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Abstract approved:

Hiroyuki Nonogaki

Seed germination *sensu stricto* is defined as the physiological events before the radicle tip ruptures the covering tissues. The ability of the radicle to elongate (or germination potential) is observed in developing embryos prior to completion of seed maturation. When embryos at early developmental stages, such as globular, heart, torpedo or walking-stick are excised and grown in media, germination potential is not observed. Following these stages, embryos start to acquire germination potential. This thesis research focused on the mechanisms of the induction of germinability in *Arabidopsis thaliana* (Arabidopsis) developing seeds. A mutant with embryo defects was also analyzed in this research to gain insights into embryogenesis and germination. The mutant named *embryo ball* (*eb*) arrests after the globular-like stage yet continued cell growth to some extent. While desiccated mature *eb* seeds were unable to germinate, if developing embryos were excised and placed on media, the main root elongated. *eb* also exhibited abnormal root hair growth from the apical

portion of the embryo. The hypocotyl and cotyledons were missing in *eb* embryos in mature seeds. When *eb* embryos were grown on media, vegetative leaf-like structures with trichomes were formed and the main and subsequent lateral roots developed normally. Some *eb* embryos exhibited desiccation tolerance which is characteristic of wild-type (WT) mature embryos. Thus, while morphological maturation does not seem essential for the induction of germination potential, physiological maturation of the embryonic cells plays an important role in determining germination potential.

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Study of *Arabidopsis thaliana* Seed Development: Occurrence of Germinability

by

Jessica Hewitt

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APPROVED:

Major Professor, representing Horticulture

Head of the Department of Horticulture

Dean of the Graduate School

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

Jessica Hewitt, Author

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This work would not have been possible without the intense dedication to teaching from my major professor Hiro Nonogaki. I am forever thankful to him for helping me through this period of growth and intellectual expansion. I am also thankful for the friendship and kindness expressed by my fellow co-workers: Tanja Homrichhausen, Jing Sun, Kyung Hong, Tammy Chan, Jennifer Coppersmith and most importantly Po-Pu (Eric) Liu. I am especially appreciative to my committee members: Sabry Elias, Kevin Ahern and Ruth Martin for not only helping me to complete my degree but guiding me through the process. Finally I would like to thank my family: my mother, Christy Hewitt; sister, Kimberley Hewitt; aunt, Deborah Koonce; grandmother, Catherine Koonce; and grandfather, Stephen Koonce. They provided me with the will to continue on and the ambition to never give up.

CONTRIBUTION OF AUTHORS

Jennifer Coppersmith, Kyung Hong, Tanja Homrichhausen, and Jing Sun assisted in growing plants, harvesting of seed, and characterization of the *embryo ball* shrunken testa phenotype. Dr. Ruth C. Martin and Dr. Po-Pu (Eric) Liu assisted in experiment preparation and completion of the following manuscript.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
abp1	*auxin-binding protein1*
ABRC	Arabidopsis Biological Resource Center
AGI	Arabidopsis Genome Initiative
ALC	Aleurone-like cell layer
Arabidopsis	*Arabidopsis thaliana* (L.) Heynh.
AS1	*ASYMMETRICAL LEAVES1*
AtML1	*Arabidopsis thaliana MERISTERM L1 LAYER*
CZE	Chalazal endosperm
d	day(s)
DAP	Days after pollination
DIC	Differential interference contrast
eb	*embryo ball*
EP	Embryo proper
h	hours
HBT	*HOBBIT*
GA	Gibberellin
gk	*gurke*
laterne	Double mutant for the gene *PINOID* and the *ENHANCER OF PINOID*
LT	Lower-tier of embryo proper

M	Mature
MCE	Micropylar endosperm
MS	Murashige-Skoog
PCR	Polymerase chain reaction
PEN	Peripheral endosperm
S	Suspensor
SCR	*SCARECROW*
sec	seconds
SHR	*SHORT ROOT*
STM	*SHOOT MERISTERM-LESS*
T	Torpedo
T-DNA	Transferred DNA
UT	Upper-tier of embryo proper
WS	Walking-stick
WT	Wild-type
WUS	*WUSCHEL*

DEDICATION

I would like to dedicate this work to my best friend and soon to be husband Evan Kristof. Without his continual love and dedication to my sometimes overambitious goals I would not be where I am today and owe him nothing but the utmost respect and love for all that he has given.

Study of *Arabidopsis thaliana* Seed Development:
Occurrence of Germinability

Chapter 1

General Introduction

Jessica Hewitt

Seeds are an important food source and a mainstay in the world's economy and health (Johnson 2000). Maize, soybean, wheat and rice are major crops in the world's economy and, consequently, humans have become dependent on seeds as a direct and indirect source of nutrients (Grant *et al.* 1983). Desired characteristics of seeds for agriculture include easy dispersal (or less dispersal in seed production), storability, longevity, rapid germination, and sustainable seedling establishment. It is important to conduct research on these seed characteristics to benefit agricultural practices. Understanding the mechanisms of seed germination is of primary importance in terms of ensuring successful seedling establishment. It is equally important to understand the mechanisms underlying seed formation including fertilization, embryogenesis and seed maturation. In this chapter, an overview of seed formation and the principles of seed germination are provided.

Fertilization of seedless plants, such as, Bryophyta (mosses) and Pteridophyta (ferns) requires water. To overcome the spores' dependence on water to complete fertilization, ancestral plants evolved pollen-receiving structures and seeds (Kenrick *et al.* 1997). Present day plants have developed diverse seed characteristics to adapt to severe environments. There are various mechanisms of seed dispersal, but most take advantage of three main forces: wind, water, and other organisms. These mechanisms are critical to prevent progeny from becoming future competitors for nutrients and space by developing structures that enable flight, forms of floatation and survival through animal/insect mobilization and digestion. Many seeds have evolved desiccation tolerance to ensure longevity and viability until optimal growth conditions are sensed. Seeds with desiccation tolerance are referred to as orthodox seed and

include Arabidopsis, wheat and maize, while seeds without desiccation tolerance are called recalcitrant seeds and include chestnut, mango and coffee. In terms of seed structure, the testa (seed coat) and the endosperm encapsulating the embryo have evolved to help withstand selection by the surrounding environment (Keeley *et al.* 1997). The endosperm is also an important storage tissue in seed. Embryos of non-endospermic seeds, such as bean and pea have evolved with high storage reserves in cotyledons to support successful seedling establishment.

Seeds generally contain three main structures (Fig. 1). The testa is the outermost tissue of seed that is exposed to the environment. The testa provides protection to the embryo and also plays important roles in seed dormancy and longevity (Debeaujon *et al.* 2000). The endosperm plays a vital role in supplying nutrients to the growing embryo (Laux *et al.* 1997; Homrichhausen *et al.* 2003). In some seeds, the endosperm has been suggested to provide mechanical resistance against the embryo (Liu *et al.* 2005). The embryo, which is derived from a single cell zygote, becomes complex in its cellular organization at maturity and will develop into a seedling after germination. The embryo is composed of three main organs. The radicle ruptures the covering tissues, such as the testa and the endosperm, and establishes the main root. The hypocotyl elongates after germination and is associated with the transfer of nutrients to and from cotyledons. Cotyledons are embryonic leaves, and in non-endospermic seeds, they are the major site of seed reserves. Depending on the species there can either be one (mono-) or two (di-) cotyledons.

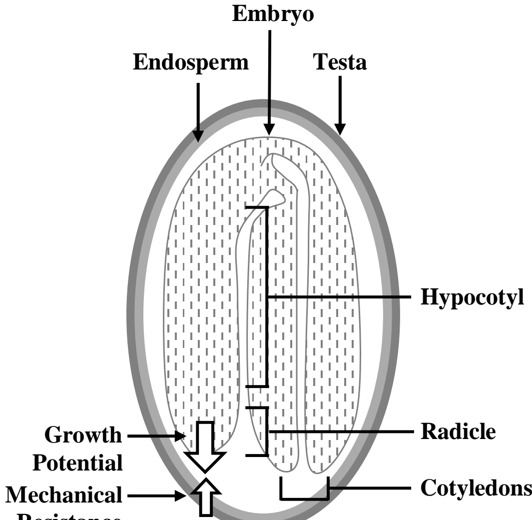

Figure 1. Schematic of Arabidopsis seed and the opposing forces important for germination. The embryo is surrounded by covering tissue such as the testa and endosperm. The occurrence of radicle emergence is determined by the balance between the growth potential of the embryo and the mechanical resistance of the endosperm. For germination to occur the growth potential of the embryo must exceed the opposing mechanical resistance of the endosperm.

Germination is initiated when nondormant seeds sense optimal conditions. Germination, in a strict sense, is defined as radicle tip rupture of the testa and the endosperm. There are many steps prior to this physical event. Increase in growth potential of the embryo induces germination. For germination to occur, embryo growth potential must exceed opposing mechanical resistance from surrounding tissues - endosperm and testa (Fig. 1). To develop this imbalance between the two forces, the seed must first exit the desiccated state by imbibition (water uptake). With rehydration, numerous molecular events are initiated and biochemical pathways become active. This stage of seed germination is defined as the germinative state. There are many cases in which seed does not germinate at optimal conditions; this is defined as seed dormancy. Seed dormancy is especially important to agriculture. Vivipary or precocious germination of seed prior to completion of the maturation program can be especially detrimental to farmers dependent on seed as a crop. Dormancy is induced by the plant hormone abscisic acid (ABA) which negatively

impacts initiation of germinative events (Finkelstein *et al.* 1990). Seed dormancy can be broken by physically damaging the testa (scarification), pre-chilling of the seed to imitate winter (cold stratification), loss of inhibitory molecules/gases through imbibition or application of germination inducing molecules. Gibberellin (GA) exhibits an action antagonistic to ABA and promotes germination by inducing enzymes required for endosperm-weakening (Chen *et al.* 2000). Active GA levels increase during early imbibition before radicle emergence (Koornneef *et al.* 1985; Ogawa *et al.* 2003). The balance between ABA and GA is thought to be important in determining if and when seed germination occurs.

In this thesis study, seed development in *Arabidopsis thaliana* (wild-type ecotype Columbia-0) (Arabidopsis) was characterized. Arabidopsis was used because of its small genome that has been sequenced, publicly available genetic resources, small plant stature, rapid life cycle, high quantity of seed production, and ease of cultivation (Reidei 1975). In addition to wild-type (WT) Arabidopsis, this study also focused on a mutant with embryo defects to obtain insights into the developmental processes of the embryo. This mutant also contributed to a greater understanding of the roles of seed dormancy and desiccation in germinability control during seed development.

Chapter 2

Seed Development in *Arabidopsis thaliana*

Jessica Hewitt and Hiroyuki Nonogaki

INTRODUCTION

Seeds become germinable during seed development. The timing of the occurrence of germinability (or germination potential) differs, depending on species. The precise timing when germination potential is acquired in an Arabidopsis embryo is not known. To understand the mechanisms of seed dormancy and germination, it is important to determine when germination potential is induced in developing embryos.

During fertilization, the pollen tube releases two sperm cells. One sperm nucleus fertilizes the diploid central cell to form the endosperm and the other sperm fertilizes the haploid egg cell in the ovule to form the diploid zygote (Fig. 2) (Faure *et al.* 2002). Embryogenesis begins with a single cell zygote. Asymmetrical division of the zygote results in apical and basal cells. The axis of division in the zygote is aligned to the chalazal-micropyle axis of the ovule and is most likely guided by orienting influences from surrounding maternal tissue (Laux *et al*. 1997). The apical or terminal cell will become the embryo proper (EP) (Long *et al.* 1998). The basal cell differentiates into the hypophysis and suspensor (S). The suspensor not only plays the physical role of attaching the embryo proper to surrounding maternal tissues but also serves as a transporter of nutrients and growth regulators to the developing embryo (Yeung *et al.* 1993; Schwartz *et al.* 1994).

The second stage of importance is the octant stage unique in that the embryo proper then divides into eight cells. The upper-tier (UT) becomes the cotyledons and shoot meristem, while the lower-tier (LT) becomes the hypocotyl and root (Berleth *et al.* 2002). The dermatogen stage then follows with tangential division of the two tiers.

The embryo proper proceeds through the early (or pre-) globular stage, the procambial stage, and the triangular (or transition) stage. Each stage is unique in the formation of cells with specific cellular functions. In early globular stages, there is complete division of the hypophysis into two root stem cells, while at the later triangular stage, future cotyledon boundaries become apparent. During globular stages the endosperm coenocyte starts to differentiate and exhibits three distinct regions: the micropylar endosperm (MCE), the peripheral endosperm (PEN) and the chalazal endosperm (CZE) (Fig. 2) (Olsen 2004). By the end of the globular stage the embryo is completely surrounded by cytoplasm of the syncytial endosperm.

Figure 2. Schematic of Arabidopsis ovule. Left, unfertilized ovule containing one egg cell and two central nuclei; right, ovule containing a globular stage embryo. MCE, micropylar endosperm; PEN, peripheral endosperm; CZE, chalazal endosperm; EP, embryo proper; S, suspensor.

Cotyledons and a shoot apical meristem are initiated during the heart stage. Late in this stage, chloroplasts become apparent. Subsequently, during the torpedo stage, a more complex differentiation of embryonic organs occurs and the suspensor undergoes programmed cell death. The developing embryo is dependent on nutrients

provided from surrounding tissues. The products from endosperm mobilization are transferred to and deposited in the cotyledons, which then provide nutrition for a germinated seedling. Intensive cell division occurs during the walking-stick and bent cotyledon stage. The final stage of embryogenesis is the mature embryo stage. The mature embryo stage is defined as the time when endosperm digestion, except for the peripheral aleurone-like cell (ALC) layer is complete, and full testa pigmentation occurs (Kitamura *et al.* 2004). It is at this stage that the induction of dormancy and desiccation tolerance takes place, however neither of these systems are well understood (Bewley 1997). Dormancy is defined as the failure of intact viable seeds to successfully germinate in favorable conditions (Bewley 1991). In this chapter, the features associated with each stage of embryo development are described. This chapter also focuses on determining when the developing embryo acquires germination potential and dormancy.

MATERIALS AND METHODS

Plant Materials

The seeds of *Arabidopsis thaliana* wild-type Columbia (Col-0) were sown in soil in small pots (2.5 x 2.5 cm) and germinated at 22°C after 3 d cold stratification at 4 °C. Seedlings were grown in an incubator (22°C) under short-day condition (8 h light/16 h dark periods) and transferred to the greenhouse (22°C) with long day condition (16 h light/8 h dark periods) to induce flowering. Days after pollination (DAP) were defined as days after the appearance of the first petals.

Embryo Excision

Embryos were excised from developing seeds at varying stages of development using fine forceps and a surgical blade under a dissection microscope (Stemi SV 11 dissection microscope, Zeiss, Jena, Germany). The excised embryos were incubated on Murashige-Skoog (MS)-medium (Murashige and Skoog 1962) with or without 1 % (w/v) sucrose. Embryo images were captured with a Pixera camera (Model #PVC 100C, Pixera Corporation, Los Gatos, CA) attached to the microscope.

Tetrazolium Staining

To examine seed desiccation tolerance, wild-type siliques containing seeds at different stages of development were desiccated at 25°C in an incubator for 2 weeks and rehydrated. Embryos were then excised and subjected to tetrazolium staining using 0.5% (w/v) 2, 3, 5-triphenyl tetrazolium chloride solution.

Differential Interference Contrast (DIC) Microscopy

Excised seeds were cleared with chloral hydrate solution (3.5 ml water, 0.5 g glycerol, 10 g chloral hydrate [#C-8383, Sigma, St. Louis, MO]) overnight according to the methods described at the *Seed Genes Projects* website (http://www.seedgenes.org:8081/Tutorial.html#Nomarski%20Optics). Samples were mounted between a microscope slide and a cover slip with Hoyer's solution (7.5 ml water, 1.3 g Glycerol, 1.9 g gum arabic and 25 g chloral hydrate) and observed with an Axioskop 2 plus microscope (Zeiss, Jena, Germany). DIC optics coupled to the Pixera

camera was used to capture images which were then processed with Pixera Visual Communication Suite software (Pixera Corporation).

RESULTS AND DISCUSSION

Different stages of developing siliques and seeds exhibited distinct colors (Fig.3). Younger seeds are smaller, lack visible testa pigmentation, and are found in siliques with green coloration (Fig. 3, 1-8). Desiccated siliques showing a light brown valve contain seeds with full testa pigmentation (Fig. 3, 16). Testa pigmentation is directly associated with the maturity of seeds (Kitamura *et al*. 2004).

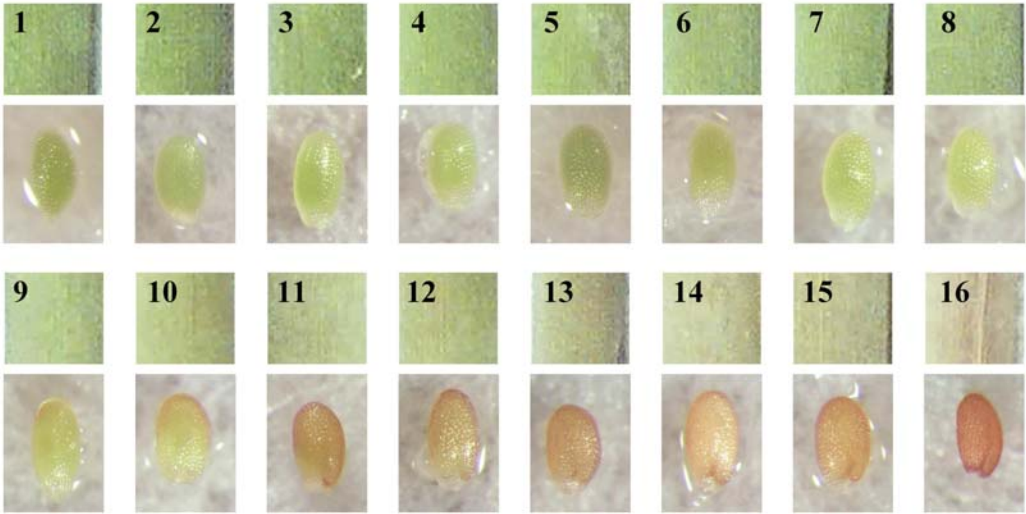

Figure 3. Coloration of siliques and seeds at different developmental stages. Each seed image corresponds to the image of silique valve. Succes-sively grown siliques (1-16) are shown with 1 being the youngest and 16 the oldest.

Embryogenesis in developing seeds was analyzed using seed clearing and DIC microscopy. Representative images of successive embryonic stages are shown in Figure. 4. There was, however, a slight variance among individual seeds found within a single silique (*e.g.*, 7 DAP silique can contain globular stage embryos, as well as a pre-globular stage embryos). The following embryogenesis stages, which were defined in previous reports (*e.g.*, West *et al.* 1993), were also observed in this study: one-cell embryo proper (~1 DAP), two-cell embryo proper (~3-5 DAP), octant (~3-5 DAP), dermatogen (3-5 DAP), early globular (~3-5 DAP), procambial (~4-6 DAP), triangular (~5-7 DAP), heart (~6-8 DAP), mid-torpedo (~8-12 DAP), walking-stick (~13 DAP), bent cotyledon (~14-16 DAP), and mature (~16-21 DAP). The suspensor was also observed in DIC images. The suspensor is an important tissue for providing nutrients and growth regulators to the embryo proper during early stages of development. Digestion of the suspensor is noted around the heart/torpedo stage (~9-11 DAP) when needed nutrients are attained from the surrounding endosperm (Yeung *et al.* 1993; Schwartz *et al.* 1994). The correspondence between DAP and the position of the silique is indicated by the schematic in Figure 4.

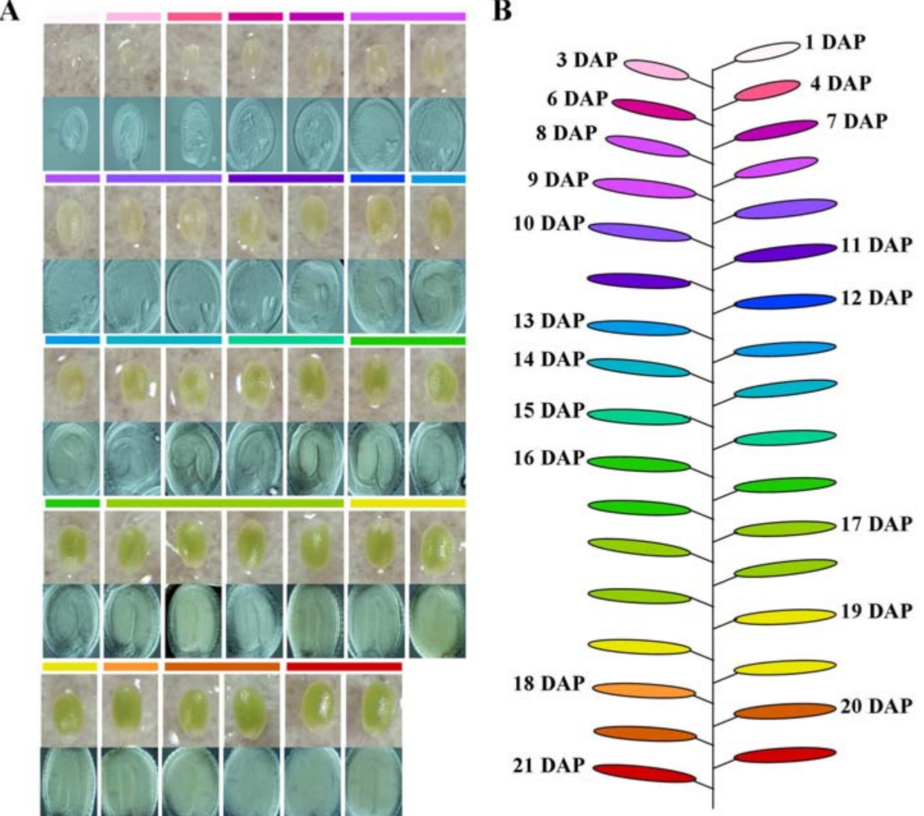

Figure 4. Embryogenesis in siliques at the different developmental stages. (A) Developing seeds excised from silique (upper tier) and embryo images in seeds observed by clearing and DIC microscopy (lower tier). **(B)** Schematic of successively growing siliques used for experiment. Colors in the scheme correspond to colors shown in panel A. Approximate time points in DAP for each silique development are shown. Mature seed has ~500 µm height by ~375 µm width.

The ability of the embryo to grow (or germination potential) is observed during seed development (Bewley *et al.* 1994; Bewley *et al*. 1997). To define when growth potential occurs during Arabidopsis embryogenesis, embryos were excised from every third silique of an inflorescence (Fig. 5A). This analysis was completed on the primary inflorescence of a single plant. The appearance of excised seeds and embryos, including DIC images of cleared seeds are shown in Figure 5. Germination potential

was not detected until A6 or late mature stage (see the numbers of seeds germinated/tested indicated below the panel in Fig. 5B).

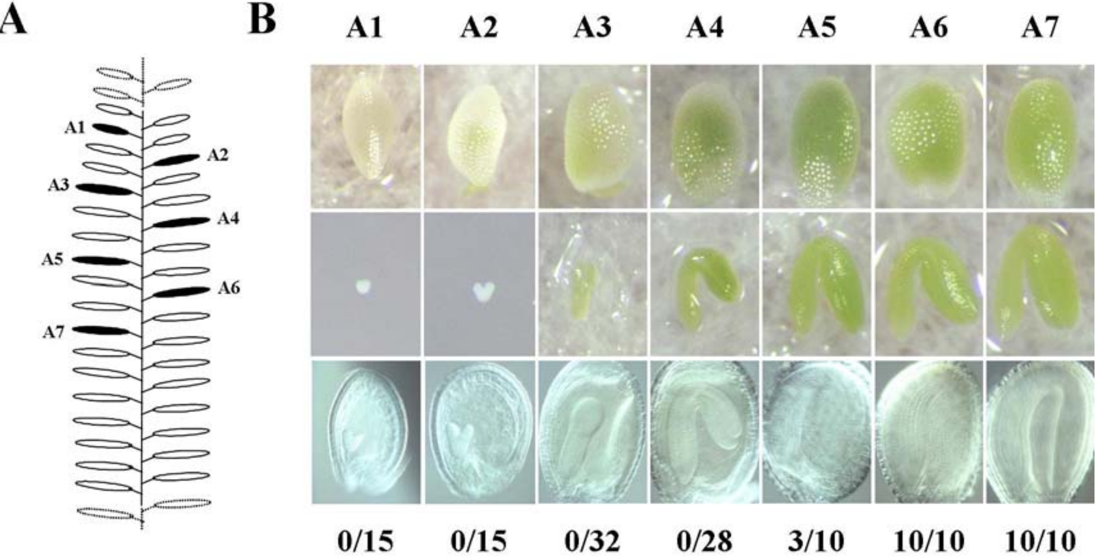

Figure 5. Germination potential of variable stages of Arabidopsis embryogenesis. (A) Schematic to indicate the position of siliques used for germination potential test (A1-A7, filled in black). **(B)** Images of excised seeds (top row), embryos (middle row) and cleared seeds (bottom row);A1-A2, A3, A4, and A5-A7, heart, mid-torpedo, walking-stick, and mature stages, respectively. Number of germinated/tested embryos are shown under the panels.

Germination potential was only observed in the mature stage. To identify more accurate timing of the induction of germination potential, developing embryos were excised from successive siliques, all of which contained morphologically indistinguishable mature embryos (Fig. 6A and B). This analysis was also completed on the primary inflorescence of a single plant. For each silique, all embryos were excised and plated on MS media to determine germination potential. The test was repeated using three biological replicates. Embryos were germinated on MS media with or without 1% (w/v) sucrose. All tests conveyed the same findings: only late mature stage embryos exhibited 100% germination potential. These results suggest

that physiological maturation, which is probably completed after reaching morphological maturation, is vital for an embryo to generate germination potential. Germination potential tests also indicate that loss of green coloration occurs in the whole embryo, axis, or cotyledons of the relatively early mature embryos that do not exhibit full germination potential (Fig. 6D and B1 to B5). However, germinable embryos at the later stages retained green color (Fig. 6D and B6 to B7). This also supports the contention that there are some differences among the mature embryos at different ages in terms of physiological maturity.

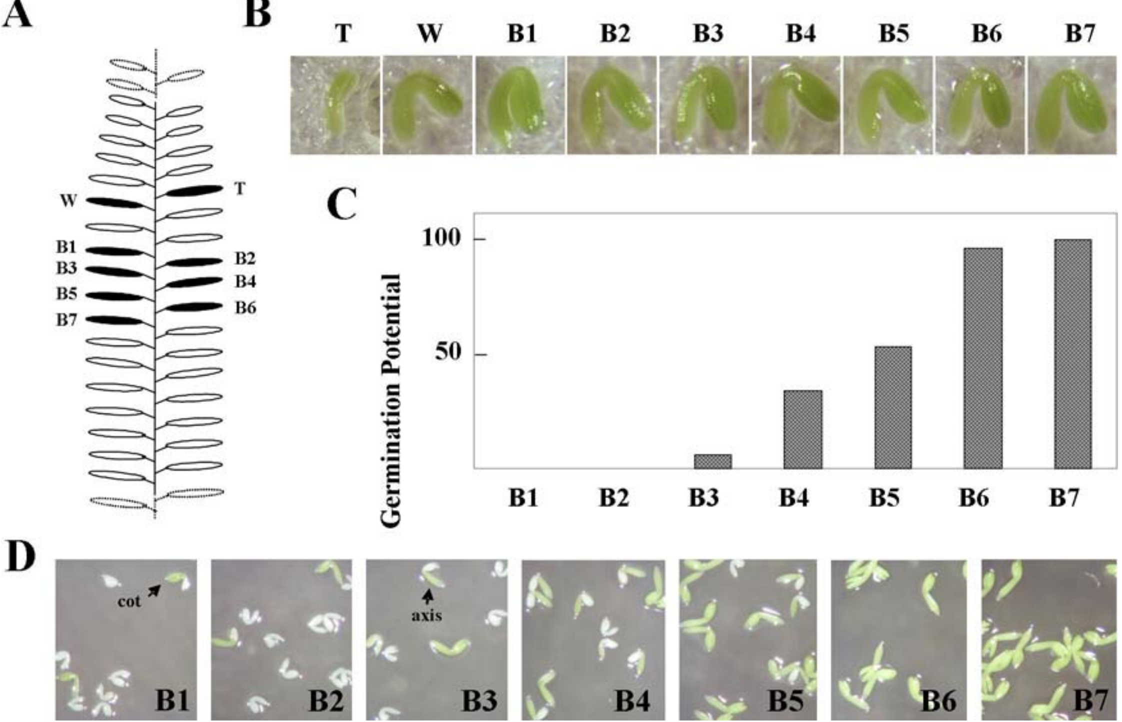

Figure 6. Induction of germination potential during mature embryo stages. (A) Schematic of the position of siliques used for experiment (B1-B7, filled in black). To provide a reference for the position of the earliest mature embryo-containing silique, the positions of siliques containing torpedo (T) and walking-stick (W) stage embryos were also shown (filled in black). **(B)** Images of embryos excised from T, W and B1-B7. (C) Germination percentage of morphologically mature embryos (B1-B7). (D) Depletion of color occurring in the embryos during germination potential test. Cotyledons (cot) and embryonic axis (axis) are indicated by arrows.

Acquiring desiccation tolerance prior to dispersal is important for orthodox seeds, such as Arabidopsis. To determine at what stage embryos gain desiccation tolerance, embryos were treated overnight with 0.5% TZ solution that stains living/respiring tissues red. Only mature stage embryos showed full red staining by TZ. This result indicates that complete desiccation tolerance is induced only at mature embryo stages. The embryos at the B6 to B7 stages exhibited full germination potential (Fig. 6D), but did not exhibit desiccation tolerance (data not shown). Therefore, the timing of the induction of desiccation tolerance is later than that of the occurrence of germination potential.

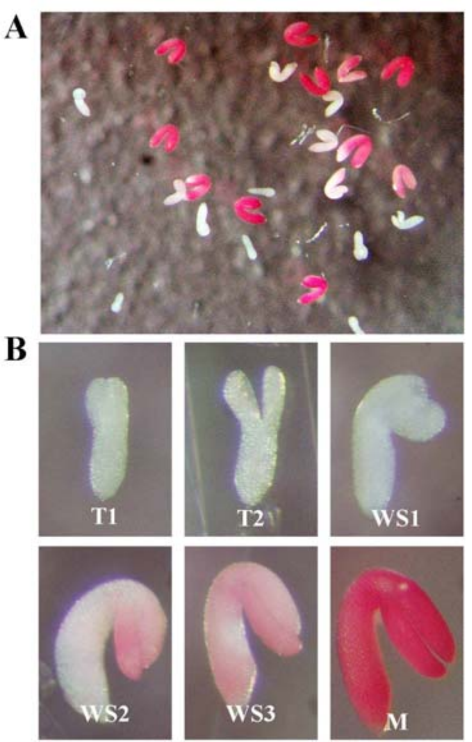

Figure 7. The induction of desiccation tolerance in developing embryos. Siliques containing different stages of developing seeds were dried, desiccated seeds were rehydrated and embryos were excised and stained with TZ. Red positive staining shows that tissue is respiring indicating that seeds are desiccation tolerant. **(A)** Multiple stages of excised embryos. **(B)** Individual embryos stained by TZ. No, partial or complete staining was observed at early torpedo to early walking-stick (T1, T2, WS1), late walking-stick (WS2, WS3) or mature (M) embryos, respectively.

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Chapter 3

Isolation of *embryo ball* Mutant

Jessica Hewitt and Hiroyuki Nonogaki

INTRODUCTION

Due to the diversification of seeds, it is difficult to generalize when and how certain mechanisms, such as germination potential, dormancy, and desiccation tolerance are induced during seed development (Bewley 1997). Mutant lines in Arabidopsis have been instrumental in determining which genes might play a critical role in seed development. The frequency of natural mutation in plants is low, with the average substitution rate per nucleotide being roughly 6×10^{-9} per year (Schultz et al. 1999). Therefore, many researchers use mutants made in an unnatural fashion. To attain random nucleotide substitutions, DNA can be treated by either chemicals or UV light. While these approaches are effective in terms of inducing mutation, it is time consuming to identify the site of mutation. Mutations can also be introduced by randomly inserting foreign DNA, called transferred DNA (T-DNA), into the genome. Since T-DNA fragments containing marker genes and/or known DNA sequences are used in this method, insertion sites can be rapidly identified with PCR. While the efficiency of mutagenesis varies depending on plant species and their genome size, many loss-of-function, gain-of-function, and alteration-of-function mutants have been isolated in Arabidopsis.

There are two main types of selection used for Arabidopsis to screen mutant phenotypes. The first is a basic developmental analysis. Plants that show irregular development or defects can be selected from the mutagenized population and further studied. The second is a positive selection which entails placing plants in adverse conditions and assessing phenotypes. Plants showing irregular reactions might contain

an advantageous mutation. To find genes associated with germination, many screenings using exogenous plant hormones, such as abscisic acid (ABA) and gibberellin (GA) have been performed. Both GA (Koornneef *et al.* 1985) and ABA (Finkelstein *et al.* 1990) insensitive mutant lines have been isolated. The problem with this method is that hormone mutants have pleiotropic effects in addition to germination phenotypes *per se*.

Microarray, a system that allows analysis of gene expression on a genome-wide scale (Schena *et al.* 1995) can also be used for seed research. By printing complementary DNAs (cDNA) on glass and administering a probe mixture made from expressed RNA, differential gene expression can be analyzed. For example, gene expression patterns can be compared between a loss-of-function mutant for a given gene and WT to determine possible downstream effects of the knocked out gene. To elucidate how endogenous GA controls seed germination, microarray analysis has been performed for WT and GA deficient seeds (Ogawa *et al.* 2003). This work identified numerous transcription factors upregulated by GA during seed imbibition.

In an effort to characterize the function of seed germination-associated genes identified by Ogawa *et al.* (2003), the SALK T-DNA insert knockout lines of these genes were ordered from the Arabidopsis Biological Resource Center (ABRC). Multiple insertion lines were ordered, if available. An interesting mutant exhibiting embryo lethality was identified during this screening and provided an excellent tool for embryogenesis research. In this chapter, isolation and initial characterization of this mutant is described.

MATERIALS AND METHODS

Plant Materials

The seeds *of Arabidopsis thaliana* wild-type Columbia (Col-0) and knockout plants were obtained from the ABRC, Ohio State University. Plants were grown in a greenhouse (22°C) under long day conditions (16 h light/8 h dark periods) to induce flowering and seed production. Siliques were harvested and dried at 25°C in an incubator for 7 d. Seeds were extracted from dry siliques and stored at 4°C until use.

Screening

To screen for germination mutants, 10 plants for each knockout line were grown. Seeds (200-300) from each knockout plant were plated on filter paper (No. 2, Whatman Inc, Clifton, NJ) moistened with 4 ml water, given 3 d cold stratification and examined for germination. Multiple seed lots (~15) were plated in a large plastic petri dish (12.5 cm diameter) without replication for the preliminary screening.

Genomic DNA Extraction

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/.

Genome-Walking PCR

Genome-walking PCR was performed using Genome Walker Kit (Clontech Laboratories, Inc, Mountain View, CA) according to the manufacturer's instructions. Briefly, genomic DNA from the mutant line (see RESULTS AND DISCUSSION) 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 left border-specific primer (LBa1: 5'-TTGGGTGATGGTTCACGTAGTGGGCCATCG-3'). Second round PCR was conducted using the nested adapter primer (5'-ACTATAGGGCACGCGTGGT-3') and the nested T-DNA left border-specific primer (LBb1: 5'-ACCAGCGTGGACCGCTTGCTGCAACTCTCT-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 a Putative T-DNA Insertion Site in the Mutant

To verify the predicted T-DNA insertion site in the At2g41940, a gene-specific primer (ZFP8 VerGSP1: 5'-CTGAAGCAGTAGTGACACTC-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 LBb1 primer and genomic DNA of the mutant

line. To verify the other predicted T-DNA insertion site in At2g31760, a gene-specific primer (ARI-like Ver: 5'-CCATCGCTGTTATTCTCCTC-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 LBb1 primer and genomic DNA of the mutant line. 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). PCR products were examined on 1% (w/v) agarose gel and DNA bands were visualized using ethidium bromide and UV light in a Fluor-S Multimage (Bio-Rad, Hercules, CA).

DNA Sequencing

DNA sequencing was done by the Central Service Laboratory in the Center for Genome Research and Biocomputing at Oregon State University. Primer LBb1 was used for sequencing the products of genome-walking PCR.

RESULTS AND DISCUSSION

GA plays a key role in Arabidopsis seed germination (Koorneef *et al.* 1985). Numerous genes are associated with GA biosynthesis and response (Ogawa *et al.* 2003). To characterize genes vital to seed germination, thirteen transcription factor genes induced by GA (from Ogawa *et al.* 2003) were chosen for further analysis (Table 1). SALK knockouts lines for each of these genes were ordered from the

ABRC. Multiple insertion lines were ordered for each gene, when available. Most knockout lines showed no germination phenotype, most likely due to gene redundancy. Only two knockout lines (At2g24790 and At2g41940, both zinc finger proteins) exhibited an embryogenesis phenotype. SALK_045674 line which exhibited an interesting embryo phenotype (see below) was chosen for further analysis.

Table 2.1 Transcription factors induced by GA during Arabidopsis seed germination (identified by Ogawa *et al.* 2003).

#	AGI	Transcription Factor	Gene Name	Knockout #
1	At1g10200	Lim Domain		SALK_016711
2	At1g22640	MYB	*MYB3*	NA
3	At1g24625	Zinc Finger (C2H2)	*ZFP7*	SALK_112481
4	At1g33240	Trihelix DNA binding	*GTL1*	SALK_005965 SALK_005966
5	**At2g24790**	**Zinc Finger (B-box)**	***CONSTANS-like***	**SALK_149951** **SALK_001005**
6	At2g28200	Zinc Finger (C2H2)		SALK_048250
7	At2g28510	Zinc Finger (DOF)		NA
8	At2g39900	Lim Domain		SALK_067765 SALK_067767
9	**At2g41940**	**Zinc Finger (C2H2)**	***ZFP8***	**SALK_045674**
10	At2g45050	Zinc Finger (GATA)		SALK_008845
11	At3g28210	Zinc Finger	*PMZ, AN1-like*	SALK_014701 SALK_021881
12	At4g32890	Zinc Finger (GATA)		SALK_080142 SALK_152156
13	At4g36540	bHLH		NA

* Numbers of available SALK_knockout lines are shown.

Out of 10 individual plants grown from SALK_045674, four showed a seed-associated phenotype. In those four individuals, reduced germination percentage (~75 %) was observed (data not shown). Upon closer analysis of dry seeds, 23.5 % (in table 2) of seeds in these individual lots turned out to be shrunken (Table 2, Fig. 8A white arrows). After 3 d prechiling at 4 ˚C, normal seeds in the same lots showed complete

imbibition (Fig. 8B). The shrunken seeds did not fully imbibe after 2 d (Fig. 6C) and still were darker brown than normally imbibed seeds after 3 d (Fig. 8D). A small ball like, arrested embryo structure was found in the fully imbibed shrunken seed (Fig. 8E). The ball like embryo was notably smaller than WT embryos (Fig. 8G). The mutant line with the arrested embryo was named *embryo ball (eb)*.

Table 2.2 Percentage of shrunken seeds found in the progeny of SALK_045674.

	Seed Number		% Shrunken
	Normal	Shrunken	
Plant 1	240	70	22.6
Plant 2	242	65	21.2
Plant 3	213	85	28.5
Plant 4	253	77	23.3

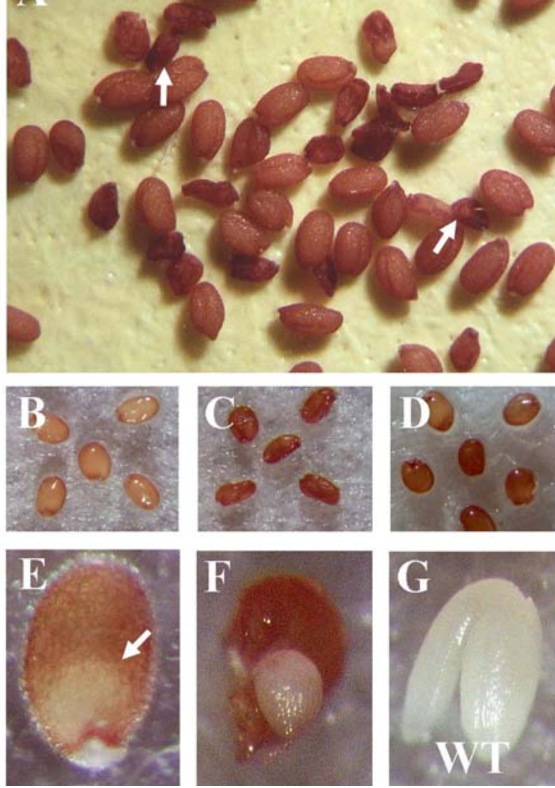
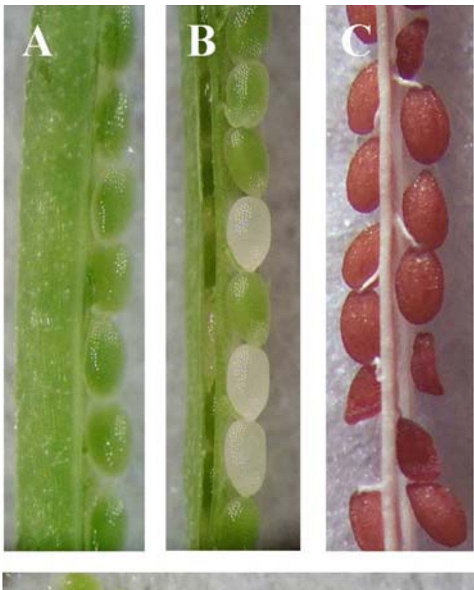


Figure 8. Shrunken seeds and arrested embryos found in SALK_045674 progeny. (A) Shrunken seeds (arrows) observed before imbibition. **(B)** WT seeds imbibed for 2 d, **(C) and (D)** *eb* seeds imbibed for 2 d and 3 d, respectively. **(E) and (F)** Embryo-like structures contained in and excised from *eb* seeds, respectively. Arrow indicates the position of embryo-like structure. **(G)** Excised WT embryo.

To investigate developmental defects that are potentially causing shrunken testa and arrested embryo phenotypes, developing siliques were dissected and seeds were examined. White seeds that were not observed in WT siliques were found in the mutant siliques (Fig. 9A, B and D). These colorless seeds became shrunken seeds at maturity (Fig. 9C). The segregation between green/round (76.9 %) and white/shrunken (23.1 %) seeds indicated that the mutation is recessive.



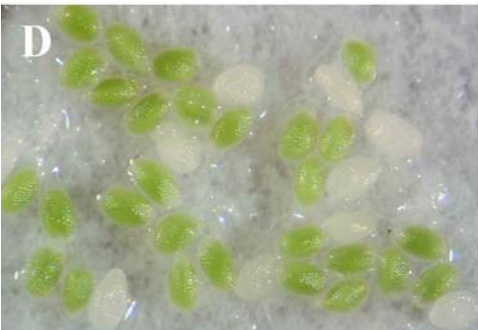


Figure 9. White and shrunken seeds found in developing mutant siliques. (A) WT developing seeds containing mature embryos in a silique. **(B)** WT (green) and mutant (white) seeds found in *eb* silique. **(C)** Mature seeds in *eb* silique. Note that white seeds have become shrunken seeds. **(D)** WT and mutant seeds excised from silique shown in B.

Homozygous *eb* mutant seeds were also noted to have a precocious pigmentation phenotype (Fig. 10B) though earlier staged *eb* mutant show no pigmentation (Fig. 10A). The precocious pigmentation seen in *eb* seeds is similar to pigmentation in late mature stage WT seeds (Fig. 10C).

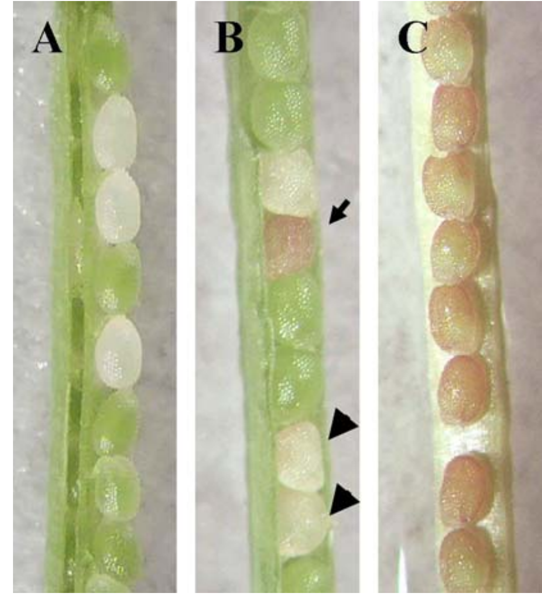


Figure 10. Precocious pigmentation in *eb* seeds. (A) Developing *eb* seeds (white) and WT seeds (green) before pigmentation. **(B)** Developing *eb* seeds showing partial (arrowhead) or full (arrow) pigmentation. **(C)** WT maturing seeds starting pigmentation.

The *eb* mutant (SALK_045674) was originally ordered for the T-DNA insertion knockout of the At2g41940 (*ZFP8*, a C2H2 type zinc finger protein). The insertion was found 92 bp upstream of the coding region of the zinc finger protein (Fig. 11A). However, genotyping results using PCR indicated that this insertion was not responsible for the *eb* phenotypes. Some plants containing the T-DNA/*ZFP8* flanking sequence, which were derived from backcrossing *eb* to WT, did not show *eb* phenotype. A second T-DNA insert was found in *eb* mutants (1kb upstream of the coding region of At2g31760, an ARIADNA-like protein) (Fig. 11B), but also turned

out not to be responsible for the *eb* phenotype. Since *eb* seeds exhibit typical characteristics of embryo lethality (see next chapter) most likely caused by recessive mutation of a single gene, they provide an excellent tool for embryogenesis and germination research. The responsible gene remains to be identified.

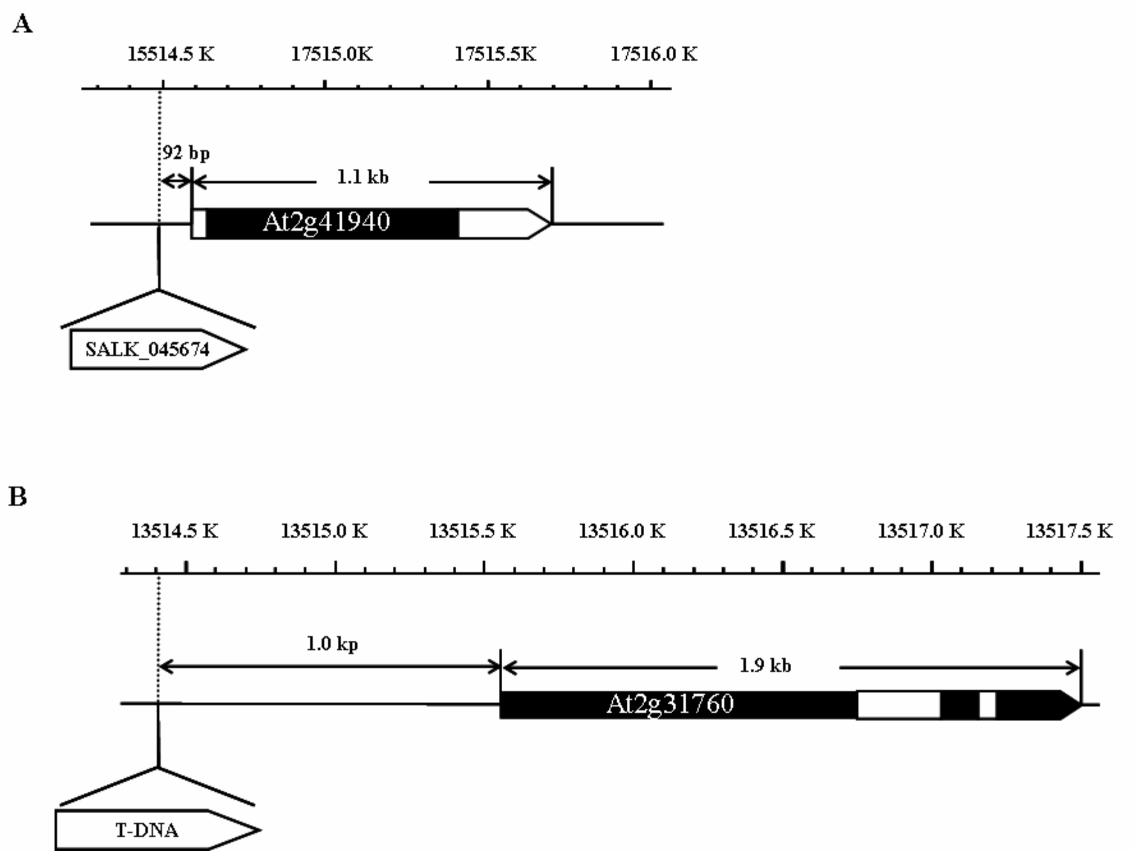


Figure 11. Schematics of SALK T-DNA insertion sites and flanking genes found in *eb* mutant. (A) The insertion site SALK_045674 flanking At2g41940 (zinc finger protein, *ZFP8*). **(B)** Another insertion site found by genome-walking PCR flanking At2g31760 (ubiquitin-protein ligase/ zinc ion binding).

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Chapter 4

Analysis of Germination Potential in Developing Arabidopsis Seeds Using *embryo ball* Mutant

Jessica Hewitt and Hiroyuki Nonogaki

INTRODUCTION

Embryogenesis is a unique developmental process that determines the capabilities of all organs to follow. Groups of cells can be distinguished during embryogenesis, organizing development of unique tissues, including the shoot meristem, epidermis, ground tissue, procambium, storage parenchyma, and root meristem (Yadegari *et al.* 1994). Each group of cells originated from a cell that uniquely differentiated during the globular stage (Jurgens 2001).

Within a globular embryo, key regulatory genes vital to the differentiation process are expressed (Fig. 12). Epidermal cell precursors are specified at the outer edge of the globular embryo by *Arabidopsis thaliana MERISTEM L1 LAYER* (*AtML1*) (Lu *et al.* 1996; Sessions *et al.* 1999). *ASYMMETRIC LEAVES1* (*AS1*) encoding a MYB-domain transcription factor is associated with leaf organ primordium initiation (Byne *et al.* 2000). *SHOOT MERISTEMLESS* (*STM*) is essential for maintaining undifferentiated shoot meristematic cells by repressing the *AS1* gene (Barton *et al.* 1993; Long *et al.* 1996; Aida *et al.* 1998). *WUSCHEL* (*WUS*) is another gene responsible for the maintenance of shoot meristem stem cells (Laux *et al.* 1996; Mayer *et al.* 1998). *SCARECROW* (*SCR*) and *SHORT ROOT* (*SHR*) expressed in vascular primordium precursor cells are probably required to maintain organization of subepidermal radial patterning (Scheres *et al.* 1995). A mutation in *HOBBIT* (*HBT*) causes a problem in root meristem formation (Willemsen *et al.* 1998; Blilou *et al.*2002).

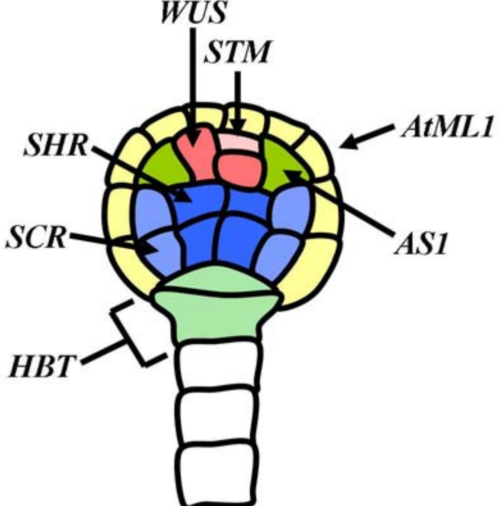


Figure 12. Schematic of a globular stage embryo and genes associated with cell fate. Cells are highlighted in colors based on their function in tissue differentiation: yellow, epidermal tissue; green, organ primordium initiation; pink, maintenance of stem cells; light pink, subsequent rate of division; light blue ground tissue, dark blue, vascular primordium; light green, root. *AtML1, Arabidopsis thaliana MERISTEM L1 LAYER* (Lu *et al.* 1996; Sessions *et al.* 1999); *AS1, ASYMMETRICAL LEAVES1* (Byne *et al.* 2000); *WUS, WUSCHEL* (Laux *et al. 1996*); *STM, Arapidopsis SHOOT MERISTEMLESS* (Aida *et al.* 1998); *SCR, SCARECROW* (Scheres *et al.* 1995); *SHR, SHORT ROOT* (Scheres *et al.* 1995); *HBT*, *HOBBIT* (Willemsen *et al.* 1998; Blilou *et al.* 2002) (Scheme modified from Jurgens *et al.* 2001).

In addition to the regulatory maintenance of cell identity by specific genes, the hormone auxin is also associated with cell division and organization of the embryo proper during embryogenesis. Mutants with irregular auxin gradient, such as *abp1*, *pin1 pin3 pin4* pin*7* and *laterne* (Chen *et al.* 2001; Friml 2003; Friml *et al.* 2003; Fu *et al.* 2003; Blilou *et al.* 2005; Treml *et al.* 2005; Weijers *et al.* 2005), exhibit defects in embryogenesis. *GURKE* (*GK*), another gene associated with the early stages of embryogenesis may be important for proper regulation of auxin within the embryo.

Different stages of Arabidopsis embryogenesis are generally classified, based on morphological characteristics. While they are over-lapping stages, each stage has

specific developmental programs. With the discovery of many genes essential to embryo development, the molecular mechanisms underlying morphological and physiological maturation are being elucidated. In this chapter, the *embryo ball* (*eb*) mutant embryo which does not require morphological maturation to attain germination potential is described. The importance of morphological and physiological maturation in determining germination potential will be discussed.

MATERIALS AND METHODS

Embryo Excision and Incubation

Embryos were excised using fine forceps and a surgical blade under a dissection microscope. Embryos were incubated in Murashige-Skoog (MS)-medium (Murashige and Skoog 1962) with 1% (w/v) sucrose.

Scanning Electron Microscopy

Excised embryos were fixed overnight in 10 mM potassium phosphate buffer, pH 7.0 containing 4% (w/v) paraformaldehyde 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 ~10 nm of 60/40% Au/Pd using an EDWARDS S150B sputter coater (Edwards High Vacuum, Ltd., West Sussex, England) operating at 1 x 10^{-2} Torr, 5 mbar Argon pressure, 1.5 kV, 20 mA plasma

current, for 60 sec. Specimens were examined with an AmRAY 3300FE SEM (AmRay, Bedford, MA) in the Electron Microscope Facility, Department of Botany and Plant Pathology, Oregon State University.

Cross Pollination

Cross pollination was performed to backcross the mutant and the GUS reporter lines (see RESULTS AND DISCUSSION). Anthers were removed from flowers prior to pollination of the stigma. Pollen was then dabbed on the stigma and pollination was confirmed by a dissection microscope. Flowers that were not cross-pollinated were removed from the inflorescence and cross-pollinated flowers were labeled. Once maturation drying was complete, cross-pollinated siliques were removed and dried further at 25°C for 7 d before seed harvest.

GUS Staining

GUS staining of embryos was performed 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) as previously described (Weigel *et al.* 2002). Staining was examined with a dissection microscope after 3 h at room temperature.

RESULTS AND DISCUSSION

As described in Chapter 3, *eb* mutant produces shrunken seeds and imbibed *eb* seeds contained arrested embryos. *eb* is unique in terms of how and when the embryo

arrests during embryogenesis. The majority (88%) of *eb* embryos dissected from imbibed mature seeds exhibited a ball-like structure (Table 3, I to III, Fig. 13, A to C), while apparently larger irregular shape embryos were found in the rest (Table 3, IV and V, Fig. 13, D to G). The smallest *eb* embryos (Table 3, I, Fig. 13A) were larger than WT heart embryos (Table 3). All irregular embryos (Table 3, IV and V, Fig. 13, D to G) were smaller than WT mature embryo (Fig. 13H). The variation in *eb* embryo types suggest that, while *eb* mutation causes impaired embryo differentiation at early developmental stages, the mutant embryos have potential for further growth.

Table 3.1 Shape and size of arrested embryos found in *eb* seeds.

Stages	Embryo (Description)	Height (μm)	Width (μm)	Number	Frequency (%)
I	(Globular-like)	~100	~100	44	52
II	(Transition-like)	~155	~130	18	22
III	(Early heart-like)	~180	~175	12	14
IV	(Asymmetrical)	~180-215	~180-315	4	5
V	(Torpedo-like)	~255	~275	6	7

*The size of WT heart, torpedo and mature embryos were measured as ~90 μM in height (H) /~80 μM in width (W), 140 μM H/75 μM W and 425 μM H/265 μM W, respectively.

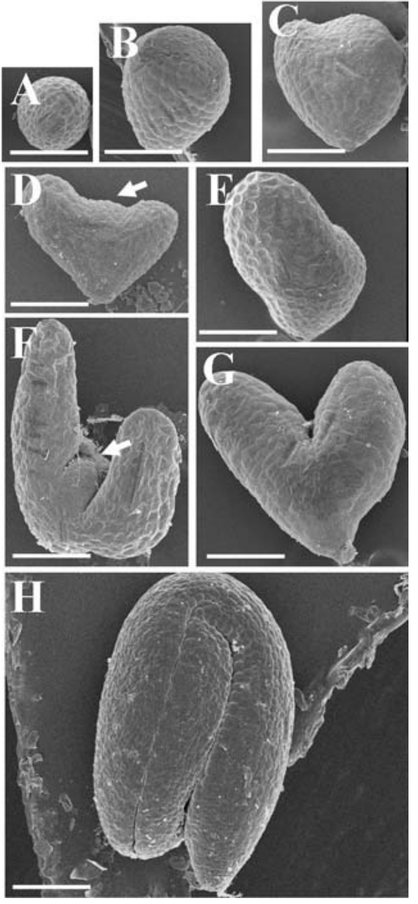


Figure 13. SEM images of arrested *eb* embryos and normal WT embryo. (A), **(B)**, **(C)**, **(D)** to **(F)** and **(G)** correspond to categories I, II, III, IV and V in Table 3, respectively. **(H)** WT mature embryo.

To determine at what stage during embryogenesis morphological abnormalities of *eb* embryos start, detailed embryo development in *eb* seeds was analyzed using DIC microscopy. At the octant (Fig. 14A), dermatogen (Fig. 4B) and early globular stages (Fig. 14, C and D), WT and *eb* embryo were indistinguishable. Although *eb* embryos initially developed at the same pace as WT embryos, a delay in *eb* embryo growth became apparent at later stages (transition/heart stages) (Fig. 14, E and F). Around these stages, mutant embryos started to show aberrant cell organization in the

procambium (Fig. 14G and H). These results suggest that the *EB* gene (unidentified) functions during early stages of embryogenesis. This is different from mutants, such as *wus*, *as1,* and *hbt* that have defects in genes responsible for the development of specific organs. In these mutants, abnormal development is restricted to the locations of gene expression. *eb* is more closely related to *gk* in terms of the extensive developmental defects early in embryogenesis and the inability to reach the morphologically normal mature embryo stage.

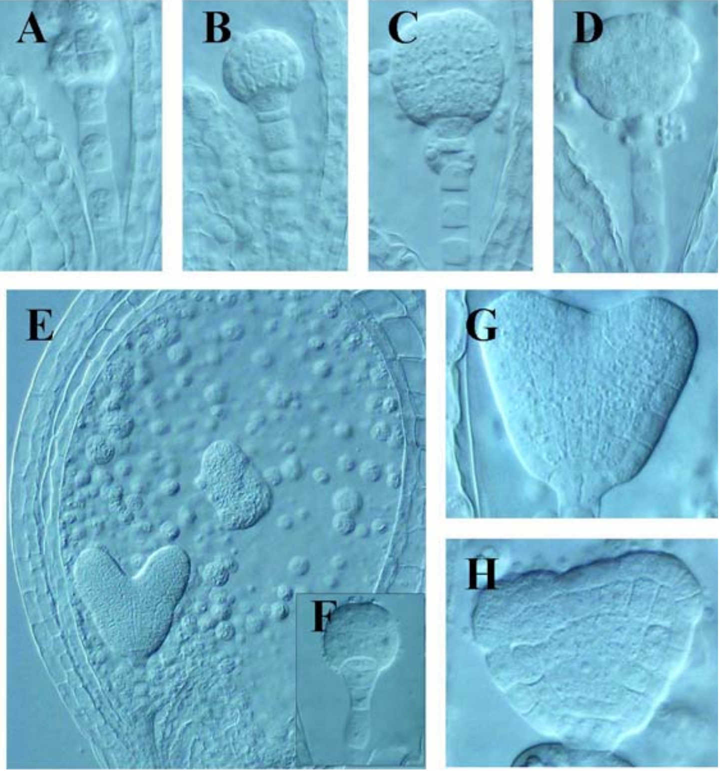


Figure 14. DIC images of developing embryos found in *eb* siliques. **(A)** Octant **(B)** dermatogen and **(C)** globular stage embryos. WT and *eb* embryos in mutant siliques show no apparent defects at these stages. **(D)** Mutant embryo exhibiting irregular cell differentiation at globular/transition stage. **(E)** Heart and **(F)** Globular stage embryos found in a single *eb* silique, respectively. Around this stage differences between WT and mutant embryos becomes apparent. **(G)** WT and **(H)** mutant heart stage embryos.

Although embryo defects were observed during early embryogenesis, the mutant embryos were not completely arrested, but continued to grow in terms of embryo size. A question is raised whether *eb* embryos still complete physiological maturation. A most prominent feature of seed maturation is the development of desiccation tolerance as described in Chapter 2. Only morphologically mature embryos exhibit desiccation tolerance in Arabidopsis (Fig. 7). When *eb* embryos (131) were excised from imbibed mature seeds (which had experienced maturation drying) and subjected to TZ test, seven embryos exhibited full positive staining (Fig. 15A) and 13 embryos exhibited partial positive staining (Fig. 15, B to D) while the majority were negative (Fig. 15, E and F). This indicates that some of *eb* embryos can complete the maturation program, suggesting that cell maturation can occur independently from morphological maturation.

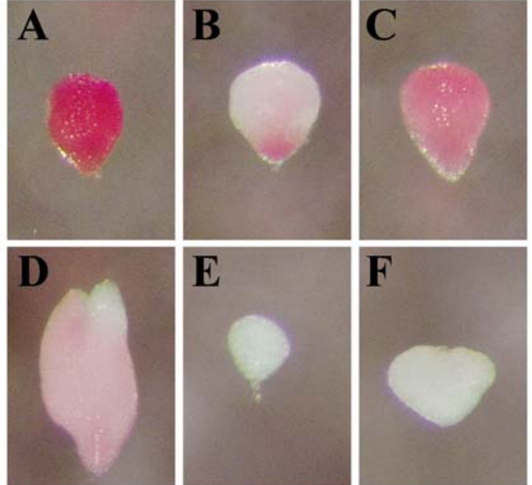


Figure 15. Desiccation tolerance of *eb* embryos excised from mature imbibed seeds stained by TZ. **(A)** Complete staining by TZ indicating desiccation tolerance of the embryo. **(B)** to **(D)** showing partial and **(E)** to **(F)** showing no staining by TZ. All WT mature embryos tested showed positive staining by TZ (refer to Fig. 7).

While partially desiccation tolerant, none of the mature *eb* seeds germinated (data not shown). However, when *eb* embryos were excised from fresh developing seeds and plated on MS media containing 1% (W/V) sucrose, many of them (77%) were able to grow. Although the degree of individual *eb* embryo growth varied, all *eb* embryos followed the same growth patterns. The globular-like embryo showed no growth but exhibited minor volume increase one day after excision (Fig. 16A). After incubation for 2-5 days, root hair-like structures emerged mainly from the apical portion of the embryo (Fig. 16B), which was followed by the initiation of the main root 5-10 d after incubation (Fig. 16C). The main root with root hairs elongated extensively (Fig. 16D), which was indistinguishable from roots of WT germinated seeds.

There are three surprising aspects of *eb* embryo growth observed here. First, as shown in Chapter 2 (Fig. 5 and 6), WT embryos show no germination potential before the mature embryo morphological stage, yet *eb* embryos with serious morphological defects exhibit germination potential at this stage. Second, the growth starts at the apical end of the embryo, which does not normally exhibit growth during germination. Third, root hair-like structures that should be specific to the basal end of the embryo, emerge at the apical portion. These observations support the notion that *eb* lacks proper cell organization. Another interesting implication from these results is that the induction of germination potential does not require morphological maturation. Cells in the basal portion of *eb* embryos might go through normal cell maturation programs that are required for radicle elongation in WT seeds, despite the serious defects in other parts of the embryo.

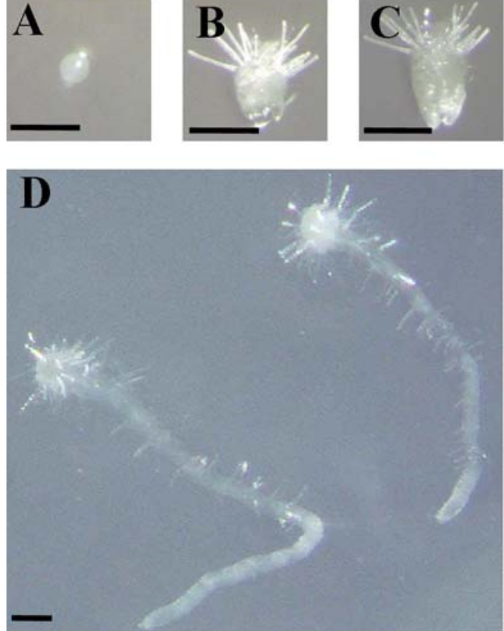


Figure 16. Growth of *eb* embryos excised from developing seeds. (A) *eb* embryo 1 d after excision, **(B)** emergence of root hair-like structure from the apical portion of the embryo after 2-5 d and **(C),** 5-10 d incubation. Note that root growth is initiated in *eb* embryo shown in panel C. **(D)** Extensive elongation of the main root from *eb* embryo (~10-14 d incubation). Black bars 250 µm.

When incubation is prolonged, leaf-like structures emerged from the apical portion of *eb* embryos (Fig. 17A). Trichomes and stomata characteristic to vegetative leaves were found on the leaf-like structures (Fig. 17, B and C). *eb* embryos seem to lack cotyledon identity which was observed in other mutants (Schnittger *et al.* 1998; Bergmann 2006). Both *gk* and *laterne* are mutants that lack cotyledon identity. Unlike *laterne, gk* and *eb* are not able to develop into normal plants. *eb* embryos did not show normal differentiation at the apical portion after growth for an extended period of time (~40 d), while basal growth was normal (Fig. 17D). This normal basal development is not seen is *gk* (data not shown).

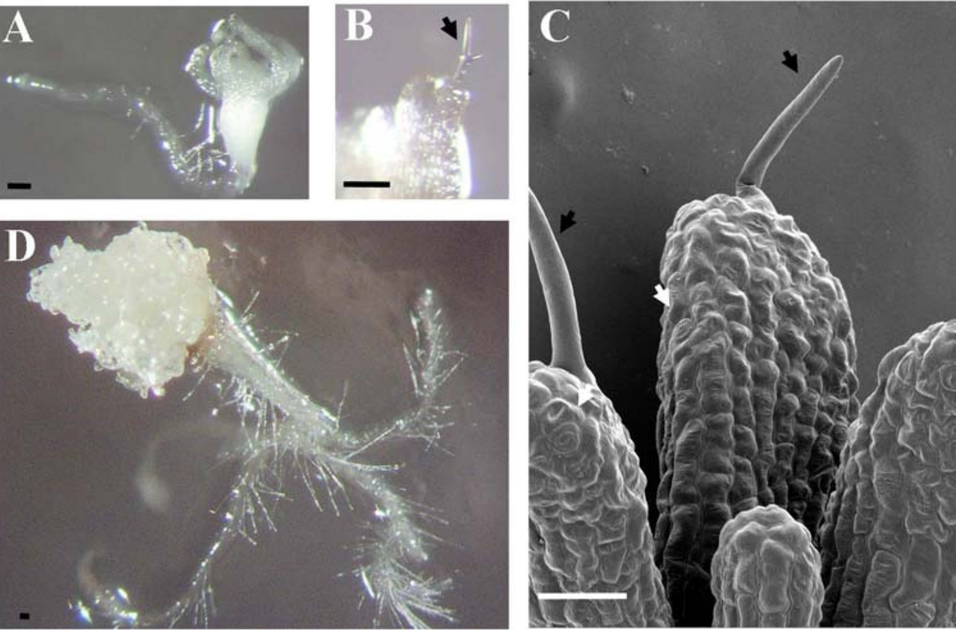
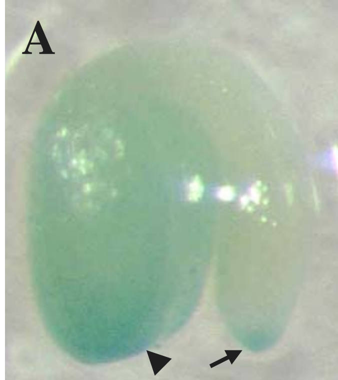


Figure 17. Abnormal apical growth in *eb* embryos. (A) Emergence of leaf-like structures from the apical portion of *eb* embryo following main root growth. **(B) and (C)** Trichomes (black arrows) found on leaf-like structures, stomata are indicated by white arrows in panel C. **(D)** Callus-like structure emerged at the apical portion of *eb* embryo (~40 d incubation). Black bar, 250 µm; white bar, 100 µm.

To further analyze the differentiation of apical and basal tissues in *eb* embryos, the expression of the marker gene *DR5:GUS*, was examined in *eb* embryos. The *DR5* promoter contains an auxin response element (AuxRE) (Friml *et al.* 2003). Auxin plays an important role in pattern formation during embryogenesis (Weijers *et al.* 2005). The *eb* mutant was cross-pollinated with a homozygous *DR5:GUS* line and the progeny were tested for mutant phenotype and GUS expression. While WT embryos showed localized GUS staining at the cotyledon tips and radicle tip (Fig. 18A), GUS staining in the *eb* embryos was detected only at the radicle tip (Fig. 18B). This data indicates that the apical portion of *eb* embryo has lost at least part of cotyledons.



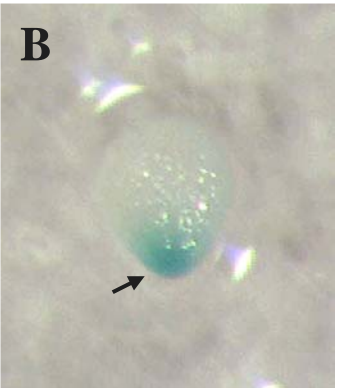


Figure 18. Expression of *DR5:GUS* marker gene in WT and *eb* embryo. (A) GUS signal from cotyledon (arrowhead) and root tip (arrow) of WT mature embryo. **(B)** GUS signal from basal portion (arrow) of *eb* embryo.

The observation above suggests that, while *eb* embryo lacks cotyledon identity, it still retains some sort of shoot meristematic tissue. In addition, the main root grows in a normal fashion. As mentioned above, the most intriguing feature of *eb* embryo is the germination potential during seed development. The mechanisms of the induction of germinability during seed development are largely unknown. This mutant will provide an excellent tool for seed germination study.

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Study of *Arabidopsis thaliana* Seed Development: Occurrence of Germinability

Chapter 5

General Conclusion

Jessica Hewitt

This research characterized embryo development in Arabidopsis by using WT, as well as *embryo ball* (*eb*) mutant embryos. During WT seed development, immature seeds that were small, consisted of embryos that still contained chlorophyll and a clear testa. In contrast, fully mature, desiccated seeds contained large white embryos. The silique color was a good indicator for seed developmental stages: siliques containing immature seeds were dark green while siliques containing mature seeds were a light brown.

Ovules with one-cell and two-cell stage embryos were observed at 1 DAP in WT. The early zygote to triangular stage was transient, while mature embryo stages were observed over a long period. Germination potential was not observed in the embryo until the late mature stage. Extensive accumulation of chlorophyll was detected only in the morphologically mature embryos. Desiccation tolerance was not induced until the late stages of embryo maturation.

Impaired development in *eb* was first observed at the procambial stage and developmental arrest was observed at varying stages during the abnormal developmental pathway. These stages included globular-like, transition-like, early heart-like, asymmetrical and torpedo-like. Even when the *eb* embryo reached the heart-like stage, cell differentiation was drastically different from the WT. *eb* had abnormal cellular organization and embryo organ development. While these stages were structurally different from anything seen in WT embryos, they were also smaller then the WT mature embryo stage.

When provided with nutrient media, *eb* continues to grow and develops normally at the basal portion of the embryo, but exhibits abnormal development at the

apical portions of the embryo. This developmental pattern plus the root tip localized *DR5-GUS* expression in *eb* embryos supports the hypothesis of *eb* cotyledon identity loss. *eb* growth suggests that normal development of the cotyledons and hypocotyl is not necessary for germination potential in root. *eb* is one of few mutants found that does not require morphological maturation to attain germination potential, however the molecular mechanisms responsible for this mutant phenotype are unknown. *eb* defies limitations in embryo development and, as such, has been a vital tool in studying Arabidopsis embryogenesis.

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