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The role of the Viviparous1 (Vp1) gene in anaerobic inhibition of maize embryo germination

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The role of the *Viviparous1* (*Vp1*) gene in anaerobic inhibition of maize embryo germination

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Abstract

An anaerobic or hypoxic environment surrounding the plant seed or embryo is thought to be a crucial factor in controlling maturation and the germination transition. In this thesis, we find a relationship between anaerobically induced germination arrest and the *Viviparous1* (*Vp1*) gene, which is a central transcription factor in the abscisic acid (ABA) hormone signaling pathway in seeds. The loss-of-function mutant and semi-quantitative RT-PCR results both demonstrate a role of *Vp1* in the anaerobic inhibition of germination. This pathway appears to be ABA-independent because an ABA-deficient mutant and an inhibitor of ABA biosynthesis both fail to release embryos from repression. Gibberellin (GA)'s ability to reverse the germination inhibition imposed by anaerobiosis indicates an interaction of this hormone with oxygen and *Vp1*. This paper provides new ideas on the physiological control of one of the most complicated programs in plant development: the transition between maturation and germination.

Keywords Maize, Maturation, Germination, Abscisic acid, *Viviprous1*, Anaerobiosis, Gibberellins

Chapter 1. Overview

The seed is the plant structure that ensures the initiation of the next generation. In it resides a developed plant embryo, separated from its surroundings by a seed coat (Koornneef et. al., 2002). Many seeds are edible and are the major source of caloric intake in people's diets. Also, cotton seed produces the world's most essential clothing fiber. Therefore, the importance of seed biology to the economy and human health can never be overstated.

Angiosperm seeds consist of three different components: (1) the embryo formed by the fusion of the egg cell with a sperm nucleus, (2) the triploid endosperm developed from two polar nuclei fusing with the second sperm nucleus, and (3) the maternally derived seed coat surrounding the outside (Kigel and Galili, 1995). The latter part of development consists of the maturation phase, during which storage compounds accumulate, growth arrests and desiccation tolerance is acquired (Vicente-Carbajosa and Carbonero, 2005; Bewley, 1997). In many plants, including some important crops like rice, wheat and barley, the seed usually enters a period of dormancy after maturation. Seed dormancy is a temporary cessation of germination of an intact viable seed under normally favorable conditions (Li and Foley, 1997).

The accurate control of dormancy and germination has significance in agriculture. Insufficient levels of dormancy can lead to germination on the mother plant during

maturation and before harvest, which is called pre-harvesting sprouting (Li et. al., 2004; Zanetti et. al., 2000). The adverse effect of the pre-harvest-sprouted grains in many cultivars of wheat and other small grains causes a serious worldwide problem resulting in huge economic losses (Gubler, Millar and Jacobsen, 2005). In contrast, long dormancy, which will prevent grain germination and re-mobilization of starch and other reserves in barley grains, will increase cost and result in potential damage for malting barley (Li et al., 2003; Gubler, Millar and Jacobsen, 2005).

Seed germination is dependent on both internal and external factors. Several environmental factors such as light, temperature, PH, oxygen and the duration of seed storage after ripening are known to have functions in controlling seed germination (Koornneef et. al., 2002; Chachalis and Reddy, 2000). Among them, the function of oxygen is complex and not well understood. With a few exceptions (rice, *Erythrina caffra* and four species of *Echinochloa),* the vast majority of species require oxygen to germinate and most of them will arrest germination under restricted oxygen supply (Corbineau and Come, 1995; Perata and Alpi, 1993). The demand for oxygen needed for germination varies significantly among species. For example, according to Al-Ani et al. (1985), the germination rate of maize seed can be stimulated slightly elevated levels of oxygen. On the other hand, treatment of complete anaerobiosis before the seeds are transferred to the air increases germination of dormant seeds in sunflower and apple. A prior period of complete oxygen deprivation is even required for cocklebur seeds to germinate (Corbineau and Come, 1995; Barthe and Bulard, 1983).

However, the level of oxygen in the atmosphere is not equal to that surrounding the embryo. The structure of the seed coat and endosperm inhibit oxygen from penetrating to the embryo. Additional protective structures such as the glumellae (lemma and palea) of barley also play important roles in limiting oxygen diffusion, (Bradford et al., 2008; Benech-Arnold et al., 1999). In 2005, Rolletschek et al. researched the oxygen depletion inside maize kernels and found that oxygen concentration gradients decline from relatively high levels at the outer surface of the embryo to undetectable levels at the embryo/endosperm interface, which complicates the analysis of oxygen conditions surrounding the maize embryo. Hypoxic conditions also surround the embryo in legumes and many other plants (Rolletschek et al., 2002; Cochrane and Duffus, 1979). Meanwhile, maize (which is the main target of this paper), has the largest cereal kernel and the embryo lacks photosynthetic activities, both of which contribute to oxygen limitation (Rolletschek et al., 2005). Hence, although most species require oxygen for germination, the embryo of developing seeds is generally in a hypoxic environment.

What is the mechanism by which seeds or embryos sense and respond to oxygen concentrations to maintain dormancy or to germinate? In Douglas-fir (*Pseudotsuga menziesii*), hypoxia can prevent oxygen from leaching to the media (Bianco et al., 1997). Also, aerobic respiration can be detected right after seed imbibition (Hourmant and Pradet, 1981), which is clear evidence that oxygen is necessary for the metabolic generation of ATP and energy for embryo germination. In addition to aerobic respiration, oxygen plays an important role in hormonal control of seed dormancy and germination. The antagonistic interaction of abscisic acid (ABA) and gibberellin (GA) is thought to be responsible for the

