ABA RESPONSIVE WHEN AFTERRIpened (ARA) MUTANTS INCREASE SEED DORMANCY, ABA SENSITIVITY, AND IMPROVE WATER RELATIONS:
TOWARDS WHEAT VARIETIES WITH IMPROVED PREHARVEST SPROUTING AND DROUGHT TOLERANCE.

By
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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of JORGEN COSTES ABELLERA find it satisfactory and recommend that it be accepted.

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ABA RESPONSIVE WHEN AFTERRIPENED (ARA) MUTANTS INCREASE SEED DORMANCY, ABA SENSITIVITY, AND IMPROVE WATER RELATIONS:
TOWARDS WHEAT VARIETIES WITH IMPROVED PREHARVEST SPROUTING AND DROUGHT TOLERANCE.

Abstract

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This thesis explores the hypothesis that mutants isolated based on ABA (abscisic acid) hypersensitive seed germination can be used to obtain drought tolerance and resistance to preharvest sprouting through increased seed dormancy in allohexaploid wheat. The plant hormone ABA is required for establishment of dormancy during seed development, stimulates stomatal closure in response to drought stress and induces expression of genes that promote osmotic adjustment. Arabidopsis mutants isolated for ABA hypersensitive seed germination have increased seed dormancy and drought tolerance resulting from increased sensitivity of stomates to ABA. Based on this result, wheat mutants showing increased sensitivity to ABA in germination of afterripened grain have been isolated and tested for improved vegetative drought tolerance. A total of 25 mutants were isolated from fast-neutron mutagenized Chinese Spring, whereas four and 27 mutants were isolated from EMS-mutagenized cv. Zak and cv.Scarlet, respectively. Of the 25 Chinese Spring mutants, 4 demonstrated a clear and reproducible hypersensitive response to ABA in dose-response germination assays, whereas 2 lines showed embryo dormancy. The remaining mutants
showed inconsistent phenotypes over seven retest experiments suggesting variable expressivity. Genetic analysis of Chinese Spring mutants showed that two are the result of a single dominant mutation, whereas three others may be single semi-dominant mutations. Mutants with a vegetative ABA-hypersensitive phenotype should close their stomates earlier in response to drought stress and have slower transpiration. Drought tolerance was evaluated by comparing the mutant rate of soil moisture loss during drought stress, stomatal conductance, and carbon isotope discrimination ($\Delta^{13}$C) to wild-type. Of eight Chinese Spring mutants tested, four showed a slower transpiration rate under drought stress, and one of the four showed reduced stomatal conductance compared to wild-type. The four mutants with ABA-hypersensitive seed germination were the same four mutants that showed lower rate of soil moisture loss under drought stress. Five of 19 mutants appeared to have reduced $\Delta^{13}$C relative to wild type, suggesting that they may have improved transpiration efficiency.
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CHAPTER ONE
INTRODUCTION

Overview

Abscisic acid (ABA) is a sesquiterpenoid phytohormone that plays important roles in many cellular processes in plants including seed development, dormancy, maturation, vegetative growth, transpiration and adoption to environmental stress responses such as drought, salinity, cold, pathogens and even UV radiation (Finkelstein and Rock, 2002; Finkelstein et al., 2002; Leung and Giraudat, 1998). ABA is required to induce dormancy during seed maturation and blocks germination of mature seeds. ABA acts antagonistically to gibberellin (GA), a hormone required in seed germination. This thesis seeks to exploit the role of ABA in stimulating seed dormancy and adaptive responses to environmental stresses to develop wheat plants with improved resistance to preharvest sprouting and drought.

Seed dormancy is the failure of a viable seeds to germinate even when beneficial conditions for growth such as light quality, moisture and temperature are realized (Bewley, 1997). Agronomically, higher dormancy strongly correlates with higher resistance to preharvest sprouting, the germination of seed on the mother plant when cool, moist conditions persist close to the time of harvest. ABA induces drought tolerance by increasing primary root growth, stimulating stomatal closure to reduce transpirational water loss, and alleviating damage through induction of genes providing osmoprotection (Leung and Giraudat, 1998). While mutants with reduced ABA biosynthesis and sensitivity show decreased seed dormancy and a vegetative wilty phenotype, mutants with increased ABA sensitivity in seed germination can display increased seed dormancy and increased tolerance
to drought. Mutants with altered ABA sensitivity have been isolated from a number of plant species including *Arabidopsis*, maize, tobacco, potato and tomato (Xiong and Zhu, 2003).

This section will provide an overview of wheat genetics and of the role of ABA in regulating seed dormancy and drought stress response. ABA signaling mutants will be discussed with particular emphasis on ABA hypersensitive mutants. The focus will then shift to the different mechanisms for obtaining drought tolerance and water use efficiency. Finally, the relationship between drought tolerance, yield and photosynthesis will be discussed.

**Wheat Genetics**

Wheat and rice are the two major human food grains widely consumed worldwide. In terms of total world production, wheat ranks next to maize and rice (www.worc.org). Unlike rice, wheat has unique protein composition making it ideal for production of bread, cookies, crackers, pasta, and noodles. It also is used for fermentation to produce alcohol and alcoholic beverages such as beer and vodka. By-products of wheat can be utilized to extract bio-fuel (Loyce et al., 2002). Bread wheat (*Triticum aestivum* L.) is an allohexaploid that arose from the convergence of three diploid progenitor species. It is a genetically complex organism, due to high genetic redundancy and large genome size. The A genome was derived from *Triticum urartu* or *T. monococcum*; the B genome from *Aegilops speltoides*; and the D genome from *Triticum tauschii* (Jiang and Gill, 1994). The haploid chromosome number of bread wheat is 21, with seven chromosomes from each progenitor (2n = 6x = 42, genomes AABBDD). The three homeologous genomes are highly similar but not identical. Wheat has one of the largest genome sizes among important crop species at nearly 16 billion base
pairs/1 Centimorgan (bp/1C) (Arumugunathan and Earle, 1991) compared to barley at 4.9 billion bp/1C and maize at 2.5 billion bp/1C. Many genes in wheat are present in multiple copies (3 copies/1C). Because of this high redundancy molecular genetic studies can be difficult.

In spite of these difficulties, genetic screens have successfully identified mutations in wheat plants altering phenotypes such as resistance to inhibitory effects of a herbicide (Newhouse et al., 1992), altered testa color (Warner et al., 2000), and resistance to diseases such as powdery mildew (Kinane and Jones, 2001), yellow and brown rust (Boyd et al., 2002), and stem rust (Kerber and Aung, 1995). These were mutations in a single gene with dominant or semi-dominant/additive phenotype, except for the single recessive mutation giving resistance to stem-rust (Kerber, 1991). These examples of success encouraged us to screen for ABA response mutants in hexaploid wheat.

Genetic studies in hexaploid wheat often use *Triticum monococcum* and barley as diploid models (Feuillet and Keller, 2002). Furthermore, both rice and barley show high degree of similarity and synteny with the wheat genome (Feuillet and Keller, 1999). The validity of using the diploid model has been proven by substantial results proving the correspondence of characters between the diploid models and hexaploid wheat. For example, a subgenome chromosome walking resulted to a ~300kb physical contig in *T. monococcum*, spanning the leaf rust resistance gene, *Lr10* in hexaploid wheat (Stein et al., 2000). Despite these successes, there are a number of questions that need to be addressed in the hexaploid. In a practical sense, the important agronomic traits for bread, cookie, or noodle making qualities must be investigated in hexaploid wheat. Finally, it is essential to isolate new genotypes with resistance to abiotic stress in hexaploid wheat in order to quickly introgress
the genotype into elite wheat varieties. With advances in molecular genetics, incorporating novel genes into modern wheat cultivars is possible. While such genes could be introduced by transformation, this possibility is hampered by social and economic barriers to acceptance of genetically modified wheat. To date, transgenic wheat has not been produced commercially in the U.S. Wheat marketers do not want to risk the loss of foreign customers since nearly 50% of U.S. bread wheat production is exported, mainly to customers who consider transgenic wheat to be unacceptable (www.worc.org).

The use of mutations has been instrumental in wheat breeding programs since 1951 (Konzak, 1987). Before the 1920s, researchers had to rely on naturally occurring mutations to study gene function. Such mutations are rare and require the collection of crop plants and wild relatives from their original habitat. The discovery of mutagens led to the use of mutation screens to increase the availability of altered genes (Konzak, 1987). Induced mutations in wheat offer the possibility of using knowledge gained in model systems like Arabidopsis to design mutant screens that should improve crop plant germplasm with the added advantage of circumventing the need for genetic transformation.

**Agricultural relevance**

Studies of ABA signaling are important because of their relevance to understanding drought tolerance in crop plants. Drought, by far, is the most significant yield-limiting factor of cereals (Araus et al., 2002). The current research interest in drought tolerance is driven by practical needs as global climate changes are predicted to cause an increase in arid regions (Petit, 1999). About 33% of the world’s surface is arid or semi-arid (Kingsford, 2000). More recent reports showed that about 40% of the world’s agricultural land lies in arid or
semi-arid regions (http://www.liv.ac.uk/~sd21/stress/drought.htm). In the U.S., up to 45% of the land surface has soils that have been subjected to continuous drought and shallow soils that are frequently subjected to water deficit (Boyer, 1982). In 2002, more than 50% of the United States suffered from moderate to severe drought conditions with record low precipitation deficits particularly in the western states (Cook et al., 2004). This long-term drought began in 1999 with severe conditions persisting until the summer of 2004 in most of the western region of the United States. In 2005, the northwestern U.S. continued to experienced moderate to severe drought. Since 1999, Washington State has experienced long-term hydrological drought conditions affecting crops, ranges and pastures (http://drought.unl.edu/dm). This drought makes the region highly vulnerable to increased aridity as a result of climate change. Global increases in temperature and aridity are predicted to have a detrimental impact on crop yields for the next 50 years. On a global scale, the increase in land aridity and elevated temperatures resulted from increased atmospheric CO$_2$ concentration, which also can stimulate crop production (Thomson et al., 2005). In the last 45 years, an increase in atmospheric CO$_2$ concentration of nearly 20% has been recorded (http://www.cmdl.noaa.gov). Increases in atmospheric CO$_2$ concentration have improved instantaneous water use efficiency (A/E = carbon assimilated/water transpired) and long-term water-use efficiency (WUE) of plants (Araus and Buxo, 1993) because less stomatal opening is required to obtain sufficient CO$_2$ for photosynthesis leading to less water loss through transpiration. On the other hand, the ever-growing world population and dependence on irrigation for agriculture has depleted water resources and exacerbated aridity (Fischer et al., 2005; Kingsford, 2000). Therefore, understanding the genetic and physiological mechanisms
controlling plant drought response is of paramount importance to plant breeders and molecular geneticists developing new cultivars with improved drought tolerance.

Understanding the relationship between ABA and seed dormancy also is of great importance in wheat because of its relevance to controlling the phenomenon of preharvest sprouting (PHS). PHS is the precocious germination of mature seeds while still attached to the mother plant. Sprouted wheat has poor end-use quality and must be sold as feed resulting in economic loss to wheat farmers. Preharvest sprouting reduces flour extraction rates (Mansour, 1993) and bread-making quality of the flour due to high \( \alpha \)-amylase content (Groos et al., 2002). High enzyme activity in flour will result in the degradation of starch into simple sugars. Breads baked from sprouted wheat will have less volume and a compact interior (Mansour, 1993). Wheat with 10% preharvest sprouting damage results in a dockage of $0.60 per bushel (www.agriculture.com). This physiological condition affects most of the world’s wheat growing regions when cool and moist conditions persist close to harvest time (Bewley and Black, 1994). Historically, the occurrence of preharvest sprouting in many crops, including wheat, was the result of many years of selection and breeding for crops with shorter dormancy period. A longer period of seed dormancy was seen as a negative trait, as it prevented uniform germination and interfered with plant stand establishment, thus reducing yield (Ringlund, 1992). As a consequence, many crops including wheat, sorghum, and barley suffer from preharvest sprouting, which is equivalent to vivipary in maize (Figure 1).

**ABA as a regulator of seed dormancy and stress response**

Seed dormancy is the failure of a viable seed to germinate even when beneficial conditions for growth such as light quality, moisture and temperature are realized (Bewley,
The ability to germinate is acquired during a period of dry storage called afterripening. Proper control of seed dormancy, afterripening, and germination are critical for the survival of most plant species. Dormancy provides an intrinsic block to germination and allows for dissemination of seeds in time and space. The failure of a dormant seed to germinate can maximize seed survival following catastrophic events such as flood or fire. Agronomically, dormancy increases resistance of seeds to preharvest sprouting. There are two categories of dormancy outlined by Bewley and Black (1994): seed coat-imposed dormancy and embryo dormancy. In seed coat-imposed dormancy, germination fails because radicle emergence is blocked by the barrier imposed by the seed coat and sometimes also the endosperm, pericarp or extrafloral organs. In contrast, seeds with embryo dormancy fail to germinate even if the seed coat is removed. Embryo dormancy occurs more often than coat-imposed dormancy. These types of dormancy can occur individually or in combination. Wheat exhibits both types of dormancy (Flintham, 2000; Paterson and Sorrells, 1990a).

Seed development can be divided into three stages of approximately equal duration (Figure 1) (Taiz and Zeiger, 2002). First, during embryogenesis the zygote undergoes pattern formation and endosperm tissue proliferation. ABA accumulation is low during this stage. During the second stage, seed storage reserve compounds (protein, starch, and oil) accumulate and cell division ceases. ABA synthesis peaks during this stage and stimulates the accumulation of storage reserves and late embryogenesis abundant (LEA) proteins. In the final stage of embryo maturation, the water content drops by up to 90%, the embryo becomes desiccation tolerant, and metabolism halts as the seed enters a quiescent (“resting”) state. Dormancy may be induced during this last stage which is associated with intermediate levels of ABA. During seed development, ABA is required (necessary but not sufficient) for
Figure 1. Stages of seed development. Seed development can be divided into three stages: (1) the zygote undergoes pattern formation and endosperm tissue proliferation, ABA accumulation is low at this stage, (2) synthesis of seed storage compounds such as protein, starch and oil, ABA peaks at this stage, and (3) seeds acquire desiccation tolerance and seed dormancy may be induced in this stage, ABA level is in the intermediate.
embryo maturation, accumulation of seed storage and LEA proteins, and seed dormancy. However, these processes are not solely regulated by ABA, but occur in concert with other hormones such as jasmonic acid, ethylene and antagonistically by GA (Xiong and Zhu, 2003).

The dormancy status of seeds is dependent on the degree of ABA sensitivity and on endogenous levels of ABA in the embryo and in the endosperm during imbibition. In Arabidopsis there is a direct correlation between the level of seed dormancy and endogenous ABA levels in the seed (Ali-Rachedi et al., 2004). In a germination-based study, Ali-Rachedi et al. (2004) found a greater decrease in ABA levels during imbibition of afterripened seeds than in dormant seeds. They established that the ability of seeds to catabolize and synthesize ABA during imbibition is associated with their ability to germinate and degree of dormancy.

