N., Heckman, J.W., and Somerville, C.R. (1992). Gibberellin is required for flowering in Arabidopsis thaliana under short days. Plant Physiol **100,** 403-408.

Windsor, J.B., Symonds, V.V., Mendenhall, J., and Lloyd, A.M. (2000). Arabidopsis seed coat development: morphological differentiation of the outer integument. Plant J **22,** 483-493.

Wu, Y., Sanchez, J.P., Lopez-Molina, L., Himmelbach, A., Grill, E., and Chua, N.H. (2003). The abi1-1 mutation blocks ABA signaling downstream of cADPR action. Plant J **34,** 307-315.

Xie, Z., Zhang, Z.L., Zou, X., Yang, G., Komatsu, S., and Shen, Q.J. (2006). Interactions of two abscisic-acid induced WRKY genes in repressing gibberellin signaling in aleurone cells. Plant J **46,** 231-242.

Xiong, L., and Zhu, J.K. (2003). Regulation of abscisic acid biosynthesis. Plant Physiol **133,** 29-36.

Xiong, L., Schumaker, K.S., and Zhu, J.K. (2002). Cell signaling during cold, drought, and salt stress. Plant Cell **14 Suppl,** S165-183.

Yamaguchi, A., Kobayashi, Y., Goto, K., Abe, M., and Araki, T. (2005). TWIN SISTER OF FT (TSF) acts as a floral pathway integrator redundantly with FT. Plant Cell Physiol **46,** 1175-1189.

Yamauchi, Y., Ogawa, M., Kuwahara, A., Hanada, A., Kamiya, Y., and Yamaguchi, S. (2004). Activation of gibberellin biosynthesis and response pathways by low temperature during imbibition of Arabidopsis thaliana seeds. Plant Cell **16,** 367-378.

Yoo, S.Y., Kardailsky, I., Lee, J.S., Weigel, D., and Ahn, J.H. (2004). Acceleration of flowering by overexpression of MFT (MOTHER OF FT AND TFL1). Mol Cells **17,** 95-101.

Yoo, S. J., Chung, K. S., Jung, S. H., Yoo, S. Y., Lee, J. S., and Ahn, J. H. (2010). BROTHER OF FT AND TFL1 (BFT) has TFL1-like activity and functions redundantly with TFL1 in inflorescence meristem development in Arabidopsis. Plant J **63**, 241-253.

Yu, H., Ito, T., Wellmer, F., and Meyerowitz, E.M. (2004a). Repression of AGAMOUS-LIKE 24 is a crucial step in promoting flower development. Nat Genet **36,** 157-161.

Yu, H., Ito, T., Zhao, Y., Peng, J., Kumar, P., and Meyerowitz, E.M. (2004b). Floral homeotic genes are targets of gibberellin signaling in flower development. Proc Natl Acad Sci U S A **101,** 7827-7832.

Zentella, R., Zhang, Z.L., Park, M., Thomas, S.G., Endo, A., Murase, K., Fleet, C.M., Jikumaru, Y., Nambara, E., Kamiya, Y., and Sun, T.P. (2007). Global analysis of della direct targets in early gibberellin signaling in Arabidopsis. Plant Cell **19,** 3037-3057.

Zhang, S., Cai, Z., and Wang, X. (2009). The primary signaling outputs of brassinosteroids are regulated by abscisic acid signaling. Proc Natl Acad Sci U S A **106,** 4543-4548.

Zhang, X., Garreton, V., and Chua, N.H. (2005). The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. Genes Dev **19,** 1532-1543.

APPENDIX

Publications during Graudate Course

Chang Liu, Wanyan Xi, Lisha Shen, Caiping Tan and Hao Yu. (2009) Regulation of floral patterning by flowering time genes. **Developmental Cell** 16, 711-722.

Wanyan Xi and Hao Yu (2009) Another flowering time gene, *FLOWERING LOCUS T*, regulates flower development. **Plant Signaling & Behavior** 4, 1142-1144.

Wanyan Xi, Chang Liu, Xingliang Hou and Hao Yu (2010) *MOTHER OF FT AND TFL1* regulates seed germination through a negative feedback loop modulating ABA signaling in *Arabidopsis*. **The Plant Cell** 22, 1733-1748.

Wanyan Xi and Hao Yu (2010*) MOTHER OF FT AND TFL1* regulates seed germination and fertility relevant to the brassinosteroid signaling pathway. **Plant Signaling & Behavior** 5