regulation of seed germination; whereas ABA inhibits seed germination and promotes seed dormancy, GA has the opposite functions (Finch-Savage and Leubner-Metzger, 2006; Seo et al., 2008; Kucera et. al., 2005; Schopfer and Plachy, 1985). In the presence of GA, the sensitivity of dormant oat grains to low oxygen concentration drops significantly (Lecat et al., 1992). Meanwhile, when oxygen availability decreases, the sensitivity of embryo germination to ABA will increase, and sensitivity to GA will drop (Bradford et al., 2008; Benech-Arnold et al., 2006). The detailed mechanism of how oxygen interacts with hormone metabolism or signaling has been studied: (1) Barthe et al. (2000) used (+)-[^{3}H] ABA and found that the products of ABA degradation decreased by lowering oxygen availability; (2) ABA 8'-hydroxylase encoded by cytochrome P450 PYP707A is responsible for the inactivation of ABA in *Arabidopsis* and the activity of this enzyme drops with lower oxygen level (Kushiro et al., 2004); (3) An increase in barley embryo ABA responsiveness in hypoxia is at least partly due to an inability of the embryo to inactivate ABA (Benech-Arnold et. al., 2006). At the same time, GA activation and deactivation are also regulated by a series of oxidation and hydroxylation reactions (Lange, 1998), which may be affected by oxygen level.

The transition from the dormant to the nondormant state of many seeds is also dependent on a decrease in ABA sensitivity (Finch-Savage and Leubner-Metzger, 2006). In maize, *Viviparous1* (*Vp1*, whose homolog in Arabidopsis is ABI3) is a key regulator in the ABA signaling pathway (McCarty et al., 1989). *Vp1* encodes a B3 domain-containing transcription factor and mutations in this gene result in reduced sensitivity to ABA and viviparous seed germination (McCarty et al., 1991; Cao et al., 2007). VP1 positively

regulates maturation and ABA response associated genes, and negatively regulates those genes associated with germination (Suzuki et al., 2003). *Vp1* expression can be stimulated or maintained by ABA, sucrose, mannitol, but not light (Cao et al., 2007). There is currently no established relationship between hypoxia and *Vp1* or any components of ABA signaling pathways in maize.

In this thesis, the function of hypoxia on regulating the germination of cultured developing maize embryos was investigated. Mutations in ABA biosynthesis and signaling reduced the effects of hypoxia. Our results also suggest that GA regulation is a major factor in the inhibition of germination under hypoxia.

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Chapter 2. Results

2.1 Introduction

Maize has great importance to humans because of its value in food, feed, energy and other uses. It is also a model plant for scientific research. The kernels, the major edible portion of maize, reside on the maize ear and the embryos are placed inside the kernels, together with the endosperm. After pollination, the embryo starts to develop in the kernel and undergo a specific stage called maturation (Bewley, 1997), during which growth is arrested and storage compounds are prepared for embryogenesis or germination. Unlike other important crops like wheat and rice, maize does not have a dormancy stage after maturation (Koornneef et al., 2002). However, the proper control of the transition between maturation and germination is still of great importance to the maize life circle.

Among many factors functioning during the transition from maturation to germination, ABA and GA's roles are thought to be crucial in the control of this transition (Finch-Savage and Leubner-Metzger, 2006; Seo et al., 2008). There is evidence showing that the ABA/GA ratio, but not their respective concentrations, regulates seed germination (White et. al., 2000). The antagonistic interaction of ABA and GA is also affected by the physical environment in which the embryo resides. The limited oxygen supply for the embryo can

affect levels or sensitivities of ABA or GA. In the presence of GA, the sensitivity of dormant oat grains to low oxygen concentration drops significantly (Lecat et al., 1992). Meanwhile, when oxygen availability decreases, the sensitivity of embryo germination to ABA will increase, and sensitivity to GA will drop (Bradford et al., 2008; Benech-Arnold et al., 2006).

Although oxygen may influence the activation or degradation of ABA and GA (Barthe et al., 2000; Kushiro et al., 2004; Benech-Arnold et. al., 2006; Lange, 1998), their results cannot fully explain why the sensitivities of embryo germination also changed under hypoxia to the existence of exogenous ABA and GA. VP1, one of the transcription factors in the ABA signaling pathway, may play a role. VP1 can positively regulate maturation and ABA response-associated genes, and negatively regulates those genes associated with germination (Suzuki et al., 2003). Also, a mutation in the *Vp1* gene would reduce the embryo sensitivity to ABA significantly and cause it to germinate viviparously (McCarty et al., 1989). The relationship between VP1 and GA (White et al., 2000) and the fact that VP1 regulates some downstream genes that are not regulated by ABA (Suzuki et al., 2003) indicate that VP1 may have an independent function outside of the ABA signaling pathway.

In this thesis, functions of ABA, VP1 and hypoxia on the germination ability of maize embryos were studied. We also tested the influence of anaerobiosis on the germination abilities of cultured maize embryos. By using the genetically deficient ABA or VP1 mutants or a chemical inhibitor of ABA, we confirmed the independent role of VP1 on anaerobiosis-

induced germination arrest. Our results also suggest that GA regulation also participates in this germination inhibition.

2.2 Materials and Methods

Plant materials

Wild type, *vp1* and *vp5* seeds, in a W22 inbred genetic background, were planted and grown in the field. Ears were harvested at 20 or 25 days after pollination (DAP) for wild type and *vp1*, and 27 DAP for *vp5*.

Embryo culture and germination assays

In most prior research, isolated embryos were cultured in liquid media with continuous gentle shaking (Cao et al., 2007;) or on a layer of support materials imbibed in desired liquid media (Benech-Arnold et al., 2006; Barthe et al., 2000; Frantz and Bugbee, 2002). Here we used solid media to culture embryos. Embryos were orientated with scutellar surfaces facing down to contact the media and allow nutrient uptake. To test the efficiency of this culture method, we compared the germination rates between liquid and solid media, and found no difference (data not shown). Therefore, we used solid media culture for convenience in tracking embryo germination.

Whole intact embryos were isolated from developing kernels and cultured in a petri dish with solid Murashige and Skoog medium (MS media with 1.5 % agar and 1% sucrose) in the dark at room temperature. Each replicate consisted of ten embryos. Embryos were placed with their scutellar surfaces facing down on the media. ABA, fluridone or GA_3 (all bought from SIGMA) were added to the media at appropriate concentrations. An anaerobic environment was provided by an anaerobiosis chamber (COY Laboratory Products). The O_2 Control Glove Box and Cabinet, also from COY laboratory Products, provided the 3% oxygen environment to the embryos (mixed with hydrogen and nitrogen). Embryos were collected and frozen in liquid nitrogen and stored at minus 50 °C. Each treatment had at least four replicates. Germination was defined by the observation of radicle emergence from the embryos (Figure 1a). Normally, a germinating embryo will be a little larger than a quiescent one.

RNA extraction and Reverse transcription (RT) -PCR analysis

The stored embryos were ground in liquid nitrogen and total RNA was isolated with a RNeasy Mini Kit (QIAGEN) following the procedures provided. The quantitated RNA was treated with DNase (Promega) to exclude DNA. The single stranded cDNA was reverse transcribed from the 1.38 μg total RNA by SuperScript® III Reverse Transcriptase (Invitrogen). PCR was conducted using Taq DNA polymerase (Promega) with gene specific primers: *ubiquitin*, TAAGCTGCCGATGTGCCTGCGTCG (forward) and CTGAAAGACAGAACATAATGAGCACAG (reverse); *Vp1*, CAAGAGCAAGGCAGTGGTTCCAG (forward) and CAAATTTAGCGTCACACAGCGGGTAG (reverse). For *ubiquitin*, amplification

conditions were 94 °C for 2min followed by 25 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 1min. For *Vp1*, amplification conditions were 94 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. PCR products were run in a 0.8% agarose gel.

2.3 Results

Relationship between maturation and germination rate

The germination ability of a maize embryo is related to its maturation level. We isolated embryos from two different stages of developing wild type maize kernels, 20 and 25 days after pollination (DAP), and compared their germination rates. In Fig. 2, the germination abilities of the embryos in an aerobic environment had obvious differences. For the 20 DAP embryos, only 10% of embryos germinated successfully after 48 hours of culture. After 3 days, less than half (40%) of the embryos showed clear germination and no more germination was observed subsequently. The 25 DAP embryos have a faster germination rate. 70% of the embryos germinated within 24 hours and all of them showed shoots and roots by 48 hours. Therefore, the embryos used in subsequent experiments were all from 25 DAP kernels except as indicated.

Function of ABA in germination arrest

ABA is known to inhibit seed germination and our control experiments confirmed this. No germination was observed on medium containing 10 μM ABA (Fig. 2). Fluridone is a widely used ABA biosynthesis inhibitor. When fluridone was added to the medium to block endogenous ABA synthesis, embryos of the two maturation stages reacted differently (Fig. 3). With 10 μM fluridone, embryos from 20 DAP maize kernels showed increased germination rates compared to the normal MS media. Around half the embryos germinated within 48 hours, compared to barely any germination without fluridone. Moreover, only 40% of the 20 DAP embryos eventually germinated in the MS media, whereas embryos germinated close to 100% with fluridone after 3 days. However, the situation is opposite in the 25 DAP embryos. The germination rate dropped after adding the ABA synthesis inhibitor. Although both treatments had the germination rate reaching 100% within 3 days, fluridone delayed the germination in the first two days. This result shows that the effect of ABA biosynthesis is dependent on the stage of embryo development.

Anaerobiosis inhibits maize embryo germination

In an anoxic culture environment, germination was arrested completely (Fig. 1b). We never observed any radicle emergence for either 20 or 25 DAP embryos. This germination arrest does not appear to depend on ABA accumulation because when fluridone was added to the medium to inhibit ABA biosynthesis, no germination was still observed.

To verify that the hypoxic inhibition of embryo germination was not a result of asphyxiation, embryo viability was tested following treatment: after 48-hours of anaerobiosis, embryos were transferred into an aerobic environment. These anaerobically-treated embryos

actually displayed a higher germination rate than embryos placed directly in aerobic culture (Fig. 4). Within the first 12 hours of aerobic culture, more than twice the pre-treated embryos started germinating than the freshly cultured embryos. By 24 hours, 95% of the pre-treated embryos had germinated compared to only 72.5% of freshly cultured ones. When cultivated in the medium with 10 μM fluridone, anaerobically treated embryos still showed a higher germination percentage at each time point. The germination curve of the anaerobically treated embryos in fluridone is very similar to the freshly cultured embryos' germination curve in MS media. This result indicates that an anaerobic treatment appears to prime embryos for rapid germination.

VP1 and ABA mutants act differently from wild type

To examine the roles of ABA biosynthesis and responses on anaerobic germination arrest, we tested the responses of maize *viviparous5* (*vp5*) and *vp1* mutants. The *vp5* gene encodes phytoene desaturase required for carotenoid and ABA synthesis and mutants are deficient in ABA production (Hable et al., 1998). The VP1 transcription factor is required for ABA-induced expression of maturation associated genes and *vp1* mutants show reduced ABA sensitivity (Suzuki, 2003; Robichaud and Sussex, 1986). Under aerobic conditions, embryos of both mutants germinated faster than wild type (Fig. 2). As has been shown previously (Robichaud and Sussex, 1986), it was confirmed that the *vp1* mutant was ABA insensitive and germinated as well in 10 μM ABA as in hormone-free medium while the germination of wild type and *vp5* embryos were inhibited completely by ABA (Fig. 4).

VP1 function is also essential for the anaerobic-arrested embryo germination because *vp1* mutant embryos showed radicle emergence after 12 hours of culture and eventually over half the embryos germinated (Fig. 1b and 5). Although germination occurred more slowly and the final percentage was lower than for embryos cultured in aerobic conditions, it was obvious that the anaerobically-induced germination arrest was impaired in *vp1* mutants. Even when applying both anaerobiosis and ABA to the cultivated embryos, germination could be observed. However, the *vp5* mutant did not display such rescue (Fig. 5). Therefore, the anaerobic inhibition of germination did not appear to require endogenous ABA synthesis in the embryo. This suggests that the *Vp1* gene function in anaerobic germination inhibition is distinct from its function in ABA signaling.

Gibberellin (GA) promotes germination in anaerobic conditions

GA functions antagonistically with ABA in controlling germination and dormancy. To investigate the effects of GA in our system, 50 μM GA_3 was included in the medium and the germination was scored. In aerobic conditions, exogenous GA_3 increased the germination of wild type embryos slightly, with higher germination percentages in the first 36 hours (Table 1). Adding fluridone to limit ABA biosynthesis did not change the effect of GA_3. Meanwhile, GA_3 did not improve the germination rates of *vp5* (Table 1) or *vp1* mutants (data not shown).

Under anaerobic conditions, GA's function was more pronounced; GA_3 promoted the germination percentage of wild type embryos to 42.5% (Table 1). The germination frequency of GA_3 treated *vp5* mutant embryos was approximately double that of wild type.

GA_3 treatment in combination with fluridone showed a similar effect on wild type embryos with an increase up to 24 hours, after which levels returned to those of GA_3 alone. A distinct characteristic of GA induced germination under anaerobiosis is that the onset of radicle emergence is delayed compared to aerobic conditions. Typically radicle emergence is apparent by 12 hours of culture but GA-induced germination under anaerobic conditions was not observed until 24 hours.

Anaerobiosis can maintain *Vp1* gene expression

The levels of the *Vp1* transcript are known to decline in maturation stage maize embryos cultured in hormone free medium, and to be induced by ABA and osmotica (Cao et al., 2007). Using RT-PCR, freshly harvested wild type embryos (25 DAP) consistently showed expression of *Vp1*. In *vp5* mutants, the level of *Vp1* transcript appeared similar to wild type levels (Fig. 6).

In our experiment, *Vp1* RNA was reduced to barely detectable levels after 24 hours of culture and expression levels were maintained by ABA to levels slightly lower than in freshly harvested embryos (Fig. 6), which is consistent with previous results (Cao et al., 2007). Interestingly, an anaerobic environment also maintained the *Vp1* transcript at levels comparable to ABA (Fig. 6).

2.4 Discussion

ABA's inhibitory function on germination has been well documented (Kureca et al., 2005; Finch-Savage and Leubner-Metzger, 2006; Karssen et al., 1983; Naumann and Dorffling, 1982; Finkelstein, 1994) and was born out here (Fig. 2). However, the level of ABA is not constant during seed maturation. According to Neill et al. (1986), ABA levels in maize kernels, peak at about 18 DAP. This level decreases markedly after the peak in maize (Rivin and Grudt, 1991) as well as soybean and wheat (Bray and Beachy, 1985; Walker-Simmons, 1988), indicating a variable rate of ABA biosynthesis during embryo maturation. We treated embryos of two different maturation stages with fluridone, the inhibitor of ABA biosynthesis (Fig. 3). The 20 DAP embryos showed much lower germination rate than 25 DAP, however, fluridone promoted germination of the younger embryos, but not the older. This is consistent with the expectation that fluridone would have a greater effect during the period of greater ABA synthesis. Similar results were obtained when the embryos were cultured under 3% oxygen (Appendix 1). Our results are consistent with Fong et al. (1983), although the delayed kernel development caused by unusually cool field conditions explains the discrepancy in timing.

Oxygen is required for seed germination in most plants, with a few exceptions such as rice (Corbineau and Come, 1995). Hypoxic conditions surrounding the embryo can be imposed by plant structures such as the pericarp, seed coat or endosperm (Rolletschek et al.,

2005), as well as the physical environment, including soil and water. Therefore, we hypothesized that an appropriately hypoxic environment might be important to mimic the endogenous physiological conditions of developing embryos. Unfortunately, the levels of hypoxia for different species and their effects on embryo development are still largely unknown. Also, oxygen levels are not uniform, with gradients of depletion within the seed and embryo (Rolletschek et al., 2005). Here we examined two levels of hypoxia, 3% O_2 and complete anoxia. Only a minor difference was observed in the rate of germination between 3% O_2 and atmospheric levels (Appendix 1) so we concentrated on anoxic conditions.

Anaerobiosis strongly inhibited the germination of maize embryos. This result is consistent with studies in wheat (Mapelli et al., 1995) and barley (Benech-Arnold et al., 2006). However, more sophisticated measurement and manipulation of oxygen levels will be needed before we can make accurate conclusions about the threshold of hypoxia level required to inhibit maize embryo germination.

The mechanism for hypoxic inhibition of germination in many plants is still a puzzle. Of course oxygen is required for aerobic respiration, but other factors are involved in this control. Oxygen is also thought to promote the rate of ABA catabolism (Barthe et al., 2000) and decrease ABA sensitivity (Benech-arnold, 2006). We tested the hypothesis that hypoxic repression of maize embryo germination was mediated by ABA. Anaerobically cultured embryos did not germinate when treated with fluridone. Furthermore, *vp5* mutant embryos, deficient in ABA synthesis, did not germinate without oxygen (Fig. 5). These results indicate

that inhibiting ABA biosynthesis is not sufficient to release embryos from the anaerobic inhibition of germination.

VP1 is a key transcription factor in ABA signaling, responsible for the regulation of many downstream genes (Suzuki, et. al., 2003; Nakashima, 2006). A deficiency of the *Vp1* gene reduces the sensitivity of the embryo to exogenous ABA (Fig. 2). In our experiment, *vp1* mutant embryos could partially germinate under anaerobiosis (Fig. 1b, 5). Moreover, *vp1* mutants germinated slightly faster than the wild type under the aerobic condition (Fig. 2). This indicates that VP1 has a function in anaerobic inhibition of germination. Furthermore, the RNA level of *Vp1* remained elevated under anaerobiosis compared to aerobic cultivation (Fig. 6). Thus the maintenance of *Vp1* RNA accumulation accompanied germination arrest. That VP1 functioned in anaerobic inhibition of germination, but ABA does not appear essential suggests that anaerobic arrest might involve independent VP1 functions on non-ABA-regulated gene expression (Suzuki, et. al., 2003). The difference between germination ability of *vp1* mutants with or without oxygen indicates that other factors function in germination arrest. Some unknown factors may be induced by a prior anaerobic treatment and promote the maize embryo germination (Fig. 4).

Exogenous GA rescued anaerobic maize embryo germination and has similarly been reported to rescue the oat seed germination arrest imposed by hypoxia (Locate et al., 1992). It is not surprising given the well know antagonism between GA and ABA in many aspects of plant development, including dormancy (Finkelstein et al., 2008; Anderson et al., 2001) and defense responses (Bari and Jones, 2009). It was reported that GA accumulates before the

peak concentration of ABA during grain maturation, which also contributes to the precocious germination of *vp5* mutant (White et al., 2000). In the current study, while GA_3 showed no obvious enhancement of germination under aerobic condition, it markedly promoted germination under anaerobiosis (Table 1). GA_3 had a mild stimulatory effect on wild type embryos under anaerobiosis, but a dramatic germination enhancement for *vp5* mutants. This suggests that in fact ABA might have a minor function in hypoxic arrest that is only apparent upon GA treatment, and reinforces the notion that GA: ABA ratios are critical for controlling maturation and germination (White et. al., 2000). .

Overcoming anaerobic germination arrest by GA links together three important factors: oxygen, GA and VP1. ABI3, which is the Arabidopsis orthologue of VP1, can inhibit GA synthesis indirectly through repression of GA3ox2, by FUSCA 3 (FUS3) (Curaba et al., 2004; Suzuki and McCarty, 2008). The inhibition of GA signaling by the AFL B3 network was also revealed by investigating the *pkl* mutant (Ogas et al., 1997; Suzuki and McCarty, 2008). More recently, researchers have reported a signal can be transported from endosperm to inhibit GA synthesis or signaling, and maintain the expression of *Vp1* (Bassel et al., 2006). This signal might be associated with the potential function of hypoxia. Additional research is required to discover all the elements of the seed environment important for regulating the maturation/germination decision.

2.5 Figure Legends

Figure 1. Comparison of germinating and nongerminating embryos

1a. On the left is a germinating embryo with clear radicle emergence. The root and shoot will continue to grow under favorable conditions. Right is a quiescent embryo failing to germinate. A typical germinating embryo is also slightly larger than the nongerminating one.

1b. Row A shows the embryos of *vp1* mutants after 36 hours of culture in aerobic conditions and showing germination and shoot growth. The *vp1* embryos grown on media containing 10 μM ABA were shown to be insensitive to ABA (row B). The *vp1* mutant also partly blocked the germination arrest induced by anaerobiosis (row C). They clearly germinated but failed to undergo further development. In row D, wild type embryos did not germinate under anaerobiosis.

Figure 2. ABA and VP1 functions in germination control

ABA concentration in the MS media was 10 μM (open circle, triangle and square), compared to simple MS media (filled circle, triangle and square). Three different genotypes of maize were used here: wild type (filled and open circles), *vp1* (filled and open triangles) and *vp5* (filled and open squares). Embryos were cultured for 72 hours. Means of four replicates +/- SD (the black bar).

Figure 3. Germination of embryos from different maturation stage kernels and the effect of fluridone

Germination curves of wild type embryos cultured for 72 hours. Embryos were from 20 DAP (diamond and square) or 25 DAP (triangle and cross) kernels. Fluridone was applied at 10 μM concentration to check the function of ABA biosynthesis (square and cross). Means of four replicates +/- SD (the black bar).

Figure 4. A prior anaerobic treatment promotes germination

A 48 hour anaerobic treatment was applied to the embryos (diamond and square) and then these embryos were transferred to aerobic conditions for 72 additional hours. The germination rates are compared with the freshly harvested embryos (triangle and cross). Fluridone concentration in the media was 10 μM in the square and cross groups. Means of four replicates +/- SD (the black bar).

Figure 5. Sensitivities of *vp1* and *vp5* mutants to germination arrest caused by anaerobiosis

Embryos from *vp1* (solid circle and triangle, open circle) and *vp5* (open triangle) mutants were cultured. The media were with (solid triangle) or without (solid and open circle, open triangle) 10 μM ABA (solid triangle). Means of four replicates +/- SD (the black bar).

Figure 6. *Vp1* transcript levels in isolated embryos can be maintained by ABA and anaerobiosis

Semiquantitative RT-PCR was performed to study the *Vp1* RNA levels. Freshly harvested embryos have relatively strong *Vp1* expression, although there is some variation between repeats. In the hormone-free media, culture for 24 hours reduced *Vp1* transcripts to barely detectable level. *Vp1* RNA levels were maintained by a10 μM ABA anaerobiosis. The *Vp1* gene is expressed at approximately the same levels in freshly harvested 25 DAP wild type and *vp5* mutant embryos. The maize ubiquitin gene was used as a control.

Table 1. GA stimulates the germination of wild type and *vp5* embryos, under anaerobic environments

Wild type embryos were cultured in aerobic or anaerobic environments with or without 50 μM GA_3 or 50 μM GA_3 plus 10 μM fluridone. *vp5* mutant embryos were cultured with or without 50 μM GA_3. Germination percentages at different time points by different treatments are listed. Means of four replicates +/- SD.

2.6 Figures and tables

Figure 1a. Comparison of germinating and nongerminating embryos

A. (*vp1*) Aerobic
B. (*vp1*) ABA
C. (*vp1*) Anaerobiosis
D. (WT) Anaerobiosis

Figure 1b. Comparison of germinating and nongerminating embryos

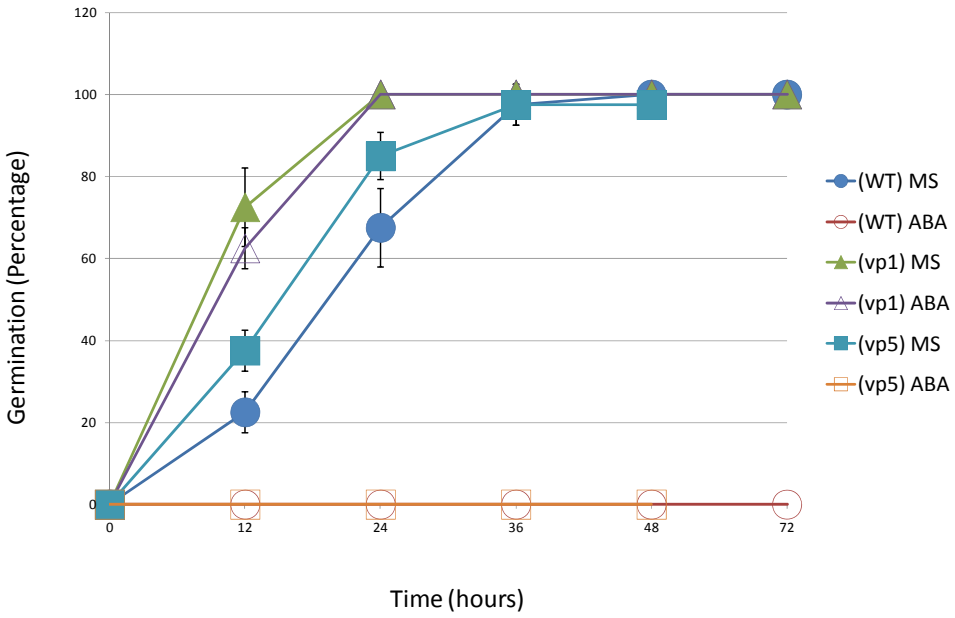

Figure 2. ABA and VP1 functions in germination control

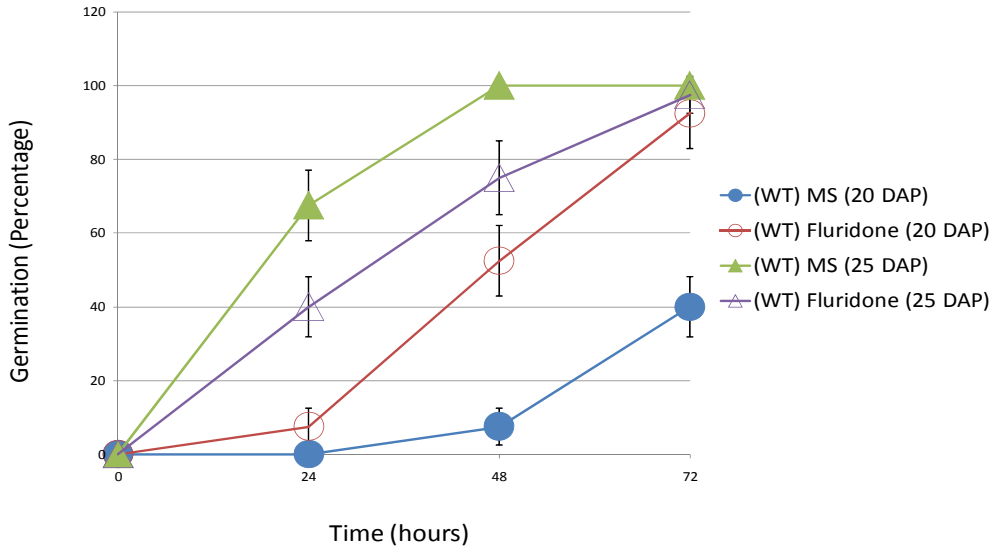

Figure 3. Germination of embryos from different maturation stage kernels and the effect of fluridone

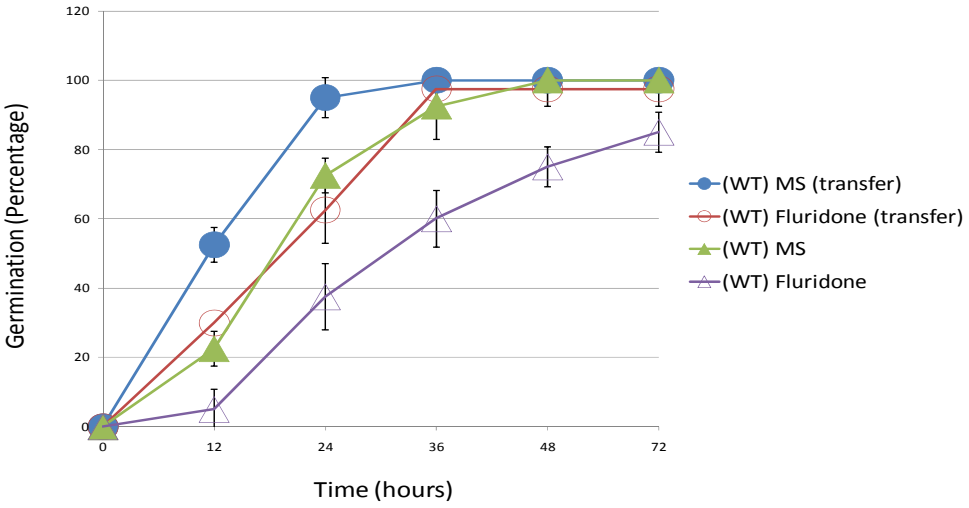

Figure 4. A prior anaerobic treatment promotes germination

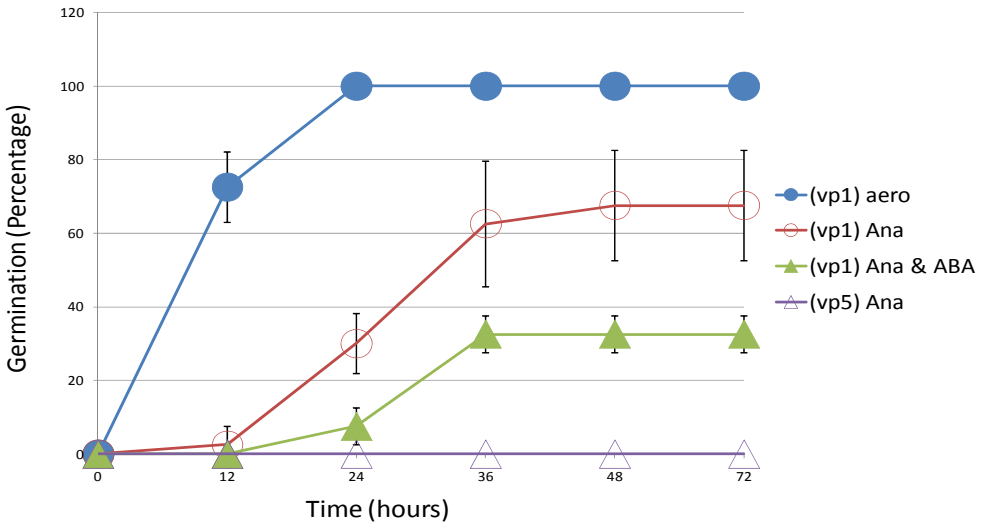

Figure 5. Sensitivities of *vp1* and *vp5* mutants to germination arrest caused by anaerobiosis

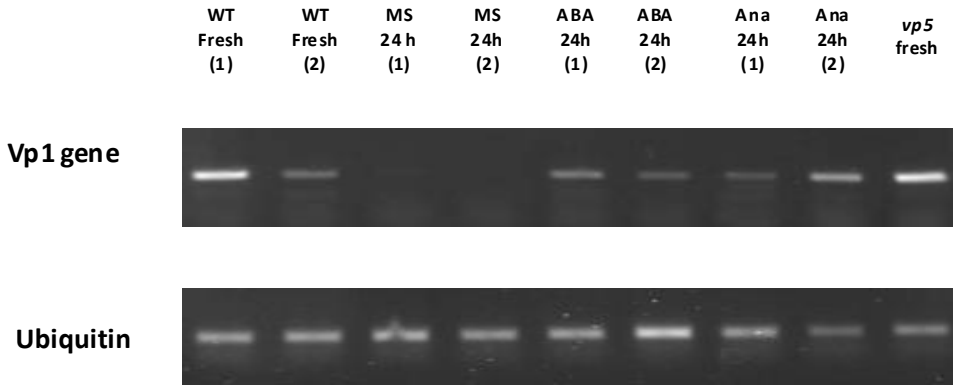

Figure 6. *Vp1* gene expression in isolated embryos can be maintained by ABA and anaerobiosis

Environment	Genotype	Treatment	Time (hours)		
			12	24	36
aerobic	WT	MS	5%	35%	87.50%
		GA	25%	62.50%	100%
		GA & Fluridone	32.50%	70%	92.50%
	vp5	MS	37.50%	80%	100%
		GA	30%	70%	100%
Anaerobic	WT	MS	0%	0%	0%
		GA	0%	12.50%	42.50%
		GA & Fluridone	2.50%	32.50%	40%
	vp5	MS	0%	0%	5%
		GA	5%	32.50%	85%

Table 1. GA stimulates the germination of wild type and *vp5* embryos, especially under anaerobic environments

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2.8 Appendix

Appendix 1. 3% oxygen level does not have obvious function on germination

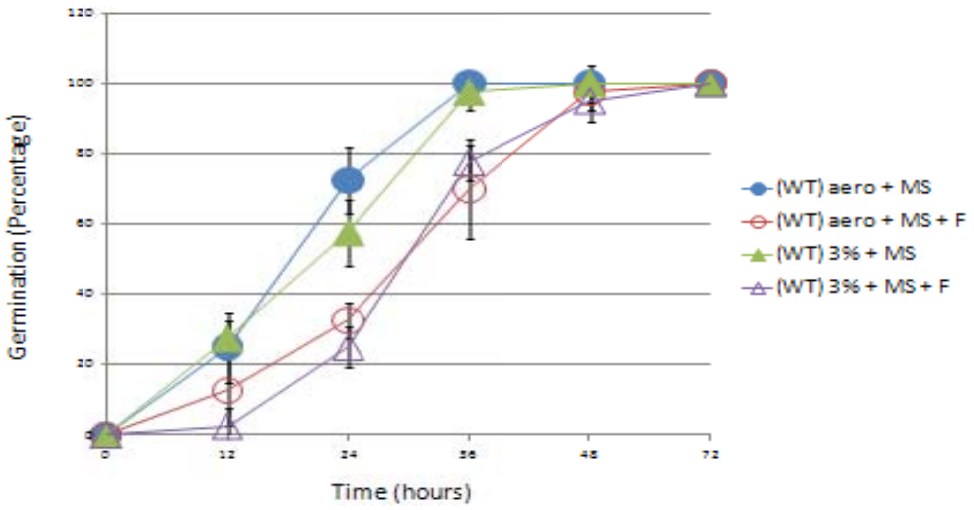
Wild embryos were cultivated on MS media under aerobic condition (solid and open circles) or 3% oxygen (solid and open triangles). 10 μM fluridone was added into the media for open circle and open triangle groups. Means of four replicates +/- SD (the black bar).

Appendix 2. Function of fluridone on osmotic pressure induced germination delay

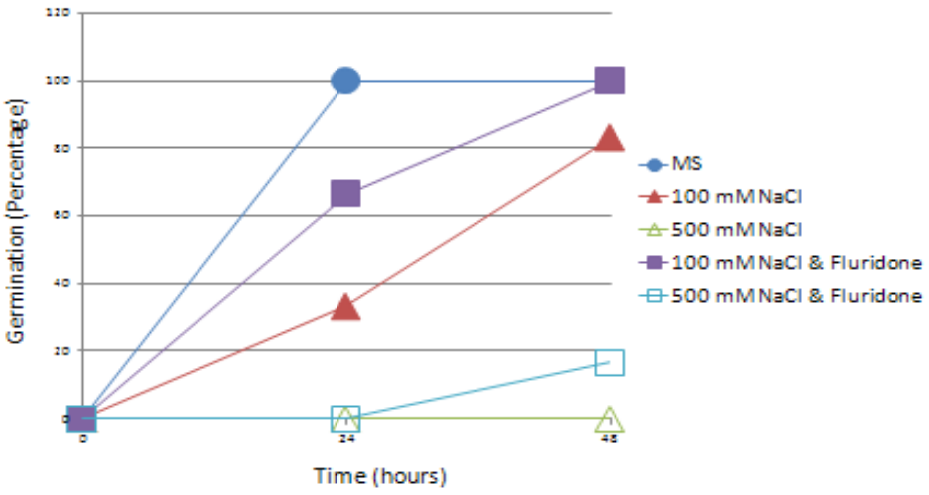
Wild embryos were cultivated on MS media (solid circle), MS media with 100 mM NaCl (solid triangle and solid square) or 500 mM NaCl (open triangle and open square). 10 μM fluridone was added into the media for solid square and open square groups.

Appendix 3. Model for the mechanistic of relationships of Anaerobiosis, GA, ABA and VP1 in the germination arrest.

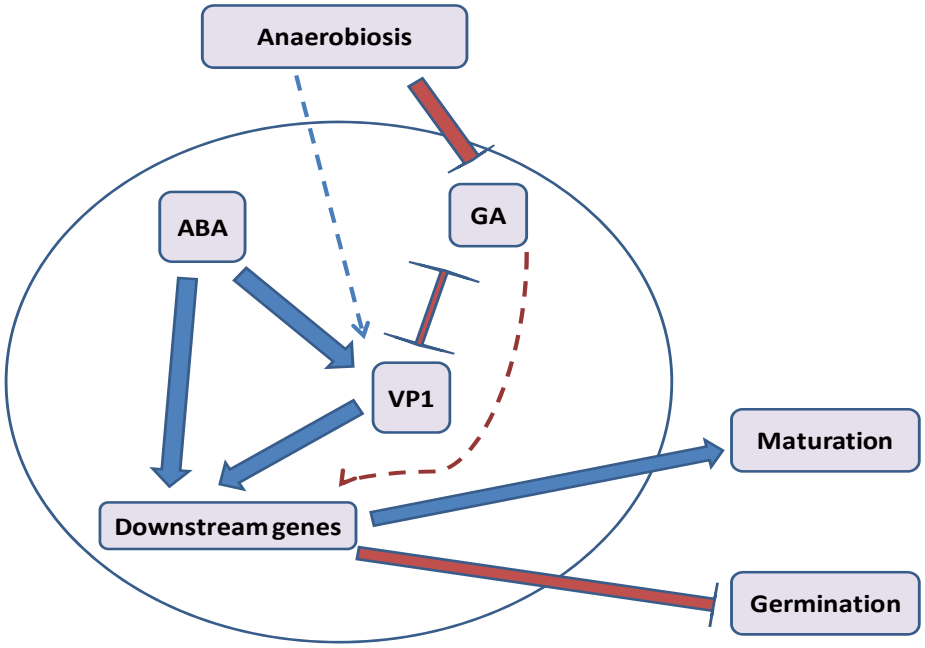
VP1 acts as a transcription factor in the ABA signaling pathway to regulate downstream maturation or germination associated genes to control the respective seed program. Meanwhile, VP1 and ABA both have independent roles in controlling downstream genes. GA and VP1 act inhibitory to each other through an indirect pathway and in the early maturation stage, GA may also function negatively on the downstream maturation genes. Besides the demonstrated role of VP1 in the anaerobiosis induced germination arrest, the fact that adding GA can overcome the arrest indicates GA's effect in the anaerobiosis-VP1 interaction. However, anaerobiosis may directly interact with Vp1 gene by some unknown means.



Appendix 1. 3% oxygen level does not have obvious function on germination



Appendix 2. Function of fluridone on osmotic pressure induced germination delay



Appendix 3. Model for the mechanistic of relationships of Anaerobiosis, GA, ABA and *Vp1* in the germination arrest.

Chapter 3. General conclusions

In this paper, we studied the germination ability of isolated maize embryos under different conditions. A de novo biosynthesis of ABA contributes to the germination inhibition at the earlier stage but not later. Exogenous ABA and complete anaerobiosis could inhibit maize embryo germination totally. The *vp1* mutant embryos with a normal content of endogenous ABA could rescue both of the germination arrests, whereas the *vp5* mutant, which is deficient in ABA and contains *Vp1* expression, failed to rescue either of them. These results suggest a central role of the *Vp1* gene in the anaerobiosis stimulated germination inhibition. The RT-PCR results further demonstrated this conclusion, while expression of *Vp1* gene was shown to be maintained at a relatively high level by limiting the oxygen supply. When applying the GA_3 in the media, germination inhibition under anaerobiosis was overcome. By summarizing the interactions between oxygen, GA and VP1, a new hypothetic model for anaerobiosis induced maturation-germination transition is proposed (Appendix 3). Anaerobiosis may inhibit GA and subsequently promote the *Vp1* level to accomplish the germination arrest, although the probability of direct interaction between anaerobiosis and *Vp1* has not been excluded yet. Future directions may focus more on the application of GA deficient mutant and GA-VP1 double mutant to get a deeper sight of this model.

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