Mutant and gene expression analysis of Arabidopsis ABA biosynthesis genes NCED6 and NCED9 (9-cis-epoxycarotenoid dioxygenases) showed that ABA accumulation in both the embryo and endosperm is important for establishment of seed dormancy (Lefebvre et al., 2006). Moreover, studies have shown that the CYP707A gene family encoding that main ABA catabolic enzyme ABA 8'-hydroxylase is required for the proper regulation of seed dormancy and afterripening (Okamoto et al., 2006). In barley, the degree of grain dormancy was found to positively correlate with the level of HvCYP707A expression suggesting that reduced ABA levels might correlate with an increased tendency towards PHS (Chono et al., 2006). In addition to overall ABA levels, the degree of ABA sensitivity of the grain also is an important determinant of grain dormancy. In wheat, PHS susceptibility was found to correlate with lower ABA sensitivity in embryos isolated during seed maturation (Walker-Simmons, 1987). Moreover, the highly dormant wheat cultivar ‘Clark’s Cream’ was found
to have greater sensitivity to ABA during embryo germination than ‘Parker 76’, a cultivar with little dormancy (Morris et al., 1989).

Analysis of ABA-deficient mutants has been instrumental in elucidating the role of ABA in plant development. ABA biosynthesis mutant phenotypes that are reversed by ABA application have been characterized in a wide range of plant species (Finkelstein and Rock, 2002) including maize (Mccarty, 1995), tomato (Burbidge et al., 1999), tobacco (Marin et al., 1996), barley (Romagosa et al., 2001) and Arabidopsis (Brocard-Gifford et al., 2004; Finkelstein, 1994b; Giraudat et al., 1992; Koornneef et al., 1984; Koornneef et al., 1982; Leung et al., 1997). These mutants exhibit germination phenotypes ranging from reduced seed dormancy in Arabidopsis and tomato to vivipary in maize. The reciprocal cross between the Arabidopsis aba biosynthesis mutant and wild-type plants resulted in dormant seeds only when the embryo itself produced ABA during maturation. Maternal ABA and exogenous ABA application were ineffective in inducing dormancy in an ABA-deficient embryo in Arabidopsis (Finkelstein, 1994a). A block in the carotenoid pathway resulted to the isolation of viviparous (vp) mutant of maize, which has reduced ABA levels and a high incidence of vivipary (Maluf et al., 1997). Vegetative phenotypes include reduced drought and salt tolerance. The loss of turgidity in leaf tissues of Arabidopsis and tomato mutants is due to the inability of stomates to close in water deficit soil (Koornneef et al., 1984; Marin and MarionPoll, 1997; Wilkinson et al., 1998).

Reduced ABA also is associated with a decrease in seed dormancy (Koornneef et al., 1984). Five ABA-insensitive (abi) mutants, abi1, abi2, abi3, abi4 and abi5, have been cloned and characterized in Arabidopsis (Finkelstein et al., 2002). ABA-insensitive mutants were selected in Arabidopsis based on their ability to germinate when exogenous ABA is applied at
3 \( \mu \)M or 10 \( \mu \)M concentrations, which inhibit wild-type seed germination (Finkelstein, 1994b; Giraudat et al., 1992; Leung et al., 1997). These mutants also show reduced seed dormancy similar to ABA biosynthesis mutants. ABA sensitivity by inhibition of seed germination in ABA-insensitive mutants, \( abi1 \) and \( abi2 \) is partially maternally controlled (Finkelstein, 1994a). Genetic analysis showed that \( abi1 \) and \( abi2 \) are semi-dominant mutations causing reduced ABA sensitivity in seed germination, root growth, and stomatal closure (Bertauche et al., 1996; Finkelstein and Somerville, 1990; Koornneef et al., 1984; Leung et al., 1997). The \( ABII \) and \( ABI2 \) genes both encode a protein serine/threonine phosphatase 2C (PP2C) with redundant yet distinct functions in ABA response in seeds and vegetative tissues (Leung et al., 1997; Meyer et al., 1994). These mutants have decreased ABA response and a permanently vegetative wilty phenotype (Koornneef et al., 1984), mainly because of their inability to close their stomates under water stress (Schroeder et al., 2001). Stomatal opening allows plants to cool because of increased transpiration. This is reflected in the fact that \( abi1 \) and \( abi2 \) mutants have cooler (by 1°C) leaf surface temperatures than wild type (Merlot et al., 2002). In contrast, the recessive \( abi3 \), \( abi4 \), and \( abi5 \) mutants are seed-specific having no detectable effect on vegetative ABA sensitivity (Finkelstein, 1994b; Finkelstein and Somerville, 1990). Recessive mutations in \( ABI3 \) result in the following seed phenotypes: reduced seed dormancy, decreased accumulation of storage reserves and LEA proteins, desiccation-intolerance, and green embryos (Finkelstein, 1994b; Finkelstein and Somerville, 1990; Koornneef et al., 1984; Nambara et al., 1992; Ooms et al., 1993). \( ABI3 \) encodes a B3 domain transcriptional activator and is an ortholog of maize and wheat \( VIVIPARY1 \) (\( VP1 \)) gene (Bies-Etheve et al., 1999; Giraudat et al., 1992; Hattori et al., 1992; Holdsworth et al., 1999; Mccarty et al., 1991). The \( ABI4 \) and \( ABI5 \) genes encode
APETALA2-like and bZIP domain transcription factors, respectively (Finkelstein, 1994b; Finkelstein et al., 1998).
Even though reduced ABA sensitivity causes reduced seed dormancy and increased drought sensitivity, increased sensitivity to ABA can result in increased seed dormancy, ABA hypersensitivity in stomatal closure and improved drought tolerance. ABA hypersensitive mutants isolated in Arabidopsis include era1, abh1, and sad1 (Cutler et al., 1996; Hugouvieux et al., 2001; Xiong et al., 2001b). Recessive mutations in the ERA1 (Enhanced Response to ABA 1) and ABH1 (ABA Hypersensitive 1) genes in Arabidopsis were isolated based on inability to germinate on 0.3 µM and 0.6 µM ABA, respectively (Cutler et al., 1996; Hugouvieux et al., 2001). Both ABA concentrations tested were too low to inhibit wild-type seed germination. ERA1 encodes a heterodimeric β-subunit farnesyltransferase that acts as a negative regulator of ABA response in seed and vegetative tissues (Cutler et al., 1996). Deletion of ERA1 or external application of farnesyltransferase inhibitors resulted in ABA hypersensitive guard cell anion channel activation, early closing of stomates, and tolerance to drought stress (Pei et al., 1998). era1 mutants have reduced water loss through transpiration in a drought treatment experiment by triggering stomatal closure. Similarly, Arabidopsis abh1 mutants show increased ABA sensitivity in stomatal closure resulting in reduced wilting during water-deficit stress. This hypersensitive stomatal closure appears to result from earlier synthesis and accumulation of ABA in guard cells. ABA Hypersensitive 1 (ABH1) encodes a component of an mRNA cap-binding protein implicated in modulation of ABA signaling at the mRNA level (Hugouvieux et al., 2001). The SAD1 gene encodes for a Sm-like protein that like ABH1 may be involved in the mRNA processing of ABA signaling genes (Xiong et al., 2001b). The inositol polyphosphate-1-phosphatase FRY1 (FRY1) gene is a negative regulator of ABA signaling that appears to have opposite effects on seed germination and drought stress in Arabidopsis (Xiong et al., 2001a).
Mutant studies suggest that genetic manipulation of ABA sensitivity can be used to improve drought tolerance of crop plants. One example was the recent successful genetic engineering of canola with improved drought tolerance and yield by reducing expression of farnesyltransferase (*ERA1*) under drought stress (Wang et al., 2005). Canola was transformed with an anti-sense *ERA1* construct under the control of the drought-inducible *RD29A* promoter. The transgenic plants were ABA hypersensitive in seed germination and showed lower stomatal conductance under water deficit. These transgenic plants displayed normal growth and development with equivalent seed yields compared to the control when grown under optimum soil moisture condition. Under water stress, transgenic canola plants out-performed control plants in terms of seed yield and quality.

ABA is considered a plant stress hormone since ABA synthesis is induced in response to abiotic stresses such as drought, salt and cold (Takahashi et al., 2004). ABA levels not only increase during seed maturation but also significantly accumulate during environmental stress. ABA is more strongly implicated in regulation of drought and salt stress responses than in cold response (Xiong and Zhu, 2003). Microarray analysis in rice shows a higher degree of overlap of ABA-regulated transcripts with drought-and salt-regulated transcripts than with cold-regulated transcripts in rice and in Arabidopsis (Rabbani et al., 2003; Shinozaki et al., 2003). Only 10% of drought-induced genes are also cold-induced indicating a partial overlap in freezing and drought tolerance mechanisms (Seki et al., 2002). (Shinozaki et al., 2003) proposed that the effect of ABA signaling on cold tolerance is due to crosstalk between these signaling pathways. This overlap in signaling pathways suggests the possibility that wheat ABA hypersensitive mutants also may enhance tolerance to salt or low temperature stress.
ABA response and preharvest sprouting resistance in wheat

The susceptibility of wheat cultivars to PHS has been correlated with both ABA sensitivity and with white kernel color (Groos et al., 2002; Walker-Simmons, 1987). Resistance, on the other hand, is associated with red testa color. Red testa is dominant over white testa color and is determined by three $R$ genes mapped on chromosomes 3A, 3B and 3D. However, testa color alone is not the only factor affecting seed dormancy and preharvest sprouting (Flintham, 2000). For example, not all red wheat cultivars have strong dormancy, and some cultivars with white testa color, such as cv Brevor and cv Clark’s Cream, have strong dormancy and excellent PHS tolerance (Gale et al., 2002; Walker-Simmons, 1987). An experiment comparing the dormancy of white-grained mutants of red-grained Chinese Spring showed that red testa color enhances but is not the sole determinant of seed dormancy (Warner et al., 2000).

Previous work has shown that the degree of ABA sensitivity in wheat positively correlates with elevated seed dormancy and resistance to preharvest sprouting (Morris et al., 1989; Walker-Simmons, 1987). Isolation of ABA-hypersensitive wheat mutants will help to test the hypothesis that increased ABA sensitivity can impart PHS tolerance and to identify wheat ABA signaling genes. For example, the $VP1$ transcription factor is an ABA signaling gene that controls vivipary resistance in maize (Mccarty et al., 1991) by promoting seed maturation while repressing seed germination (Hoecker et al., 1995; McCarty et al., 1989). The $VP1$ homologue in wheat has been found to impose resistance to PHS regardless of testa color (McKibbin et al., 2002). A wheat homologue to maize $VP1$ has bee identified and proved that the level of gene expression correlates with the degree of seed dormancy and
ABA sensitivity (Nakamura and Toyama, 2001). This previous work suggests that it should be possible to increase the PHS tolerance of white wheat cultivars using mutants with increased sensitivity to ABA and increased seed dormancy.

**ABA sensitivity of wheat grain is dependent on the dormancy status of the grain.**

An interesting property of wheat is that the degree of ABA sensitivity in seed germination is dependent on the dormancy status of wheat grain (Strader, 2004). This unique property of wheat is not shared with model organisms like *Arabidopsis thaliana*. In *Arabidopsis* (ecotype Columbia) dormant and afterripened seeds have the same dose-response to the inhibitory effect of ABA on seed germination (Figure 2a). For the wheat cultivar Brevor (soft white winter), the germination of dormant whole seed will not occur within 72 hours even in the absence of ABA, whereas whole afterripened seeds are highly ABA-insensitive germinating fully on 100μM ABA (Figure 2b). Once seeds are cut in half (referred to here as embryos), the germination of dormant embryos is stimulated and show a dose-dependent response to ABA in inhibition of seed germination (Figure 2c). In the absence of ABA, dormant embryos germinate to about 85% and show reduced germination of 60%, 50%, 30% and 10% at 0.1 μM, 0.5 μM, 1.0 μM and 5 μM ABA, respectively, after 72 hours of incubation. Complete inhibition of germination is reached at ABA concentrations of 50 μM and 100 μM (Figure 2c).
Figure 2. ABA sensitivity is dependent on the dormancy status of wheat seeds. Percent seed germination on varying concentrations of ABA is shown. (a) No difference in percent germination of whole dormant and afterripened *Arabidopsis* seeds is seen following 120 h incubation in the absence of presence of increasing concentration of (+/-)-ABA in 0.5xMS/0.8% agar/5 mM MES. (b) Comparison of percent germination of whole dormant and afterripened wheat seeds following 72 h incubation on filter paper soaked with (+)-ABA in 5 mM MES, pH 5.5. (c) Comparison of percent germination of dormant and afterripened wheat embryos following 72 h incubation on (+)-ABA. Y-axis error bars represent standard error for 3 samples of 10 seeds each (Strader, 2004)
Mechanism of acquiring drought tolerance and water-use efficiency

Plants use a wide range of strategies to adapt to drought stress at different times in the life cycle and in different environments. The different strategies by which plants acquire drought resistance have been placed into different classifications (Levitt, 1980). This review discusses the strategies of drought escape and drought tolerance, which is further divided into desiccation postponement and desiccation tolerance. Plants escape drought by completing the entire life cycle, or critical portions of the development, during drought-free periods in an otherwise drought-dominated environment. In arid regions, plants with the ability to escape drought may combine a short life cycle with high growth rates and high photosynthetic activity when soil moisture is still available (Maroco et al., 2000). Drought escape in cereals can result in improved grain yield based on ability to partition and mobilize assimilates more efficiently to developing grains by storing reserves in some organs (Nilsen and Orcutt, 1996).

Drought tolerance is the ability of plants to yield or endure drought conditions despite a deficit in available soil water required for growth and production. Plants may tolerate drought by desiccation postponement and desiccation tolerance.

In desiccation postponement plants can resist damage under water-limited conditions by maintaining high tissue water potential (Salisbury, 1996; Taiz and Zeiger, 2002). This can be achieved by reducing water loss and increasing water uptake. In vegetative tissues, endogenous ABA levels increase under water stress conditions and initiate plants responses to the stress at the cellular and whole-plant levels. Increased concentration of ABA in the leaf during the early part of soil drying can induce stomatal closure and reduce leaf expansion (Bahrun et al., 2002). Evidence suggests that this effect of ABA on the leaf may decrease the rate of water loss through transpiration by closing stomates. ABA stimulates primary root growth.
elongation while inhibiting lateral root formation, a strategy that may lead to use of moisture deeper in the soil profile (Sharp et al., 2004). Changes in the relationship between atmospheric vapor pressure and stomatal conductance can lower transpiration during periods of high evaporative demand. The main drawback of the desiccation postponement strategy is that closing stomates to reduce water loss may reduce the availability of CO₂ for photosynthesis.

