Plant Science 179 (2010) 620–629

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Isolation of ABA-responsive mutants in allohexaploid bread wheat (*Triticum aestivum* L.): Drawing connections to grain dormancy, preharvest sprouting, and drought tolerance

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ARTICLE INFO

Article history:
Received 30 October 2009
Received in revised form 18 March 2010
Accepted 16 June 2010
Available online 30 June 2010

Keywords:
Abscisic acid
ABA
Wheat
Dormancy
Preharvest sprouting
Drought

ABSTRACT

This paper describes the isolation of Wheat ABA-responsive mutants (W*arm*) in the Chinese Spring background of allohexaploid *Triticum aestivum* L. Because the hormone abscisic acid (ABA) is required for the induction of seed dormancy, stomatal closure, and drought, cold and salt tolerance, ABA-hypersensitive wheat mutants were expected to show increased seed dormancy and decreased leaf transpiration in drying soils. Lack of wheat grain dormancy is associated with a propensity for preharvest sprouting (PHS), the germination of seed on the mother plant when moist conditions persist before harvest. PHS tolerance correlates with higher seed dormancy resulting from red grain color, higher ABA accumulation and sensitivity. Wheat grain loses dormancy and sensitivity to ABA inhibition of seed germination with after-ripening. W*arm* lines maintained higher ABA sensitivity when partially after-ripened. The W*arm1* and W*arm4* mutants showed the strongest and most reproducible increase in ABA sensitivity, accompanied by a requirement for more prolonged after-ripening to break dormancy. W*arm2*, W*arm3*, W*arm5*, and W*arm6* showed normal germination without ABA but increased sensitivity to inhibition of germination by applied ABA. The W*arm4* mutant showed decreased whole plant transpiration in drying soil, consistent with the role of ABA in inhibiting vegetative leaf transpiration.

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1. Introduction

Preharvest sprouting (PHS), the germination of mature seeds on the mother plant when moist cool conditions occur before harvest, is a problem in many cereals including wheat [1], sorghum [2], barley [3], and rice [4]. Wheat PHS can occur in most wheat-growing regions of the world resulting in economic hardship for farmers when grain is rated as feed and for millers since high alpha-amylase expression breaks down starch resulting in low quality baked goods [5–8]. The tendency to sprout is associated with lack of seed dormancy [3,9]. Seed is considered dormant when it fails to germinate under conditions that normally stimulate germination [reviewed by 10]. Seed dormancy is lost during a period of dry storage called after-ripening. Persistent seed dormancy can also be a negative trait when it prevents uniform germination resulting in poor plant stand establishment and reduced yield [11]. Seed dormancy can also be broken by cold stratification, in which seeds imbibe in the cold. Wheat also appears to lose sensitivity to germination inhibition by abscisic acid (ABA) when cold stratified, which may be another factor in PHS susceptibility [12]. Wheat seed dormancy and PHS resistance have been correlated with physiological traits of the inflorescence, red testa pigmentation, and sensitivity or accumulation of the plant hormones gibberellic acid (GA) or ABA. This paper will first review the known factors controlling wheat grain dormancy with a focus on ABA, and then will describe the isolation of novel Wheat ABA response mutants (W*arm*) based on the role of ABA in inhibiting grain germination.

Dormancy mechanisms can be divided into two types: seed coat-imposed dormancy and embryo dormancy [13]. In seed coat-imposed dormancy, parts of the seed, including the seed coat, surrounding the embryo contribute to dormancy. There are several mechanisms by which this can take place; germination can be prevented by an inability to take up water, by physical constraint, by gas exchange interference, or by presence of inhibitors, such as ABA or pigments [reviewed by 13]. Seeds with embryo dormancy, however, will not germinate even if the embryo is removed

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0168-9452/$ – see front matter. Published by Elsevier Ireland Ltd.
doi:10.1016/j.plantsci.2010.06.004

from the surrounding tissues. Wheat exhibits both of these types of dormancy, either in combination or separately [1,9,14].

Dormancy is not the sole factor determining PHS resistance. PHS is triggered by environmental conditions, namely moisture (in the form of rain or humidity) and cool temperatures after physiological maturity. Some morphological traits can protect the grain from moisture. Several inflorescence characteristics have been found to influence sprouting in barley and wheat. Barley lines with absence of epicuticular waxes on the spike have been associated with an enhanced tendency to take up water compared with lines with glaucous spikes, as well as less ability to shed water from the spike in shaking tests designed to mimic windy conditions [15]. In wheat, the presence of awns enhanced spike wetting and subsequent sprouting compared to awnless spikes [15,16]. Club head type in wheat also enhanced water uptake compared to common head type [16].

In wheat, resistance to PHS has historically been associated with the red seed coat color controlled by the *R* (red grain color) genes. Because wheat is an allohexaploid, there are three copies of the *R* genes (*R-A1*, *R-B1*, and *R-D1*), one on each of the homeologous group 3 chromosomes. The *R* genes are dominant maternally expressed genes that have an additive effect on red coloration resulting from the deposit of red phlobaphene isoflavones in the testa or seed coat [17,reviewed by 18]. Although red color has been used as a selection tool to breed for better PHS resistance, not all red wheat lines have strong dormancy and white wheat can show good dormancy and PHS tolerance [9,19]. QTL analysis has identified loci affecting PHS tolerance on all 21 homeologous groups in wheat. Many of these QTLs are background-specific and have not been confirmed in multiple genotypes. However, some loci have been identified in multiple studies. The long arm of chromosome 4A has been implicated in sensitivity of germination to inhibition by ABA [20], and also includes the *PHS1* locus, a major QTL conferring enhanced dormancy and sprouting resistance independent of red testa color in multiple backgrounds [21–23]. In addition, QTLs identified on chromosome 2B by Anderson et al. [24], Liu et al. [25], and Munkvold et al. [26] appear to be located in the same region of chromosome 2B. Several studies have identified QTLs on homeologous group 3, but the resolution of QTL analysis was insufficient to prove that the sprouting tolerance associated with *R* was not the result of a linked gene.

Studies examining red and white near-isogenic lines and induced mutations in the single *R*-gene of Chinese Spring revealed the loss of red color does not completely eliminate, but does reduce mature grain dormancy and sensitivity to ABA in seed germination [27,28]. During grain development, the red lines showed only a slight decrease in germination capacity, but a larger increase in ABA sensitivity [28]. Thus, the *R* genes are not the sole determining factor of wheat grain dormancy. The *R* genes encode Myb-type transcription factors involved in the transcriptional activation of genes resulting in flavonoid synthesis [29]. One hypothesis regarding the role of *R* genes is that precursors to the red color in the flavonoid biosynthetic pathway might affect dormancy and/or ABA sensitivity in a seed coat-dependent manner [30]. Clearly, more research is needed to clarify the relative roles of seed pigmentation and hormone signaling in the control of wheat PHS tolerance.

ABA is a sesquiterpenoid phytohormone that induces seed dormancy and embryo desiccation tolerance during embryo maturation, stomatal closure in response to drought stress, and other adaptations to environmental stress such as drought, salinity, cold, pathogens and UV radiation [31–33]. In addition to inducing seed dormancy during embryo maturation, ABA also acts antagonistically to the seed germination-inducing hormone GA and can block the germination of mature seeds [10]. It is known that increased ABA sensitivity results in increased seed dormancy and drought tolerance in Arabidopsis and canola [34–36]. The role of ABA in establishing seed dormancy has been characterized using ABA biosynthesis and signaling mutants in Arabidopsis, maize, tobacco, wheat and tomato [31]. Reduced ABA accumulation and ABA-insensitivity result in reduced seed dormancy in many species, and results in vivipary in maize, the germination of developing embryos on the mother plant [37–40]. The maize *VIVIPAROUS1* (*Vp1*) gene is a homologue of *Arabidopsis ABI3* and encodes a B3 domain transcription factor required for ABA response [41]. It is possible that the QTLs on chromosomes 3A and 3D identified by Munkvold et al. [26] are located near *Vp1* on the long arm of homeologous group 3 [42]. Missplicing of *TaVp1* in the wheat cultivar 'Soleil' has been associated with low dormancy and the tendency to sprout [43]. Transformation of wheat with *Vp1* from wild oat resulted in increased dormancy and PHS resistance [43]. However, examination of *TaVp1* splicing in different cultivars and genetic backgrounds in subsequent studies has revealed cultivar-specific differences in *Vp1* splicing, with some cultivars showing a higher abundance of properly spliced transcripts than Soliel [44,45]. However, McKibbin et al. [43] examined splicing of *Vp1* in imbibed mature seeds whereas Yang et al. [44] and Utsugi et al. [45] examined *Vp1* in dry seeds, so direct comparison may not be possible. Imbibition of embryos in water resulted in a decrease in *Vp1* transcript levels compared to dry seeds, while imbibition in ABA resulted in transcript levels that remained high [44,45]. These results suggest that *Vp1* may be involved in the maintenance of dormancy in wheat.

In barley, the degree of grain dormancy was found to positively correlate with the level of ABA catabolic gene *HvABA8'OH* expression suggesting that reduced ABA levels might correlate with decreased dormancy and an increased tendency towards PHS [46,47]. Moreover, dormancy-releasing treatments are associated with a decline in ABA content in cereals [reviewed by 48]. Imbibing dormant and after-ripened barley seeds both show rapid decreases in ABA, but the dormant embryos maintain a higher level of ABA after the initial drop [49]. Similar results have been reported in the dormant Arabidopsis ecotype Cvi [50]. In wheat, PHS tolerance has been shown to be associated not with higher ABA levels in dry seeds, but with higher ABA sensitivity during embryo maturation [51] and during seed germination in some cultivars [52]. No one has yet determined, however, whether there are differences in ABA levels of imbibing wheat grain.

Although no base pair change in a known ABA signaling gene has yet been associated with differences in seed dormancy or PHS tolerance in wheat, ABA signaling genes have been identified and differences in dormancy have been shown to correlate with differences in ABA signaling gene expression. The protein kinase ABA signaling gene *PKABA1* was first identified in wheat based on the fact that it was ABA-induced in developing wheat embryos [53]. *PKABA1* is located on the long arm of homeologous group 2 in wheat and is also induced by dehydration, cold, and salt stress [54,55]. The role of *PKABA1* in ABA signaling and suppression of GA signaling was subsequently elucidated using the barley aleurone system [56,57]. A wheat member of the ABA response-element binding factor (TaABF) was identified as a PKABA1 substrate that regulates ABA-responsive gene expression in barley aleurone [58,59]. There are three copies of *TaABF* located on the short arms of chromosomes 2A, 2B, and 2D that show differential expression patterns in developing and germinating grain, seedling, roots, and flag leaves [60]. The ABA signaling gene homologs *TmABF*, *TmVp1*, *TmABI8*, and *TmERA1* mapped to chromosome 3A whereas *TmERA3/EIN2* mapped to 5A of the diploid wheat *Triticum monococcum* [61]. *TmABF* and *TmABI8* are candidate genes for seed dormancy genes because their map positions correspond to those of seed dormancy QTLs. However, proof that wheat seed dormancy QTLs are the result of mutations in ABA signaling genes will require proof that specific

base pair changes result in changes in seed dormancy or PHS tolerance phenotypes. Such proof may come from studies of induced mutations in ABA signaling genes.

This study reports the recovery of ABA-hypersensitive mutants in Chinese Spring Dv418 wheat based on the unique relationship between wheat dormancy and wheat ABA sensitivity. Induced mutations represent an alternative approach to studying the role of ABA sensitivity in determining grain dormancy in wheat. This approach can provide new germplasm for breeding efforts as well as the potential to clone specific ABA signaling genes controlling wheat grain dormancy. A total of 25 mutants exhibiting increased ABA sensitivity once after-ripened were identified. Among these, five were chosen for in-depth characterization. Embryos of the W*arm1* and W*arm4* (Wheat ABA-responsive mutant) lines showed the strongest increase in embryo sensitivity to ABA inhibition of germination. This sensitivity was associated with a tendency to after-ripen more slowly than wild-type Chinese Spring grain. The W*arm1* line appeared to segregate as a dominant mutation and was associated with a small decrease in plant height. The W*arm4* mutation was associated with a vegetative decrease in transpiration suggesting that this mutation may also result in increased vegetative ABA sensitivity, a trait that may be useful in the study of wheat drought tolerance.

2. