AN ABSTRACT OF THE DISSERTATION OF

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Abstract approved:

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Hiroyuki Nonogaki

In mature Arabidopsis seeds, the testa (seed coat) is no longer a living tissue. Thus, major sites of gene expression in imbibed seeds are the internal living tissues such as the embryo and the endosperm. To elucidate the molecular mechanisms of seed germination, the gene regulation in these two tissues during germination needs to be investigated. An enhancer-trap approach was used in this study to identify tissue- and stage-specific gene expression in seeds. The Arabidopsis enhancer-trap lines (Thomas Jack's population, CS31086) from the Arabidopsis Biological Resource Center (ABRC) were screened for β-glucuronidase (GUS) expression in imbibed seeds in order to identify and characterize seed germination-associated genes. One hundred twenty one independent lines exhibiting diverse tissue-specific GUS expression patterns were isolated and kept as the *Seed-GUS-Expression* enhancer-trap library. This library was donated to the ABRC (stock no. CS24362–CS24480), and is now available to the international seed research

community. Ninety one lines showed GUS expression predominantly in the micropylar end of the seed (named *BME* [*Blue Micropylar End*] lines), indicating that the micropylar region of Arabidopsis seed is selectively activated during germination. One of these lines, *BME3*, had a T-DNA insertion site in the 5' upstream region of a GATA-type zinc finger transcription factor gene (termed *BME3-ZF*). The *BME3-ZF* mRNA accumulated in seeds during cold stratification, suggesting its involvement in the physiological changes that accelerate the release of dormancy. *BME3-ZF* was expressed just prior to the expression of two GA biosynthesis genes, *AtGA20ox3* and *AtGA3ox1* which are also induced by cold stratification. The *BME3-ZF* knockout plants produced seeds exhibiting increased dormancy, which showed reduced response to cold stratification. The ungerminated knockout seeds exhibited testa rupture, but failed to penetrate the endosperm layer. Application of gibberellic acid (GA_3) rescued impaired germination of the knockout seeds without cold stratification, indicating that the normal GA signal transduction pathway is present in the knockout seeds. These results indicate BME3 GATA zinc finger protein is a positive regulator of Arabidopsis seed germination. In this study, we provide the proof-of-concept study for the *Seed-GUS-Expression* enhancer trap library and the detailed procedures to use a combination of gene-expression analysis of wild-type seeds and functional analysis using knockout plants for encouraging international collaborations to utilize this library which is a useful tool to identify the genes critical for seed germination.

Study of Seed Germination-Associated Genes Using Arabidopsis Enhancer-Trap

by

Po-Pu Liu

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Approved:

Major Professor, representing Horticulture

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Po-Pu Liu, Author

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CONTRIBUTION OF AUTHORS

Dr. Nobuya Koizuka, Ms. Tanja M. Homrichhausen and Ms. Jessica R. Hewitt assisted in harvesting seeds from the *Seed-GUS-Expression* enhancer trap lines and identifying the insertion sites of individual lines using genome-walking PCR. Ms. Jessica R. Hewitt also contributed the Scanning Electron Microscopy image (Figure 2-1) in Chapter 2. Dr. Ruth C. Martin assisted in the preparation of all manuscripts.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
aba	*aba deficient* mutant
abi	*aba insensitive* mutant
ABP	Auxin-Binding Protein
ABRC	Arabidopsis Biological Resource Center
ABRE	ABA Responsive Elements
ACT	Actin
AP	APETALA
Arabidopsis	*Arabidopsis thaliana* (L.) Heynh.
BCT	Blue Cotyledon Tips
βGlu I	Class I β-1,3-glucanase
bHLH	basic Helix-Loop-Helix
BME	Blue Micropylar End
CaMV 35S	Cauliflower Mosaic Virus 35S
CAP	Chromosome Associated Protein
CO	CONSTANS
Cvi	Cape Verde Island
DELLA	Aspartic Acid-Glutamic Acid-Leucine-Leucine-Alanine
etr	*ethylene insensitive* mutant
FR	Far-Red
GA	Gibberellins

LIST OF ABBREVIATIONS (Continued)

ga1	*ga deficient* mutant
ga3	*ga biosynthesis* mutant
GA3ox	GA 3-oxidase
GA20ox	GA 20-oxidase
GSP	Gene-Specific Primer
GUS	*β*-glucuronidase
HAN	HANABA TARANU
HD-ZIP	Homeobox-Leucine-Zipper
LB	Left Border
MARD	MEDIATOR OF ABA-REGULATED DORMANCY
MP	Minimal Promoter
MS	Murashige-Skoog
NCEDs	9-*cis*-epoxycarotenoid dioxygenases
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
Pfr	Far-red-light-absorbing form of PHY
PHY	Phytochrome
PIL	PHY-INTERACTING-FACTOR3-LIKE
pil	*pil* mutant
PWWP	Proline-Tryptophan-Tryptophan-Proline
RB	Right Border

LIST OF ABBREVIATIONS (Continued)

RGL	REPRESSOR OF *ga1-3* LIKE
rgl	*rgl* mutant
RING	Really Interesting New Gene
RT-PCR	Reverse Transcription-PCR
RNAi	RNA interference
RPR	Regulation of nuclear Pre-mRNA processing
SDS	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy
SCF	Skp1-cullin-F box protein
SLY	SLEEPY
sly	*sly* mutant
SPT	SPATULA
TAIR	The Arabidopsis Information Resource
TPS	Trehalose-6-Phosphate Synthase
TSP	T-DNA-Specific Primer
TZ	Tetrazolium
X-Gluc	5-Bromo-4-chloro-3-indolyl b-D-glucuronide-cyclohexylamine salt
XTH	Xyloglucan endoTransglycosylase/Hydrolase
ZIM	Zinc finger protein expressed in Inflorescence Meristem
ZF	GATA-type Zinc Finger transcription factor

ZFKO	*bme3-zf* mutant

DEDICATION

To my father and mother:

Cheng-Chung Liu and Chiu-Chin Huang

Study of Seed Germination-Associated Genes Using Arabidopsis Enhancer-Trap

Chapter 1

General Introduction

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Seeds are dispersal units of plants and an excellent delivery system of genetic information. Dispersal units of some species such as the Composite family (e.g. sunflower, lettuce), the grass family (e.g. rice, wheat and barley) and the Umbelliferae family (e.g. carrot) are botanically fruits but are also called seeds. An important function of seeds that has evolved over a long time is to provide survival mechanisms during unfavourable climatic periods. In many cases, mature seeds enter desiccation in a dormant state. Deep dormancy is usually observed in wild species that avoid germination under unfavourable environmental conditions (e.g. winter). When the favourable state (e.g. spring) arrives, seed germination takes place and the next generation of plants are established. Successful and uniform germination is also important in agriculture. The quality of crops depends on optimal germination and plant development. Understanding seed formation and germinative events will advance the crop production. In this chapter, current knowledge related to the mechanisms of seed formation and development, the regulation of germination by plant hormones and environmental signals, and the repression and de-repression of the germinative program is reviewed.

Seed formation and development

Pollination which is mediated by pollinators such as insects, birds or wind is an initial process proceeding fertilization that, in turn, leads to seed development. Following pollination and pollen germination, a pollen tube elongates through the transmitting tissue of the style. Two sperm nuclei are generated through mitosis of

the pollen grain germ cell and thereafter are transported to the micropyle of the ovule. Gymnosperms undergo a single fertilization event where one sperm nucleus fuses with an egg cell nucleus to form a diploid zygote that develops into an embryo and the fertilization does not involve in the megagametophyte (Baroux *et al.* 2002). In contrast, angiosperms have evolved mechanisms to undergo double fertilization. The diploid embryo is derived from the fusion between one sperm nucleus and an egg cell nucleus while the other sperm nucleus fuses with two central nuclei to form the triploid endosperm (Baroux *et al.* 2002). A mature ovule containing an embryo is generally considered a seed. Seeds of most gymnosperms such as those from the pine family (Pinaceae), the cypress family (Cupressaceae) and the ginkgo family (Ginkgoaceae) are not enclosed by a ripened ovary (called "naked" seeds), while angiosperm seeds are surrounded by an ovary wall which becomes the pericarp. A mature ovary containing mature ovules develops a fruit such as tomato and watermelon. Although fertilization is required for seed development, seed formation can also occur without fertilization (Ohad *et al.* 1996; Chaudhury *et al.* 1997; Grossniklaus *et al.* 1998; Kinoshita *et al.* 1999; Kiyosue *et al.* 1999). This process, which is referred to as apomixis, may have potential applications in agriculture such as the reproduction of the genetic architecture that is identical to the mother plant.

In angiosperm seeds, embryogenesis takes place after an egg cell nucleus has been fertilized. Based on embryo morphology, embryogenesis is usually classified into the globular, transition, heart, torpedo, walking-stick and mature

embryo stages. This continuous process involves cell division, differentiation and enlargement. When the embryo is fully developed morphologically, the mature seed which contains reserves such as carbohydrates, lipids and proteins, goes through maturation drying, develops desiccation tolerance and then becomes dormant. Embryogenesis has been intensively studied (Harada and West 1993 for review) and a substantial amount of information concerning the mechanisms of embryo pattern formation has also been obtained by using a genetic approach in Arabidopsis over the past few decades. Defects in important embryogenesis-associated genes such as *ABP1*, *CAP-C* and *TPS1* cause embryo lethality (e.g. Chen *et al.* 2001; Eastmond *et al.*; 2002; Siddiqui *et al.* 2006; Satu *et al.* 2006). Genes essential for maintaining a normal embryonic program required for seed production have been identified in these studies.

Mechanisms involved in the control of seed germination

To understand the molecular mechanisms involved in seed germination, it is essential to identify the function and regulation of key genes expressed during seed germination. The process of seed germination commences with the uptake of water during imbibition of dry seeds and ends with radicle protrusion through all covering layers (Bewley and Black 1994; Bewley 1997b). This process usually contains a three-phase pattern of water uptake. Rapid initial uptake is followed by a plateau phase where activation of metabolic processes including gene expression takes place. The visible sign of the completion of germination where the embryo

axis breaks through the surrounding tissues is observed when there is a further increase in water uptake. In a typical endospermic seed, the embryo is surrounded by two tissues, the endosperm which provides nutrition for seedling development and the testa (seed coat) which is derived from the integuments of the ovule. Two important forces, growth potential of the embryo and mechanical resistance of the endosperm and testa, play antagonistic roles in the completion of germination (Figure 1-1). It is known that the embryo cells elongate as a result of growth potential increase during imbibition and then the radicle penetrates the surrounding tissues (Kucera *et al.* 2005). The growth potential of the embryo can be generated from two potential pathways; the accumulation of solutes such as sugars and amino acids which reduces the water potential inside the cell and results in cell enlargement, and cell wall loosening which allows cells to elongate without changes in turgor pressure. Seed germination is also regulated by the testa and endosperm which provide opposing forces to embryo growth potential. Studies of Arabidopsis testa pigmentation mutants have shown that pigmentless seeds exhibit reduced seed dormancy (Debeaujon and Koornneef 2000; Debeaujon *et al.* 2000). It suggests that pigments may provide the physical strength to the testa and alterations of the properties of testa can improve seed germination. Other studies, using tomato seeds, have shown that endosperm weakening through cell-wall modification by hydrolytic enzymes or other cell wall proteins such as expansins occurs prior to the radicle protrusion (Nonogaki and Morohashi 1996; Chen and Bradford 2000; Chen *et al.* 2002). There are a number of experiments that have

used puncture force analyzers to directly measure the resistance of endosperm with either thin (e.g. lettuce, cress) or thick (e.g. tomato tobacco, coffee, *Datura ferox*) cellular layers (Ikuma and Thimann, 1963; Bewley 1997a; Leubner-Metzger 2003; da Silva *et al.* 2004; Müller *et al.* 2006; Nonogaki 2006). The results support the idea that the micropylar endosperm provides a barrier to the radicle and needs to be weakened for germination to occur in these species.

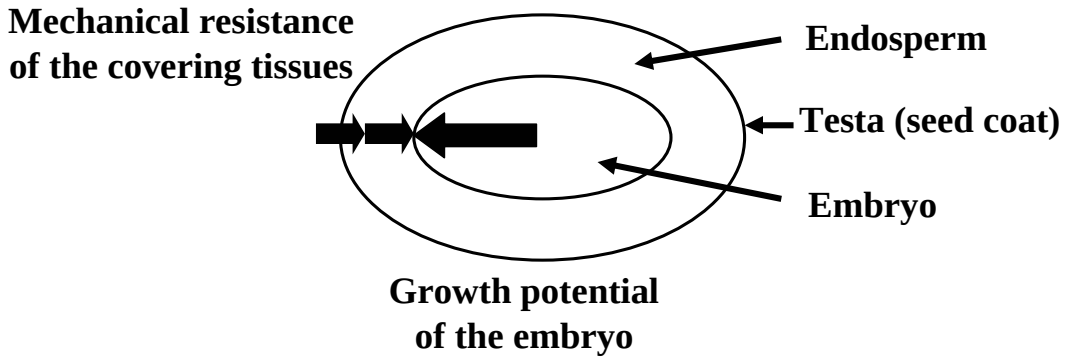

Figure 1-1. A model of the mechanisms of seed germination.
Completion of seed germination is determined by the balance between two opposite forces, the growth potential of the embryo and mechanical resistance of the covering tissues such as the endosperm and testa. The embryo growth potential has to be increased and/or the mechanical resistance of the endosperm needs to be decreased for germination to occur.

Liu *et al.* (2005a; Chapter 2) observed that the testa ruptures just above the embryonic axis prior to endosperm rupture in the processes of Arabidopsis seed germination (Figure 1-2). Since testa rupture does not occur during cold stratification at 4 ^{o}C to release dormancy, the testa must be ruptured by other physiological processes in seed such as embryo expansion. Testa and endosperm ruptures have also been reported in other species, such as *Lepidium sativum* (Müller *et al.* 2006), *Nicotiana attenuata* (Leubner-Metzger *et al.* 1995), *Chenopodium album* (Karssen 1976), and *Trollius ledebouri* (Hepher and Roberts 1976). Since endosperm elongation is observed in Arabidopsis seeds prior to the completion of germination, the micropylar endosperm may also provide mechanical resistance to the embryonic axis that needs to be weakened during germination (Figure 1-2). A number of cell wall-modifying proteins and hydrolases have been identified in tomato, tobacco and lettuce seeds. These proteins have been partially characterized and implicated in the endosperm weakening process. However, functional analysis using loss-of-function (knockout or RNAi) or gain-of-function (overexpression) mutants has not yet been performed. Only βGlu I, a typical pathogenesis-related protein, has been characterized in terms of its biological role in the regulation of tobacco seed germination using a transgenic approach (Leubner-Metzger and Meins 2000; Leubner-Metzger 2002). In imbibed tobacco seed, induced *βGlu I* expression is localized specifically to the micropylar endosperm where radicle protrusion will occur and is mainly involved in the promotion of the endosperm rupture (Leubner-

Metzger 2002). These results support with the hypothesis that the endosperm weakening-associated enzymes may also play important roles in Arabidopsis seed germination. To complete germination, an appropriate balance between embryo growth potential and the dissection of mechanical resistance of the endosperm and testa must be achieved.

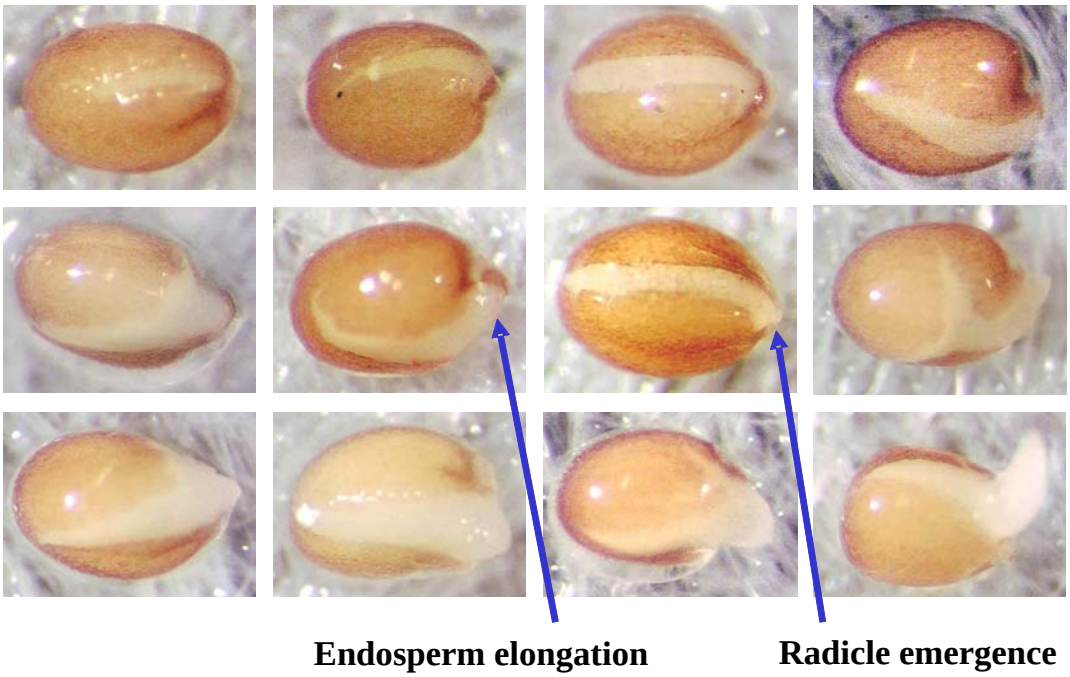

Figure 1-2. Time courses of Arabidopsis seed germination.
The occurrence of radicle emergence at the micropyle region is preceded by the process of two-step germination, testa rupture and endosperm rupture. Endosperm elongation takes place before the radicle penetrates the endosperm, implying the micropylar endosperm provides barrier to the embryo axis and needs to be weakened during germination.

Regulation of seed germination by plant hormones and environmental signals

The balance of plant hormones such as ABA and GA which inhibit and promote seed germination, respectively, is important in regulating seed germination. Koornneef *et al.* (1982) reported that the Arabidopsis *ga1* mutant exhibiting dwarf plant and non-germinating seed phenotypes can be rescued by a double-mutant approach with an additional *aba1* locus. In the presence of GA inhibitors such as paclobutrazol and uniconazole, the embryonic axis of ABA-deficient Arabidopsis mutants can penetrate the covering tissues (Nambara *et al.* 1991; Léon-Kloosterziel *et al.* 1996). In ABA-insensitive mutants such as *abi1* and *abi3*, seeds can germinate in the absence of GA synthesis and action (Nambara *et al.* 1991). It is well documented that the ABA content increases at the end of maturation during the seed development, eventually decreases and then maintains a level necessary to prevent precocious germination during the dormant state (Karssen *et al.* 1983). Recently, mutant and overexpression analysis of *AtNCED6* and *AtNCED9*, ABA biosynthesis genes, revealed their importance during Arabidopsis seed development (Lefebvre *et al.* 2006). Furthermore, ABA synthesized in both the embryo and endosperm was shown to play important roles in the hormonal balance (between ABA and GA) and the regulation of seed germination. By comparing transcriptome profiles obtained from a range of dormant and after-ripened seeds of Arabidopsis ecotype Cvi, genes with the ABRE motifs were found to be significantly over-represented in the dormant state when

compared to those expressed in the after-ripened seeds, indicating that the dormant state is associated with ABA biosynthesis genes and regulated by the balance between ABA and GA (Cadman *et al.* 2006). In the presence of GA, ABA levels increased in dormant Cvi seeds (Ali-Rachedi *et al.* 2004), implying that a high ratio of ABA/GA is maintained in this Arabidopsis ecotype. These studies also support the idea that the ratio of ABA to GA plays an important role in the regulation of seed germination.

Gene expression profiles during seed germination *sensu stricto*, which is defined as the period between the start of imbibition of the dry seed and radicle protrusion, may differ from those present after germination or during seedling stages. Physiological processes occurring during seed germination *sensu stricto* are also different from those during other stages of the plant development. For example, after radicle emergence, seeds can not be dehydrated and stored again; while imbibed seeds can survive desiccation before radicle emergence. Although many studies have identified critical genes involved in seed development, dormancy and germination, the molecular mechanisms of seed germination *sensu stricto* are not well understood.

Seed germination is also mediated by environmental factors such as light and cold. Light is required to promote Arabidopsis seed germination, suggesting that PHY is an important factor in the regulation of seed germination (Shinomura 1997). Although the molecular mechanisms of PHY involvement in the induction of seed germination are not fully elucidated, PIL5, a bHLH transcription factor,

was the first protein identified that is involved in the regulation of PHY signaling during Arabidopsis seed germination (Oh *et al.* 2004). It is known that the PHY photoreceptor responds to light signal when PIL5 is localized in the nucleus and binds to Pfr. When *PIL5* knockout and overexpression mutant seeds are germinated under red and far-red light conditions, seeds of *pil5* can germinate under far-red light treatment while *PIL5* overexpression lines are inhibited from germination. Thus, *PIL5* overexpression seeds do not respond to red light, while *pil5* seeds exhibit a normal response in germination. These data suggest that the biological function of this protein is the repression of seed germination through regulation by PHY.

Both light and low-temperature increase bioactive GA levels (Derkx *et al.* 1994; Yamaguchi *et al.* 2004). The final and rate-limiting step of GA biosynthesis is catalyzed by GA3ox. PHY promotes the expression of *GA3ox1* and *GA3ox2* (Yamaguchi *et al.* 1998). In many species, seed dormancy is broken by the exposure of imbibed seeds to low temperature (cold stratification at 4°C). Expression of *GA3ox1* significantly increases in a low-temperature dependent manner and is detected in the embryonic axis (Yamaguchi *et al.* 2004), suggesting that the release of seed dormancy by cold-stratification is at least partly due to GA biosynthesis and the subsequent increase in growth potential of the embryo. Ogawa *et al.* (2003) also reported the increase in the expression of GA-dependent transcription factors and cell wall proteins during imbibed Arabidopsis seeds prior to radicle emergence, suggesting that *de novo* GA biosynthesis is involved in the

generation of embryo growth potential or endosperm weakening. These studies indicate that the accumulation of GA during imbibition of Arabidopsis seeds is important in the promotion of seed germination *sensu stricto*. Recently, SPT, a bHLH transcription factor, was shown to negatively regulate seed germination responses to cold stratification and red light and to repress expression of *GA3ox* in a dormant state (Penfield *et al.* 2005). This work has also revealed the potential interaction between SPT and PIL5 mediated by cold and light in the regulation of seed germination. Both SPT and PIL5 negatively regulate seed germination in dark conditions, but the repressive activities of these two proteins are low in the presence of the light and cold environments. The repressive activities of PIL5 are reduced when imbibed seeds are exposed to light. In a dark and cold environment, the repression of SPT promotes seed germination.

Repression and de-repression in the germinative program

Initiation of vegetative growth of plant takes place upon completion of successful germination. Seed germination, a decision for this initiation is précised controlled by repression and de-repression of gene expression. Repressors, such as SPT and PIL5, which are involved in regulating *GA3ox* expression during seed germination, need to be repressed in order to maintain the normal germinative program. Repression of repressors (de-repression) has recently been proposed to be important for GA signal transduction during seed germination. RGL2 is a transcription factor that belongs to the DELLA protein family and plays a critical

role in Arabidopsis seed germination in response to GA (Lee *et al.* 2002; Tyler *et al.* 2004). GA-deficient seed germination phenotype is rescued by the deletion of the *rgl2* locus, suggesting RGL2 is a repressor of seed germination (Lee *et al.* 2002). Although RGL2 transcripts increase during seed imbibition and decline after radicle protrusion, a quick protein degradation of RGL2 occurs after GA treatment in Arabidopsis GA-deficient mutant seeds (Lee *et al.* 2002; Tyler *et al.* 2004), suggesting the rapid turnover of the RGL2 protein, rather than reduction in *RGL2* mRNA levels, promotes germination. Degradation of RGL2 protein is mediated by ubiquitination by SLY, a SCF E3 ubiquitin ligase and the 26S-proteasome pathway (McGinnis *et al.* 2003). The *sly* mutant has a dwarf phenotype, produces highly dormant seeds, and can not be rescued by GA application. This indicates that this protein potentially acts at or downstream of GA perception (Steber *et al.* 1998). RGL2 degradation does not occur when GA is applied to imbibed *sly* seeds (Tyler *et al.* 2004), suggesting that SLY is involved in the turnover of the RGL2 protein during seed germination. It has been recently hypothesized that degradation of one of the repressors involved in the regulation of PIL5 is controlled by PHY through the 26S proteasome pathway. When PIL5 is degraded under light conditions, transcription of *GA3ox1* and *GA3ox2* increases and transcription of *GA2ox2* is decreased, effectively increasing bioactive GA and allowing seeds to germinate (Oh *et al.* 2006). Seo *et al.* (2006) demonstrated that PHY inhibits ABA biosynthesis and enhances ABA inactivating enzymes, and decreases ABA content during photoreversible seed

germination. Decreased ABA levels promote GA biosynthesis enzymes and repress GA inactivation enzymes which results in increased GA levels allowing germination to proceed. It is well documented that *aba2-2* seeds can germinate in the dark when irradiated with FR light and that this process enhances expression of GA biosynthesis and response genes and increases active GA levels (Seo *et al.* 2006). GA biosynthesis inhibitor paclobutrazol can inhibit the germination ability of the *aba2-2* seeds under FR light conditions. This suggests that GA biosynthesis may be increased in the absence of the ABA-deficient locus (Seo *et al.* 2006). Similarly, the light/GA pathway induces tobacco *βGlu I* expression in the micropylar endosperm and enhances endosperm rupture (Kucera *et al.* 2005). Exogenous ABA can reduce the expression of *βGlu I* and inhibit endosperm rupture but not testa rupture (Leubner-Metzger 2002). These data suggest that ABA degradation mediates the interconnection between GA and ABA biosynthesis pathways during seed germination.

The existing knowledge of the regulation of GA and ABA biosynthesis-, deactivation- and signal transduction-associated genes mentioned above has contributed significantly to the understanding of the molecular and genetic mechanisms of seed germination. However, genes associated with *sensu stricto* germination involved in increasing embryo growth potential and endosperm weakening need to be further studied in order to better elucidate the mechanisms of seed germination. Although important genes that are associated with either promotion or inhibition of seed germination have been identified using

mutagenesis in various model plant species, these expressed genes are limited and the tissue specificity of them is not well understood.

In this study, we identified and investigated more genes associated with seed germination *sensu stricto* using an enhancer-trap approach. We took advantage of the reporter gene expression to directly search for genes expressed in either the endosperm or embryo prior to the completion of germination. Tissue- and stage-specific expression was analyzed and the biological function of identified genes was also investigated. The enhancer-trap vector which contained a reporter gene fused to a MP was randomly inserted into Arabidopsis plant genome to generate the enhancer-trap population (Campisi *et al.* 1999). This reporter gene is only activated when the MP::GUS construct is inserted near the enhancer or promoter region of a gene. This genetic tool was applied for seed germination research to screen for tissue-specific gene expression during *sensu stricto* germination. Diverse reporter gene expression patterns in germinating and germinated seeds and some potential trapped genes are summarized in Chapter 2. Characterization of one of the trapped genes involved in seed germination is presented in Chapter 3. The biological function of this trapped gene was extensively studied using knockout mutants. This zinc finger (GATA type) protein gene, whose expression increases during cold stratification plays important roles in breaking seed dormancy and germination. To encourage the utilization of the *Seed-GUS-Expression* enhancer-trap lines by the seed research community, detailed procedures for the identification the T-DNA insertion site and for the

functional characterization of candidate/trapped genes are documented in Chapter 4. Characterization of more seed germination-associated genes will advance our understanding of the molecular mechanisms of seed germination.

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Chapter 2

Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes

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ABSTRACT

Enhancer trap is a powerful approach for identifying tissue- and stage-specific gene expression in plants and animals. For Arabidopsis research, GUS enhancer-trap lines have been created and successfully used to identify tissue-specific gene expression in many plant organs. However, limited applications of these lines for seed germination research have been reported. This is probably due to the impermeability of the testa to the GUS substrate. By focusing on the stages between testa and endosperm rupture, we were able to circumvent the testa barrier to the GUS substrate and observe diverse tissue-specific gene expression during germination *sensu stricto*. One hundred and twenty-one positive subpools of 10 lines out of 1,130 were isolated. Approximately 4,500 plants from these subpools were grown in a greenhouse and one to seven individual plants exhibiting GUS expression in seeds were isolated for each subpool. This library of the Arabidopsis seed enhancer-trap lines is an efficient tool for identifying seed germination-associated genes. The individual lines from this library will be provided to the international seed biology research community. International collaboration to identify the trapped genes using genome-walking PCR and to characterize the gene functions using knockout plants will significantly enhance our understanding of the molecular mechanisms of seed germination.

Introduction

The Arabidopsis testa mutants have advanced our knowledge of seed biology, revealing that the chemical and physical properties of Arabidopsis testa significantly affect seed dormancy. The critical genes which determine the properties of testa are expressed in the endothelium of developing seeds (Debeaujon *et al.*, 2001, 2003; Sagasser *et al.*, 2002).

In mature Arabidopsis seeds, the testa is no longer a living tissue. The major sites of gene expression in imbibed Arabidopsis seeds are in the internal living tissues such as the embryo and the endosperm. Therefore, to comprehensively understand the mechanism of seed germination, it is necessary to characterize gene expression in these two tissues during imbibition. cDNA microarrays and proteomics have provided emerging data on gene expression in germinating Arabidopsis seeds (Gallardo *et al.*, 2001, 2002; Ogawa *et al.*, 2003). Although these are robust approaches to identify seed-expressed genes, the tissue specificity of the expression of these genes is unknown.

Enhancer- and gene-trap techniques using the GUS reporter gene have successfully been applied to identify tissue- and stage-specific gene expression in Arabidopsis leaves and inflorescences (Campisi *et al.*, 1999; He *et al.*, 2001) and carrot somatic embryos (Ko and Kamada, 2002). This technique has also been used to identify embryo- and endosperm-specific gene expression in developing Arabidopsis seeds (Stangeland *et al.*, 2003). However, to our knowledge, only one successful application of gene-trap technique for seed germination studies in

Arabidopsis has been reported (Dubreucq *et al.*, 2000). The GUS substrate cannot penetrate the testa of Arabidopsis seeds to probe the sites of gene expression (i.e. the embryo and the endosperm) in germinating seeds of Arabidopsis because the testa is impermeable to the substrate. Therefore, it may be difficult to detect GUS activity and identify seed germination-associated genes in germinating seeds of the enhancer-trap lines. To investigate the mechanism of seed germination *sensu stricto*, it is essential to characterize gene expression in imbibed seeds before radicle protrusion, which is the first visible sign that germination is complete.

We characterized the morphological changes during Arabidopsis seed germination and identified the lag phase between testa and endosperm rupture. This lag phase has also been observed in lettuce (Cantliffe *et al.*, 1984; Pavlista and Haber, 1970; Pavlista and Valdovinos, 1978) and tobacco seeds (Leubner-Metzger, 2002; Leubner-Metzger *et al.*, 1996). We focused on the lag phase between testa and endosperm rupture and successfully detected GUS activity in the embryo and the endosperm of germinating Arabidopsis seeds. In this chapter, we discuss the isolation of many positive enhancer-trap lines showing tissue-specific GUS expression in seeds and demonstrate the feasibility of using these lines for identification and characterization of seed-expressed genes.

Results

Arabidopsis seed germination and the timing of GUS staining

The predominant tissue in mature Arabidopsis seed is the embryo whose large cotyledons are the major sites of seed reserves, which are mobilized during seedling establishment. Arabidopsis seed contains only a single cell layer of endosperm tissue that is poorly visible when performing an ordinary germination test (Figure 2-1a). However, the endosperm is distinguishable from the embryo using SEM (Figure 2-1b). The endosperm and the embryo have distinct cell surface structures: the epidermal cells of the radicle are elongated while the endosperm cells appear more hexagonal (Figure 2-1c, d).

We conducted careful inspection of morphological changes in imbibed wild-type Arabidopsis seeds under a dissection microscope every 3 h and we also fixed seeds at different stages of germination for SEM to observe fine structural changes. The first apparent morphological change in germinating seed was testa rupture which generally occurred just above the embryonic axis, indicating that the expansion of the embryonic axis was a major driving force to open the testa. Testa rupture started 18 h after incubation at 22°C. There was at least a 12-h delay before endosperm rupture (or radicle emergence; Figure 2-2). During this period, the endosperm enclosing the embryo gradually elongated and emerged from the opened testa, and the radicle tip subsequently penetrated the endosperm layer in the micropylar region (endosperm cap; Figure 2-1e). Thus, the elongation of the endosperm was observed between the occurrence of testa and endosperm rupture,

suggesting that endosperm weakening occurs and allows the radicle to elongate inside the endosperm before the completion of germination. We observed a similar pattern of seed germination in the Arabidopsis enhancer-trap lines (Thomas Jack lines, 1130 pools of 10 lines, CS31086, ABRC at Ohio State University) and focused on this lag phase for GUS staining. By staining the seeds showing testa rupture (22-h imbibition at 22°C), we were able to detect tissue-specific GUS expression in seeds. The application of GUS substrate after testa rupture allowed penetration of GUS substrate into the endosperm and the embryo early enough to detect gene expression during germination *sensu stricto*.

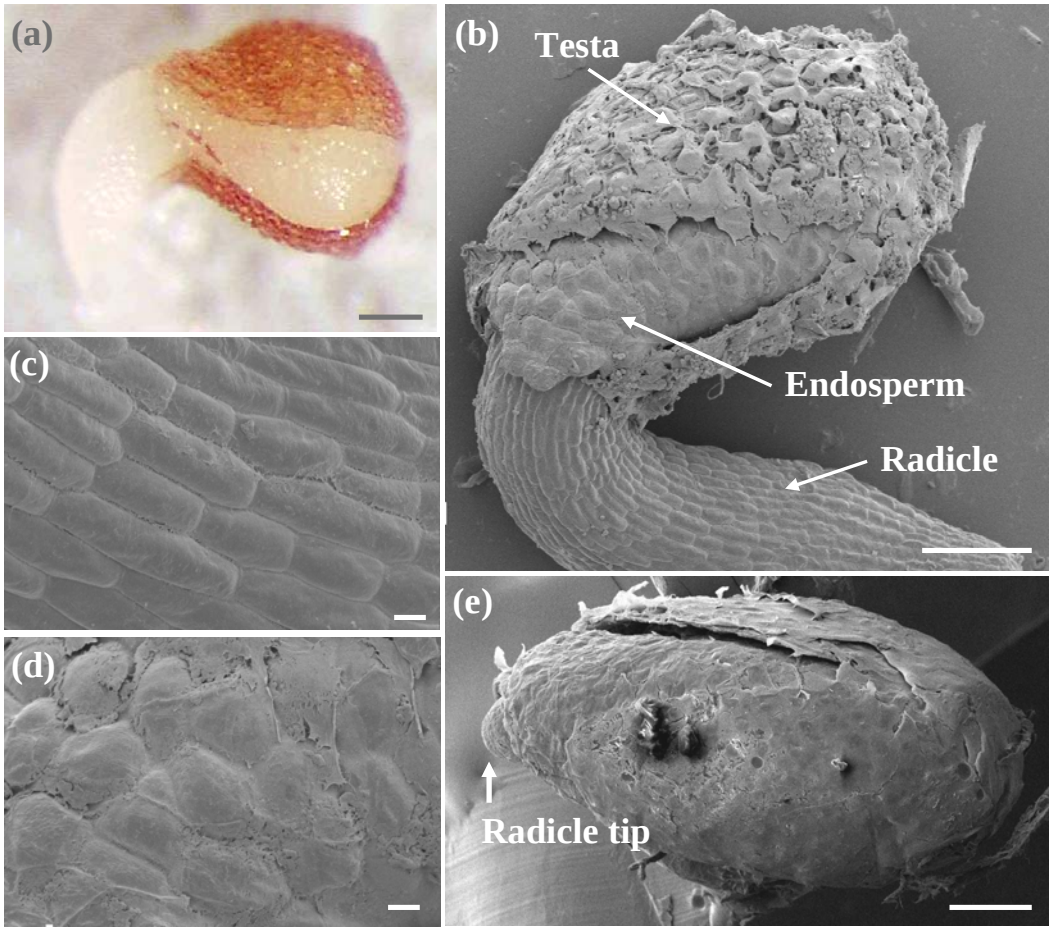

Figure 2-1. Arabidopsis seed structure following germination.
(a) Germinated Arabidopsis seed under a dissection microscope.
(b) SEM of germinated Arabidopsis seeds.
(c) Close up view of the surface cells of the radicle.
(d) Close up view of the surface cells of the endosperm.
(e) SEM of germinated seed immediately after radicle emergence. Note that the endosperm elongates and emerges from the opened testa, the radicle tip penetrates the endosperm layer.
Scale bars = 100 μm for (a), (b) and (e); 10 μm for (c) and (d).

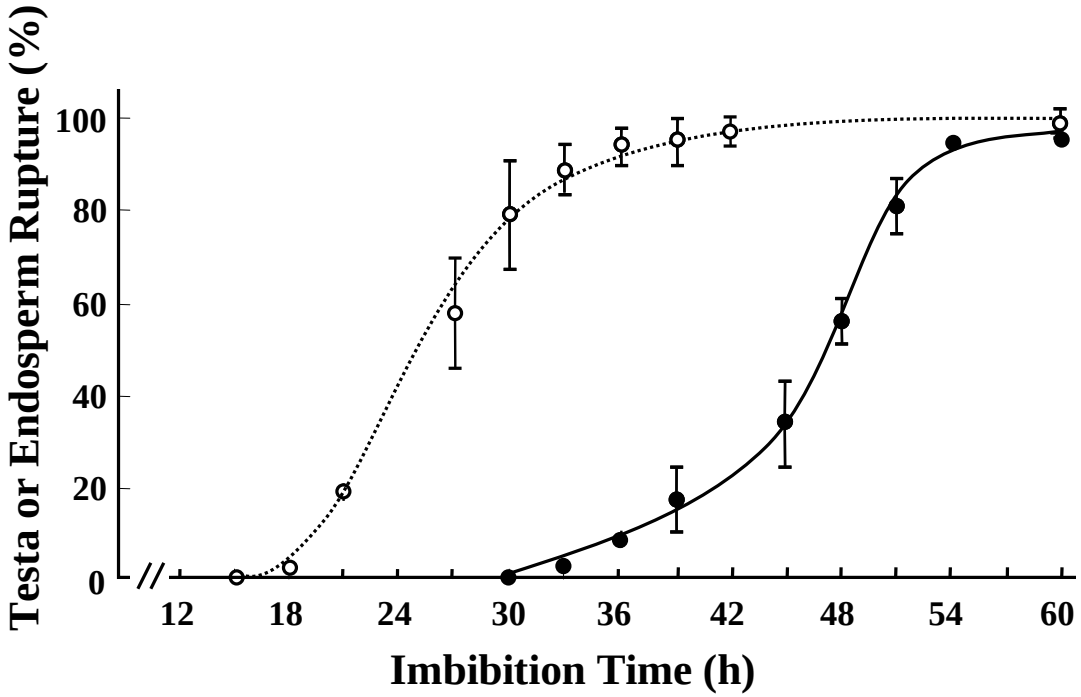

Figure 2-2. Germination time course of wild-type Arabidopsis (Columbia-0) seeds. Completion of germination, which was defined as penetration of radicle through the endosperm layer (endosperm rupture; closed symbol), was recorded together with the occurrence of testa rupture (open symbol). Imbibition time indicates time of incubation at 22°C. Each data point represents the average of three replicates; vertical bars indicate SD.

Tissue-specific GUS expression in germinating seeds

The Arabidopsis enhancer-trap population was screened for seed-expressed genes. A total of 121 positive pools showing GUS expression in germinating and germinated seeds were identified. GUS activity expressed in seeds was generally strong and easily detected using a dissection microscope. The patterns of GUS staining in seeds varied. In some lines, the whole seed (except for testa) showed GUS activity (Figure 2-3a) including the embryo and the endosperm (Figure 2-3b, c, respectively), whereas other positive lines displayed tissue-specific GUS expression in the axis, hypocotyl and hypocotyl plus cotyledons (Figure 2-3d–f, respectively). One line displayed GUS expression exclusively at the tips of the cotyledons (Figure 2-3g). Post-germinative GUS expression was also detected (Figure 2-3h).

Overall, GUS expression localized in the micropylar region (endosperm plus radicle tip) was the predominant pattern in germinating Arabidopsis seeds (Figure 2-4). Of the 121 positive subpools, 91 subpools exhibited GUS activity exclusively in the micropylar region of the seed. Micropylar-localized GUS staining was found mostly in seeds with a large testa rupture, however, a small percentage of seeds with a slight testa rupture also showed micropylar staining. GUS staining was detected in both the endosperm cap and the radicle tip in some lines. It is possible that the GUS signal in these lines was transferred between tissues in close contact to each other. Other lines having micropylar-localized GUS activity exhibited the expression in either an embryo-specific or an endosperm-

specific manner. In embryo-specific expression lines, the GUS activity was detected only in the radicle tip, which was enclosed by the endosperm layer (Figure 2-4a–c). The GUS staining in these seeds was less intense compared with other lines because the site of activity was still enclosed by the unstained endosperm layer (Figure 2-4b). However, when the embryo was dissected from the seed, an intense color was observed in the radicle tip, which confirmed the specificity of embryo expression (Figure 2-4c). In contrast, in other lines with endosperm cap-specific GUS expression, the GUS signal was clearly visible from the outside of testa. The specificity of GUS expression in these lines was confirmed by dissecting seeds into micropylar and lateral halves and also into embryo and endosperm parts (Figure 2-4d–g).

It is possible that the micropylar-localized expression is a reflection of limited infiltration of the GUS substrate during staining. The micropylar region of the seed is the first to take up the substrate because testa rupture is initiated in this part of the seed. To examine this possibility, we scratched and removed the testa at the lateral part of the seed and then stained with GUS substrate. The GUS activity was still restricted to the micropylar region in scratched seeds, while the control tetrazolium staining was detected in the entire seed except for the testa (Figure 2-4h). These results indicated that the micropylar-localized GUS staining was not a consequence of limited substrate uptake but that it represents tissue-specific gene expression. Diverse patterns of tissue-specific GUS expression in germinating and germinated Arabidopsis seeds are summarized in Figure 2-5.

To isolate individual positive plants showing GUS expression in seeds, 30 plants from each positive subpool were grown in the greenhouse and seeds were harvested from each plant. The second screening for GUS expression using these seeds allowed us to isolate one to seven GUS-positive individual plants for each subpool. The seed-GUS-expressing lines have been kept as a library.

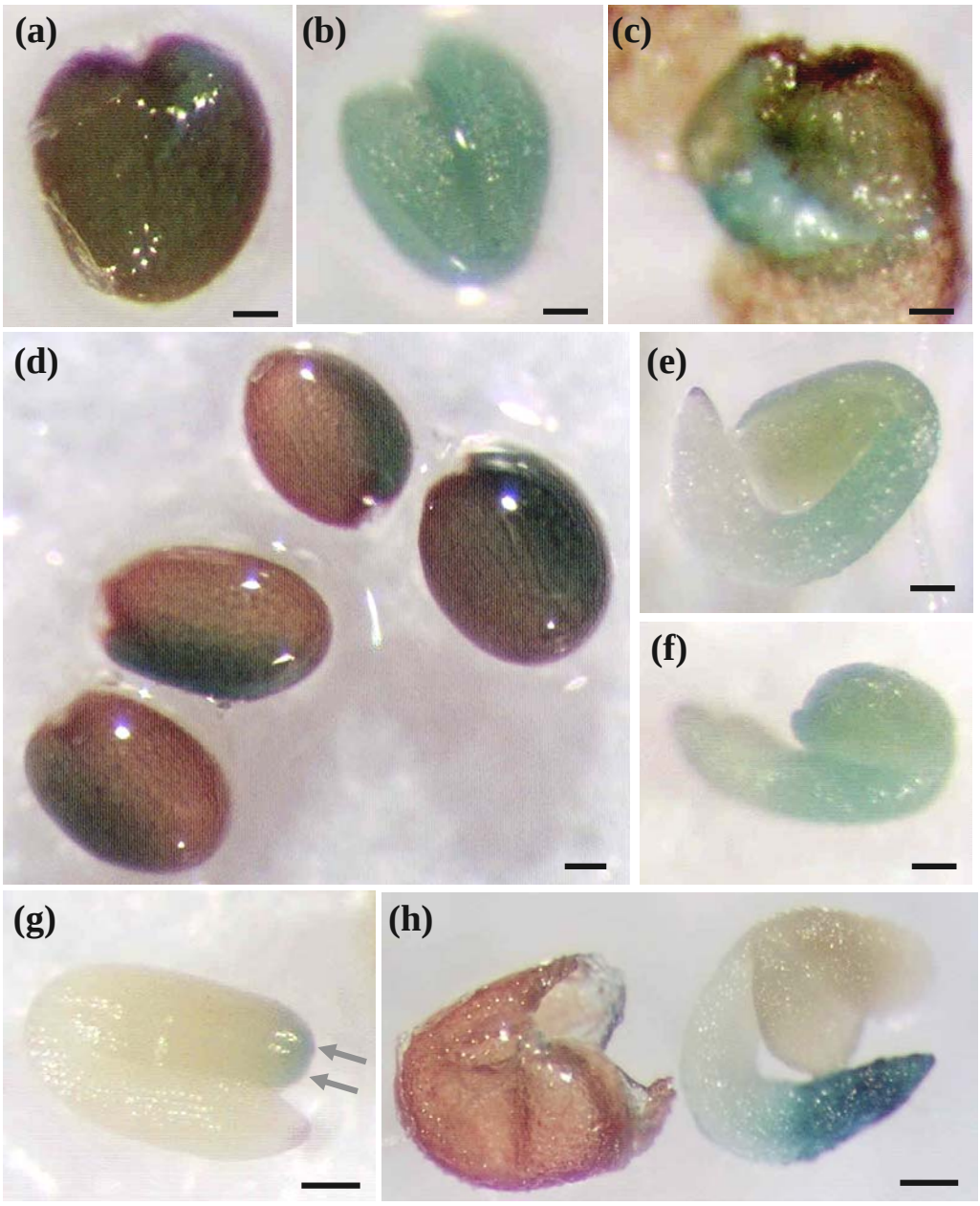

Figure 2-3. Variable tissue-specific GUS expression in germinating and germinated seeds of the enhancer-trap Arabidopsis lines.
(a–c) GUS expression in the whole seed (except for testa), the embryo and the endosperm in the same seeds, respectively.
(d–g) GUS expression in the whole axis, hypocotyl, hypocotyl plus cotyledons and cotyledon tips (arrows) in germinating seeds, respectively.
(h) GUS expression in whole radicle of germinated seed.
Scale bars = 10 μm.

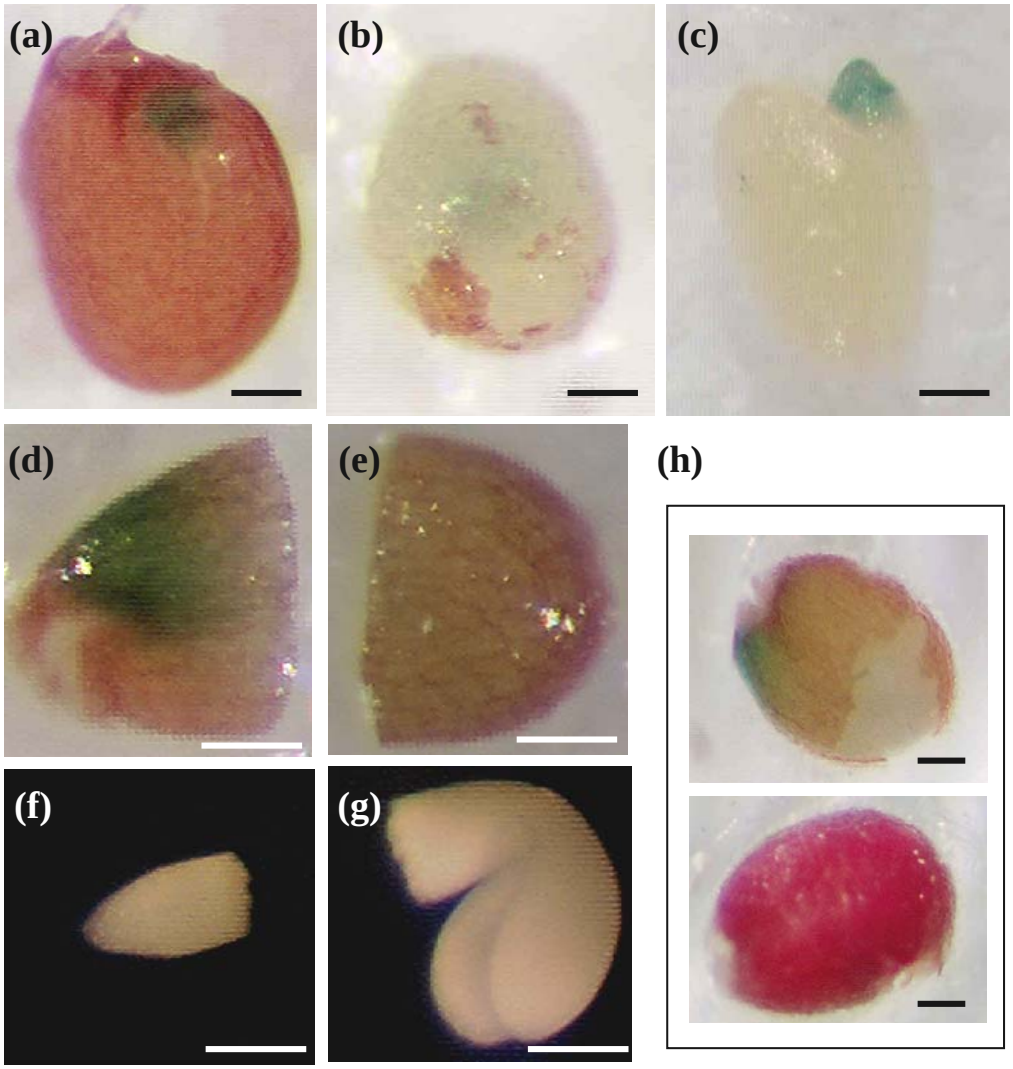

Figure 2-4. Micropylar region-localized GUS expression in germinating Arabidopsis seeds.
(a–c) Embryo-specific GUS expression. The intensity of the GUS staining is relatively weak because only the embryo has expression. Note that weak GUS staining is visible through the unstained endosperm layer in (b). The isolated embryo is shown in (c).
(d–g) Endosperm-specific GUS expression. GUS activity is detected only in the micropylar half of the endosperm (with testa; d), but not in the lateral half of the endosperm (e), radicle (f) and hypocotyls plus cotyledons (g).
(h) GUS and TZ staining of 'scratched' seeds. Part of testa was removed before staining to enhance the penetration of the substrate or dye to test the specificity of GUS staining.
Scale bars = 10 μm.

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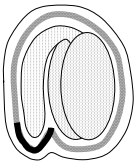
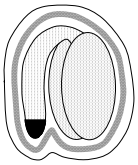
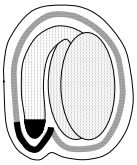
(91)</td><td> 
Endosperm cap</td><td> 
Radicle tip</td><td> 
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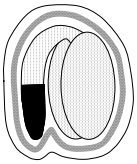
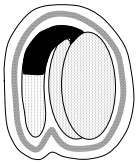
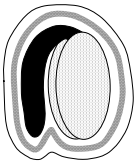
(21)</td><td> 
Radicle</td><td> 
Hypocotyl</td><td> 
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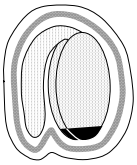
Endosperm</td><td> 
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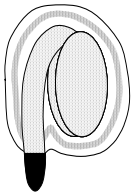
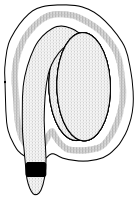
(9)</td><td> 
Root</td><td> 
Elongation Zone</td><td></td></tr>
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Figure 2-5. Summary of tissue-specific GUS expression patterns in germinating and germinated seeds of the Arabidopsis enhancer-trap lines.
The positions of GUS activity are highlighted in the schematic representations of seeds by black filling. The number of lines that showed micropylar-localized or other patterns of GUS expression in germinating seeds and root-specific expression in germinated seeds are shown in the figure.

The feasibility of identifying and characterizing the trapped genes

To evaluate the feasibility of the enhancer-trap approach for identifying seed-expressed genes, gene expression in one of the positive lines TMH1 that showed root-specific GUS expression was characterized. All TMH1 progeny exhibited GUS expression when germinated. GUS expression detected in the next generation of seeds indicated that the T-DNA lines were stable transformants. To identify the T-DNA insertion site in TMH1, genome-walking PCR was conducted using T-DNA RB-specific primers and adapter primers (see Materials and Methods). An approximately 0.7 kb DNA fragment was amplified from genome-walking PCR, sequenced, and found to contain the T-DNA RB (data not shown). The DNA sequence flanking the T-DNA RB was analyzed using the SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress) and predicted to be in the Arabidopsis chromosome V. The putative insertion site was located in the intergenic region between At5g02750 (zinc finger protein) and At5g02760 (protein phosphatase 2C) (Figure 2-6a). To verify the insertion site, PCR was conducted using a TSP and a GSP designed to part of the intergenic sequence of the putative insertion site. A DNA fragment of the predicted size (0.6 kb) was amplified from the genomic DNA of TMH1 but not from wild-type Arabidopsis genomic DNA (Figure 2-6b). The DNA sequence of the PCR product verified the junction between the genomic DNA and the T-DNA, confirming the predicted insertion site (data not shown).

The insertion site was detected in the intergenic region of the two genes *At5g02750* and *At5g02760*, indicating that GUS expression detected in the TMH1 is most likely driven by the enhancer(s) of one of these two candidate genes. RT-PCR was conducted to examine the potential mRNA expression of these two genes in wild-type Arabidopsis seeds. No RT-PCR product was obtained with *At5g02750*-specific primers, whereas a product of the predicted size (1.1 kb) was amplified with *At5g02760* primers (Figure 2-6c, RT). The DNA sequence of the RT-PCR product of *At5g02760* matched the coding region of the genomic DNA sequence of the same gene. Both sets of primers amplified a DNA fragment of the predicted size (0.7 and 1.4 kb for *At5g02750* and *At5g02760*, respectively) from wild-type Arabidopsis genomic DNA (Figure 2-6c, gDNA). These results indicate that the *At5g02760* gene is expressed in Arabidopsis seeds. Thus, isolation of an enhancer-trap line carrying GUS expression in seeds, and characterization of the T-DNA insertion site and gene expression analysis allowed us to identify the seed-expressed protein phosphatase 2C gene. This demonstrates the feasibility of using the enhancer-trap lines isolated from our large-scale screening to identify tissue-specific genes in Arabidopsis seeds. The T-DNA insertion sites identified for some other lines are shown in Figure 2-7.

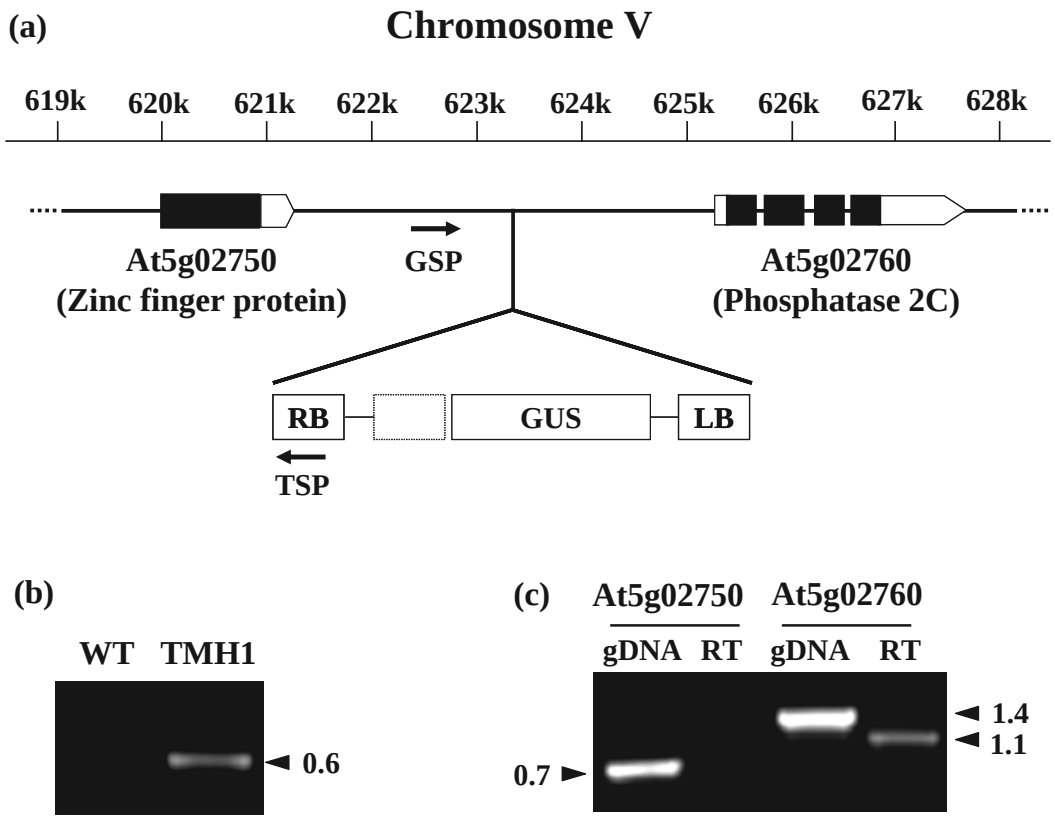

Figure 2-6. Identification of the T-DNA insertion site in the TMH1 enhancer-trap line.
(a) Schematic representation of the putative T-DNA insertion site in the TMH1 line. The positions of GSP and TSP used to verify the predicted insertion site (see below) are shown.
(b) PCR product amplified with the GSP and TSP from wild type (WT) and the enhancer-trap line (TMH1) genomic DNA.
(c) PCR products amplified using *At5g02750* and *At5g02760* gene-specific primers (see Materials and Methods) with wild-type Arabidopsis genomic DNA (gDNA) and reverse transcription products (RT) of total RNA extracted from germinated (36 h) wild-type seeds. The sizes (kb) of the amplified DNA fragments are shown on the right of panel (b) and on both sides of panel (c).

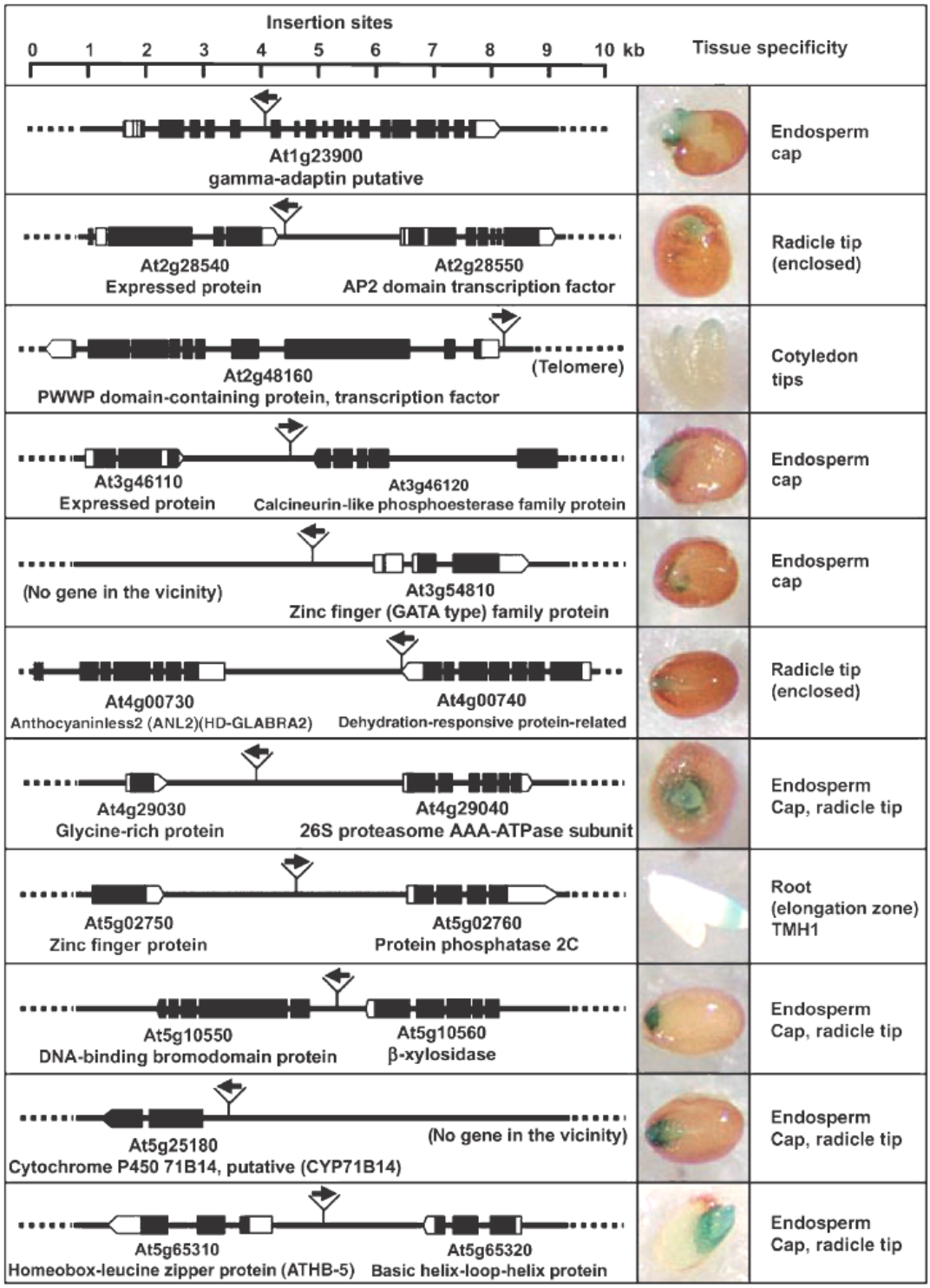
Insertion sites
0 1 2 3 4 5 6 7 8 9 10 kb
Tissue specificity
At1g23900
gamma-adaptin putative
Endosperm cap
At2g28540
Expressed protein
At2g28550
AP2 domain transcription factor
Radicle tip (enclosed)
At2g48160
PWWP domain-containing protein, transcription factor
(Telomere)
Cotyledon tips
At3g46110
Expressed protein
At3g46120
Calcineurin-like phosphoesterase family protein
Endosperm cap
(No gene in the vicinity)
At3g54810
Zinc finger (GATA type) family protein
Endosperm cap
At4g00730
Anthocyaninless2 (ANL2)(HD-GLABRA2)
At4g00740
Dehydration-responsive protein-related
Radicle tip (enclosed)
At4g29030
Glycine-rich protein
At4g29040
26S proteasome AAA-ATPase subunit
Endosperm Cap, radicle tip
At5g02750
Zinc finger protein
At5g02760
Protein phosphatase 2C
Root (elongation zone) TMH1
At5g10550
DNA-binding bromodomain protein
At5g10560
β-xylosidase
Endosperm Cap, radicle tip
At5g25180
Cytochrome P450 71B14, putative (CYP71B14)
(No gene in the vicinity)
Endosperm Cap, radicle tip
At5g65310
Homeobox-leucine zipper protein (ATHB-5)
At5g65320
Basic helix-loop-helix protein
Endosperm Cap, radicle tip

Figure 2-7. The T-DNA insertion sites identified in the enhancer-trap lines and their GUS expression patterns.
The genomic DNA region (10 kb) in the vicinity of the T-DNA insertion sites (arrows) containing the candidate trapped genes is shown in the table. Arrows at the insertion sites indicate the direction of GUS gene relative to the Arabidopsis genes in the genome. Gene structures are based on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).

Discussion

A substantial amount of information on the biochemical and molecular mechanisms of seed germination and tissue-specific gene expression was obtained using tomato seeds as a model system (Chen and Bradford, 2000; Chen *et al.*, 2001, 2002; Nonogaki *et al.*, 2000; Wu and Bradford, 2003; Wu *et al.*, 2001). However, only a limited number of germination-associated genes have thus far been characterized in tomato seeds. The detailed regulatory mechanisms controlling expression of these genes remain unknown.

Recent publications demonstrate that Arabidopsis can also be used as a powerful tool for seed germination research (Clerkx *et al.*, 2004; Sattler *et al.*, 2004; Yamauchi *et al.*, 2004). We have screened Arabidopsis enhancer-trap lines with detailed attention to seed morphology during germination and GUS staining. Morphological characterization of germinating seeds led to identification of a lag phase between testa and endosperm rupture, suggesting that the Arabidopsis endosperm cap also provides mechanical resistance to the radicle and is weakened before radicle protrusion. It is plausible that specific sets of genes are activated in the endosperm cap and the radicle tip to complete the prerequisite processes of radicle emergence (i.e. endosperm weakening and generation of growth potential of the radicle). The predominant GUS expression pattern detected in the enhancer-trap Arabidopsis seeds was micropylar-specific. One identified T-DNA insertion site was in the vicinity of the *β*-xylosidase gene (Figure 2-7), although its expression needs to be verified. Hydrolases such as xylosidase, which could be

involved in cell wall modification and could cause endosperm weakening and embryo expansion, are possibly expressed during germination. In tomato seeds, XTH genes are expressed exclusively in the endosperm cap and the radicle of germinating seeds (Chen *et al.*, 2002). Further characterization of the remaining lines may identify other cell wall-modifying proteins.

Expression of hydrolases and other cell wall proteins occurs in a micropylar-specific manner in multiple species including tomato (endo-*β*-1,3-glucanase and chitinase, Wu *et al.*, 2001; endo-*β*-mannanase, Nonogaki *et al.*, 2000; XTH, Chen *et al.*, 2002; expansin, Chen and Bradford, 2000), tobacco (endo-*β*-1,3-glucanase, Leubner-Metzger *et al.*, 1996), coffee (endo-*β*-mannanase, da Silva, 2002) and Arabidopsis (extensin, Dubreucq *et al.*, 2000). It is plausible that the activation of the micropylar region of seed is a widespread phenomenon for the induction of seed germination. However, the activation mechanism is unknown. The regulatory mechanism controlling expression of the genes encoding the hydrolases and other cell wall proteins is not yet fully understood.

Some T-DNA insertion sites that we identified for the enhancer-trap lines exhibiting micropylar-specific GUS expression were found in the vicinity of transcription factor genes, with one insertion site located between two transcription factors (HD-ZIP protein and bHLH protein; Figure 2-7). Further characterization of these lines could potentially provide useful information concerning upstream events during seed germination. Some of these transcription factors might be involved in determining tissue- and stage-specific expression of cell wall proteins

in germinating seeds. GUS signals in these lines were also detected in rosette and cauline leaves, inflorescences and siliques, indicating that some of the transcription factors which are potentially important for seed germination could also play roles in other stages of plant development (P.-P.L., N.K., T.M.H., J.R.H. and H.N., unpublished data). Only three of the 12 lines that we are currently characterizing exhibited GUS expression only in seeds, indicating that genes essential for seed germination may not necessarily be seed-specific.

Using the enhancer-trap screening protocol, we have created a library of 121 individual lines carrying GUS expression in seeds. Campisi *et al.* (1999) conducted segregation analyses for kanamycin resistance and suggested that no more than 25% of the enhancer-trap lines are likely to contain more than one insertion. We conducted Southern blot analyses for 40 lines and found that 24 plants contained a single insertion with the rest carrying mainly two or three insertions. At least 60% of our lines are most likely single insertion lines. For the lines with more than a single insertion, backcrossing to the wild type will be necessary to identify which insertion is responsible for seed-specific expression (He *et al.*, 2001).

As our experiments have demonstrated, it is feasible to utilize these lines for identifying seed-expressed genes. One line showed a T-DNA insertion site in the coding region of the gene, which potentially prevents gene expression (Figure 2-7, gamma-adaptin). This potential knockout line could be directly utilized for gene function analysis. The function of other identified genes can also

be characterized by obtaining the knockout plants from available resources. However, characterization of all the lines will require a tremendous amount of work. Genome-walking PCR and gene function analyses for all the isolated lines will require much time. Detailed gene expression analyses under different conditions such as dormant or non-dormant states and different hormonal treatments will also need to be carried out for each line to examine the physiological roles of the gene products. Therefore, we will not analyze all the isolated lines of the library but will focus on the genes that we have already identified. The remaining lines will be donated to the ABRC at Ohio State University and provided to the international research community. The detailed information on this library is provided on the website NSF-funded Integrative Seed Biology at Oregon State University (http://www.science.oregonstate.edu/isb/).

Materials and Methods

Plant materials and growth conditions

For germination, Arabidopsis (wild type Columbia-0) seeds were placed in 9 cm plastic petri dishes on two layers of filter paper (no. 2; Whatman Inc., Clifton, NJ) moistened with 4 ml water and incubated at 4°C for 3 days in the dark and at 22°C for 3 days under the light. The seeds were examined for testa rupture and radicle protrusion through the endosperm under a dissection microscope every 3 h.

Scanning electron microscopy

Germinating and germinated Arabidopsis seeds at different stages were fixed in 10 mM potassium phosphate buffer, pH 7.0 containing 4% (w/v) paraformaldehyde overnight and then dehydrated in an increasing ethanol series (30–100% v/v). Specimens in 100% ethanol were critical point dried with carbon dioxide in a Balzer CPD-020 dryer (Balzers Union, Ltd, Balzers, Liechtenstein) according to Anderson (1951). The dried specimens were mounted on an aluminum planchette and coated with approximately 10 nm of 60/40% Au/Pd using an Edwards S150B sputter coater (Edwards High Vacuum, Ltd, West Sussex, UK) operating at 1×10^{-2} Torr, 5 mbar argon pressure, 1.5 kV, 20 mA plasma current, for 60 sec. Examination was performed using the AmRAY 3300FE SEM (AmRay, Bedford, MA) in the Electron Microscope Facility, Department of Botany and Plant Pathology, Oregon State University.

Screening of Arabidopsis seeds for GUS expression

The Arabidopsis enhancer-trap lines (Thomas Jack lines, 1130 pools of 10 lines, CS31086, ABRC; Campisi *et al.*, 1999) were used for screening. Approximately 50 seeds from each subpool were placed on a small round filter paper (approximately 7 mm in diameter) moistened with water and placed on a metal stage for SEM specimens. The metal stage was cleaned thoroughly each time before placing another subpool of seeds to avoid contamination. One-hundred subpools of 50 seeds placed on small filter papers were incubated on two layers of larger (15 cm in diameter) filter papers placed in a plastic petri dish and incubated as described above. After 3-day pre-chilling at 4°C and 22 h incubation at 22°C, the small filter papers supporting the seeds were briefly blotted on dry filter papers to remove excessive water and then soaked in GUS substrate solution. GUS staining of seeds was performed as previously described (Weigel and Glazebrook, 2002) using 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100 and 2 mM X-Gluc (RPI Co., Mount Prospect, IL). Staining was examined with a dissection microscope after overnight incubation at room temperature (approximately 22°C).

Genome-walking PCR

Genomic DNA was extracted from Arabidopsis leaves using phenol extraction according to the QUICK-PREP method described at http://www.biotech.wisc.edu/NewServicesandResearch/Arabidopsis/ and used for

genome-walking experiments. Genome-walking PCR was performed using Genome Walker Kit (Clontech Laboratories, Inc, Palo Alto, CA) according to the manufacturer's manual. Briefly, the genomic DNA of the enhancer-trap TMH1 line was digested with *Dra*I. Adapter DNA provided in the kit was ligated to the *Dra*I-digested genomic DNA fragments. The first PCR was conducted using *Ex Taq* DNA polymerase (Takara, Madison, WI), an adapter primer (5'-GTAATACGACTCACTATAGGGC-3') provided with the kit and a T-DNA RB-specific primer (RB1: 5'-TCTAGAGTCGACCTGCAGGCATGCAAGCTT-3'). Second round PCR was conducted using the nested adapter primer (5'-ACTATAGGGCACGCGTGGT-3') and the nested T-DNA RB-specific primer (RB2: 5'-TCCCAACAGTTGCGCACCTGAATGGCGAAT-3'). The conditions used for the first PCR were: one cycle at 94°C (4 min), 80°C (2 min); seven cycles at 94°C (25 sec), 72°C (3 min); 32 cycles at 94°C (25 sec), 67°C (3 min) followed by one cycle at 67°C (7 min). The conditions used for the second PCR were: one cycle at 94°C (4 min), 80°C (2 min); five cycles at 94°C (25 sec), 72°C (3 min); 20 cycles at 94°C (25 sec), 67°C (3 min) followed by one cycle at 67°C (7 min).

PCR to verify the putative T-DNA insertion site

To verify the predicted T-DNA insertion site, a GSP (5'-ACGTTCCAAGGCCACATGTG-3') was designed for part of the intergenic sequence that was located upstream of the putative T-DNA insertion site and used in PCR with RB2 primer and genomic DNA from TMH1. The following

conditions were used for PCR: the initial denaturation at 94°C (4 min), touchdown cycles [94°C (15 sec), 67→ 61°C (15 sec), and 72°C (30 sec)] (one cycle for each temperature) and 25 cycles at 94°C (15 sec), 60°C (15 sec) and 72°C (30 sec) followed by extension at 72°C (7 min).

RNA extraction and RT-PCR

Total RNA was extracted from 36-h-imbibed Arabidopsis seeds using standard phenol-SDS extraction (Sambrook *et al.*, 1989). Two micrograms of DNase-treated total RNA was used for RT with a RETROscript Kit (Ambion, Austin, TX). The RT product was subjected to PCR using the primers for *At5g02750* (5'-ATGGAAGACGAAAACACCAC-3' and 5'-TTACGCCACGTGTAACTCG -3') and *At5g02760* (5'-ATGGTTAAACCCTGTTGGAG-3' and 5'-TCATGATGTTGAATGCATCG-3'). The following conditions were used for PCR: the initial denaturation at 94°C (4 min), touchdown cycles [94°C (15 sec), 62→ 56°C (15 sec), and 72°C (30 sec)] (one cycle for each temperature) and 25 cycles at 94°C (15 sec), 55°C (15 sec) and 72°C (30 sec) followed by extension at 72°C (7 min).

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Chapter 3

The BME3 GATA zinc finger transcription factor is a positive regulator of Arabidopsis seed germination

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ABSTRACT

In many plant species, seed dormancy is broken by cold stratification, a pre-chilling treatment of fully imbibed seeds. Although the ecological importance of seed response to cold temperature is well appreciated, the mechanisms underlying the physiological changes during cold stratification is unknown. In this chapter, we show that the GATA zinc finger protein expressed in Arabidopsis seeds during cold stratification plays a critical role in germination. Characterization of an enhancer-trap population identified multiple lines that exhibited GUS expression in the micropylar end of the seed (named *BME* lines). One of these lines, *BME3*, had a T-DNA insertion site in the 5' upstream region of a GATA-type zinc finger transcription factor gene (termed *BME3-ZF*). The *BME3-ZF* mRNA accumulated in seeds during cold stratification. Characterization of the *BME3-ZF* promoter indicated that this gene was activated specifically in the embryonic axis, which was still enclosed by the endosperm. The zinc finger gene knockout plants produced seeds exhibiting deeper dormancy, which showed reduced response to cold stratification. The ungerminated knockout seeds exhibited testa rupture, but failed to penetrate the endosperm layer. Application of gibberellic acid (GA_3) rescued impaired germination of knockout seeds without cold stratification, indicating that the normal GA signal transduction pathway is present in the knockout mutants. Expression of GA_{20}-oxidase and GA_3-oxidase genes was greatly reduced in the knockout seeds, suggesting the potential involvement of the

zinc finger protein in GA biosynthesis. These results suggest that the GATA zinc finger protein is a positive regulator of seed germination.

Introduction

The biological functions of GATA zinc finger proteins are well characterized in fungi and animals. Recent studies have provided emerging evidence that plant GATA zinc finger transcription factors also play significant roles in developmental control and responses to the environment. *HAN* encodes a GATA-3-like zinc finger protein containing the typical 18-aa-residue zinc finger loop ($CX_2CX_{18}CX_2C$) found in plants, and is important in determining the organ boundaries in the flower, shoot apical meristem and developing embryos. *HAN* controls the number and position of cells expressing *WUSCHEL*, and affects cell proliferation and differentiation (Zhao *et al.*, 2004). ZIM, a novel Arabidopsis GATA zinc finger transcription factor, has a zinc finger loop with 20 aa residues ($CX_2CX_{20}CX_2C$). ZIM is involved in hypocotyl and petiole elongation. The domain structure of the ZIM protein is found exclusively in plants (Shikata *et al.*, 2004).

GATA zinc finger transcription factors are also associated with plant responses to environmental stimuli and defense mechanisms. Many light-responsive promoters found in plants contain GATA motifs (Teakle *et al.*, 2002), suggesting that GATA zinc finger proteins are general regulators in light signal transduction. It is known that Arabidopsis GATA-1, GATA-2, GATA-3 and GATA-4 zinc finger genes are not developmentally regulated, but are responsive to light signals (Teakle *et al.*, 2002). The Arabidopsis GATA zinc finger protein CO (Putterill *et al.*, 1995) and its homologues in rice (*Oryza sativa*; Song *et al.*,

1998), wheat (*Triticum aestirum*; Nemoto *et al.*, 2003), and perennial ryegrass (*Lolium perenne*; Martin *et al.*, 2004) are associated with photoperiodic control of flowering. Expression of the moss (*Physcomitrella patens*) *CO/COL* homologue *PpCOL1* is controlled by the circadian clock, and is regulated photoperiodically at the gametophore stage when the rate of sporophyte formation is affected by day length. This suggests that the GATA zinc finger protein is also involved in the photoperiodic regulation of reproduction in moss (Shimizu *et al.*, 2004).

In tobacco (*Nicotiana tabacum*), the AGP1 GATA zinc finger protein binds to the specific motif AGATCCAA in the promoter region of *NtMyb2*, which is a regulator of the *PAL* gene. Both *PAL* and *NtMyb2* are induced by various stresses such as wounding and elicitor treatments (Sugimoto *et al.*, 2003). These results suggest that GATA zinc finger proteins also play critical roles in the defense response of plants. Thus zinc finger proteins are required for both developmental and environmental controls in plants.

Seed germination, which is a highly specialized phase of plant development, is also controlled by environmental signals such as cold temperature, light and soil nitrate concentration. Little is known about the involvement of GATA zinc finger proteins in seed germination. We found a GATA-type zinc finger transcription factor which was expressed in the micropylar end of germinating Arabidopsis seeds. Activation of genes in the micropylar end in both embryo and endosperm plays a significant role in seed germination of other plant species. Hydrolases and cell-wall proteins, which are involved in endosperm

weakening or generation of embryo growth potential, are induced in a micropylar-specific manner in tomato (*Lycopersicon esculentum*; Chen and Bradford, 2000; Chen *et al.*, 2002; Nonogaki *et al.*, 2000; Wu *et al.*, 2001), tobacco (Leubner-Metzger *et al.*, 1996), and coffee (*Coffea arabica*; da Silva, 2002). In this chapter, we characterized a micropylar-specific GATA zinc finger protein in Arabidopsis seeds. The biological function of this protein in breaking seed dormancy and positively regulating seed germination in Arabidopsis is discussed.

Results

Isolation of the BME enhancer-trap lines

Arabidopsis enhancer-trap lines [Thomas Jack lines, CS31086, ABRC, Ohio State University] were screened for seed germination-associated genes, and 121 lines showing tissue-specific GUS expression in seeds were isolated and donated to ABRC (Liu *et al.*, 2005a; Chapter 2). In this screen we found multiple independent lines exhibiting strong GUS signals at the micropylar end of the seed (Figure 2-5; Figure 3-1a). These were designated *BME* lines. GUS expression in these lines was detected when the radicle tip was still enclosed by the thin endosperm (stages I–III), indicating that the trapped genes are associated with *sensu stricto* germination, which is defined as physiological events before radicle emergence.

We further characterized one of these *BME* lines, *BME3*, which exhibited a relatively weak GUS signal in the embryo and endosperm (Figure 3-1b). GUS expression in *BME3* started before radicle emergence. GUS expression was not detected in developing seeds, but the top and bottom of developing and mature siliques in *BME3* exhibited GUS expression (Figure 3-1c). The progeny of the *BME3* line also showed the same expression patterns, indicating that the putative T-DNA insertion was transmitted genetically.

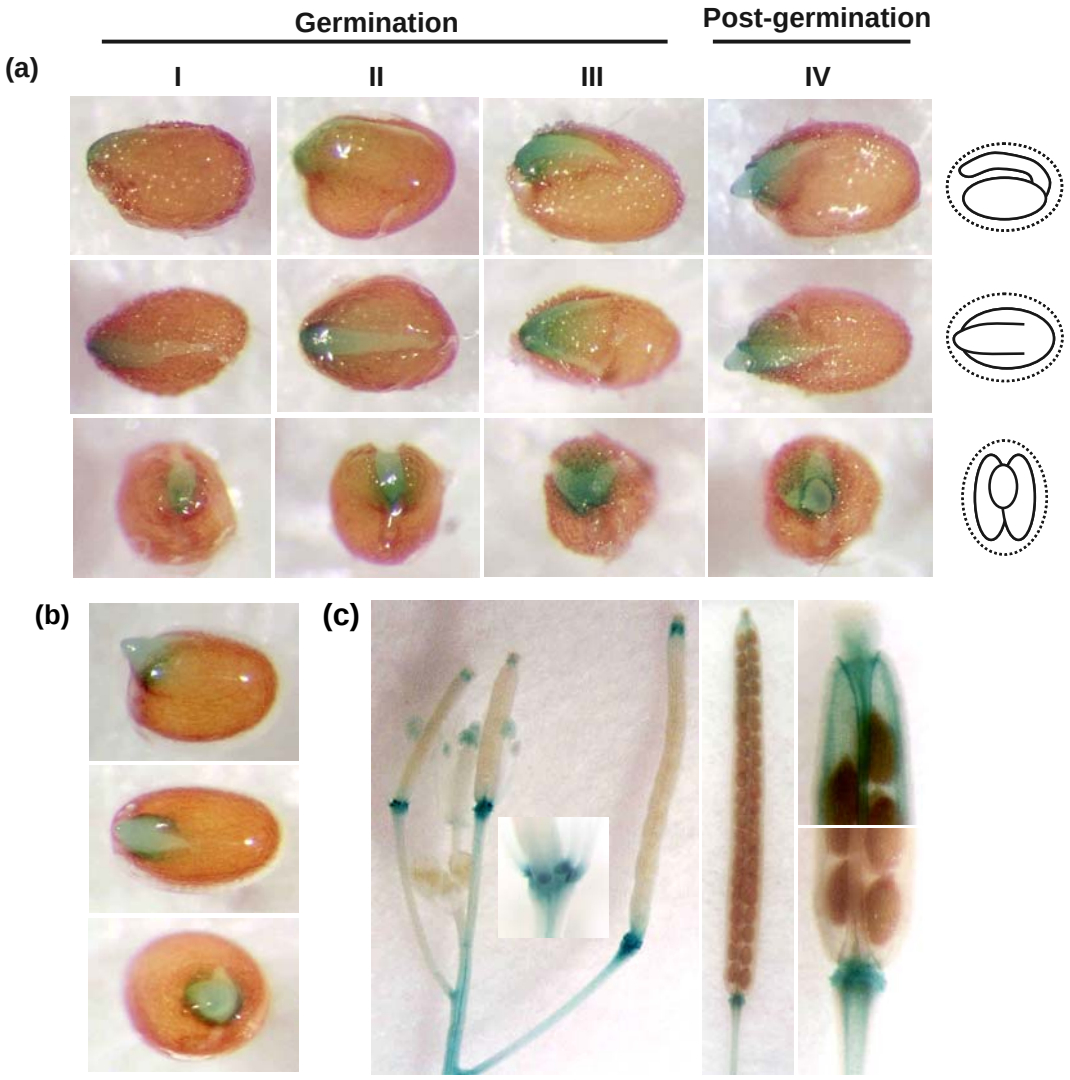


Figure 3-1. GUS expression in *BME* Arabidopsis enhancer-trap seeds.
(a) Representative images of GUS expression observed in *BME* lines. Three different views of seeds of a *BME* line (ABRC, stock number CS24447) are shown. In most *BME* lines GUS activity was detected in both embryo and endosperm before (stages I–III, germination) and after radicle emergence (stage IV, post-germination). Note that the radicle tip has penetrated the thin endosperm layer at stage IV.
(b) GUS expression detected in *BME3* seeds. Relatively weak expression was observed in this line.
(c) Left, GUS activity detected at top and bottom of developing siliques (inset: close-up view of receptacle); middle, GUS activity in mature silique; right, close-up views of top and bottom of mature silique.

Identification of the T-DNA insertion site in the BME3 line

To identify the *BME3* gene in the enhancer-trap line, genome-walking PCR was conducted using T-DNA RB-specific primers (RB1 and RB2) and adapter primers (see Materials and Methods). A DNA fragment of approximately 0.3 kb was amplified in the genome-walking PCR reaction (data not shown) and sequenced. Analysis of sequence data using the *SIGnAL T-DNA Express* Arabidopsis *Gene Mapping Tool* (http://signal.salk.edu/cgi-bin/tdnaexpress) indicated that the T-DNA insertion was located in Arabidopsis chromosome III. The T-DNA was inserted in the 5' upstream region of *At3g54810* (GATA-type zinc finger family protein) (Figure 3-2a). The nearest gene, *At3g54800* (lipid-binding START domain-containing protein), was located approximately 5 kb distant from the insertion site (data not shown). To verify the predicted T-DNA insertion site, PCR was conducted using the T-DNA RB-specific primer (RB2) and a GSP (Figure 3-2a). The predicted size of DNA fragment (0.5 kb) was amplified from the genomic DNA of the *BME3* line, but not from wild-type Arabidopsis genomic DNA (data not shown). The sequence of this PCR product contained the junction sequence between genomic DNA and the T-DNA right border, confirming the position of the predicted insertion site.

To examine potential expression of the candidate gene in seeds, RT-PCR was performed using RNA extracted from wild-type Arabidopsis seeds with gene-specific primers for *At3g54800* and *At3g54810*. An RT-PCR product of the predicted size (0.8 kb) was obtained for the *At3g54810* gene, while no PCR

product was obtained for the *At3g54800* gene (Figure 3-2b, RT). This RT-PCR product was sequenced and found to match the coding region of *At3g54810*. DNA fragments of the predicted size (1.9 and 1.1 kb for *At3g54800* and *At3g54810*, respectively) were amplified for both genes using wild-type Arabidopsis genomic DNA (Figure 3-2b, gDNA). These results indicate that the *At3g54810* gene is expressed in germinating Arabidopsis seeds.

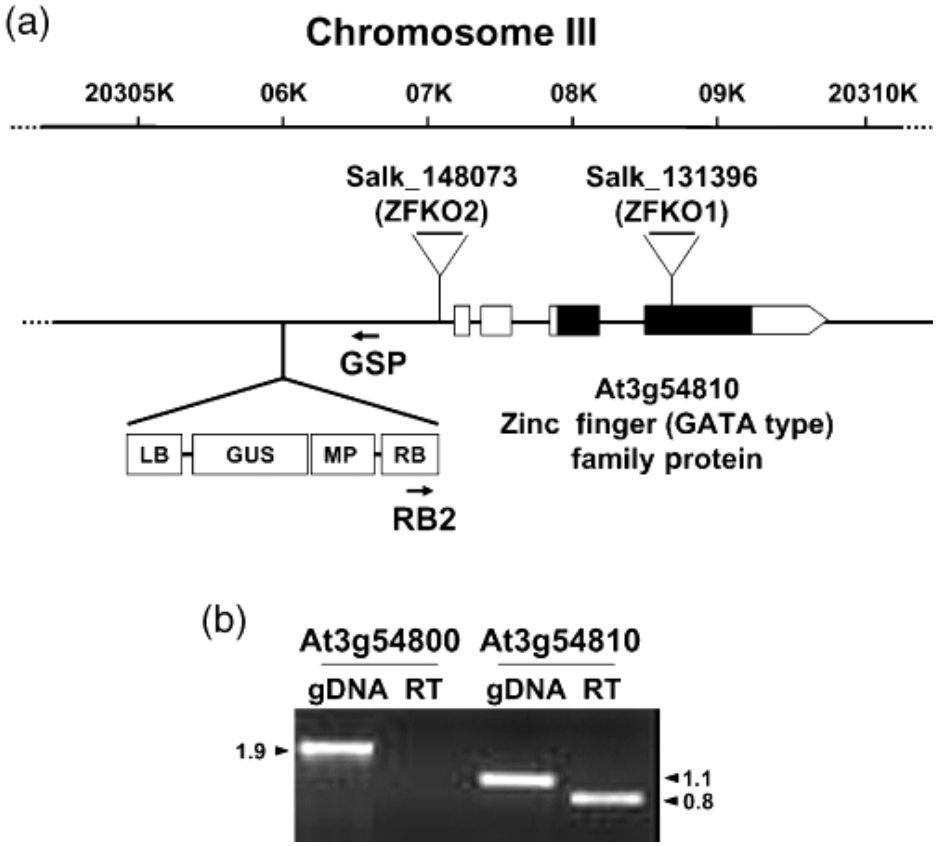


Figure 3-2. Identification and verification of the T-DNA insertion site and trapped gene in the *BME3* enhancer-trap line.
(a) Schematic representation of the predicted T-DNA insertion site in *BME3* line. The T-DNA flanking region was amplified by genome-walking PCR using T-DNA RB-specific primers RB1 and RB2 and adapter-specific primers (see Materials and Methods). The putative insertion site was identified by analyzing the sequence of the amplified DNA fragment with *SIGnAL T-DNA Express* Arabidopsis *Gene Mapping Tool* (http://signal.salk.edu/cgi-bin/tdnaexpress). The positions of the two primers, GSP and RB2, used for verification of the T-DNA insertion site and the T-DNA insertion sites of two putative SALK knockout lines [SALK_131396 (ZFKO1) and SALK_148073 (ZFKO2)], used for gene-function analysis (Figures 3-6 and Figure 3-7), are also shown in this scheme.
(b) PCR products amplified using *At3g54800* and *At3g54810* GSPs with wild-type Arabidopsis gDNA and with RT products of total RNA extracted from germinating seeds (12 h imbibition following 3 days' cold stratification). The predicted size (0.8 kb) of RT-PCR product was obtained from *At3g54810* but not from *At3g54800*, while PCR using gDNA with the same sets of primers amplified the predicted size (1.9 and 1.1 kb for *At3g54800* and *At3g54810*, respectively) of DNA fragments. Sizes (kb) of amplified DNA fragments are shown at both sides of the panel.

BME3 encodes a GATA zinc finger transcription factor

The *BME3* (*At3g54810*) gene encodes a transcription factor with a GATA-type zinc finger (termed *BME3-ZF*). The zinc finger domain was found in the second exon of the BME3-ZF gene (Figure 3-3a). The amino-terminal region of BME3-ZF contains an acidic domain which is enriched with Asp (D) and Glu (E) (Figure 3-3a,b). The predicted isoelectric point for the amino-terminal 100 aa of BME3-ZF was calculated to be approximately 3.5. The characteristic Ser and Lys repeats were found in the amino acid sequence. The BME3-ZF protein contained the motif typical of the $CX_2CX_{18}CX_2C$-type zinc finger (Figure 3-3b). Amino-acid sequence alignment of BME3-ZF and other related GATA zinc finger proteins in plants and fungi showed identical amino acids in the highly conserved regions of the C_2C_2 zinc finger domain. The BME3-ZF homologues in *N. tabacum* (NTAGBP-5) and *Oryza sativa* (OsGATA1) have extensive regions of amino acids identical to those found in BME3-ZF (Figure 3-3c).

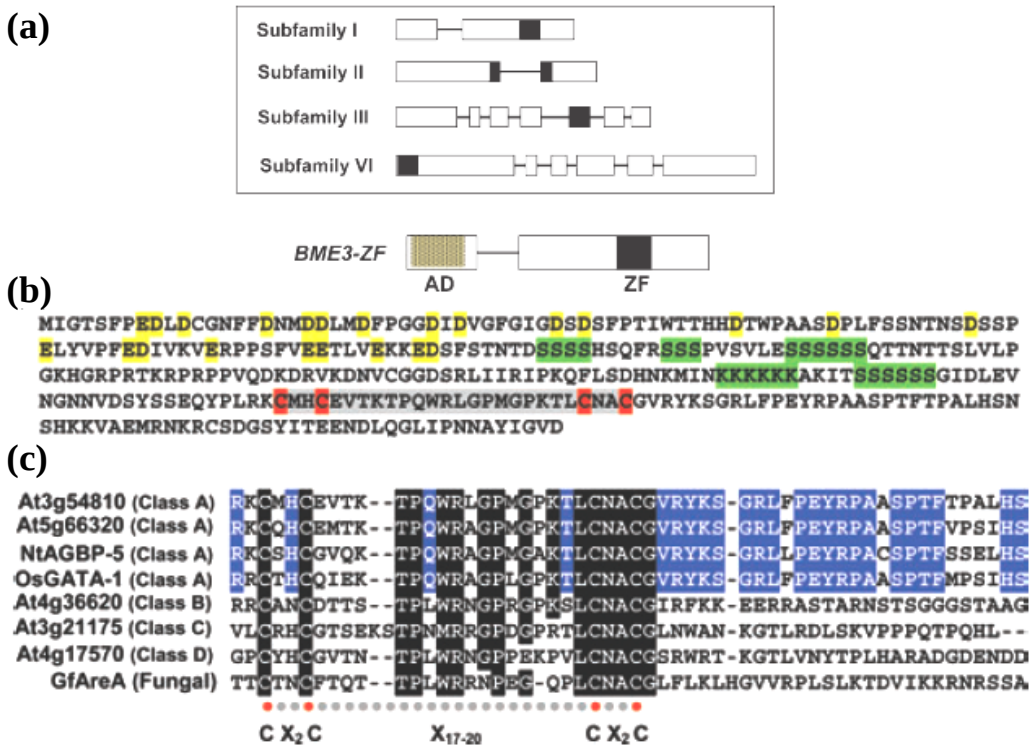


Figure 3-3. Gene structure and amino acid sequence of BME3-ZF.
(a) Schematic representation of gene structures of four different subfamilies of plant GATA zinc finger proteins (upper panel). Representative exon–intron organization in each subfamily shown according to Reyes *et al.* (2004). Black boxes, position(s) of zinc finger domains. The gene structure of the *BME3-ZF* gene is shown (lower). Yellow and black, positions of the acidic domain (AD) encoded in the first exon and the zinc finger domain encoded in the second exon of *BME3-Z*F, respectively.
(b) Amino acid sequence of BME3-ZF. Asp (D) and Glu (E) in the amino-terminal acidic region; yellow and green, Ser- (S) and Lys- (K) repeats, respectively. Red, Cys (C); gray, other amino acids in the zinc finger domain.
(c) Amino acid sequence alignment of zinc finger domains with flanking regions from BME3-ZF (At3g54810) and four other Arabidopsis GATA zinc finger proteins representing class A (At5g66320), B (At4g36620), C (At3g21175) and D (At4g17570) zinc finger proteins. BME3-ZF homologues in tobacco NtAGBP-5 and rice OsGATA-1, and a fungal GATA zinc finger protein *Gibberella fujikuroi* GfAreA, are also compared. Black, identical amino acids in the zinc finger domain of all proteins. Blue, extended identical amino acids found in the flanking region of the zinc finger domain in class A proteins. Red dots, Cys (C); gray dots, other amino acids in the zinc finger domain.

The BME3 zinc finger transcription factor gene is expressed during cold stratification

To examine the temporal expression of *BME3-ZF* in Arabidopsis seeds, semi-quantitative RT-PCR was conducted. *BME3-ZF* mRNA accumulation was detected in dry seeds at a very low level, increased during imbibition at 4°C (cold stratification) for the first 18 h, and decreased thereafter (Figure 3-4a). Relatively low, but constant, levels of mRNA accumulation were detected in seeds transferred to 22°C after 24 h cold stratification (data not shown). These results suggested that the biological function of BME3-ZF might be associated with physiological changes during cold stratification. The timing of *BME3-ZF* expression was a little earlier than that of the GA biosynthesis-enzyme genes *AtGA20ox3* and *AtGA3ox1*, which are also induced during cold stratification (Yamauchi *et al.*, 2004).

It is possible that *BME3-ZF* is a cold-inducible gene and is not associated with germination *per se*. To examine whether gene expression is correlated with seed germination, we characterized *BME3-ZF* expression in dormant Arabidopsis seeds kept in our laboratory. The dormant seeds had been harvested, dried and immediately stored at −80°C to maintain deep dormancy. The seeds were still capable of germinating without cold stratification, but at slower rates (Figure 3-4b). To determine if cold treatment is essential for the induction of *BME3-ZF*, we analyzed gene expression in dormant seeds incubated at 22°C without cold stratification. As shown in Figure 3-4(c), *BME3-ZF* expression was induced before radicle emergence. These results indicate that induction of *BME3-ZF* is not

absolutely dependent on cold induction, and suggest that the gene is probably associated with, and a prerequisite for, the induction of seed germination.

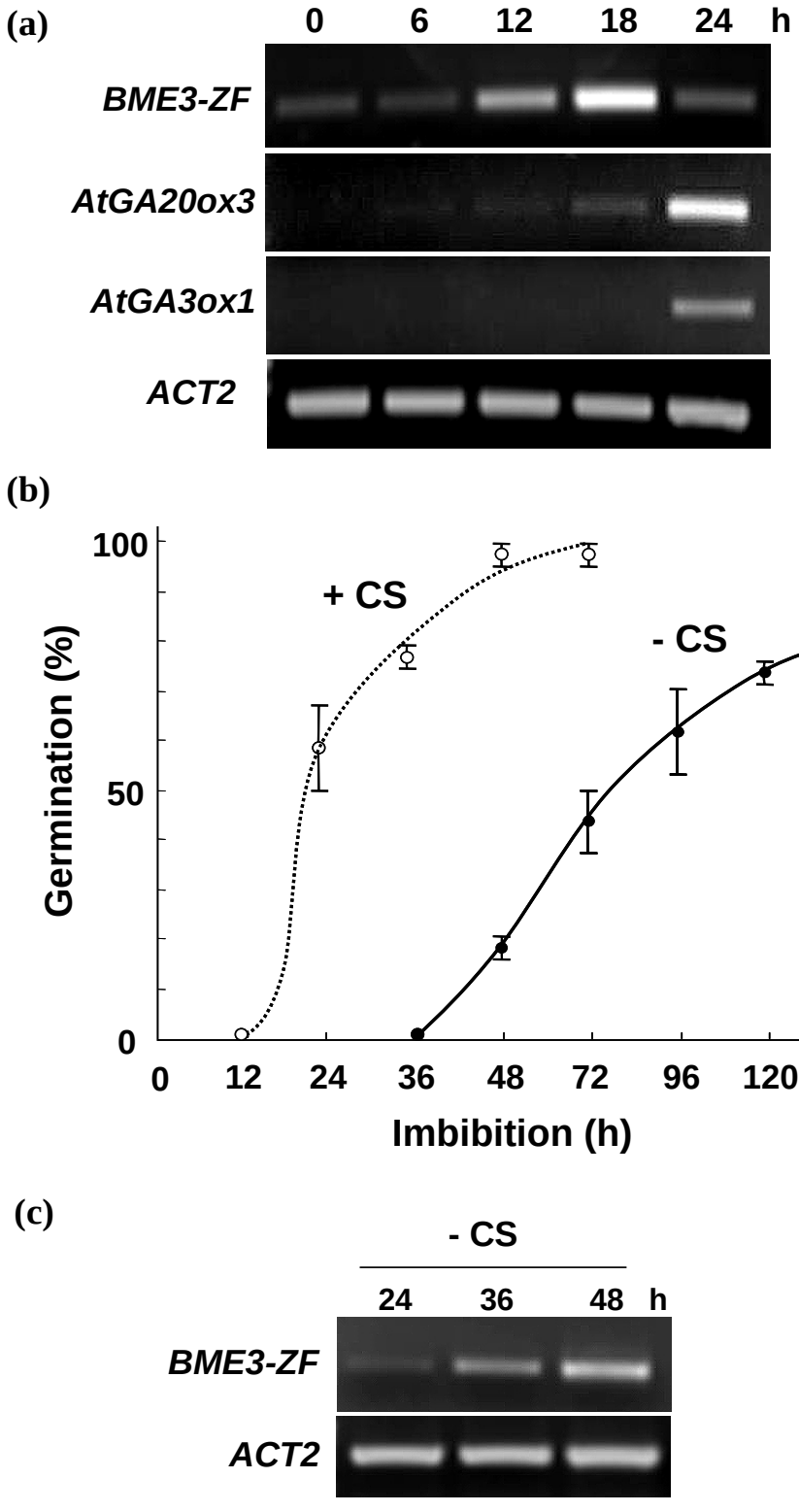

(a)
0
6
12
18
24
h
BME3-ZF
AtGA20ox3
AtGA3ox1
ACT2
(b)
100
50
0
Germination (%)
+ CS
- CS
0
12
24
36
48
72
96
120
144
Imbibition (h)
(c)
- CS
24
36
48
h
BME3-ZF
ACT2

Figure 3-4. Expression of *BME3-ZF* gene and germination in dormant Arabidopsis seeds.
(a) Semi-quantitative RT-PCR of *BME3-ZF*. RNA was extracted from seeds during cold stratification (0, 6, 12, 18 and 24 h) and used for reverse transcription. The expression of GA_{20}-oxidase (*GA20ox3*, *At5g07200*) and GA_3-oxidase (*GA3ox1*, *At1g15550*) genes, expressed in Arabidopsis seeds during cold stratification (Yamauchi *et al.*, 2004), was also examined. *ACT2* (*At3g18780*) was used as a control.
(b) Germination of dormant Arabidopsis seeds with (+CS) or without (−CS) 3 days' cold stratification. These seeds were used for experiments in (c). '+CS (cold stratification)' indicates exposure to 4°C for 3 days and an additional imbibition period.
(c) Semi-quantitative RT-PCR of *BME3-ZF*. RNA was extracted from dormant Arabidopsis seeds imbibed for 24, 36 and 48 h without cold stratification (−CS) and used for RT-PCR. *ACT2* was used as a control.

BME3-ZF transcription factor gene is activated in the embryonic axis

Although characterization of the *BME3* enhancer-trap line allowed us to identify expression of *BME3-ZF* in Arabidopsis seeds, Southern blot analysis using a GUS-specific probe indicated that this enhancer-trap line might have multiple T-DNA insertions (data not shown). There was a possibility that GUS expression detected in *BME3* enhancer-trap seeds reflected the promoter activity of other genes. To examine the spatial pattern of *BME3-ZF* expression, we amplified the 5' upstream region (−1075 to +19) of *BME3-ZF* by PCR, fused the promoter sequence to the GUS gene, and transformed Arabidopsis with this promoter::reporter construct. Seeds were produced from the transgenic plants and tested for GUS expression.

Germinating seeds showed very weak GUS activity after testa rupture, reproducing the characteristic expression of *BME3* seeds (Figure 3-5a). The GUS signal was detected in the embryo, which was enclosed by the thin, transparent endosperm layer (Figure 3-5b). The presence of GUS activity in the embryonic axis was confirmed by inspecting the excised embryo; little or no activity was observed in the endosperm (Figure 3-5c). These results indicate that the *BME3-ZF* gene is activated mainly in the embryo. The low-intensity GUS signal detected in the endosperm of *BME3* enhancer-trap seeds (Figure 3-1b) might have diffused from embryonic tissues, or could reflect the expression of another trapped gene in the *BME3* line. GUS activity was also detected at the top and bottom (receptacle)

of siliques (data not shown) of the transformants, which was consistent with the GUS-expression patterns observed in the original enhancer-trap line. The GUS gene of the inserted T-DNA in the enhancer-trap line was in opposite orientation to the *BME3-ZF* gene. Probably, the trapped enhancer can function in both directions.

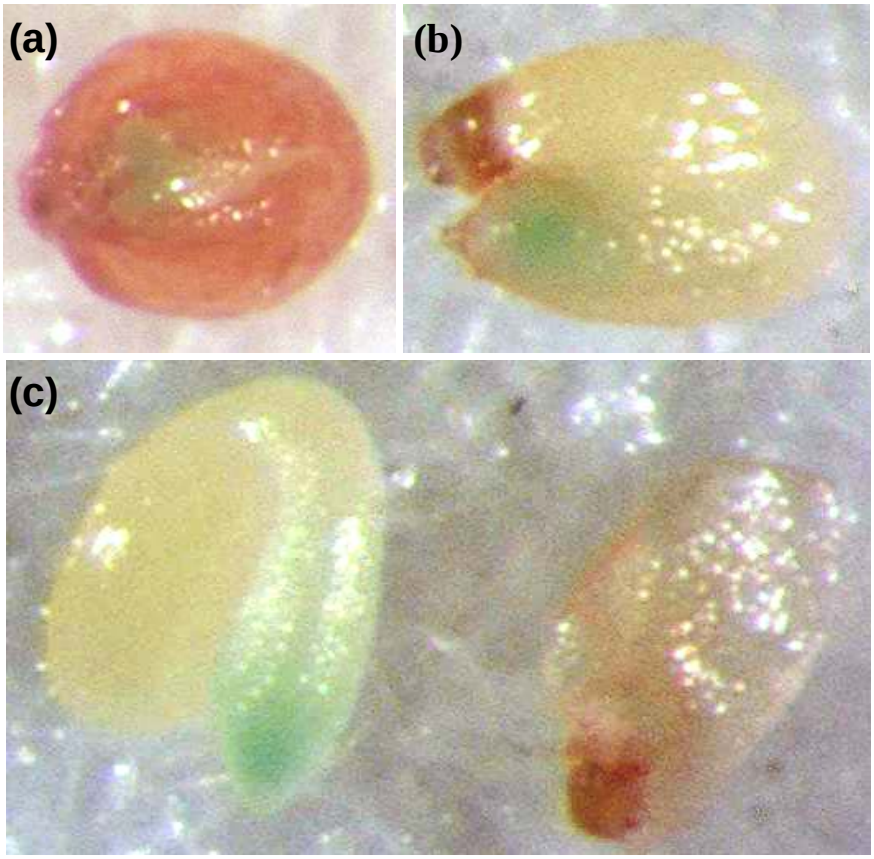


Figure 3-5. GUS expression driven by the 5' upstream region of *BME3-ZF* gene. The promoter region (−1075 to +19) was fused with the GUS reporter gene and used to transform wild-type Arabidopsis.
(a) GUS activity in germinating seed with testa rupture. A very weak signal is seen inside seed.
(b) The same seed with testa removed. The embryo is still enclosed by the thin, transparent endosperm. Note that a GUS signal is detected in the axis inside the de-coated seed.
(c) Embryo (left) and endosperm (right) excised from the same seed.

Disruption of BME3 zinc finger transcription factor gene causes impaired seed germination

Two putative knockout lines of the *At3g54810* GATA zinc finger gene, Salk_131396 (termed ZFKO1) and Salk_148073 (ZFKO2), which had T-DNA insertion sites in the second exon and promoter region, respectively, were obtained to investigate gene function (see Figure 3-2a for insertion sites). Homozygous plants were identified by PCR screening. No apparent phenotype was observed in seedlings, in developing siliques, or at later stages of plant growth in the homozygous lines. Seeds were harvested from homozygous plants of ZFKO1 and ZFKO2. Expression of *BME3-ZF* in these putative knockout lines was analyzed by semi-quantitative RT-PCR using RNA extracted from seeds treated at 4°C for 18 h. The expression levels of *BME3-ZF* were drastically reduced in both ZFKO1 and ZFKO2 compared with expression in wild-type Arabidopsis seeds (Figure 3-6a).

Germination of wild-type and knockout Arabidopsis seeds was initially compared without cold stratification. The final germination percentage was less in ZFKO1 and ZFKO2 seeds compared with that of wild-type Arabidopsis seeds (Figure 3-6b), suggesting that disruption of the zinc finger gene caused a deeper dormancy. Cold stratification induced full germination of wild-type Arabidopsis seeds, but the final germination percentage of ZFKO1 and ZFKO2 seeds reached <70% even after 5 days' incubation at 22°C following 3 days' cold stratification (Figure 3-6c). Further incubation did not increase the germination percentage. The ungerminated ZFKO1 and ZFKO2 seeds were still viable, as they showed positive

staining by a TZ test (data not shown). These results demonstrated that ZFKO1 and ZFKO2 seeds had a reduced response to cold stratification.

As >30% of ZFKO1 and ZFKO2 seeds still failed to germinate, even with cold stratification, we examined the effect of potassium nitrate (0.2% w/v), which is typically used instead of cold stratification to break seed dormancy in seed-testing laboratories. Although germination was slightly enhanced by potassium nitrate, seeds of the two knockout lines still showed reduced germination levels compared with wild-type seeds after this treatment (Figure 3-6d). Gibberellic acid (GA_3, 10 μM) stimulated germination of the knockout seeds, with over 90% of seeds germinating (Figure 3-6d). It is interesting that the deep dormancy of ZFKO1 and ZFKO2 seeds, which was not even overcome by 3 days' cold stratification, was broken by exogenous GA without cold stratification. This result suggests that the normal GA signal transduction pathway is present in these two mutant lines.

The impaired germination phenotype was observed consistently in both ZFKO1 and ZFKO2 knockout seeds. The degree of seed dormancy, however, can also be affected by the physiological state of the maternal plants. That is, the environment that the maternal plants experience during seed development could affect germinability of the progeny seeds. It was possible that the difference in wild-type and knockout seed-germination performance was due to small differences experienced by their maternal plants during seed production, although the wild-type, ZFKO1 and ZFKO2 seeds were produced side-by-side under the

same conditions. To confirm the low germination phenotype of ZFKO1 and ZFKO2 lines, the next generation of seeds from these two mutant lines and wild-type Arabidopsis were produced again under the same conditions and tested for the germination phenotype. The impaired germination phenotype was reproducible in the second generation of ZFKO1 and ZFKO2 lines (termed ZFKO1-2nd and ZFKO2-2nd, respectively). More than 35% of seeds in both lines failed to germinate after 5 days incubation at 22°C following 3 days cold stratification (data not shown). Most of the seeds that failed to germinate exhibited testa rupture (Figure 3-7a), although there was a delay in the timing of testa rupture in the mutant seeds. This result indicates that the mutant embryos had at least a small increase in the growth potential to cause testa rupture, but could not penetrate the endosperm layer. When the remaining ungerminated seeds were transferred to GA_3 solution (10 μM) they germinated within 1 day (data not shown), which was consistent with experiments on the previous generation of seeds. These results led to the hypothesis that impaired germination of the knockout seeds may be due to reduced GA synthesis rather than decreased sensitivity to GA. To test this hypothesis, we examined the expression of *GA20ox3* and *GA3ox1* genes, which are the major GA-biosynthesis enzymes induced by cold stratification (Yamauchi *et al.*, 2004). Strikingly, we found that both genes are downregulated in the progeny of both ZFKO1 and ZFKO2 lines (Figure 3-7b), indicating that BME3-ZF is probably associated with the upstream event(s) of GA biosynthesis.

We also found that, when the ungerminated knockout seeds were plated on agarose medium with MS salt (Murashige and Skoog, 1962) and 1% (w/v) sucrose, they were able to germinate. The promotion of knockout seed germination on agarose medium was also observed in the absence of MS salts and sucrose, suggesting that higher retention of water around seeds, rather than nutrition of the medium, enhanced radicle emergence. However, comparison of wild-type and knockout seed germination on agarose medium also showed large differences in germination speed (Figure 3-7c).

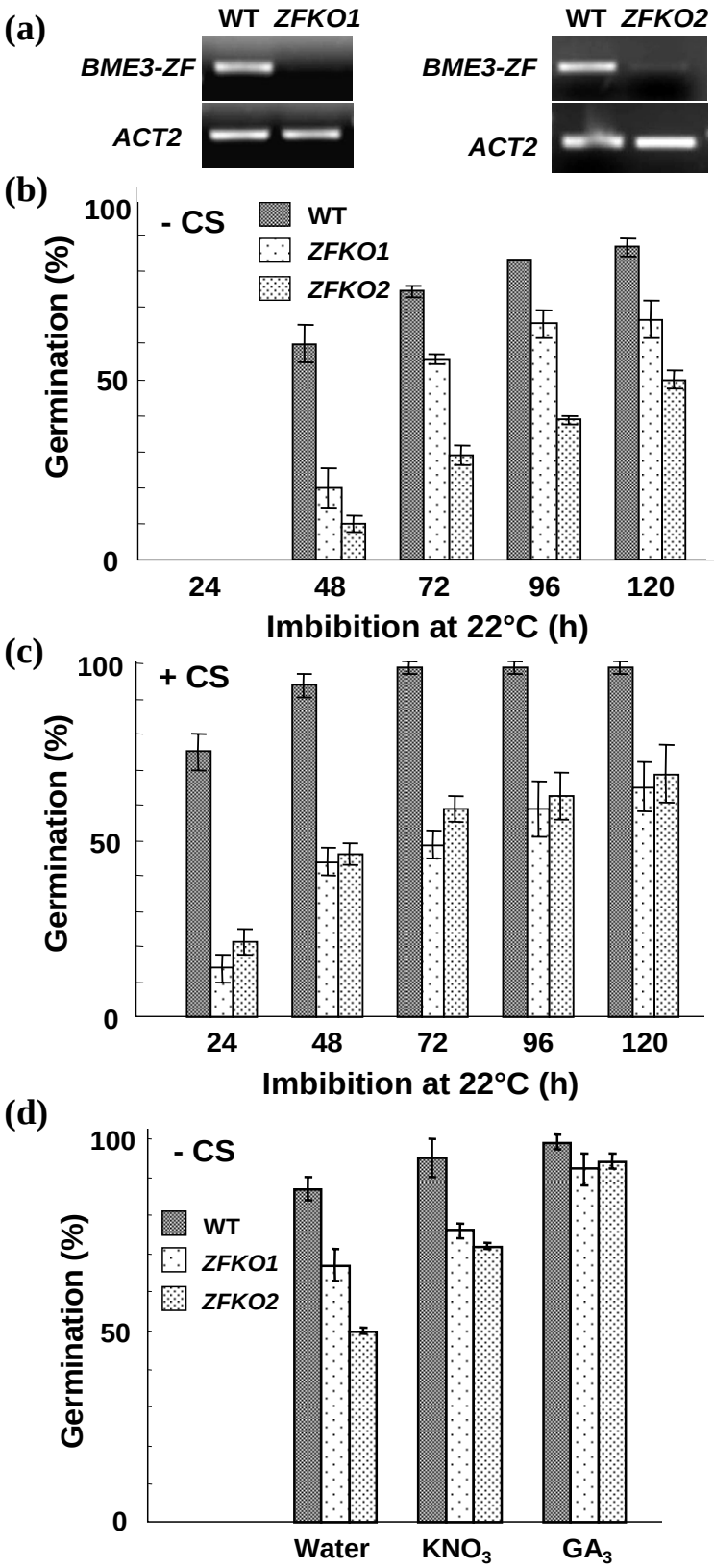
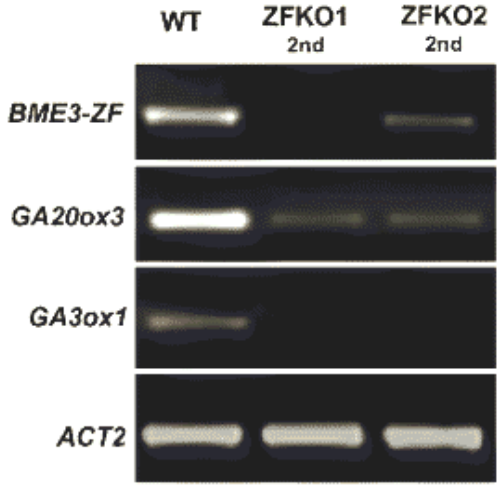

(a)
WT ZFKO1
BME3-ZF
ACT2
WT ZFKO2
BME3-ZF
ACT2
(b)
- CS
WT
ZFKO1
ZFKO2
Germination (%)
100
50
0
24
48
72
96
120
Imbibition at 22°C (h)
(c)
+ CS
Germination (%)
100
50
0
24
48
72
96
120
Imbibition at 22°C (h)
(d)
- CS
WT
ZFKO1
ZFKO2
Germination (%)
100
50
0
Water
KNO3
GA3

Figure 3-6. Gene expression and germination phenotype of knockout plants.
(a) Semi-quantitative RT-PCR of *BME3-ZF* gene for wild-type Arabidopsis (WT) and the two putative SALK knockout lines [SALK_131396 (ZFKO1) and SALK_148073 (ZFKO2)]. RNA was extracted from seeds treated at 4°C for 18 h. *ACT2* was used as a control.
(b) Germination of wild-type Arabidopsis (WT) and the ZFKO1 and ZFKO2 knockout seeds without cold stratification (−CS).
(c) Germination of wild-type Arabidopsis (WT) and the ZFKO1 and ZFKO2 knockout seeds with cold stratification (+CS). '+CS' indicates exposure to 4°C for 3 days and an additional imbibition period.
(d) Germination of wild-type Arabidopsis (WT) and the ZFKO1 and ZFKO2 knockout seeds after 120 h in water, 0.2% (w/v) potassium nitrate (KNO_3) and 10 μM gibberellic acid (GA_3) without cold stratification (−CS).
Each data point in panels (a–c) represents the average of three replicates; vertical bars, SD.

(a)

(b)


WT
ZFKO1
2nd
ZFKO2
2nd
BME3-ZF
GA20ox3
GA3ox1
ACT2

(c)

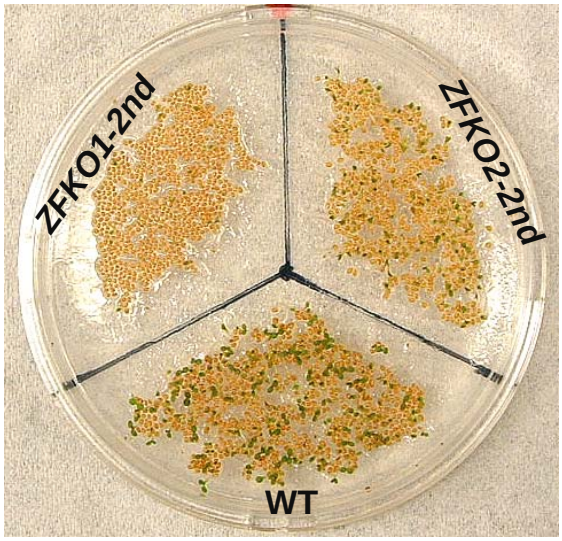

ZFKO1-2nd
ZFKO2-2nd
WT

Figure 3-7. Germination phenotype and expression of *BME3-ZF* and GA biosynthesis enzyme genes in the second generation of knockout seeds.
(a) Photograph of knockout seeds that failed to germinate after 5 days' incubation at 22°C following 3 days' cold stratification. Note that testa rupture and slight elongation of the endosperm occur in the ungerminated seeds.
(b) Semi-quantitative RT-PCR of *BME3-ZF*, *GA20ox3 and GA3ox1* genes for wild-type (WT) and knockout (ZFKO1-2nd and ZFKO2-2nd) seeds. RNA was extracted from seeds treated at 4°C for 24 h. The same sets of reverse transcription products were used for four different genes, including *ACT2* control.
(c) Photograph of wild-type (WT) and knockout (ZFKO1-2nd and ZFKO2-2nd) seeds at the early post-germinative stage (3 days) on an agarose plate without cold stratification.

Discussion

Potential of the '*Seed-GUS-Expression*' enhancer-trap library for seed germination research

We previously reported the generation of a library of '*Seed-GUS-Expression*' enhancer-trap lines (Liu *et al.*, 2005a; http://www.science.oregonstate.edu/isb; Chapter 2), one of which (*BME3*) was used in the present study. The seeds, which have been donated to the ABRC at Ohio State University, are now available (CS24362–CS24480) for the international research community to identify seed germination-associated genes. We previously reported the identification of insertion sites in the vicinity of genes encoding signal transduction proteins and several types of transcription factors in these enhancer-trap lines (Liu *et al.*, 2005a; Chapter 2); however, examples of the successful application of the enhancer-trap lines for functional analysis of seed germination-associated genes were yet to be demonstrated. The present work provides the proof-of-concept study for the '*Seed-GUS-Expression*' enhancer-trap lines. Although the *BME3* line had several T-DNA insertions (data not shown), characterization of the 5' upstream sequence of the GATA zinc finger gene verified the tissue-specific GUS expression detected in the *BME3* seeds. This study also shows that a combination of gene-expression analysis using wild-type seeds and functional analysis using knockout plants allows us to identify genes crucial for seed germination.

BME3-ZF is a subfamily I GATA-type zinc finger protein

In eukaryotes, zinc finger proteins are an abundant group of transcription factors which have a wide range of biological functions (Laity *et al.*, 2001). The zinc finger genes are by far the largest family of transcription factors (762 members), followed by homeobox genes (199 members) and bHLH genes (117 members) (Messina *et al.*, 2004). Analysis using the *SIGnAL 'T-DNA Express'* Arabidopsis *Gene Mapping Tool* (http://signal.salk.edu/cgi-bin/tdnaexpress) indicates that >800 putative zinc finger genes are present over five chromosomes of Arabidopsis. Zinc finger proteins can be classified into several types, such as C_2H_2; C_2HC; C_2C_2; $C_2HCC_2C_2$; and $C_2C_2C_2C_2$ types, based on the number and position of Cys and His residues in the zinc finger domains (Huang *et al.*, 2004). BME3-ZF is classified as a C_2C_2 GATA zinc finger protein which recognizes the consensus sequence motif (A/T)GATA(A/G). The GATA-type zinc finger proteins were originally identified in vertebrates (Evans and Felsenfeld, 1989; Evans *et al.*, 1988) and are also present in fungi, animals and plants (Reyes *et al.*, 2004). The DNA-binding domains were typically defined as $CX_2CX_{17-20}CX_2C$, where X is any amino acid. The $CX_2CX_{17}CX_2C$ and $CX_2CX_{18}CX_2C$ configurations are found in fungal GATA-type zinc finger proteins (Teakle and Gilmartin, 1998). Most animal zinc finger proteins contain $CX_2CX_{17}CX_2C$ and have two zinc finger domains (Patient and McGhee, 2002; Reyes *et al.*, 2004). Zinc finger domains with 18–20 residue loops ($CX_2CX_{18-20}CX_2C$) can be found in plants (Nishii *et al.*, 2000; Reyes *et al.*, 2004). In Arabidopsis, 29 GATA zinc finger protein genes have

been identified (Reyes *et al.*, 2004). The coding region of the *BME3-ZF* gene contains two exons, with the last exon encoding the complete zinc finger motif (Figure 3-3a). The exon–intron organization seen in the *BME3-ZF* gene structure is also found in 13 Arabidopsis genes which are categorized as subfamily I GATA zinc finger proteins. Another characteristic of subfamily I GATA zinc finger proteins is the presence of an acidic amino-terminal domain with pI < 4 (Reyes *et al.*, 2004). Although the function of an acidic domain is unknown, the presence of this characteristic in BME3-ZF also indicates that BME3-ZF falls into subfamily I (Figure 3-3a,b). Reyes *et al.* (2004) conducted comprehensive alignments of $CX_2CX_{18}CX_2C$ (and $CX_2CX_{17}CX_2C$) GATA zinc finger domains and their flanking amino acid sequences of Arabidopsis and rice. They grouped the plant $CX_2CX_{18}CX_2C$ zinc finger domains into four classes (A–D) based on the conservation of sequences in the flanking region. According to their classification, BME3-ZF is a class A GATA zinc finger which also includes NtAGBP-5 in tobacco and OsGATA-1 in rice (Figure 3-3c).

BME3-ZF is a positive regulator of seed germination

Seeds exhibit no apparent morphological changes during imbibition, except for seed-size change following water uptake, and testa rupture which occurs only during the later stages of seed imbibition. Apparent embryo growth is a post-germinative event and occurs only after rupture of the endosperm layer by the radicle. However, generation of embryo growth potential, which is a prerequisite

for the completion of germination, takes place while the embryo is enclosed by the endosperm during imbibition. The molecular mechanisms controlling this early event in seed germination are not comprehensively understood.

Gibberellic acid releases seed dormancy in many different species, including Arabidopsis. The mechanisms of the GA-signaling pathway in seed-dormancy release are emerging. The DELLA transcription factors play significant roles in GA signal transduction during seed germination (Lee *et al.*, 2002; Peng and Harberd, 2002; Tyler *et al.*, 2004; Wen and Chang, 2002). The DELLA proteins are repressors of seed germination and are probably degraded by the ubiquitin-26S-proteosome pathway (Dill *et al.*, 2004; Itoh *et al.*, 2003; McGinnis *et al.*, 2003).

Only a few factors involved in seed dormancy and *sensu stricto* germination have been characterized, other than DELLA transcription factors. Dof zinc finger proteins DAG1 and DAG2 are examples of well-characterized transcription factors associated with cold stratification and light response for seed germination. These two Dof zinc finger proteins share high sequence homology and an identical zinc finger domain, but they exhibit completely opposite functions in terms of response to cold stratification, light and GA. DAG1 and DAG2 play negative and positive roles, respectively, in Arabidopsis seed germination. Both genes are expressed in the maternal tissues, such as funiculus, during seed development (Gualberti *et al.*, 2002; Papi *et al.*, 2000). A novel-type zinc finger

protein MARD1, which is a negative regulator of seed germination, is associated with maintenance of seed dormancy imposed by ABA (He and Gan, 2004).

The GATA zinc finger protein was identified as another member of *sensu stricto* germination-associated genes in the present study. Disruption of the *BME3-ZF* reduced the response of mutant seeds to cold stratification and caused a deeper dormancy. This suggests that BME3-ZF positively mediates the developmental shift from dormancy to germination. The mutant seeds showed very slow germination, and >30% of seeds failed to germinate even when cold-stratified (Figure 3-6c). Ungerminated knockout seeds exhibited testa rupture and slight elongation of the endosperm out of the testa (Figure 3-7a). The rupture of testa is not simply due to physical changes following water uptake, because seeds imbibed during cold stratification or completely dormant seeds imbibed at 22°C do not show testa rupture. Generation of growth potential of the embryo is probably required for testa rupture. Therefore the embryos of the mutant ungerminated seeds generated a minimal growth potential, enough for testa rupture but insufficient for the embryo to penetrate the endosperm layer. It is not known (and technically difficult to examine) whether a single cell layer of the Arabidopsis endosperm provides significant mechanical resistance to prevent radicle protrusion. The thin endosperm seems to have some resistance that could not be overcome by the radicle of the mutant embryo. The *BME3-ZF* promoter was activated in the axis in terms of seed expression (Figure 3-5). The function of BME3-ZF is probably associated with a change in the embryonic axis, providing further growth

potential increase which is necessary for the radicle to overcome the residual mechanical resistance of the endosperm after testa rupture.

The impaired germination phenotype of the knockout seeds was rescued by exogenous GA_3 (Figure 3-6d), which is associated with cell enlargement and elongation of the embryo in Arabidopsis seeds (Yamaguchi *et al.*, 2001). The conversion of an inactive form of GA_9 to bioactive GA_4, which is catalyzed by GA_3-oxidase, is a rate-limiting step for the synthesis of bioactive GA in Arabidopsis seeds (Yamaguchi and Kamiya, 2001; Yamaguchi *et al.*, 1998, 2001). The GA_3-oxidase genes *GA4* (*GA3ox1*) *and GA4H* (*GA3ox2*), which are regulated by a plant photoreceptor phytochrome (Yamaguchi *et al.*, 1998), play a crucial role in stimulating Arabidopsis seed germination. Yamauchi *et al.* (2004) discovered that cold stratification also induces GA-biosynthesis genes *GA20ox3* and *GA3ox1*, suggesting that the promotional effect of cold stratification on seed germination is mediated by GA synthesized in seeds.

The knockout seeds showed sensitivity to exogenous GA_3 (Figure 3-6d). Impaired germination of ZFKO mutant seeds is probably due to a reduced level of GA biosynthesis. Expression of the GA-biosynthesis genes *GA20ox3* and *GA3ox1* was downregulated in the knockout seeds (Figure 3-7b). BME3-ZF probably acts upstream of GA biosynthesis. Expression of *BME3-ZF* starts just prior to expression of the GA-biosynthesis genes during cold stratification (Figure 3-4a). *GA3ox1* is also expressed in the axis of Arabidopsis seeds (Yamaguchi *et al.*, 2001). These findings also support the potential function of BME3-ZF in GA

biosynthesis during and following cold stratification. The knockout plants did not show phenotypes typical of GA-deficient mutants such as dwarfism. The regulation of GA biosynthesis by BME3-ZF could be seed-specific.

More than 60% of the knockout seeds were still capable of germinating despite the drastic reduction in *BME3-ZF* expression (Figure 3-6). This can be explained by a partial reduction in GA biosynthesis. Alternatively, it is also possible that there is redundancy in *BME3-ZF* gene function. The possibility that BME3-ZF also modulates germination by regulating other plant hormones cannot be excluded. Studies using the GA signal transduction mutant *sly* and GA-biosynthesis mutant *ga3* (Steber and McCourt, 2001) and the ethylene-insensitive mutant *etr-1–2* (Chiwocha *et al.*, 2005) clearly showed cross-talk between GA and other plant hormones such as brassinosteroids, ethylene and cytokinins. Identification of potential interacting partner(s) of BME3-ZF and other germination-associated transcription factors will provide a clear picture of the biology of seed germination.

Materials and Methods

Screening of the Arabidopsis enhancer-trap lines

The Arabidopsis enhancer-trap lines (Thomas Jack lines, 1130 pools of 10 lines, CS31086, ABRC) (Campisi *et al.*, 1999) were used for screening. Approximately 50 seeds from each sub-pool were placed on a small, round filter paper (approximately 7 mm diameter), moistened with water, and placed on a metal stage for SEM specimens. The metal stage was cleaned thoroughly each time before placing another sub-pool of seeds, to avoid contamination. One hundred sub-pools of 50 seeds placed on small filter paper were incubated on two layers of larger (15-cm diameter) filter paper placed in a plastic Petri dish, and incubated as described above. After 3 days' pre-chilling at 4°C and 22 h incubation at 22°C, the small filter papers holding seeds were briefly blotted on filter papers to remove excessive water; then examined for GUS expression.

GUS staining

GUS staining of seeds and other tissues was performed as described previously (Weigel and Glazebrook, 2002) using 100 mM sodium phosphate buffer pH 7.0 containing 0.1% (v/v) Triton X-100 and 2 mM X-Gluc (RPI Co., Mount Prospect, IL). Staining was examined after overnight incubation at room temperature (approximately 22°C).

Identification and verification of T-DNA insertion site and trapped gene

Genomic DNA was extracted from Arabidopsis leaves using phenol extraction according to the QUICK-PREP method described at http://www.biotech.wisc.edu/NewServicesandResearch/Arabidopsis and used for genome-walking PCR experiments to identify the T-DNA insertion site. Genome-walking PCR was performed using Genome Walker Kit (Clontech, Palo Alto, CA) according to the manufacturer's manual. Briefly, genomic DNA of the *BME3* enhancer-trap line was digested with *Dra*I. Adapter DNA provided with the kit was ligated to the *Dra*I-digested genomic DNA fragments. The first PCR was conducted using *Ex Taq* DNA polymerase (Takara, Madison, WI), an adapter primer (5'-GTAATACGACTCACTATAGGGC-3') provided with the kit, and a T-DNA RB-specific primer (RB1: 5'-TCTAGAGTCGACCTGCAGGCATGCAAGCTT-3'). Second-round PCR was done using the nested adapter primer (5'-ACTATAGGGCACGCGTGGT-3') and the nested T-DNA RB-specific primer (RB2: 5'-TCCCAACAGTTGCGCACCTGAATGGCGAAT-3'). The conditions used for the first PCR were: one cycle at 94°C (4 min); one cycle at 80°C (2 min); seven cycles at 94°C (25 sec), 72°C (3 min); 32 cycles at 94°C (25 sec), 67°C (3 min); followed by one cycle at 67°C (7 min). The conditions used for the second PCR were: one cycle at 94°C (4 min); one cycle at 80°C (2 min); five cycles at 94°C (25 sec), 72°C (3 min); 20 cycles at 94°C (25 sec), 67°C (3 min); followed by one cycle at

67°C (7 min). The amplified DNA fragment was sequenced at the Center for Gene Research and Biotechnology, Oregon State University.

To verify the predicted T-DNA insertion site in the *BME3* line, a GSP (5'-ACGACGTTTACGCGTACATG-3') was designed for the 5' upstream sequence of the *At3g54810* gene (located downstream of the putative T-DNA insertion site) and used in PCR with RB2 primer and genomic DNA from the *BME3* line. The following conditions were used for PCR: initial denaturation at 94°C (4 min); touchdown cycles [94°C (15 sec), 69 → 63°C (15 sec), 72°C (30 sec)] (one cycle for each temperature) and 25 cycles at 94°C (15 sec), 62°C (15 sec) and 72°C (30 sec) followed by extension at 72°C (7 min).

To examine the expression of the candidate trapped genes identified by genome-walking PCR in imbibed seeds, specific primers were designed for *At3g54800* (5'-TTATTGCTGCGACGCTACTG-3' and 5'-TTGAGCCGACAAGTTCCTAG-3') and *At3g54810* (5'-CTACCATCTGGACCACTCAT-3' and 5'-TACCATCACTGCATCTCTTG-3'), and RT-PCR was conducted using RNA extracted from 12-h imbibed seeds. The following conditions were used for PCR: initial denaturation at 94°C (4 min); touchdown cycles [94°C (15 sec), 67 → 61°C (15 sec), and 72°C (30 sec)] (one cycle for each temperature) and 25 cycles at 94°C (15 sec), 60°C (15 sec) and 72°C (30 sec), followed by extension at 72°C (7 min).

Semi-quantitative RT-PCR

Total RNA was extracted from Arabidopsis seeds imbibed for 0, 6, 12, 18 and 24 h at 4°C, and for an additional 6, 12 and 18 h at 22°C, using standard phenol–SDS extraction (Sambrook *et al.*, 1989). Two μg DNase-treated total RNA were used for RT with a RETROscript Kit (Ambion, Austin, TX). The RT product was subjected to semi-quantitative PCR using the primers for the *At3g54810* gene (described above). The conditions for semi-quantitative PCR were essentially as described above, except that 20 cycles were used at 94°C (15 sec), 60°C (15 sec) and 72°C (30 sec). An *ACT2* (An *et al.*, 1996) was used as a control in the semi-quantitative PCR with specific primers (5'-GCCATCCAAGCTGTTCTCTC-3' and 5'-GAACCACCGATCCAGACACT-3').

Construction of BME3-ZF promoter::GUS reporter cassette

The 5' upstream region of *BME3-ZF* (−1075 to +19) was amplified using forward (5'-CTGAGCTCCGTCCTAGGGGAAAAACTCA-3') and reverse (5'-CAGTCGACGGAGTGGGGAGAAGTGAAGA-3') primers, which contained *Sac*I and *Sal*I restriction enzyme sites at their 5' and 3' ends, respectively. The PCR products were digested with *Sac*I and *Sal*I and cloned into the *Sac*I and *Sal*I sites of the shuttle vector pRJG23 (Grebenok *et al.*, 1997) that contained the *uidA* (GUS) gene. The promoter-GUS construct from pRJG23 was removed with *Sac*I and *Spe*I and subcloned into *Sac*I and *Xba*I sites in pGPTV-KAN binary vector (Becker *et al.*, 1992) to produce the *BME3-ZF* promoter::GUS binary vector.

Arabidopsis transformation and screening

For transformation, 200 ml YEP medium [1% (w/v) yeast extract, 1% (w/v) peptone, 0.5% (w/v) NaCl] (Weigel and Glazebrook, 2002) containing 50 μg ml^{-1} kanamycin was inoculated with 5 ml overnight culture of *Agrobacterium tumefaciens* EHA 105 strain (Hood *et al.*, 1993) harboring the *BME3-ZF* promoter::GUS binary vector and grown for an additional 16 h at 28°C with vigorous shaking. Cells were harvested by centrifugation at 6000 *g* with a Sorvall RC-5B centrifuge (DuPont Instruments, Wilmington, DE) at ambient temperature, resuspended in 400 ml 5% (w/v) sucrose solution containing 0.02% (v/v) Silwet L-77 detergent (Lehle Seeds, Round Rock, TX), and used for transformation with the floral dip method as described previously (Clough and Bent, 1998). Seeds were harvested and stored at room temperature (approximately 22°C). For screening, seeds were sterilized in 70% (v/v) ethanol for 1 min, and in 50% bleach solution containing 0.1% (v/v) Tween 20 for 10 min, followed by several washes with sterile water. Kanamycin-resistant plants were selected by growing plants for 14 days on 0.7% (w/v) agarose plates containing 4.3 mg ml^{-1} MS salts, 1% (w/v) sucrose and 25 μg ml^{-1} kanamycin.

Germination test

Fifty seeds were germinated in a Petri dish containing two layers of filter paper moistened with 4 ml water or test solution (three replicates per treatment). Germination and seedling establishment were also tested on 0.7% (w/v) agarose

plates with or without 4.3 mg ml^{-1} MS salts and 1% (w/v) sucrose. Cold stratification was conducted at 4°C in the dark. Germination was performed at 22°C with 16-h light and 8-h dark periods.

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Chapter 4

A *Seed-GUS-Expression* Enhancer-Trap Library for Germination Research

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ABSTRACT

Enhancer-trap lines are used to identify tissue- and stage-specific gene expression. An Arabidopsis enhancer-trap population from the ABRC at Ohio State University has been screened for GUS expression in germinating seeds. Application of these enhancer-trap lines for functional analysis of seed germination-associated genes has led to the successful identification of a GATA zinc finger transcription factor crucial for seed germination. A *Seed-GUS-Expression* enhancer-trap library was donated to the ABRC (stock no. CS24362–CS24480), and is now available to the international seed research community. This library can be used for seed germination research by combining northern blotting and RT-PCR expression analysis with functional analysis using knockout plants. In this chapter, an overview of the procedures for utilizing the enhancer-trap library for germination research including some potential pitfalls and hints for troubleshooting experiments are presented.

Introduction

A substantial amount of information on the biochemical and molecular mechanisms of seed germination has been obtained using tomato (*Lycopersicon esculentum* Mill.) as a model system (Bradford *et al*., 2000). The tomato seed is an excellent system for seed germination research, because it has a distinct endosperm and an embryo at maturity, and therefore provides an excellent tool for analysing the physical and chemical interactions between these two tissues. Since the tomato seed is larger than the seeds from many other plant species, such as tobacco (*Nicotiana tabacum* L.) and Arabidopsis, it is feasible to dissect it. The molecular mechanisms of endosperm weakening and the generation of embryo growth potential are best understood in this model system. However, the number of genes identified in tomato seeds is still limited and the regulatory mechanisms of gene expression are not fully understood. Although multiple genes are expressed in tomato seeds in a tissue-specific manner, the mechanisms regulating tissue specificity are largely unknown. Therefore, characterization of the promoter regions of the germination-associated genes by identifying hormone responsive and tissue-specific *cis*-elements and isolating the DNA-binding proteins specific to these elements is necessary. However, this is difficult in tomato due to limited availability of genomic resources.

Although Arabidopsis seeds are too small to analyze embryo–endosperm interactions in a physiological way, a large number of genetic tools are available for this model species. Considering the genomic resources available for

Arabidopsis, and ongoing genome synteny between Arabidopsis and tomato (Ku *et al*., 2000, 2001), the identification of seed germination-associated genes in Arabidopsis will significantly enhance characterization of their orthologues in tomato seeds. The transfer of information between Arabidopsis and tomato seeds has been applied successfully in studies of tomato testa (Downie *et al*., 2003).

On the other hand, there is only limited information on *sensu stricto* germination-associated genes in Arabidopsis. Testa mutants of Arabidopsis seeds have advanced our knowledge of seed biology; however, the critical genes that determine the chemical and physical properties of the testa are expressed in the endothelium of developing seeds (Debeaujon *et al*., 2001, 2003; Sagasser *et al*., 2002). Gene expression in germinating Arabidopsis seeds needs to be characterized. There are emerging data from cDNA microarrays and proteomic studies concerning gene expression in germinating Arabidopsis seeds (Gallardo *et al*., 2001, 2002; Ogawa *et al*., 2003); however, the tissue specificity of the expressed genes is still unknown.

Enhancer-trap technology has been successfully applied to identify tissue and stage-specific gene expression in *Drosophila* (Akimoto *et al*., 2005), zebrafish (*Danio rerio* (Ham. Buc.); Balciunas *et al*., 2004) and also in plants including Arabidopsis (Campisi *et al*., 1999; He *et al*., 2001; Vroemen *et al*., 2003; Endo *et al*., 2005; Engineer *et al*., 2005), rice (*Oryza sativa* L.; Johnson *et al*., 2005) and carrots (*Daucus carota* L.; Ko and Kamada, 2002). Enhancer-trap lines are generated by randomly integrating a cassette that contains a MP fused to a reporter

gene into the genome. If the MP–reporter fusion integrates adjacent to an enhancer (or promoter proximal element), the expression of the reporter gene is detected in an organ-, tissue-, or cell-specific pattern as directed by the enhancer (Campisi *et al*., 1999).

In a previous study, Arabidopsis enhancer-trap lines (Thomas Jack lines, 1130 pools of 10 lines, CS31086, ABRC at Ohio State University) were screened for GUS expression in imbibed seeds and a library of *Seed-GUS-Expression* enhancer-trap lines was generated (Liu *et al*., 2005a; Chapter 2). The seeds have been donated to ABRC by the international research community to identify seed germination-associated genes. *BME3-ZF*, whose expression is induced during cold stratification, was identified in this library. Seeds from two independent T-DNA knockout lines of *BME3-ZF* showed reduced germination, suggesting that this gene is a positive regulator of seed germination (Liu *et al*., 2005b; Chapter 3). This study is a proof of concept that clearly demonstrates the utility of the *Seed-GUS-Expression* library. An overview of the seed enhancer-trap approach and the resources useful for conducting experiments is presented.

Results and Discussion

Seed Order and Production

Seed-GUS-Expression enhancer-trap lines (CS24362–CS24480) are now available from ABRC (http://www.Arabidopsis.org/abrc). Detailed information about individual lines is also described at the NSF-funded Integrated Seed Biology web site at Oregon State University (http://www.science.oregonstate.edu/isb/). The general scheme of utilizing this library is summarized in Figure 4-1. The initial step for using the enhancer-trap approach is to order seeds from ABRC. This can be done through TAIR web site (http://www.arabidopsis.org/). Ordering multiple lines (5–10) is recommended since the identification of trapped genes may not always be successful, as discussed below. Each of the individual stock pools of *Seed-GUS-Expression* should express GUS in germinating or germinated seeds. However, the available seeds are a mixture of wild-type, heterozygous and homozygous enhancer-trap seeds; therefore, more than ten plants from each pool should be grown for seed production. Seeds need to be prechilled at 4°C for 3 days to break dormancy. When plants start to produce mature seeds, a few siliquae are collected and dried in a 25°C incubator for 5 days. Seeds are extracted from siliquae, given 3 days of prechilling treatment, incubated at 22°C for 22 h and tested for GUS activity. By conducting this preliminary GUS expression analysis, positive enhancer-trap individual plants are identified. In this way, seeds for future experiments should be harvested from the GUS-positive plants only. Several

rosette leaves need to be collected, frozen and saved to extract gDNA for gene identification before terminating these positive individual plants.

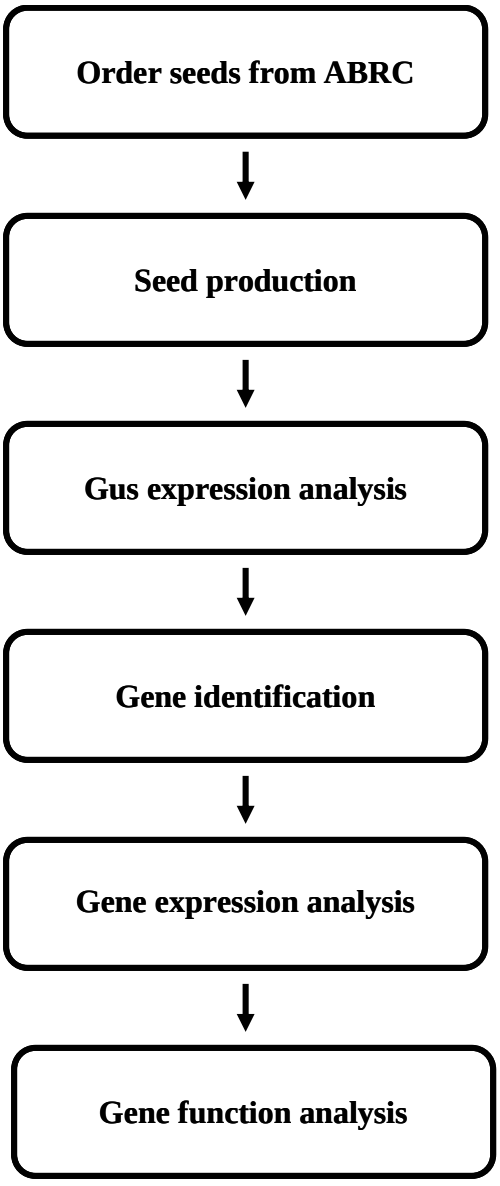


Figure 4-1. A flow chart for identifying germination associated genes using the *Seed-GUS-Expression* enhancer-trap library.

Reporter Gene Analysis

After thoroughly drying the harvested seeds, ~50 seeds from each plant are placed on a small round filter paper (~7 mm in diameter), which has been moistened with water and placed on a metal stage for SEM specimens (Figure 4-2a). The metal stage is cleaned thoroughly after each specimen to avoid contamination. A large plastic Petri dish (15 cm in diameter) with two layers of filter paper (No. 2, Whatman Inc., Clifton, NJ) can hold about 50 seeds from each of 100 lines (e.g. ten individual plants × ten ABRC stocks) (Figure 4-2b). After seeds are imbibed at 4°C for 3 days and incubated at 22°C for 22 h, the small filter papers holding seeds are briefly blotted on dry filter papers to remove excessive water and thereafter transferred to GUS substrate solution. GUS staining of seeds is performed as previously described (Weigel and Glazebrook, 2002) using 100 mM sodium phosphate buffer (pH 7.0) containing 0.1% (v/v) Triton X-100 and 2 mM X-Gluc (RPI Co., Mount Prospect, IL). Samples can be examined for GUS staining after an overnight incubation at room temperature (approximately 22°C).

The timing of GUS staining is critical for identifying germination-associated genes in the enhancer-trap lines. Imbibed Arabidopsis seeds initially exhibit only testa rupture, which is then followed by endosperm rupture indicating that germination is complete (Figure 4-3a). If staining is done after endosperm rupture, the majority of GUS-positive samples may actually reflect only postgermination-associated gene expression. On the other hand, it seems difficult to penetrate the GUS substrate into the endosperm and the embryo before testa

rupture, probably due to the impermeability of the testa to the GUS substrate. Therefore, GUS staining needs to be focused on the lag phase between testa and endosperm rupture (Liu *et al*., 2005a; Chapter 2). Initiation of GUS staining after 20 h of incubation at 22°C appears most appropriate to fit this narrow lag phase window of time (Figure 4-3b).

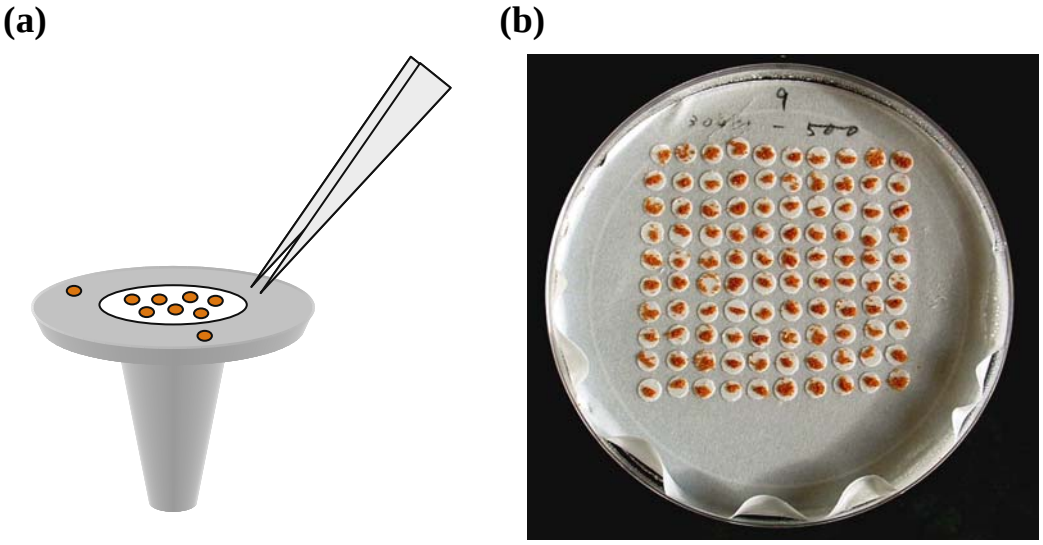
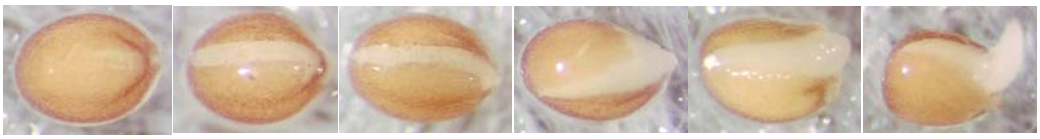


Figure 4-2. Procedures for plating seeds to screen for GUS expression during germination.
(a) Schematic representation of seed plating on the metal stage, which is generally used for SEM specimens. Small filter paper (~7 mm) moistened with water is placed on the stage and ~50 seeds are sown.
(b) A 15 cm plastic Petri dish with two layers of filter paper moistened with 8 ml water, containing 50 seeds (placed on small filter paper) from each of 100 lines.

(a)



(b)

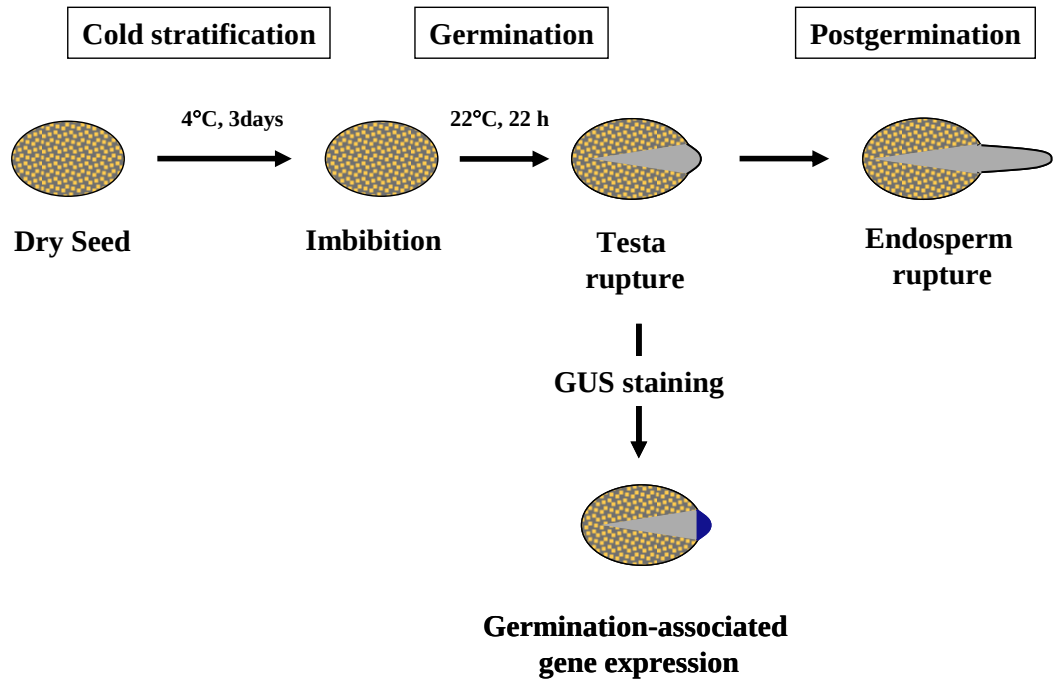


Figure 4-3. Timing for GUS staining in germinating Arabidopsis seeds.
(a) Morphological changes in imbibed Arabidopsis seeds (left to right). First visible change during germination is the rupture of testa; the radicle then penetrates a single cell layer of endosperm following some lag phase.
(b) Schematic representation of the timing of GUS staining to identify germination-associated genes. Germinating seeds need to be transferred to GUS substrate solution immediately after testa rupture but before radicle emergence.

Identification of Trapped Genes

The leaves collected from the GUS-positive individual plants are used for gDNA extraction. The QUICK-PREP method (http://www.biotech.wisc.edu/NewServicesandResearch/Arabidopsis/) works well for a scale of one to several leaves. The gDNA is run on a 1% (w/v) agarose gel with ethidium bromide staining to determine the quality of the DNA. Genome-walking PCR analysis is performed using a Genome Walker Kit (Clontech Laboratories, Inc., Palo Alto, CA), according to the manufacturer's manual. Briefly, gDNA extracted from the enhancer-trap lines is digested with *Dra*I. Adapter DNA provided with the kit is ligated to the *Dra*I digested gDNA fragments. The gDNA region flanking the T-DNA insert is amplified using an adapter primer provided with the kit and a T-DNA RB-specific primer (RB1: 5'-TCTAGAGTCGACCTGCAGGCATGCAAGCTT-3'). Second round PCR is performed using the nested adapter primer and the nested T-DNA RB-specific primer (RB2: 5'-TCCCAACAGTTGCGCACCTGAATGGCGAAT-3'). Three other enzymes (*Eco*RV, *Pvu*II and *Stu*I) contained in the kit can also be used for the same procedure, but *Dra*I gave the best results in our laboratory. Detailed methods can be found in Liu *et al*. (2005a) or Chapter 2. Figure 4-4a shows the examples of amplified DNA fragments from genome-walking PCR. The product is usually 0.1–2 kb long and can be directly sequenced. The DNA sequence flanking the T-DNA RB is analyzed using the *SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool* (http://signal.salk.edu/cgi-bin/tdnaexpress). An example showing

the identification of a putative T-DNA insertion site is shown in Figure 4-5. In this program, the direction of the submitted sequence is displayed so that the direction of the T-DNA insertion relative to the trapped candidate gene is clear. The example shows a putative T-DNA insertion site in the vicinity of a *cytochrome P450* gene (*At5g25180*) with the right border facing the gene.

To verify the predicted T-DNA insertion site in the enhancer-trap line, a GSP is designed in the region ~0.5 kb upstream of the putative insertion site. The primer is used together with the RB2 primer (described earlier) to perform PCR for both the trapped line and wild-type Arabidopsis. The predicted size of DNA fragment should be amplified from the enhancer-trap line, but not from wild-type gDNA. The junction between the gDNA and T-DNA is verified by sequencing this amplified product.

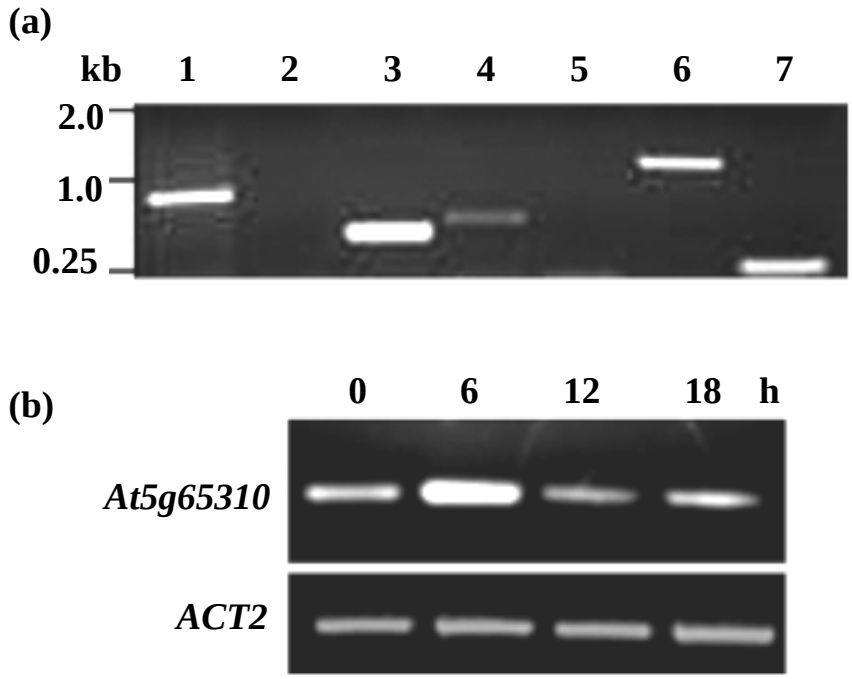


Figure 4-4. Amplification of DNA fragments from genome-walking PCR and expression analysis of the potential trapped gene.
(a) Example of PCR products obtained by genome-walking PCR. gDNA from seven independent enhancer-trap lines (1–7) are examined. No amplification was detected in lines 2 and 5. Successfully amplified products were sequenced to identify the putative T-DNA insertion sites.
(b) Example of semi-quantitative RT-PCR to characterize stage-specific expression of the identified trapped genes (*At5g65310* in this case). An *ACT2* (An et al., 1996) is used as a control.

Gene Expression Analysis

The presence of a T-DNA insertion immediately adjacent to the candidate gene does not necessarily prove that the GUS expression detected in the enhancer-trap line represents the expression patterns of this gene. One also needs to examine expression of the candidate gene during seed germination. Forward and reverse primers are designed in the coding region of the candidate gene and used for RT-PCR analysis to examine gene expression. A few time points (e.g. 12 and 24 h) during cold stratification at 4°C and incubation at 22°C can be tested for expression analysis. When expression is confirmed, detailed analysis is performed using semi-quantitative RT-PCR to characterize stage-specific expression (Figure 4-4b).

It is possible to have multiple T-DNA insertion sites in an enhancer-trap line; therefore, it is important to characterize the 5' upstream regulatory sequence of the candidate gene using a promoter–reporter construct. If the candidate gene is the correct one, the tissue-specific GUS expression obtained from the plants transformed with the promoter–reporter construct should be the same as that observed in the enhancer-trap line. This approach has been used successfully in previous work (Liu *et al*., 2005b; Chapter 3).

Gene Function Analysis

The biological function of the genes identified from the enhancer-trap lines can be determined using knockout plants. For example, three independent knockout lines (SALK_026675, SALK_057045 and SALK_127456) for the cytochrome *P450* gene were found using the *SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool* web site (Figure 4-5). Knockout lines that have T-DNA insertions in the coding regions are the best candidates, although those carrying an insertion in the promoter region have also exhibited knockout phenotypes (Liu *et al*., 2005b; Chapter 3). Seeds of these putative knockout lines can be ordered through the TAIR web site. These seeds are used to grow plants from which the next generation of seeds will be harvested. To identify homozygous lines, segregation ratios can be determined by growing plants on media containing antibiotic (e.g. kanamycin). When putative homozygous lines showing 100% antibiotic resistance are obtained, target gene expression levels in the potential mutant seeds need to be compared with the level of gene expression in wild-type seeds. Knockout lines may not be available for some genes identified. In these cases, an RNAi approach can be used to produce and examine loss-of-function mutants. It is possible that an apparent phenotype in seed germination or in other phases of plant development may not be observed due to redundancy in gene function. In this case, overexpression of the candidate genes using CaMV 35S or inducible promoters or examination of double mutants may be necessary. The

knockout approach worked well for characterization of the BME3-ZF expressed in germinating Arabidopsis seeds (Liu *et al*., 2005b; Chapter 3).

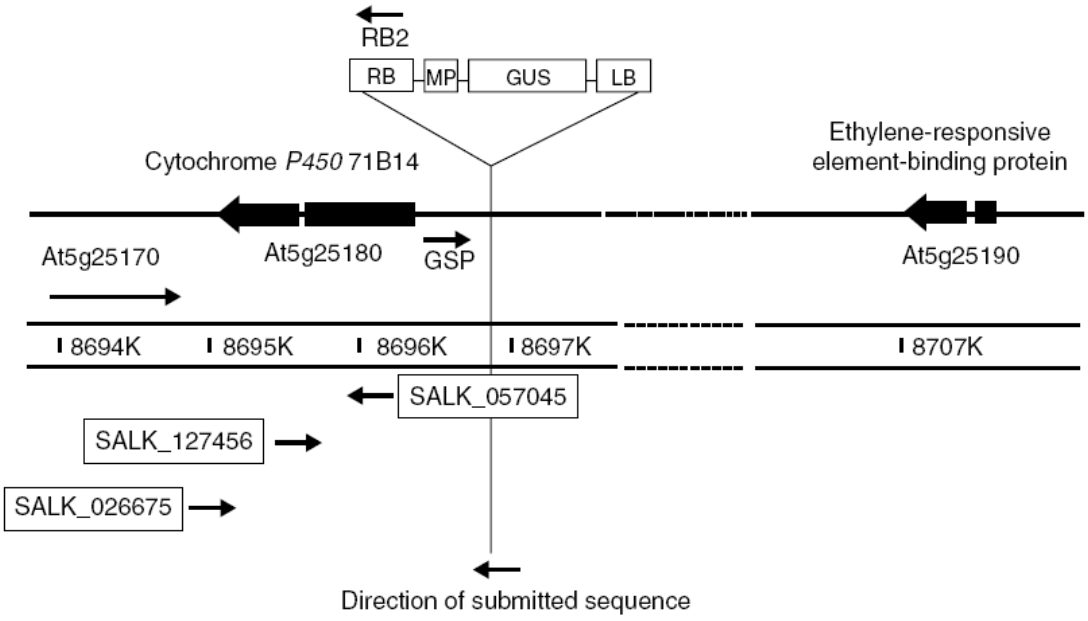


Figure 4-5. Identification of the T-DNA insertion site and potential trapped genes in enhance-trap line.
Schematic representation of the process involved in identifying a putative T-DNA insertion site using *SIGnAL T-DNA Express Arabidopsis Gene Mapping Tool* (http://signal.salk.edu/cgi-bin/tdnaexpress). Simplified display of the web site is shown with an example of an enhancer-trap line that had a T-DNA insertion site in the vicinity of *cytochrome P450* gene. The sequence obtained from genome-walking PCR is copied, pasted and submitted to this web site to identify the putative insertion site. The program on the web site indicates the location of the genome where the submitted sequence matches (vertical bar in the middle). It also shows the direction of the submitted sequence relative to the genome sequence (arrow at the bottom) so that the direction of T-DNA RB, MP, GUS and LB relative to the insertion site can be deduced. The positions of T-DNA insertions in three putative knockout lines (SALK_026675, SALK_057045 and SALK_127456) are shown with arrows. RB2: right border-specific primer. GSP: gene-specific primer.

Perspectives

The enhancer-trap approach is well utilized to identify potentially important factors of seed germination. Multiple transcription factors have been identified as candidate trapped genes (Liu *et al*. 2005a; Chapter 2). Use of Arabidopsis as a model plant is accelerating gene discoveries in seed biology. However, it is still important to identify and characterize homologues of these Arabidopsis genes in model species for germination research, such as tomato and lettuce (*Lactuca sativa* L.), and also in seeds of other agricultural crops. This approach is very important to elucidate universal mechanisms underlying seed dormancy and germination.

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Study of Seed Germination-Associated Genes Using Arabidopsis Enhancer-Trap

Chapter 5

General Conclusion

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In this study, the Arabidopsis enhancer trap has been applied to seed germination research and has successfully isolated 121 independent lines exhibiting diverse gene expression patterns in germinating and germinated seeds (Chapter 2). GUS expression in these lines was localized predominantly at micropylar region of the seed. The micropylar region is the area that radicle protrusion occurs. This indicates that the selective activation of gene expression in the micropylar region of the seed plays an important role in germination.

Trapped genes in the *Seed-GUS-Expression* enhancer-trap lines were identified using genome-walking PCR. Examples of trapped genes activated in imbibed seeds before endosperm rupture include several transcription factors such as zinc finger (GATA type, and C_3HC_4-type RING finger) family proteins, an AP2 domain containing protein, a PWWP domain containing protein, a HD-ZIP protein and a bHLH family protein. These transcription factors may be involved in the activation of downstream genes such as hormone biosynthesis and deactivation genes and genes associated with cell wall modification. Understanding the upstream events in detail provides information to better understand the regulation of seed germination. Three transcription factors identified from the *Seed-GUS-Expression* library have been extensively characterized for their biological functions in seed germination using knockout and overexpression mutants.

Gene expression analysis has shown that the zinc finger (GATA type) gene (*BME3-ZF*) is expressed during cold stratification just prior to the expression of two GA biosynthesis genes, *AtGA20ox3* and *AtGA3ox1*, suggesting the possible

involvement of this gene in the physiological events before GA biosynthesis (Chapter 3). The other zinc finger (C_3HC_4-type RING) gene (termed *XERICO*) has been studied by a different research group. Overexpression of *XERICO* caused hypersensitivity to exogenous ABA, salt, and osmotic stresses during seed germination and early seedling growth (Ko *et al.* 2006). In our study, we also found an enhancer trap line exhibiting GUS activity at the tips of cotyledons in germinating seeds before radicle emergence. This enhancer-trap line carried a single T-DNA insertion in the promoter region of *At2g48160* and was designated as *BCT*. The *BCT* encodes a transcription factor with PWWP and RPR domains. This research has been carried out in collaboration with Dr. Oscar Lorenzo at University of Salamanca, Salamanca, Spain. The peak of *BCT* gene expression occurred 6 h after the start of incubation at 22°C during the early stage of germination-inducible conditions. This indicates that the *BCT* gene plays an important role in the regulation of early seed germination events. Two independent knockout mutants (Salk_013479/Salk_013480 and Salk_029629) were used to characterize the biological function of *BCT*. Disruption of the BCT protein gene causes reduced sensitivity to exogenous ABA and increased sensitivity to exogenous auxin during seed germination and seedling development, suggesting the potential involvement of *BCT* gene in crosstalk between auxin and ABA response pathways (Liu P-P, González-García MP, Homrichhausen TM, Hewitt JR, Martin RC, Nonogaki H, Lorenzo O, unpublished data).

Proof-of-concept studies for the usefulness of *Seed-GUS-Expression* enhancer-trap lines for the identification of the genes critical for seed germination are demonstrated by the functional analysis of genes identified in this study. Chapter 4 documents the detailed procedures for the identification a T-DNA insertion site and the potential characterization of biological functions of a candidate/trapped gene in order to guide the seed research community to utilize these enhancer-trap lines in the library. Thus, the *Seed-GUS-Expression* library is a useful tool to identify the important genes essential for seed germination *sensu stricto* and will help to elucidate the molecular mechanisms of seed germination.

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