Ideally, plants could postpone desiccation by increasing transpiration efficiency (TE), the amount of CO₂ fixed per amount of water transpired (A/T). Increased TE should reduce transpiration but sustain carbon fixation while maintaining high water potential during drought (Condon et al., 2002b; Nilsen and Orcutt, 1996). A plant under drought stress can reduce water loss by preferential abscission of large leaves in order to reduce total transpiring leaf area without affecting carbon gain. This process is largely the result of increased production and sensitivity to the plant hormone ethylene (Aharoni, 1978). Morphological adjustments may include leaf rolling to minimize light absorbance, increasing light reflectance by trichomes or modifying leaf angles (Taiz and Zeiger, 2002). When water uptake is limited, leaf expansion is inhibited, thereby reducing the use of carbon and energy which allows a greater portion of assimilates to be allocated to the root system to support root growth. This enhances water uptake since roots can extend deeper into the soil profile to reach moisture (Gowing et al., 1990). This mechanism can enhance water uptake. Hydraulic lift by plant communities of deep water to the soil surface at night increases availability of water in some species (Jackson et al., 2000). Most plants have the capacity to store water in various root and stem tissues. During drought stress, plants utilize these reserves to maintain transpiration and to minimize the reduction in water potential.
Desiccation tolerance is defined as the ability to continue physiological functions despite reduction in plant water potential (Salisbury, 1996). The mechanisms used by plants to continue metabolic activity under low plant water potential differ from those used by plants that have acquired the ability to maintain high water potential during water deficit. These mechanisms can be used by plants in combination with other techniques to limit decreases in tissue water potential and to continue metabolizing at lower water potential. ABA is involved in the activation of stress-response genes that provide osmoprotection to plant cells by activation of enzymes that synthesize compatible solutes and LEA-like proteins (Finkelstein and Gibson, 2002; Hasegawa et al., 2000). Osmotic adjustment, or the accumulation of solutes by cells, is a process by which water potential can be decrease without an accompanying decrease in turgor or decrease in cell volume (Kusaka et al., 2005). Osmotic adjustment occurs when the solute content increases without any change in the proportion of water in the symplast. Accumulation of solutes during osmotic adjustment appears to be restricted to the vacuoles. A high concentration of solutes in the cytoplasm can inhibit enzymes of plant cells. Instead, vascular plants synthesize and accumulate low molecular weight, soluble compounds referred to as ‘compatible solutes’ because they accumulate in high concentration in the cytoplasm to maintain high water potential without interfering with cellular functions. A number of researchers have described the role of compatible solutes in enhancing tolerance to drought and salinity stress including mannitol (Stoop et al., 1996), trehalose (Goddijn and van Dun, 1999), proline (Yamada et al., 2005) and glycine betaine (McNeil et al., 2001). Moreover, the synthesis of LEA proteins by ABA aid in the preservation of embryo viability when seeds are subjected to extreme dry environments and provides protection in vegetative tissues from desiccation.
Measurements to determine plant water status and stomatal conductance

One of the goals of this study is to determine if ABA-hypersensitive mutants will have increased drought tolerance as a result of increased ABA sensitivity of stomates to close earlier in response to soil water deficit. This section examines methods for examining plant water status and stomatal responses to water deficit including stomatal conductance and carbon isotope discrimination ($\Delta^{13}\text{C}$).

Stomatal aperture is controlled by the uptake and loss of water from guard cells, and is a function of light, atmospheric $\text{CO}_2$ levels, and water status via (Schroeder et al., 2001). Stomatal opening is stimulated by light via the blue light receptor phototropin (Matsuoka et al., 2006). Stomates open in response to low $\text{CO}_2$ and close in response to excess atmospheric $\text{CO}_2$. Various mechanisms cause stomates to close in response to stress stimuli. Stomates close when guard cells lose their turgor pressure, for example, due to loss of water by evaporation into the atmosphere in response to low humidity. Guard cells also will lose turgor and close stomates in response to ABA synthesized by dehydrated leaves and roots. This mechanism depends on the metabolic activity and solute content of guard cells which determine the degree of loss of water and turgor. When water is depleted, reduced guard cell solute content causes loss of guard cell turgidity resulting in stomatal closure (Davies et al., 2002). ABA is synthesized in the roots and transported to the shoots via the xylem and triggers stomatal closure (Davies et al., 2002). Elevated ABA levels whether in the cell or in the apoplast during water stress appears to cause the reduction in turgor pressure of the guard cells that lead to shrinkage and closure to minimize transpiration (Wilkinson and Davies,
The degree of stomatal aperture is directly linked to photosynthesis and productivity because stomatal opening is required for CO$_2$ uptake for photosynthesis.

Stomatal conductance is a method for estimating the vapor loss through the stomata by transpiration and is expressed in millimoles of water loss per meter squared seconds (mmol m$^{-2}$ s$^{-1}$) (Weyers and Meidner, 1990). Transpiration is the passage of water from the soil through the plant into the atmosphere by evaporation from the mesophyll layer of the leaf through the stomata (Nilsen and Orcutt, 1996). The stomatal conductance can be used as an indicator of the overall transpiration rate of the plant. Stomatal conductance is a function of stomatal density, size, and degree of aperture. A direct measurement of stomatal conductance can be obtained using a leaf porometer (Parkinson, 1985). Changes in transpiration rate also can be estimated by measuring the rate at which soil moisture is lost by weight in potted plants. The rate of soil moisture loss depends on the stomatal conductance over the course of the experiment.

Stomatal closure reduces water loss from transpiration, and as a consequence, it also reduces uptake of carbon dioxide. A reduction in carbon dioxide uptake can limit the photosynthetic capacity of the plant and subsequently lead to reduced yield. The balance between photosynthesis and water conservation is often discussed in terms of water use efficiency (WUE), which is defined as the ratio of the amount of carbon dioxide fixed over the expense of water transpired (A/T). Agronomists also define WUE in terms of the unit of production as the yield of product harvested from the water made available to the crops through natural precipitation and/or irrigation (Condon et al., 2004). More interesting for this research is instantaneous WUE, which refers to the ratio of A/T or transpiration efficiency (TE) by individual leaves or canopies. An increase in WUE indicates that more carbon can
accumulate for growth with the use of less water. Studies in a number of species including wheat demonstrated a positive correlation between WUE and increase in total biomass of plants grown under drought conditions (Ehdaie and Waines, 1993). However, increasing WUE of crops may not always translate into higher crop yield, especially under well-watered conditions. In barley, genotypes with increase WUE resulted in decreases yields (Acevedo et al., 1991). Measurement of instantaneous WUE by gas exchange techniques poses difficulties in the field because of variable microclimates around leaves and canopies over time. Use of gas-exchange to determine whole plant WUE of potted greenhouse plants also is labor intensive and varies with changing light conditions (Nilsen and Orcutt, 1996).

Theoretical and empirical studies have shown that carbon isotope discrimination ($\Delta^{13}C$) can provide a powerful alternative tool for estimating transpiration efficiency and improving and instantaneous WUE (Condon et al., 2004). Analysis of carbon isotope discrimination $\Delta^{13}C$ has conceptual and practical advantages over measuring WUE by instantaneous gas exchange measurements and whole-plant harvest (Condon et al., 2004). Carbon has two naturally occurring stable isotopes; $^{12}C$ is more abundant and accounts for 98.9% whereas $^{13}C$ is only about 1.1%. During photosynthesis, $C_3$ species fix more $^{12}C$ and discriminate against the heavier isotope $^{13}C$. This discrimination results from differences in diffusion of $^{12}CO_2$ and $^{13}CO_2$ into the substomatal cavity, and from enzymatic discrimination during the fixation of $CO_2$ into sugars in the mesophyll. This preferential use of $^{12}C$ isotope is dependent on stomatal conductance. When stomatal conductance is high (stomates are open), discrimination is high because both forms of $CO_2$ freely enter the substomatal cavity. $\Delta^{13}C$ is low when stomatal conductance is low (more stomata are closed) and the plant is forced to discriminate less against $^{13}CO_2$ as the supply of $^{12}CO_2$ decreases. Thus you can
select for a more conservative plant (closes stomata more readily under drought stress) by screening for lower $\Delta^{13}C$ under drought stress. We expect ABA hypersensitive plants to have a lower $\Delta^{13}C$.

This section discusses the utility of measuring $\Delta^{13}C$ as a means of improving yield in crop plants. A number of studies described a positive correlation between $\Delta^{13}C$ and yield in wheat (Araus et al., 1993; Condon et al., 1993; Merah et al., 2001), whereas others describe a negative correlation (Condon et al., 2002a; Rebetzke et al., 2002). Seedlings with low $\Delta^{13}C$ (high WUE) tended to grow slower than plants with high $\Delta^{13}C$ which reduced plant biomass and yield under well-watered conditions but not under water deficit (Condon et al., 2002b). The correlation between $\Delta^{13}C$ and yield depends on the organ sampled, the growth stage, and timing of drought stress. Monneveux et al. (2006) attempted to resolve this controversy by examining the $\Delta^{13}C$ in different tissues harvested from wheat plants exposed to drought stress at different times in their life cycle. They found that the $\Delta^{13}C$ of mature grain correlated positively with yield under postanthesis and residual moisture water stress whereas $\Delta^{13}C$ of leaves only positively correlated with yield under postanthesis water stress.

This study explores the possibility of using mutants with increased ABA sensitivity to understand the role of ABA in seed dormancy and in drought tolerance in hexaploid bread wheat. Specifically, this study will determine whether the mutants recovered are recessive and genetically stable. This project also examines whether ABA hypersensitivity in seed germination can be used to prevent the lack of seed dormancy associated with preharvest sprouting. Lastly, experiments will investigate whether ABA hypersensitive mutants have improved drought tolerance due to increased sensitivity to ABA in stomatal closure.
CHAPTER TWO

MATERIALS AND METHODS

Plant material

To isolate ABA hypersensitive mutants, the hard red spring wheat genotype ‘Chinese Spring’, Dv418 (constructed by Jan Dvorak) was used as the primary population (Warner et al., 2000). This cultivar was chosen as the wild-type genetic background because of its high level of homozygosity as a doubled haploid-derived line. Seeds were fast-neutron mutagenized using 4 grays, an optimum treatment to allow a number of lines to produce seeds without compromising fertility (Redei and Koncz 1992). Fast neutron uses physical irradiation that creates random deletions or lesions in the genome (Li and Zhang, 2002). These seeds were provided by R. Warner (Warner et al., 2000). The first generation of mutagenized seeds (M₁), approximately 800 plants were planted in the field and allowed to self-fertilize and grown to maturity to generate the M₂ (second generation of mutagenized seed) progeny. One head from each M₁ plant was hand-harvested to retain an intact head and maximize the number of alleles known to be independent. A total of 22,250 M₂ seeds were obtained from all M₁ heads. They were stored at room temperature and afterripen for two years (arbitrary period prior to acquisition, allowed maximum afterripening) before screening.

Zak (Kidwell et al., 2002) and Scarlet (Kidwell et al., 1999), a soft white and hard red spring wheat cultivar, respectively, also were mutagenized with ethylmethane sulphonate (EMS) to isolate ABA hypersensitive mutants. EMS mutagenesis results with high degree of
point mutational densities that does not result to chromosome breaks that cause aneuploidy and reduced fertility (Greene et al., 2003). Five hundred and 400 M₁ seeds of cv Zak and Scarlet, respectively, were advanced to M₂ following self-fertilization and grown to maturity in the greenhouse. A total of 15,000 and 12,000 M₂ seeds were harvested from M₁ heads of cv Zak and Scarlet, respectively. M₂ heads were hand-threshed and stored at room temperature to afterripen for six months (maximum period of afterripening of cv. Brevor) prior to the screen (Strader, 2004).

**Growth condition**

Mutant and wild-type plants were grown in the greenhouse or in the field at Spillman Farm in Pullman, WA. Seed increases (M₂ to M₃ and so on) were performed in the greenhouse with a photoperiod of 16 hours at a PPDF of 400-450 µmol m⁻² s⁻¹ with a daytime temperature of 18-22°C and a nighttime temperature of 15-17°C. During winter months, supplemental light with 1000 watt high pressure sodium lamps were used (Sullivan Co.). Two seedlings were transplanted in one six-inch-wide polyvinyl pot using Sunshine Mix II garden potting soil mixture (Sun Gro Horticulture). Pots were watered to saturation every three days during noontime from seedling stage to stem elongation and every two days from then on until plants reached physiological maturity. A nutrient solution (20-20-20 peters product) at 100 parts per million (ppm) of nitrogen (N) was supplied once a week.

**Identification of ABA hypersensitive wheat genotypes**

Heads from greenhouse-grown mutants and wild-type plants were harvested at the same time with wild-type and hand-threshed to avoid scarification, which may have impacted
seed germination. Seeds harvested from each head were divided, and were stored at 20-25°C to afterripen, and the remaining half were frozen at −20°C to maintain dormancy. Chinese Spring M₂ seeds afterripened for two years at room temperature were utilized to isolate ABA hypersensitivity mutations. A pooling strategy was devised to allow for screening large number of seeds where each pool represented progeny from 10 M₁ plants (Strader, 2004). A pool consisted of four M₂ seeds coming from 10 M₁ heads were hand-threshed and plated in a disposable plastic 9-cm Petri dish (Falcon No. 35-1029, Becton and Dickinson Labware, Franklin Lakes, NJ, USA) lined with a single moistened germination disc (3.38” diameter, **www.seedpeper.com** Catalog no. SAB3.375) in an aqueous solution containing 6 ml of 5 μM (+)-ABA buffered to pH5.5 with 5 mM 2-[N-morpholino] ethane sulfonic acid (MES) (Sigma Chemical Co., St. Louis, MO, USA) solution to allow ABA to cross the apoplast (Haubrick et al., 2006). Optically pure (+)-ABA, the form that is biologically active, was obtained as a gift from S. Abrams of the National Research Council of Canada (NRC) and maintained as a 0.1 M stock in dimethyl sulfoxide (DMSO) or methanol (Abrams et al., 1997). Each plate contained three replications with 10 seeds per replication. The plates were sealed with Parafilm, wrapped with aluminum foil and incubated at 30°C in the dark. Seeds were scored for germination every 24 hours until 96 hours had passed. A seed is scored as germinated when both the radicle and coleoptiles grew in excess of 1 mm. Unerminated seeds were rescued and transferred to new petri dish containing a single germination disc moistened with 6 mL of 10 μM of GA₃ (Sigma Co.) to promote germination. GA₃ was maintained as a 10 mM stock in ethanol. Seeds that failed to germinate after 72 hours despite the presence of GA₃ were discarded, whereas those that germinated on GA₃ were transplanted.
to soil and grown in the greenhouse for advancement to M₃ generation along with wild type Chinese Spring as a control.

**M₃ retest**

A retest for ABA hypersensitivity was conducted on whole M₃ seeds that were harvested from M₂ heads and were afterripened for 6 months following the same screening protocol mentioned above using 6 ml of 5 µM (+)-ABA in MES, a dose so low, germination of the wild type Chinese Spring was promoted. Two scoring schemes were employed for seed germination. A seed was scored as germinated when the radicle reached ~1 mm in length. The second scheme allowed scoring for all three-point roots emergences. Results are presented as the mean percent seed germination of three replicates with 10 seeds per replicate. Ungerminated seeds from the M₃ retest were rescued following the protocol described above and transferred in the greenhouse for advancement to M₄. Wild type Chinese Spring also was planted together with the M₃ mutants.

**ABA dose-response in seed germination**

ABA dose-response experiments were based on a procedure developed by Walker-Simmons (1987). An ABA dose-response germination experiment were performed on M₃ seeds by L. Strader (Strader, 2004). The assay compared percent germination of cut half-grains (referred to here as embryos), dormant seeds, and whole afterripened seeds on varying concentrations of (+)-ABA (Walker-Simmons, 1987). J. Abellera used homozygous M₆ seeds for a similar second dose-response experiment using five (+)-ABA concentrations to characterize putative mutants for sensitivity in seed germination: 1.0 µM, 5.0 µM, 10.0 µM,
and 25.0 μM of (+)-ABA in MES with a control consisting only of solvent in MES. Each plate contained 30 seeds representing 3 subsamples of 10 seeds each. Germination was scored as percent germination at radicle emergence and as three-point seminal roots emergence every 24 hours for 5 days. The second dose-response experiment was conducted using the carefully selected M₆ individual plants that were assumed homozygous for the ABA hypersensitivity phenotype. These plants were selected from a plating experiment for using M₄ afterripened seeds on 5 μM ABA incubated for 96h at 30°C. This assay was done to select for seeds with ABA hypersensitivity in seed germination. A maximum of 4 plants per M₄ mutant were harvested separately and ABA sensitivity in seed germination phenotype was evaluated for 10 dormant embryos. ABA hypersensitive and increased embryo dormancy mutant classes were plated on 5 μM ABA. Taking into account the wild-type ABA sensitivity of seed coat-imposed dormancy class, ABA concentration was increased to 10 μM. The most promising plants for ABA hypersensitivity based on percent germination were increased in the greenhouse. For details concerning seed germination results from this experiment are found in Appendix 1.

**Backcrossing and F₂ segregation analysis**

Each mutant was backcrossed to the wild-type parent to determine the pattern of inheritance of ABA hypersensitivity trait among the offspring. Wheat spikes were emasculated by cutting one-third part of the glumes and removing the immature anthers with tweezers. Emasculated spikes were bagged using a glassine bag and were fastened with a metal clipped. Pollinations were performed one or two days after emasculation depending on the receptivity of the stigmas. Mature anthers were taken from Chinese Spring just before
shedding of the pollen sacs, and at least one anther was placed on each stigma of the mutant wheat spikes. The spikes were bagged again after pollination. Emasculation and pollination works were made in the morning between 8:00 to 11:00. Chinese Spring wild-type plants were used as male parent and the mutants as the female parent constantly for all crosses. Initial characterization of these mutants based on a single dose-response germination experiment suggested that there were two classes of ABA hypersensitivity (data presented in the Result section). All BC₁F₁ seeds out from the backcross in the ABA hypersensitive class were scored for failure to germinate on 5 µM of (+)-ABA using dormant embryos. If failure to germinate on ABA segregated 1:1 in the F₁ seeds, then we concluded that the mutant parent was heterozygous for a possibly dominant allele. Germination was scored every 24 hours for 5 days. Ungerminated seeds were transferred to petri dish with GA to stimulate germination and germinated seeds were transplanted to soil in pots in the greenhouse to obtain F₂ seeds. The second mutant class, referred to as seed coat-imposed dormancy mutants, needed six months to afterripen before seed germination screens could be conducted. This afterripening step was necessary because they are mutants that have apparent ABA hypersensitivity only when whole seeds were used, but showed wild-type ABA sensitivity when seed coats were cut (Strader, 2004). A more detailed description of the two classes is presented in the Results section.

Each F₂ head from every BC₁F₁ plant that was self-fertilized was harvested separately and treated as an independent entry for genetic analysis. The ABA hypersensitive class mutants were germinated using the same protocol as described above on 5 µM of (+)-ABA using cut, dormant seeds. The number of BC₁F₁ seeds per spike ranged from eight to 29 (Table 4). Consequent to the number of BC₁F₁ seeds, approximately 400 to 1700 F₂ seeds for
each mutant were analyzed. Segregation analysis for seed coat-imposed dormancy mutants was conducted using whole seeds after 6 months of dry storage. Chi-square test ($\chi^2$) for goodness of fit was used to examine $F_2$ gene segregation following the formula, $\chi^2 = \Sigma [d^2/e]$, where ($d$) is the deviation of the observed from expected value ($e$) and the sum ($\Sigma$) (Falconer and Mackay, 1996). If the mutant parents used to generate the backcross population was homozygous dominant, then every individual in the $F_1$ generation was heterozygous. In the $F_2$, the expected segregation ratio was 3:1 for mutant phenotype to wild type phenotype for a dominant trait. The level of significance was set at 5% with degrees of freedom ($df$) of 1 (two phenotypic classes minus 1). If the calculated chi square value was less than the expected chi square value of 3.84 (5%, $df=1$) then the hypothesis was accepted. In cases where the $F_2$ segregation ratio did not fit the 3:1 or 1:3 segregation ratios, both ungerminated and germinated seeds were advanced to $F_3$ for further analysis. It is possible that mutations resulting in ~50% seed germination in the $F_2$ consist either of a single semi-dominant mutation or of two recessive mutations (9:7). If the line contains a single semi-dominant mutation, we expect that $F_3$ descendents of $F_2$ seeds that failed to germinate will either be homozygous (25% of total $F_2$) or segregating (number of $F_2$ in excess of 25%) for a semi-dominant trait.

**Drought tolerance experiments**

**A. Plant transpiration experiment**

Analysis of drought tolerance was performed based on estimation of plant transpiration rate based on the percentage of soil moisture loss over time (based on Pei, et al., 1998 and Wang, et al. 2005 with modifications for wheat). Seeds were plated on a Petri dish
with a single germination disk moistened with 6 ml of 5 µM (+)-ABA in MES solution grown for 120 hours at 30°C in the dark. Ungerminated seeds were transferred to new plates lined with a germination disk containing 6 mL of 10 µM GA₃ in MES solution for 48 hours before they were transplanted in the greenhouse. Three independent experiments were carried out to test all 12 ABA hypersensitive mutants and only two were conducted thus far. Four mutants, wild-type Chinese Spring and positive drought control cv. Alpowa composed an experiment. In each experiment, a minimum of 15 plants per genotype (one plant per pot) including wild type Chinese Spring were grown in the greenhouse using three-inch-wide, square polyvinyl pots filled with 430 grams of uniformly damped mixture of vermiculite and potting soil. Plants were grown in the growth chamber (Controlled Environment Limited, Winnipeg, Canada) and watered every three days with non-nutrient water and a nutrient solution was supplied once a week. Growing conditions were set at 16-hour day with 350-400 umol light intensity using 50/50 mixture of 400-watt high pressure sodium and metal halide lamps (Sullvain Co.) at temperatures of 22°C during the day and 15°C at night. In the first experiment, plants were moved from growth room condition to greenhouse condition at the four-leaf-two-tiller stage according to Zadok Scale = 14, 22 (Nelson et al, 1988). They were allowed to acclimate for 7 days with a photoperiod of 16 hours at a PPDF of 400-450 µmol m⁻² s⁻¹ with a daytime temperature of 18-22°C and a nighttime temperature of 15-17°C. Supplemental light with 1000 watt high pressure sodium lamps were used (Sullivan Co.). Plants in the second experiments were moved to the greenhouse at fifth-leaf-two-tiller stage (Zadok Scale = 15, 22) (Nelson et al, 1988). Pots were bottom-watered to saturation a day before the experiment started and excess water was allowed to drip overnight, leaving pots evenly moist on day 1 based on weight. At least 10 plants of approximately the same
developmental stages were selected to minimize the effects on the differences in soil water loss due to subtle differences in plant size (Wang, et al., 2005). To avoid evaporation of water from the soil surface, pots were covered with heavy-duty transparent cellophane wrap (autoclave bags cut to fit, Fisher Scientific, Pittsburgh, PA) and every pothole was sealed with a clear tape. At least two plants of each mutant genotype and wild-type Chinese Spring were watered normally during the experiment as a well-watered control. At this point, watering ceased and the initial pot weights (in grams) were taken. Initial weight was within 430 grams for pots of all genotypes. Water loss was monitored by weighing individual pots every 12 hours for the first 48 hours at 12 pm and 12 am and every 24 hours thereafter for 14 days at 12 pm. At the end of the experiment, above ground plant parts were weighed before and after oven-drying to 70°C for 48 hours to obtain dry weights. Dry weights were subtracted from the daily pot weights to calculate percent water loss. The results were expressed in terms of percent of soil water loss over the number of days of drought treatment. Root biomass also was evaluated at the end of the experiment. Roots were separated from soil by soaking in water and water pressure was applied to thoroughly clean the roots. Roots were pat-dried in absorbent paper towel and initial fresh weights were recorded prior to oven drying. The condition for drying was set at 70°C for 48 hours. Root dry weights were subtracted from the initial fresh weights to obtain root biomass.

**Stomatal conductance measurements using leaf porometer**

Stomatal conductance was measured using a steady-state leaf porometer (Leaf Porometer® SC-1, Decagon Devices Inc., Pullman, WA) using plants during the transpiration time-course experiment described above. Stomatal conductance is a function of
stomatal density, size and degree of aperture (Weyers and Meidner, 1990). The device measures the vapor pressure at two locations in the diffusion path, then determines flux and gradient from the vapor pressure measurements and the known conductance of the diffusion path (Parkinson, 1985). Measurements were taken on a single, young, fully-grown unshaded leaf that was perpendicular to a light source. Leaves were of approximately the same age and similar placement in the plant. Data were obtained only from the adaxial surface of the leaf. Five plants of each genotype were examined. Over the first seven days of the time course, the stomatal conductance of a single leaf (tagged) from each plant was measured 3-7 times between noon and 2 pm. In order to avoid changes in environmental conditions that might alter plant stomatal aperture, variation in greenhouse lighting and temperature was reduced by closing the shaded panels to minimize effects of cloud cover, and by using supplemental high pressure sodium lights (details described above) during the entire period of measurements. The automatic mode for leaf porometer measurements was used; the sensor was clamped onto a leaf and held steady until the automatic 30 second measurement of conductance was completed (http://www.decagon.com/manuals/Poromanual.pdf). The conductance was recorded in units of mmol m\(^{-2}\)s\(^{-1}\) (millimoles per meter squared seconds).

**Carbon isotope discrimination (\(\Delta^{13}C\))**

Measurements were performed on single, young fully-grown leaves at four-leaf-two-tiller stage (Zadok Scale = 14, 22) (Nelson et al., 1988) from well-watered, field-grown plants (Condon et al., 2004). Nineteen and four mutants of ‘Chinese Spring’ (M\(_5\)) and ‘Zak’ (M\(_4\)), respectively, including their wild type genotypes and a positive cultivar check ‘Alpowa’ (soft white spring wheat), were grown in a well-watered field. Ten plants of each
genotype (mutants and wild-type) were planted in a 30 x 60 cm single, unreplicated plot. Non-nutrient water was supplied twice a week. Sampling protocols were followed after carbon isotope analyzes using seedlings (Condon et al., 2002b; Rebetzke et al., 2002) with modifications to age of the leaves being sampled. A single leaf from four M₅ plants from each M₄ genotype were sampled independently and oven-dried at 70°C for 72 hours. Dry leaf tissue samples were placed in 2 mL Eppendorf microcentrifuge tube and ground to ~1 mm particle using polypropylene pestle. Each of the four independent leaves from each genotype was sampled. Carbon isotope composition was determined on 1.90 to 2.10 mg ground leaf subsamples with Isotopic Ratio Mass Spectrometer (IRMS) at the Bioanalysis Stable Isotope Core Laboratory at the School of Biological Sciences, Washington State University. Results were presented as means of the eight replicate leaf tissue samples of each genotype and calculated as $\delta^{13}C \, (\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 1000$, where $R$ is the ratio of $^{13}C/^{12}C$. A secondary standard calibrated against Pee Dee Belemnite (PDB) carbonate was used for comparison. The precision of analysis (final $\delta^{13}C$ +1 standard deviation) ranged between 0.02 to 0.04‰. The discrimination ($\Delta$) was calculated using the following formula: $\Delta(\text{‰}) = \left[\left(\delta_a - \delta_p\right) / \left(1000 + \delta_p\right)\right] \times 1000$, where $\delta_p$ is the $\delta^{13}C$ of the samples and $\delta_a$, the $\delta^{13}C$ of atmospheric CO₂. Atmospheric CO₂ has a current deviation of approximately -8‰ (Farquhar et al., 1989).

**B. Field tests**

A preliminary field test was carried out in April 2005 at the Washington State University Spillman Farms, Pullman, Washington to evaluate the performance of all 25 ABA hypersensitive mutants in terms of yield at two sites representing drought and well-watered
conditions. Wild-type Chinese Spring and five other spring wheat cultivars including ‘Scarlet’, ‘Zak’, ‘Alpowa’, ‘Calorwa’, and ‘Eden’ (Kidwell et al., 2004) were included in the test as check. Seeds were directly planted without prior screening for ABA sensitivity in seed germination. Twenty seeds were each entry is planted to two rows in a 30 x 90 cm plot size and replicated 3 times. Heavy stripe rust (*Puccinia striiformis*) pressure during the growing season necessitated the application of fungicide (Tilt® Fungicide, Syngenta) at concentration of 4 oz/acre at six-leaf with four tillers stage and at post-anthesis stage. Plant height was recorded from 5 tillers of 5 different plants at maturity by measuring distance of the tallest tiller from the soil surface to spike tips, excluding awns (del Blanco et al., 2000). Total biomass was calculated by harvesting and weighing above ground plant parts divided by the number of plants in a plot. Heads were hand-threshed and seeds were weighed from each plot to obtain gross seed yield. Finally, harvest index was determined as the ratio of grain weight to total above-ground weight (del Blanco et al., 2000).
CHAPTER THREE

RESULTS

This chapter presents data for the isolation, genetic analysis, and characterization of ABA hypersensitive genotypes of wheat. The mutant screens performed by Lucia Strader and Sven Nelson prior to the beginning of this masters thesis are described in this section for the sake of continuity in description. The characterization of Chinese spring dormancy and ABA response were performed by a fellow graduate student, Elizabeth Schramm. Transpiration experiments were performed in collaboration with Elizabeth Schramm. The remaining experiments were solely the work of Jorgen Abellera.
The effect of seed dormancy and afterripening on Chinese Spring ABA sensitivity in seed germination.

Previous work showed that the ABA sensitivity of wheat cv. Brevor (soft white winter) is dependent on the dormancy status of the grain (Figure 3). To determine if the same is true for the hard red spring wheat germplasm Chinese Spring, ABA sensitivity was compared in dormant and afterripened whole seeds and embryos. Similar to the Brevor background, whole dormant seeds of Chinese Spring did not germinate in the presence or absence of ABA, whereas dormant embryos showed dose-dependent inhibition of germination by ABA (Figure 3a). Afterripened whole seed and afterripened embryos germinate fully in the absence of ABA and in the presence of high concentrations of ABA (Figure 3b). While dormant Brevor embryos germinated at 100% in the absence of ABA, Chinese Spring dormant embryos showed 55% germination at 0.0 μM after 72 hours of incubation (Figure 3b, Figure 2a). Dormant Chinese Spring embryos also showed a lower percentage of seed germination at each ABA concentration tested. For example, Chinese Spring showed 7% germination at 10 μM ABA, whereas Brevor showed 30% germination at 10 μM ABA. It appears that white dormancy in Brevor is entirely seed coat imposed, whereas Chinese Spring has a combination of seed coat imposed and embryo dormancy. Based on this result, care has been taken to briefly afterripen Chinese Spring to obtain seeds showing mostly seed coat imposed dormancy. Nevertheless, ABA sensitivity is dependent on the dormancy status of the grain in wheat in both genotypes with afterripened grain showing a high degree of ABA insensitivity. This ABA insensitivity is a unique characteristic of afterripened wheat grain.
Figure 3. Seed germination experiment showing sensitivity to ABA is dependent on dormancy status in Chinese Spring wheat. Percent germination following incubation on varying concentrations of ABA is shown. (a) Comparison of percent germination of whole dormant and afterripened wheat seed following 72 h incubation at 30°C on filter paper soaked with (+)-ABA in 5 mM MES, pH 5.5. (b) Comparison of percent germination of dormant and afterripened wheat embryos following 72 h incubation on filter paper soaked with (+)-ABA in 5 mM MES, pH 5.5. Y-axis bars represent standard error for 3 samples of 10 seeds each. (data provided by E. Schramm)
Afterripened seeds of other species, such as Arabidopsis, continue to show dose-dependent inhibition of seed germination by ABA (Strader, 2004).

This distinctive property of wheat ABA response in seed germination formed the basis for screening strategy used to isolate ABA hypersensitive mutants in Chinese Spring wheat. Based on experiments with Brevor and Chinese Spring, 5 μM ABA was found to be a concentration that inhibited dormant seed but had no effect on afterripened seed germination. For this reason, whole afterripened seeds were screened for mutations causing inability to germinate on 5 μM of ABA.

**Screening for ABA hypersensitive mutants in wheat**

ABA hypersensitive mutants were identified in allohexaploid wheat using a germination-based screen for wheat mutants with increased responsiveness to ABA following 6 months afterripening (Figure 4). M$_2$ fast-neutron mutagenized (4 grays) Chinese Spring seeds were allowed to afterripen for two years prior to screening for mutants with increased sensitivity to exogenous ABA in seed germination. Any M$_2$ seeds that failed to germinate on plates containing 5 μM ABA after 96 hours were classified as putative ABA hypersensitive mutants. A total of 22,520 M$_2$ seeds were screened. From these 89 M$_2$ putative ABA responsive mutants were isolated of which 39 independent mutants passed the retest for failure to germinate on 5 μM ABA performed on M$_3$ seeds afterripened for 6 months (Table 1). Of the 39, 25 mutants were further characterized and 14 were discarded due to problems with fertility or lethality. The 25 mutants showing ABA sensitivity when
Figure 4. Schematic diagram of the screen for wheat ABA-responsive when afterripened (ARA) mutants. Seeds of Chinese Spring were fast-neutron mutagenized. M₁ seeds were grown in the field to generate M₂ progeny. Screen was performed on whole afterripened (2 years) M₂ seeds. Ungerminated seeds were rescued moved to plates containing 10 mM GA₃ to stimulate seed germination. Germinated seeds were transplanted to soil in the greenhouse and advanced to M₃.
afterripened will be referred to as *ARA (ABA Responsive when Afterripened)* mutants in the remainder of the thesis.

Numerous retests have been performed during the course of this thesis (Table 1, Appendices 2 and 3). It appears that some of these mutants were originally isolated as heterozygous dominant or semi-dominant mutations and subsequent generations have shown some segregation. I have attempted to isolate homozygous plants by identifying plants showing a germination phenotype in consecutive generations. However, some genotypes also appear to have incomplete penetrance or variability in expressivity of the trait in different generations. This is not unusual in mutants involving dormancy state. For example, problems were encountered in the genetic analysis of the *Arabidopsis reduced dormancy (rdo)* mutants due to the variability in the germination phenotype (Leon Kloosterziel et al., 1996). Some of these seed germination experiments were performed to assure the plants used for drought studies still showed the seed germination phenotype. Plating has been done using cut dormant seeds as well as whole afterripened seeds so as to avoid having to wait 6 months for afterripening.

The seven retests (Table 1) were evaluated to determine the number of times each mutant has retested. Table 2 shows that a single mutant retested in all 7 experiments, whereas 4 retested in 5 of 7 experiments, 3 retested in 4 of 7 experiments, and 3 retested in 3 of 7 experiments. The remaining mutants showed a phenotype in 2 or less experiments. One concern is that some mutations may be lost to segregation and selection or may have lost the original phenotype due to silencing. This will be considered in the Discussion section.

Phenotypic evaluation revealed a number of secondary phenotypes. In the greenhouse, mutants 1314-115, 78-7 and 1314-42 had normal tillers but reduced seed set and
Table 2. Frequency at which retested with 40% or less seed germination on ABA. Mutants in bold font are dwarves.

<table>
<thead>
<tr>
<th>Retested in 7 out of 7 experiments</th>
<th>Retested in 5 out of 7 experiments</th>
<th>Retested in 4 out of 7 experiments</th>
<th>Retested in 3 out of 7 experiments</th>
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<tr>
<td>1314-45</td>
<td>910-13</td>
<td>78-68</td>
<td>78-15</td>
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<tr>
<td>910-22</td>
<td>1314-28</td>
<td>1314-76</td>
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<tr>
<td>1314-16</td>
<td>1314-130</td>
<td>1314-82</td>
<td></td>
</tr>
<tr>
<td>1314-64</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
seed characteristics varying from wrinkled to normal-sized seeds. In addition, these mutants have delayed transition to flowering relative to wild-type. Seed size varied between genotypes. By visual comparison, larger seeds were observed consistently in mutants 1314-26B, 78-39, 78-68, and 910-22 relative to wild-type Chinese Spring. An unknown sweet, viscous substance was always observed at the embryo end of 1314-34 grain. Based on the first transpiration experiment and subsequent observations in the greenhouse, mutant 910-55 headed ~10 days earlier than wild-type. Field test revealed that some mutants transitioned to flowering earlier than wild-type including 910-55, 1314-1, 1314-26A, 1314-26B, 1314-35, and 1314-76. Among this subset of mutants, 910-55, 1314-26A, 1314-26B and 1314-35 appear to have high pollen load relative to wild-type Chinese Spring. Conversely, mutants 1314-115, 78-7, and 78-39 had delayed transition to flowering relative to wild-type Chinese Spring. A similar condition was observed in the first 3 mutants when they were grown in the greenhouse. Phenotypic evaluations revealed that 6 of the 25 mutants appeared to be dark green, dwarves including 78-68, 910-22, 910-55, 1314-45, 1314-76, 1314-115 and (Figure 5, mutants 1314-45 and 910-55). Dwarf mutants are shown in bold in Table 2. These may be GA response mutants rather than mutants directly affecting ABA signaling. However, this observation has yet to be tested.

Classification of mutants based on ABA dose-response curves

Mutants were placed into classes based on their dose-response to varying concentrations of ABA during seed germination. An original dose-response experiment was conducted by L. Strader using material that is now known to be segregating. Dose-response
Figure 5. Comparison of the dwarf phenotype of *ARA* mutant lines 1314-45 (left) and 910-55A (right) with wild-type Chinese Spring. Photographed at milk stage (Zadok scale, 70)
curves have been repeated in this thesis using homozygous seed lots. Data from both experiments are described here for comparisons sake.

The screen recovered ABA mutants showing increased sensitivity to 5 μM ABA after 6 months of afterripening as whole grain. In order to compare the dose-response of mutants to wild-type Chinese Spring, plating experiments were performed using cut dormant half-seeds. If afterripened grain had been used, Chinese Spring would be fully insensitive to ABA. The experiment conducted by L. Strader was performed on cut dormant M₃ seeds allowed to afterripen at room temperature for at least 2 months prior to the experiment. I performed a second ABA dose-response experiment using cut dormant M₆ grain allowed to afterripen at room temperature for 2 weeks prior to the experiment.

L. Strader’s results from the ABA dose-response experiments for the 25 of the independent ARA mutants will be discussed first. Based on this first experiment, mutants were divided into 3 phenotypic classes (Table 3). The first class of mutants has prolonged seed coat-imposed dormancy. The second class displaying a clear ABA hypersensitive response and were classified as ABA hypersensitive. Lastly, three mutants form a class that appeared to have increased embryo dormancy.

Mutants classified as having prolonged seed coat-imposed dormancy showed failure to germinate on 5 μM ABA in the M₃ retest of whole afterripened seeds but showed a normal (similar to wild-type Chinese Spring) ABA dose-response as dormant embryos (Figure 6). There were two possible explanations for this phenotype: 1) the mutants require more time to afterripen and so appear ABA sensitive as whole grains following 6 months of afterripening, or 2) there is no consistent change in dormancy or afterripening and these lines fail to retest. The first explanation was chosen as a working hypothesis. Ten mutants showed this seed
Table 3. Phenotypic classification of ABA hypersensitive mutants based on ABA dose-response in seed germination in two independent experiments. Mutants in bold font indicate a confirmation of the phenotype in both experiments.

<table>
<thead>
<tr>
<th>Mutant Class</th>
<th>L. Stader data</th>
<th>J. Abellera data</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABA-hypersensitive</td>
<td>78-7 \textbf{78-15} 78-39 910-13 910-22 \textbf{1314-28} 1314-42 1314-46 \textbf{1314-64} 1314-82 \textbf{1314-130}</td>
<td>78-15 1314-28 1314-64 1314-130</td>
</tr>
<tr>
<td>Embryo dormancy</td>
<td>78-68, \textbf{1314-16, 1314-45}</td>
<td>1314-16, 1314-45</td>
</tr>
<tr>
<td>Wild-type prolonged seed-coat imposed dormancy</td>
<td>1314-26A 910-55 46-17 1314-26 1314-35 1314-1 78-112 1314-115 1314-93 1314-34 1314-73</td>
<td>78-68 910-22 1314-76 1314-82 remaining not retested</td>
</tr>
</tbody>
</table>
Figure 6. ABA dose-response of selected ARA mutants of the seed coat-imposed dormancy class. 1314-35 and 1314-1 show a similar response to ABA compared to WT Chinese Spring as dormant embryos incubated for 96h incubation at 30°C on filter paper soaked with increasing concentrations of (+)-ABA in 5 mM MES, pH 5.5. Error bars represent SE for 3 samples of 10 grains. (Strader, 2004)
Mutants in the \textit{ABA hypersensitive} class show wild-type seed germination in the absence of ABA but exhibit increased ABA sensitivity in seed germination as dormant embryos. The ABA dose-responses of five of these eleven mutants (Table 3) in this class were compared to wild-type Chinese Spring over a 72-96 h germination time course. A dose-dependent response is evident in this class, increasing the ABA concentration limits germination to less than 20\%, whereas wild-type reached 80\% germination at 25 \(\mu\text{M}\) ABA. The original goal of the mutant screen was to recover mutants with this ABA-hypersensitive phenotype.

The ABA dose-response experiment revealed that three mutants show \textit{increased embryo dormancy} that resulted in reduced germination relative to wild-type both in the presence and absence of ABA. These mutants include 78-68, 1314-16, and 1314-45, shown in Figure 7. For example, 78-68 germination is reduced to \(~60\%\) after 96 h in the absence of ABA. Strong inhibition of seed germination is further observed over increasing concentrations of ABA. At the highest ABA concentration (25 \(\mu\text{M}\)), 78-68 germination is reduced to only about 10\% after 96h, whereas wild-type Chinese Spring germination is 100\%. In the absence of ABA, seeds with seed coat imposed dormancy germinate when the seed coat is cut, whereas seeds with embryo dormancy fail to germinate even after the seed coat is cut. Afterripening or cold stratification can be used to overcome both embryo and seed coat imposed dormancy. One problem with this class of mutants is that it is difficult to judge if failure to germinate on low concentrations of ABA is the result of a true increase in
Figure 7. ABA dose-response of selected *ARA* mutants of the ABA-hypersensitive class. Mutants 1314-64, 910-22, 1314-130, and 78-15 are compared to WT Chinese Spring as dormant embryos incubated for 96h at 30°C on increasing concentrations of (+)-ABA in 5 mM MES, pH 5.5. Error bars represent SE for 3 samples of 10 grains. (Strader, 2004)
ABA sensitivity or is the result of an overall decrease in seed germination due to increased embryo dormancy.

In order to obtain a clearer picture of the ABA dose-response, plating experiments were used to obtain homozygous mutants. Plating experiments suggested that many of the independent mutants were segregating for the desired phenotype in the M₃ (Table 1). Ideally, a series of backcrosses to the wild-type parent and selection of phenotype in subsequent generation by single-seed-descent could clean-up the genetic background. Single-seed-descent selection is the selection in which the F₂ plants and their progeny are advanced by single seed (one seed from each plant) until genetic purity is virtually reached (Poehlman and Sleper, 1995). Unfortunately, genetic studies can take time before fully homozygous material can be obtained. Thus, careful selection of homozygous individual plants was conducted in the M₄. Whole afterripened M₄ seeds were plated on 5 μM ABA and incubated for 96h at 30°C to select individual grains that failed to germinate. Four M₄ plants (known to express the desired phenotype) were harvested separately for each genotype and segregation of the ABA sensitive seed germination phenotype was evaluated using 10 M₅ dormant embryos from each plant. *ABA hypersensitive* and *increased embryo dormancy* mutant classes were plated on 5 μM ABA as cut dormant grain or embryos (Table 1, M₅ 14-Oct-05). Taking into account the wild-type ABA sensitivity of the seed coat-imposed dormancy class, these mutants were plated as whole grain and as cut dormant grain on 10 μM ABA (Table 1, M₅ 14-Oct-05). These mutants also will be screened as whole afterripened grain in July 2006 (by E. Schramm). The progeny of those M₄ plants that showed 0% seed germination on ABA in the M₅ were advanced as single plants for seed-increase in the greenhouse. In cases where more than 0% seed germination was observed on ABA, the plant(s) showing the
lowest % seed germination on ABA was selected. The progeny of these plants will need to be re-examined for possible segregation in the M₆. Seeds from plants where more than one plant exhibited ABA sensitivity were bulked for use in transpiration experiments described later in this Chapter.

A second round of dose-response experiment was conducted using selected M₆ derived from homozygous M₅ plants based on ABA hypersensitive seed germination. Only the ABA hypersensitive and increased embryo dormancy classes were examined since the seed coat-imposed dormancy mutants require at least 6 months afterripening to have a meaningful dose-response experiment using whole afterripened seeds. Results indicate that four mutants showed ABA dose-response similar to the first experiment. Mutants 1314-64, 78-15B, 1314-130, and 1314-28 showed dose-dependent hypersensitive response to exogenous ABA application allowing their classification as ABA-hypersensitive mutants (compare Figure 8 and Figure 9). 78-15 and 1314-130 demonstrate wild-type seed germination on plates without ABA and on plates with 1 μM ABA, but showed increased inhibition of seed germination by ABA at 5 μM to 25 μM range of concentration. 1314-64 displayed strong inhibition of seed germination even at the lowest ABA concentration of 1 μM but fully germinated without ABA after 120h of incubation at 30°C. 1314-28 germinated at over 80% in both 0 μM and 1 μM ABA treatments but showed strong inhibition of seed germination at higher ABA concentrations suggesting that this is an ABA hypersensitive mutant that can sometimes show embryo dormancy. In this experiment, wild-type Chinese Spring germinated fully on plates without ABA but showed a higher degree of ABA sensitivity as the ABA concentration (Figure 9) increased compared to L. Strader’s experiments (Figure 6, 7, 8). This may be due to the fact that L. Strader allowed her
Figure 8. ABA dose-response of selected ARA mutants of the ABA-hypersensitive class. Mutants 1314-64, 910-22, 1314-130, and 78-15 are compared to WT Chinese Spring as dormant embryos incubated for 96h at 30°C on increasing concentrations of (+)-ABA in 5 mM MES, pH 5.5. Error bars represent SE for 3 samples of 10 grains. (Strader, 2004)
Figure 9. ABA dose-response of *ABA Responsive when Afterripened (ARA)* mutants showing ABA hypersensitivity. Percent germination of mutants is compared to WT Chinese Spring as dormant embryos incubated for 96h at 30°C on increasing concentrations of (+)-ABA in 5 mM MES, pH 5.5. Mutants showed similar percent germination to wild-type at 0 μM or 1 μM ABA and decreased germination at concentrations as low as 1 μM ABA. Error bars represent SE for 3 replicates of 10 grains. (J. Abellera data)
“dormant” seed to afterripen for approximately 2 months, whereas seed used in this experiment was allowed to afterripen for 2 weeks to overcome embryo dormancy.

Some mutants that showed ABA-hypersensitive seed germination in L. Strader’s experiment, showed wild-type ABA dose-response in the second experiment (Figure 10). Genotypes 910-13, 910-22, 1314-46, 1314-82A, and 1314-76 previously were categorized as ABA hypersensitive mutants, whereas 78-68 was initially categorized as having increased embryo dormancy. It may be that the degree of embryo dormancy in 78-68 is variable or that these mutations have been lost in segregation. Two of the mutants, 1314-16 and 1314-45, showed a consistent increased seed dormancy phenotype (compare Figure 8 and Figure 11). Seed germination is permitted to ~50% without ABA and further inhibited as concentration is increased. This also is true in the case of mutant 1314-16 and confirmed the increase in embryo dormancy noted in the first experiment.

**Genetic analysis ABA-hypersensitive mutants**

Each mutant was backcrossed to the wild-type parent to clean up the genetic background following mutagenesis and to determine whether the mutations were dominant or recessive (by J. Abellera). Multiple crosses were made to all 12 mutants but only 7 mutants have successfully produced BC$_1$F$_1$ progeny including 1314-16, 78-68, 1314-28, 78-15, 1314-130, 1314-82, and 1314-45. Backcrossing of the remaining 5 mutants will be repeated by E. Schramm. ABA sensitivity in seed germination was scored in all BC$_1$F$_1$ at 5 μM ABA following 96h incubation at 30°C using dormant embryos (Table 4, columns 3 and 4). Due to growth space restrictions single BC$_1$F$_1$ plant was advanced to generate F$_2$ progeny for segregation analysis. A complete summary of all BC$_1$F$_1$ tested for ABA sensitivity in seed
Figure 10. ABA dose-response of mutants that do not appear to have a reproducible ABA hypersensitive phenotype. Percent germination of mutants is compared to WT Chinese Spring as dormant embryos incubated for 96h at 30°C on increasing concentrations of (+)-ABA in 5 mM MES, pH 5.5. These genotypes have an ABA dose-response similar to wild-type this experiment, but showed an ABA-hypersensitive phenotype in L. Strader 2004. Error bars represent SE for 3 replicates of 10 grains. (J. Abellera data)
Figure 11. ABA dose-response of *ARA* mutants that show increased embryo dormancy. Percent germination of mutants is compared to WT Chinese Spring as dormant embryos incubated for 96h at 30°C on increasing concentrations of (+)-ABA in 5 mM MES, pH 5.5. Mutants showed a reduced percent germination compared to wild-type at all ABA concentration examined and in the absence of ABA. Error bars represent SE for 3 replicates of 10 grains. (J. Abellera data)
Table 4. Seed germination phenotype of ABA hypersensitive mutants backcrossed to wild-type Chinese Spring at BC$_1$F$_1$ on 5 μM ABA after 48h and at F2 on 96h incubation at 30°C in dark. n represents the number of seeds tested; a test for 1:3 (wild-type : mutant) segregation and b test for 3:1 (wild-type : mutant) segregation at $P>0.05$.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Genotype</th>
<th>BC1F1 seed germination</th>
<th>F2 segregation analysis</th>
<th>Genetics of Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutant</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>1314-16 x WT</td>
<td>ARA/ARA</td>
<td>26</td>
<td>0.00</td>
<td>873</td>
</tr>
<tr>
<td>1314-76 x WT</td>
<td>ARA/ara</td>
<td>24</td>
<td>58.33</td>
<td>1246</td>
</tr>
<tr>
<td>78-68 x WT</td>
<td>ARA/ara</td>
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<td>60.00</td>
<td>1689</td>
</tr>
<tr>
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<td>23</td>
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<td>1314-130 x WT</td>
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<tr>
<td>1314-82A x WT</td>
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</tbody>
</table>
germination is presented in Appendix 4. However, for the purpose of discussion a single example for each mutant is shown in Table 4. If the mutation were recessive, we would expect all of the F₁ seeds to germinate. The fact that the mutant BC₁F₁ seeds showed between 0% and 60% suggests that all are either the result of a single dominant (0%) or semi-dominant (40-60%) mutant gene. Note that a gene is dominant when the heterozygote (BC₁F₁) is more like one parent (ARA mutant) than the other (wild-type Chinese Spring). For example, the BC₁F₁ heterozygotes from the ABA hypersensitive line 1314-16 showed complete inhibition of seed germination when grown in 5 μM ABA for 96h at 30°C whereas wild-type germination was 72% and 83% after 48h and 96h respectively. The nomenclature for different species refers to a genotype as semi-dominant, partially dominant, or additive if the heterozygote shows a phenotype intermediate between the two parents (Poehlman and Sleper, 1995). An added complication in this study resulted from the fact that some of the mutant parents were heterozygous for the ARA genotype. Thus, BC₁F₂ segregation analysis was used to examine whether mutants were dominant or semi-dominant. In the BC₁F₁, three mutants namely 78-68, 1314-28 and 78-15 exhibited above 40% germination suggesting that the mutant parents were either homozygous for a semi-dominant trait or heterozygous for a dominant trait. All ungerminated and germinated BC₁F₁ progeny from one or two heads were transferred and grown on new petri plates wetted with an aqueous solution containing 10 μM of GA₃ for 96h at 30°C. Germinated BC₁F₁ and wild-type seeds were then sown in the greenhouse and allowed to self-fertilize to generate F₂ progeny.

BC₁F₂ segregation analysis was used to further evaluate whether mutations are recessive, dominant, or semi-dominant. The total number of seeds tested for F₂ segregation ranged from 400 to 1700 harvested and tested independently from each BC₁F₁ plant. Table 4
(columns 5 to 9) summarizes the segregation analysis of F₂ progeny from a backcross of ARA mutants to wild-type Chinese Spring. When the heterozygous BC₁F₁ plants self-pollinate, the F₂ offspring are expected to have a genotypic ratio of 1 homozygous dominant: 2 heterozygous dominant: and 1 homozygous recessive. However, the seed germination phenotype cannot distinguish between the homozygous and heterozygous dominant seeds. Hence a phenotypic ratio of 1 seed of wild type phenotype to 3 seeds of mutant phenotype is expected for dominant mutations, and a ratio of 3 wild-type to 1 mutant is expected for recessive mutations. Two cases were consistent with a dominant trait; the F₂ progeny showed a 1:3 segregation for ABA sensitivity (5 μM) in seed germination phenotype (Table 4). Based on the χ² value (P<0.05), mutants 1314-16 and 1314-76 fit the 1:3 segregation ratio for increased sensitivity to ABA in seed germination conferred by a dominant mutation in a single gene. Based on the χ² value a single mutant, 1314-130, fit the 3:1 segregation ratio expected for a single recessive gene in one of three experiments. However, the BC₁F₁ seeds derived from this mutant showed a high degree of dormancy, raising the possibility that this is a semi-dominant trait.

The F₂ segregation analysis of the remaining four mutants, 78-68, 1314-28, 78-15, and 1314-82, did not give a segregation ratio consistent with either a single dominant or single recessive gene (Table 4). The germination segregation data could be explained either by a single semi-dominant/additive gene or by segregation of two or more genes. Given that these mutants were recovered in a genetic screen of fast-neutron mutagenized material, the multiple-gene hypothesis seems less likely. However, these plants are being advanced to the F₃ generation to distinguish between these two possible explanations. The criteria for considering these hypotheses are described in the materials and methods section.
Increased sensitivity to ABA in seed germination correlates with drought tolerance

One of the objectives of this thesis is to determine whether ABA hypersensitive mutations in wheat result in increased drought tolerance. Previous studies in Arabidopsis have isolated mutants with ABA hypersensitive seed germination that also show ABA hypersensitivity in stomatal closure including \textit{abh1}, \textit{sad1} and \textit{era1} (Xiong et al., 2001a). This vegetative ABA hypersensitive phenotype altered plant water relations and improved drought tolerance. Of the 25 wheat ARA mutants, 14 independent mutants exhibited increased inhibition of seed germination in both afterripened seeds and dormant embryos relative to wild type in the original dose-response experiment. These mutants were further examined to determine if these ABA-hypersensitive wheat mutants show altered vegetative drought response by closing their stomates earlier in response to water stress. Four methods were used to evaluate the drought tolerance: (1) estimation of transpiration rate by soil moisture loss, (2) measurement of stomatal conductance using a leaf porometer, (3) measurement of carbon isotope discrimination as a general indicator of transpiration efficiency, and (4) preliminary field tests to evaluate yield and harvest index under different watering regimes.

\textbf{Transpiration experiment.} To determine if ABA-hypersensitive mutants have improved drought tolerance compared to wild type by closing their stomates earlier during the onset of drought stress, eight mutants were further tested to determine the rate of soil moisture loss through plant transpiration during a 14-day period without watering (J. Abellera collaborated with E. Schramm). Two separate experiments were conducted...
examining 4 mutants, 1 positive control cultivar (drought tolerant cv. Alpowa) and the wild-type in each experiment. The experiments were conducted in collaboration with E. Schramm. The first experiment was composed of mutants 1314-45, 1314-130, 1314-16, 1314-64, wild-type Chinese spring and Alpowa. The second experiment was composed of mutants 1314-28, 910-22, 1314-82, 1314-46, including wild-type and Alpowa. Loss of soil water was significantly slower than wild-type in three out of eight ABA hypersensitive mutants tested (Figure 12a, 13a, 14a). In the first experiment, mutants 1314-64 and 1314-130 showed significantly less soil moisture loss in 7 of 16 timepoints (Figure 12a). In the second experiment, mutant 1314-28 showed significantly less soil moisture loss than wild-type Chinese Spring in 12 out of 16 timepoints (Figure 12a).

All mutants placed in the ABA hypersensitive class based on both ABA dose-response germination assays (Table 3) including 1314-130, 1314-64 and 1314-28 displayed vegetative drought tolerance as young plants in terms of ability to retain water (Figure 12a) and maintain a green healthy vegetative appearance (Figure 12 b,c) during the water stress treatment. Recent preliminary transpiration data suggest that another ABA hypersensitive mutant, 78-15, also shows a reduced rate of water loss compared to wild-type (E. Schramm, unpublished). During the entire course of the experiment, these mutants showed higher soil water content than wild-type, and appeared to stay greener and more turgid than wild-type plants after 14 days without water.

Mutants classified as ABA hypersensitive based only on the first ABA dose-response experiment (L. Stader data, Table 3) were observed to have either no significant difference compared to wild-type (mutant 910-22) or a faster rate of soil water loss (mutants 1314-46 and 1314-82A) (Figure 13a). The rapid loss of soil water in these mutants corresponds to
Figure 12. ABA-hypersensitive *ARA* mutants appear to have a reduced rate of soil moisture loss. (a) Comparison of percent soil moisture loss of ABA hypersensitive *ARA* mutants compared to wild-type Chinese Spring over a 14 day time course. Soil moisture was determined by weight at 0h, 12h, 24h, and every 24h thereafter for 14 days. Error bars represent standard errors with *n*=11. On the following page, (b) appearance of plants of each genotype, and (c) three random samples of each genotype after 14 days of drought treatment with a well-watered control.
Figure 13. *ARA* mutants with an inconsistent ABA-hypersensitive phenotype did not cause a decrease in the rate of soil moisture loss compared to WT. (a) Comparison of percent soil moisture loss of *ARA* mutants compared to wild-type Chinese Spring over a 14 day time course. Soil moisture was determined by weight at 0h, 12h, 24h, and every 24h thereafter for 14 days. Error bars represent standard errors with $n=11$. On following page, (b) appearance of all plants of each genotype, and (c) three random samples of each genotype after 14 days of drought treatment with a well-watered control.
Figure 14. ARA mutants with increased embryo dormancy showed a similar rate of soil moisture loss to WT. (a) Comparison of percent soil moisture loss of ARA mutants compared to wild-type Chinese Spring over a 14 day time course. Soil moisture was determined by weight at 0h, 12h, 24h, and every 24h thereafter for 14 days. Error bars represent standard errors with \(n=11\). On following page, (b) appearance of all plants of each genotype, and (c) three random samples of each genotype after 14 days of drought treatment with a well-watered control.
their dry appearance following 14 days of water-stress (Figure 13b,c). Mutants with increased embryo dormancy, 1314-45 and 1314-16, displayed a rate of soil water loss similar to wild-type Chinese Spring (Figure 14a). Both mutants showed a wilting phenotype similar or more severe than the wild-type during 14 days of water stress (Figure 14bc).

The spring wheat cultivar Alpowa developed for use in semi-arid regions of Washington shows good drought tolerance in the field and was used as a positive control. In two independent experiments, Alpowa showed a similar pattern of soil moisture loss to wild-type Chinese spring (Figure 15a). However, some Alpowa plants showed greener and more turgid leaves suggesting that this cultivar may attain drought tolerance through a different mechanism. The variability observed in Alpowa may be due to genetic heterogeneity within the seed lot used in the experiment or may be due to heterogeneity in the penetrance of the phenotype within the cultivar. Caution must be exercised in interpreting this data because Alpowa and Chinese Spring are of two different genetic backgrounds, making direct comparison imprudent.

The data in the transpiration experiment also were evaluated by calculating the ratio of water loss to shoot dry weight (SDW) after 5 days of water-stress treatment (Figure 16). The ratio of water loss per SDW was calculated to explain any variation in soil water content owing to slight differences in plant size and number of leaves. In addition to slower water loss and a more turgid phenotype after drought treatment, the three ABA hypersensitive mutants showed less water loss per unit of shoot dry weight than wild-type (Figure 16). It should be noted that wild-type had inconsistent results between the two separate experiments. The lower rate of water loss/SDW in the second experiment can be explained by a difference in plant size; plants in the second experiment (including wild-type and mutants) were slightly
**Figure 16.** Evaluation of water loss following 5 days without watering. Loss of water (grams on day 1 minus grams on day 5) divided by the final shoot dry weight (SDW) from the first transpiration experiment at Zadok=15 (a) and second experiment at Zadok=16 (b). Error bars represent standard errors with $n=11$. 

![Graph](image-url)
older than the first. Nevertheless, results reveal that mutants 1314-64, 1314-130 (Figure 16a) and 1314-28 (Figure 16b) lost less water per unit of shoot dry weight than wild-type during the first 5 days of drought treatment experiment. These 3 mutants displayed ABA hypersensitivity in both ABA dose-response experiments. To determine whether the lower transpiration rate was associated with altered root mass, the plant roots were washed clean and weighed at the conclusion of the transpiration experiment. The root mass of Alpowa and of ABA-hypersensitive mutants was lower than that of wild-type Chinese Spring at the conclusion of the experiment (Figure 17). Since ABA inhibits lateral root formation and stimulates primary root elongation, this reduction in root mass might be the result of decreased lateral root formation (Sharp et al., 2004).

**Stomatal conductance by leaf porometer measurement.** Stomatal conductance will be used in this experiment as a method to measure the closing of stomates in ABA-hypersensitive mutants under drought stress. The original premise of this project was that some of the mutants isolated for ABA-hypersensitive seed germination also should show increased sensitivity to ABA in stomatal closure. Such increased ABA sensitivity in stomatal closure should result in more rapid closure of stomates in response to mild drought stress. This method will evaluate if increased apparent drought tolerance in the transpiration experiment (by means of slower soil water loss) correlates with more rapid decrease in stomatal conductance resulting from increased ABA sensitivity of stomates. Measurements were recorded together with E. Schramm during the two water loss experiments described in the previous section. To evaluate this parameter, leaf porometer measurements were performed daily on single leaves of plants subjected to drought treatment over 7 days of
Figure 17. Root biomass (g) of ARA mutants compared to wild-type. Plants used for the experiments in Figures 14, 15, and 16 were examined to determine for differences in root biomass. Roots were removed from the soil following growth in 6-inch square pots for 21 days with normal watering followed by 14 days of drought treatment and weighed after oven drying at 70°C for 48h. Above: Results from the first experiment at Zadok=15. Bottom: Results from the second experiment at Zadok=16. Error bars represent standard errors with n=11.
water-stress treatment. Under constant light conditions, a lower stomatal conductance may result from increase sensitivity to ABA under drought stress. Thus we expected ABA-hypersensitive mutants to show lower stomatal conductance under drought stress. ABA hypersensitive mutants having reduced water loss during the drought treatment period showed varying stomatal conductance compared to wild-type. In the ABA hypersensitive class (Figure 18), lower stomatal conductance was observed in the mutants 1314-28 and 1314-130, although only mutant 1314-130 is significantly lower than wild-type in most time points. Mutant 1314-64 had slightly higher stomatal conductance compared to wild-type, but error bars indicate no significant difference during the first 4 days of measurements. The data for the wild-type were from the two independent experiments conducted and it can be noticed that wild-type stomatal conductance started ~100 points lower than that in the first experiment. Nevertheless, wild-type conductance was slightly higher compared to mutant 1314-28, but error bars indicate that this was not a significant difference. The mutants that did not appear to have improved drought tolerance in Figure 13 appeared to have similar or higher stomatal conductance compared to wild-type (Figure 19). Thus, the drought-sensitive phenotype did correlate with higher stomatal conductance. Interestingly, these mutants showed wild-type ABA sensitivity in the second ABA dose-response experiment (Figure 10). The mutants classified with increased embryo dormancy and cv. Alpowa showed a striking similarity to wild-type Chinese Spring in stomatal conductance (Figure 20). Moreover, these two mutants (1314-16 and 1314-45) exhibited drought sensitivity similar to wild-type 14 days after drought treatment (Figure 14).

**Measurement of carbon isotope discrimination as an indicator of water-use efficiency.** Measurement of carbon isotope discrimination (Δ¹³C) was used as a method for
Figure 18. Leaf stomatal conductance of ABA hypersensitive ARA mutants was either less or similar to wild-type. Stomatal conductance was measured daily using a single leaf from 5 plants of each genotype before and during water-stress for 7 days. Error bars represent standard error with $n=5$. 
Figure 19. Leaf stomatal conductance of *ARA* mutants with inconsistent ABA hypersensitive dose-response was similar to wild-type. Stomatal conductance was measured daily using a single leaf from 5 plants of each genotype before and during water-stress for 7 days. Error bars represent standard error with *n*=5.
Figure 20. Leaf stomatal conductance of ARA mutants with increased embryo dormancy and Alpowa (bottom) were compared to wild-type. Stomatal conductance was measured daily using a single leaf from 5 plants of each genotype before and during water-stress for 7 days. Error bars represent standard error with n=5.
estimating the water use efficiency of *AR4* mutants compared to wild-type. The great irony of drought tolerance is that closing the stomates to reduce water loss can reduce yield because of the reduction in CO₂ availability for photosynthesis. Transpiration efficiency (TE) or instantaneous WUE is defined as the carbon fixed per water transpired. Ideally, we would improve the TE of wheat. The use of carbon isotope discrimination (Δ₁³C) as a method to evaluate drought tolerance, offers a general indicator to measure water-use efficiency of leaf gas exchange in wheat, a C₃ species. Lower Δ₁³C in young well-watered plants has been shown to correlate with improved water-use efficiency in wheat (Condon 2004). Generally speaking, a lower Δ₁³C measurement in such an experiment should indicate a more “conservative” plant that should have higher yield under drought conditions, but may have lower yield under well-watered conditions. It is expected that ABA-hypersensitive mutants should have a lower due to a tendency to close stomates more readily. Closed stomates allow less CO₂ to enter the stomatal cavity forcing the cells to use up ¹³CO₂ due to reduced availability of ¹²CO₂.

J. Abellera measured Δ₁³C using leaves of 3-4 leaf stage seedlings grown in the field under well-watered conditions. Mutants with significantly lower Δ₁³C relative to wild-type include 1314-82A, 1314-76, 910-22, 78-15B, and 1314-130 (Figure 21). The last two mutants showed increased sensitivity to ABA in seed germination and vegetative drought tolerance (no data yet for 78-15B) (Figure 9). If such mutant plants with lower Δ₁³C are grown under well-watered conditions, they should have lower biomass and this may possibly translate into lower yield (Condon et al, 2004). Conversely, plants with higher Δ₁³C relative to wild-type should tend to have higher biomass production and thus better yield. In this experiment only mutant 1314-16 has significantly higher Δ₁³C compared to wild-type,
Figure 21. Carbon isotope discrimination $\Delta^{13}C$ of young leaves from well-watered plants of mutants ABA hypersensitivity in seed germination (green), wild-type ABA sensitivity (blue), increased embryo dormancy (brown) and wild-type Chinese spring and positive control, Alpowa (red). Error bars represent standard errors with $n=8$. 
whereas the remaining mutants have either an insignificant increase or decrease in mean Δ\textsuperscript{13}C (n=8) compared to wild-type. Interestingly, cultivar Alpowa showed a markedly lower Δ\textsuperscript{13}C compared to the other genotypes, suggesting that some of the drought tolerance in this cultivar may result from a “conservative” strategy at an early growth stage.

**Using ABA hypersensitive mutants for germplasm improvement**

This study aimed to incorporate isolated ABA hypersensitive mutations into modern cultivars either by traditional breeding or by repeating the screen in the desired background. To determine whether ABA hypersensitive mutations can be isolated in a red or white cultivated variety, the ABA hypersensitive screen was repeated in spring wheat cultivars Zak and Scarlet.

*Enhanced Response to ABA (ERA) mutants in cv. Scarlet.* Mutants with *Enhanced Response to ABA (ERA)* were identified in cultivar Scarlet using germination-based screen similar to the protocol used to isolate Chinese Spring mutants. *Enhanced Response to ABA* refers to the previously isolated mutants in *Arabidopsis* showing enhanced sensitivity to ABA hence the name *ERA*. From ~12,000 seeds screened 97 were identified as putative *ERA* mutants of which 27 passed the retest performed on M\textsubscript{3} seeds for failure to germinate on 5 µM ABA after 96 hours. The screen also resulted in interesting secondary phenotypes that include ScERA-10a named ‘crawling mutant’ which is characterized by its loss of erect phenotype but has the ability to produce normal heads and seeds (Appendix 5). At BC\textsubscript{1}F\textsubscript{1} segregation analysis, this phenotype co-segregates with enhanced ABA response (data not shown). Another interesting mutant is ScERA-38c, dubbed ‘Spotty’, appeared to have physiological leaf spots on all 8 plants of this genotype observed in the greenhouse
Unfortunately, no seeds were recovered from the backcrosses. Future work will characterize ABA dose-response in the \( M_4 \) generation. All 27 of \( ERA \) mutants were backcrossed to wild-type but only 19 successfully produced \( F_2 \) progeny intended for segregation analysis. This thesis will not cover the results of the analysis.

Thirteen out of 27 Scarlet \( ERA \) mutants and 2 wild-type plants were examined for stomatal conductance using a leaf porometer. Flag leaf measurements revealed 3 \( ERA \) mutants with significantly lower stomatal conductance namely, ScERA-41B, ScERA-41C and ScERA-45 (Figure 22). Future work will determine if this is indicative of a vegetative ABA-hypersensitive phenotype.

**Enhanced Response to ABA in white-grained Zak.** In wheat, evidence from past studies suggest testa color has a profound effect on seed dormancy and seed germination encouraged us to screen for ABA hypersensitive mutants in white-grained cultivar Zak. We screened \( \sim15,000 \) EMS-mutagenized \( M_2 \) seeds and identified 35 putative mutants. Only 4 independent mutants passed the retest for failure to germinate on 5 \( \mu \text{M} \) ABA after 96 hours using \( M_3 \) seeds. In ratio, there will be 1 \( ERA \) mutant for every 3,750 seeds screened (1:3,750) in white-grained cultivar specifically cv Zak. This number is lower than the two red-grained cultivars; Scarlet had 1 \( ERA \) mutant per 545 mutagenized seeds screened (1:545) and Chinese Spring 1 \( ARA \) mutant in every 890 screened (1:890). It appears that testa color does affect seed dormancy and/or ABA hypersensitivity in seed germination.

All four Zak ERA mutants were further examined for their ABA dose-response in germination. Mutants ZakERA-33A showed wild-type seed germination, and ZakERA26A showed increased sensitivity to ABA as whole afterripened grain not as dormant embryos (data not shown). ZakERA-0 and ZakERA-19A showed reduced seed germination in the
Figure 22. Flag leaf stomatal conductance of Scarlet ERA mutants recorded during head emergence stage. Bars represent average of single-plant repeated 5 times of ERA mutants (blue) and two Scarlet wild-type plants in (red). Error bars represent standard error (n=5).
presence and absence of ABA both as dormant embryos (Figure 23) and whole afterripened seeds. In whole afterripened (6 months) seeds, this mutant had strong inhibition of seed germination suggesting increased seed dormancy. These mutants do eventually afterripen within about 10-12 months. If this apparent ABA sensitivity is exclusive to whole afterripened seeds, then increased embryo dormancy may explain the lack of germination.
Figure 23. ABA dose-response of wild-type Zak (above) and ERA mutant ZakERA-0 (below) in dormant embryo mutants. ZakERA-0 showed enhanced ABA sensitivity in whole afterripened seeds (not shown) compared to wild-type sensitivity of Zak.
CHAPTER FOUR
DISCUSSION

Isolation of ABA hypersensitive mutations in allohexaploid wheat

The objectives of this research were to 1) identify and characterize wheat ARA mutants with increased sensitivity to the plant hormone abscisic acid (ABA) during germination of afterripened seeds, 2) determine if ARA mutants cause increased seed dormancy with a long term view to obtaining resistance to preharvest sprouting, and 3) determine whether ARA mutant show increased drought tolerance. Bread wheat has a large allohexaploid genome with high genetic redundancy (Galili et al., 2000). Due to this redundancy, it was expected that most of the induced mutations isolated in wheat would be dominant or semi-dominant. Thus, it was questionable whether ABA-hypersensitive mutants could be isolated at all in this organism given that all of the ABA-hypersensitive mutants isolated by mutagenesis of Arabidopsis thus far have been recessive (reviewed by Finkelstein and Rock, 2002). In spite of the redundancy of the allohexaploid wheat genome, this project successfully isolated mutants displaying the ABA-responsive when afterripened (ARA) phenotype.

The screen not only identified mutations showing altered ABA sensitivity, but also altered grain dormancy and afterripening. Because ABA sensitivity in seed germination is dependent on the dormancy status of the grain in wheat, the screen was designed to recover mutations that retained sensitivity to 5 μM ABA after 6 months of afterripening. The screen for ARA seed germination was performed in three different spring wheat backgrounds including Chinese Spring (red spring), and the two widely-cultivated varieties cv. Scarlet...
(hard red spring) and cv. Zak (soft white spring). Of the 25 ‘Chinese Spring’ ARA mutants originally isolated (Strader, 2004), 11 showed a reproducible ARA phenotype (Table 2), four showed a reproducible hypersensitive ABA dose-response in seed germination (Figure 9), and three have shown an increased embryo dormancy phenotype (Figure 8 and 11). Of the four ERA mutants recovered in cv. Zak, two appear to result from enhanced response to ABA in germination (Figure 23). A total of 27 ERA mutants have been recovered in cv ‘Scarlet’, and have not yet been characterized for ABA dose-response in seed germination. Mutants with altered seed dormancy characteristics are rare due to difficulties in screening directly for this trait. Thus, ARA mutants with altered dormancy and afterripening represent a unique resource for research on seed dormancy.

Genetic analysis of 7 of the Chinese Spring ARA mutants revealed that two result from a single dominant mutation and the remaining may be due to a single semi-dominant mutation (Table 4). Future work will need to evaluate the apparent semi-dominant ARA mutations in the F3 to confirm this interpretation. A single ARA mutant, 1314-82 appeared to be recessive by F2 segregation analysis, but failed to germinate on 5 μM ABA as a heterozygous F1 plant. If the F1 seeds were exhibiting embryo dormancy, it is possible that this is actually a recessive mutation. However, this experiment will have to be repeated to confirm this interpretation. Segregation analysis is also needed in the cv. Zak and cv. Scarlet backgrounds. F2 seeds of Scarlet and Zak backcrosses have yet to be scored for segregation. It would be interesting to compare the genetic segregation in other spring wheat backgrounds with the current results in Chinese Spring. In the future, crosses for complementation tests will determine and characterize the number of genes responsible for the phenotypes under study.
One observation made during the retests of the *ARA* mutants is that many mutants show a variable phenotype (*Table 2 and 3*). This causes problems for genetic characterization and raises some interesting questions about mutation studies in wheat. It is possible that some of variation in the *ARA* phenotype is due to variation in growing conditions. Differences in watering regime during seed maturation may influence the acquisition of primary dormancy. It is also possible that some mutations may have been lost due to a mistake in selection. It is difficult to distinguish if the lack of germination of dormant embryos on low concentrations of ABA is the result of increased ABA sensitivity or is the result of increased embryo dormancy. Alternatively, the fact that wheat has a complex and redundant genome invites the speculation that phenotypes may show differences due to epigenetic effects and changes in the expression of homeologous genes. Wheat, as an allohexaploid must have attained exquisite dosage control in order to survive the “genomic stress” of a large polyploidy genome (Madlung and Comai, 2004). One possibility that must be considered in the characterization of induced wheat mutations is that wheat plants might be able to adapt to loss of gene function by up-regulating wild-type homeologous genes or silencing mutant copies.

**Mutation breeding as a tool for translational genomics**

The extensive research done in model plant systems provides a plethora of information about the genetic mechanisms controlling plant traits of agricultural importance (reviewed in Finkelstein, R.R. and C.D. Rock, 2002). It is important to find methods to apply this abundant knowledge to agricultural crops such as wheat. With advent of advanced molecular genetic techniques, genetic transformation is a clear choice for translational
genomics. Crop plants that have been genetically modified, however, have problems gaining acceptance for social and economic reasons. This project provides evidence that mutation breeding can provide an alternative tool for applying information from basic research to crop plants. Using this “old-fashioned” technology, modified wheat germplasm can be incorporated into current wheat breeding program without crossing the “GMO” line.

Use of ARA mutants for increasing seed dormancy and resistance to preharvest sprouting

One of the long term goals of this research is to use *ARA* mutants with increased seed dormancy or increased ABA sensitivity in seed germination to control the risk of preharvest sprouting in wheat. In wheat, increased ABA sensitivity in seed germination is correlated with increased resistance to preharvest sprouting in white wheat (Walker-Simmons, 1987). The germination-based screen described here was used to identify *ARA* mutants in three different genetic backgrounds. Interestingly, this screen recovered not only *ARA* mutants with a hypersensitive ABA dose-response in seed germination, but also *ARA* mutants with altered seed dormancy and afterripening.

Most of the Chinese Spring mutants that appeared to retest for the *ARA* phenotype as whole afterripened grain failed to show a consistent ABA-hypersensitive dose-response in germination assays performed on cut grain. It is likely that most of these are actually “wild-type” lines demonstrating a variable degree of dormancy. However, another possibility is that these *ARA* mutants are exhibiting *prolonged seed coat-imposed dormancy*. However, the variability in this phenotype discourages further study. These mutants require at least 6 months of afterripening before a meaningful seed germination assay is realized. However, this does point out the importance of determining the length of time required to afterripen all
mutants compared to wild-type. A time-course seed germination experiment will determine specific time requirements of these mutants to afterripen using newly-harvested seed batch to examine whole and cut afterripening and dormant seedlots.

Three of the ARA mutants isolated in Chinese Spring exhibit increased embryo dormancy. Based on ABA dose-response these mutants have reduced germination relative to wild-type both in the presence and in the absence of ABA in whole afterripened seeds. The increased embryo dormancy phenotype of two ARA mutants, 1314-45 and 1314-16, proved highly reproducible. (Table 2, Figure 8 and 11). All four cv. Zak ERA mutants showed increased dormancy or retarded seed germination without showing any change in ABA sensitivity. Embryo dormancy may be a component of this increased dormancy.

Four ARA mutants in Chinese Spring showed a reproducible ABA-hypersensitive phenotype as cut grains (Figures 7 and 9). An initial concern was that such true ABA-hypersensitive mutants might not be detectable in lines that also showed increased embryo dormancy. However, this classification has been corroborated by the fact that only these four ARA lines, not the ARA lines showing increased embryo dormancy show increased drought tolerance (Figure 12). True ABA hypersensitivity appears only in ARA lines with seed-coat imposed dormancy not embryo dormancy. This suggests that ABA stimulates seed coat dormancy, but not embryo dormancy.

An interesting question raised by this research, is whether the ABA-hypersensitive phenotype may partly depend on seed coat color in wheat. Wheat varieties with white seed coat color are more prone to preharvest sprouting due to reduced seed dormancy (Groos et al., 2002). The association between white-grained wheat cultivars and lack of seed dormancy raises the doubt of isolating mutants in any white genetic background. We did succeed in
isolating $ARA$ mutants in a white-grained cultivar Zak. However, we were unable to recover true ABA hypersensitive $ARA$ mutants in this background. Future work will determine if the ABA hypersensitive $ARA$ mutant phenotype is expressed after crosses into the white grain Chinese Spring background developed by induced mutation (Warner et al., 2000). Nevertheless, it is possible that any mutant with increased seed dormancy will help prevent preharvest sprouting in white-grained wheat cultivars. A study comparing near isogenic lines (NILs) with red and white test color suggested that the $R$ gene increased seed dormancy in wheat by enhancing ABA sensitivity (Himi et al., 2002). In agreement to this finding, is that fact that 27 and 25 $ARA$ mutants were recovered in the red-grained Scarlet and Chinese Spring backgrounds, whereas four were recovered in white-grained Zak. All of the four cv. Zak $ARA$ mutants were the result of increased embryo dormancy rather than hypersensitive ABA dose-response. Future work will have to examine the relative efficacy of ABA-hypersensitivity and increased embryo dormancy in enhancing PHS tolerance of wheat varieties.

ABA responsiveness in seed germination may be altered by the degree of embryo and seed coat-imposed dormancy. This raises the concern that many of the isolated mutants have altered seed dormancy or altered germination kinetics, but are not true ABA hypersensitive response mutants. The presence of different forms of dormancy and mutations with variable expressivity in this screen should be considered when one decides to incorporate increased seed dormancy to combat preharvest sprouting by wheat breeding. Naturally occurring genes for sprouting tolerance may also show a high degree of variability causing difficulties during segregation analysis and selection. Failure of seeds to germinate in plating experiments may be highly dependent on environmental conditions during embryo maturation.
It is possible that the *ARA* mutants isolated result in apparent ABA hypersensitivity through altered ABA signaling or through altered accumulation of endogenous ABA. For example, a defect in ABA catabolism could lead to increased endogenous ABA levels and higher apparent sensitivity to applied ABA. Defects in both ABA synthesis and reduced sensitivity of embryos to ABA are associated with low dormancy of whole seeds in wheat (Hugouvieux et al., 2001; Morris et al., 1989) and yellow cedar (Schmitz et al., 2002). In the future, it will be important to determine the embryonic ABA levels of mutants using either the immunoassay developed by Walker-Simmons (1987) or by mass spectrometry (Chiwocha et al., 2003). It is expected that true ABA signaling mutants will exhibit increased ABA sensitivity regardless of embryonic tissue ABA levels.

In the future both *ARA* and the two dormancy class mutants will be evaluated using assays that can be used to evaluate preharvest sprouting resistance in wheat including α-amylase assays and assays of preharvest sprouting using the spike-wetting method (Paterson and Sorrells, 1990b). Whereas no direct field experiments testing preharvest sprouting have yet been performed, it is clear that the approach used in this screen has resulted in increased seed dormancy in plating experiments. In fact the design of the screen recovered not only mutants with ABA hypersensitive germination but also mutants with increased seed dormancy. It is expected that the true ABA hypersensitive mutants will exhibit preharvest sprouting tolerance. The apparent ABA hypersensitivity of whole afterripened seeds observed in mutants should provide resistance to preharvest sprouting. Further investigation will determine if mutants showing prolonged seed coat-imposed dormancy or increased embryo dormancy will show preharvest sprouting resistance.
The effect of ABA hypersensitive mutants on wheat drought tolerance

Another long-term objective of this project is to use ABA hypersensitivity to improve the drought tolerance of cultivated wheat. An ABA-hypersensitive phenotype in vegetative tissues should result in drought tolerance by causing stomates to close earlier under water deficit in response to lower levels of ABA accumulation in the guard cells. During drought stress, more frequent or earlier closure of stomates limits water loss by transpiration, thereby postponing desiccation. Whereas closing stomates reduces water loss, it also reduces uptake of carbon dioxide. Reduction in CO$_2$ uptake will result to lower photosynthetic rate, and eventually lead to reduced yield. The ideal is to improve transpiration efficiency (TE), that is fix more CO$_2$ per unit of water lost. In a water-limited environment, an improvement in TE should improve yield as long as the efficiency of conversion of biomass into grain or harvest index (HI) does not decrease. Differences in TE of wild-type and ARA mutant lines have been estimated by carbon isotope discrimination ($\Delta^{13}$C, Figure 21).

Based on mutant studies in other plant species, a subset of mutants with increased ABA sensitivity in seed germination should show increased vegetative ABA sensitivity and drought response. Water loss through transpiration was measured in young wheat plants during a time course of 14 day without watering. It was expected that only ARA mutants with increased ABA sensitivity (as opposed to increased seed dormancy) could show a decrease in transpiration water loss due to increased sensitivity to ABA in stomatal closure. As expected, only the four ARA mutants that reproducibly showed an ABA-hypersensitive dose-response in seed germination, 1314-28 1314-64, 1313-130 (Figure 12), and 78-15 (E. Schramm, unpublished) showed an apparent reduction in the rate of soil moisture loss
relative to Chinese Spring. Similar results have been described by Pei et al. (1989) and Hugovieux et al. (2001) in *Arabidopsis* and by Wang et al. (2005) in transgenic canola.

The expectation that the apparent change in the rate of soil moisture loss was due to reduced stomatal conductance was examined using leaf porometer measurements of stomatal conductance over the first 7 days of the same water restriction time course. Only the *ARA* mutant 1314-130 showed a significant decrease in stomatal conductance compared to wild-type Chinese Spring (Figure 18). *ARA* mutant 1314-28 showed a slightly lower stomatal conductance, and 1314-64 showed a slightly higher stomatal conductance compared to wild-type (Figure 18). In water deficit conditions, these mutants may have the ability to postpone desiccation by maintaining high water potential thereby preventing plant damage. *ARA* mutants that showed inconsistent ABA-hypersensitive response in seed germination showed greater water loss through transpiration, and in some cases higher stomatal conductance relative to wild-type (Figure 13 and Figure 19). Mutants that showed increased embryo dormancy showed a markedly similar rate of soil moisture loss and similar stomatal conductance to wild-type (Figure 14 and Figure 20). This suggests that the increased embryo seed dormancy may be completely unrelated to ABA signaling per se. If so, these are a unique class of mutants affecting an ABA-independent component of dormancy.

Differences in transpiration efficiency of mutants relative to wild-type were estimated using carbon isotope discrimination ($\Delta^{13}\text{C}$) analysis of well-watered young plants. Low $\Delta^{13}\text{C}$ corresponds with high TE in young well-watered wheat (Farquhar and Richards, 1994). Five out of 11 mutants examined so far resulted in lower $\Delta^{13}\text{C}$ relative to wild-type (Figure 21). Taking into account the environment where these plants were grown, we anticipated that *ARA* mutants should show lower $\Delta^{13}\text{C}$ compared to wild-type if they resulted in a vegetative
ABA-hypersensitive phenotype. Of the ABA-hypersensitive mutants examined, 1314-130 showed a lower $\Delta^{13}C$ whereas 1314-64 and 1314-28 showed similar $\Delta^{13}C$ to wild-type. These results correlate well with the stomatal conductance of these mutants. Of the three, only 1314-130 shows a significantly lower stomatal conductance relative to wild-type. Of the ARA mutants with inconsistent ABA-hypersensitive dose-response, three showed reduced $\Delta^{13}C$, 910-22, 1314-76, and 1314-82. One possible explanation for this inconsistency is the fact that plants that were used to measure stomatal conductance were not the same generation used to evaluate $\Delta^{13}C$ ($M_4$). Moreover, stomatal conductance was measured in greenhouse-grown plants using a more homozygous set of plants ($M_5$, Table 1). All three of the ARA lines that have shown increased embryo dormancy show similar $\Delta^{13}C$ to wild-type (1314-16, 1314-45, and 78-68, Figure 21).

Additional $\Delta^{13}C$ measurements were taken for cv. Alpowa and mutations in the cv. Zak background. The $\Delta^{13}C$ drought tolerant cultivar Alpowa was quite low compared to Chinese Spring at the 3 leaf stage (Figure 21). This suggests that young cv. Alpowa plants are conservative and should survive under water-limited growing environment. In contrast, the stomatal conductance of 5-6 leaf stage cv. Alpowa is quite high. This is consistent with a previous observation that cv. Alpowa is slow-growing when young, and later grows quite vigorously (K. Kidwell, personal communication). Low $\Delta^{13}C$ and high TE may explain the better yield of Alpowa in dry environments. The ZakERA-19A mutant showed a much lower $\Delta^{13}C$ than wild-type or the remaining three ZakERA mutants examined (Figure 24). Scarlet mutants have not yet been examined for carbon isotope discrimination.
Figure 24. Carbon isotope discrimination $\Delta^{13}$C of young leaves from well-watered plants of Zak mutants with (blue), wild-type Zak (green), Chinese Spring and positive control cv Alpowa (red). Error bars represent standard errors with $n=8$. 
In this experiment, low $\Delta^{13}$C-mutants should show higher TE where water is not limiting for plant growth. My results are baseline data that can be used to predict the possible $\Delta^{13}$C in future experiments. Out from my preliminary $\Delta^{13}$C measurements we can predict mutants with low $\Delta^{13}$C should show high TE in well-watered environments. Conversely, high-$\Delta^{13}$C mutants should have low TE in the same environment. The implication of having low $\Delta^{13}$C in the absence of soil water deficit is the tendency toward a ‘conservative’ growth that may affect total biomass and subsequently yield. If low-$\Delta^{13}$C mutants are grown under drought stress, they may yield better than high-$\Delta^{13}$C mutants because of their higher TE. Fortunately, we have two ARA mutants that showed high $\Delta^{13}$C in the pioneering experiment. High-$\Delta^{13}$C mutants should have better yield in environments without water limitation because TE is not a factor in this case. These predictions are based on fairly preliminary data. Future experiments will measure $\Delta^{13}$C of mutants comparing plants grown in contrasting water regimes. Grain yield and harvest index are two primary parameters that are essential to correlate $\Delta^{13}$C to TE. A replicated controlled-irrigation field test is currently conducted for this objective. Plant tissue samples will include leaves from seedlings and flag leaves taken from pre- and post- anthesis stages, and whole mature grain (Monneveux et al., 2006).

**Future Directions**

Greenhouse and field experiences during the course of this project have underscored the limitations in measuring stomatal conductance by leaf porometer. Although we have shown the efficacy of leaf porometer in estimating stomatal conductance in our greenhouse drought experiments, the length of time required to replicate measurements on the same leaf of multiple plants posses a major problem as numbers vary over the course of the day. In the
field, stomatal conductance can vary due to changes in light intensity, temperature and cloud cover. This problem can be addressed by the use of multiple leaf porometer readings taken simultaneously to limit the time-imposed errors. However, this approach is limited by the cost of personnel and instruments. In the future, stomatal closure assay will be used to directly assess the sensitivity of stomates to ABA. This will complement the present methods being employed.

While field data is ideal for determining the effect of a genotype on behavior of the crop, it is with difficulty to restrain the effects of multiple stresses provided by nature. Previous field trial had witnessed the vulnerability of our experimental plots to animal feeding and disease pressure. Experience also tells us that the same can happen in the greenhouse. Estimating water loss through series of experiments revealed large variation between separate trials mainly due to seasonal differences and greenhouse temperature. Despite the feeding pressure to animals coupled with heterozygote plant materials being used, the test provided an idea how they behave in the field.

In the preliminary field experiment, released cultivars have higher yield in both dry and wet locations. Mutant 1314-76 is a probable candidate germplasm for improved yield under drought (Figure 25). Interestingly, mutant 1314-76 had one of the lowest $\Delta^{13}$C. Future work will have to examine this $ARA$ mutant using the transpiration experiment. Oddly, cv. Alpowa showed low yield under drought stress presumably due to stripe rust pressure or animal feeding problem during that field test.

In conclusion, the results of the present study are a first step in validating the hypothesis that improved drought tolerance can be achieved in hexaploid wheat by increasing
Figure 25. Grain yield (g) of Chinese Spring ABA hypersensitive mutants in wet (blue bars) and dry (yellow bars) locations, August 2005, Spillman Farms, Pullman, WA. Scarlet, Eden, Calorwa and Alpowa are spring wheat cultivars used as yield check. Error bars represent standard errors \((n=3-4)\)
ABA sensitivity in seed germination. This study had also identified seed dormancy mutants that might augment resistance of wheat germplasm against preharvest sprouting.
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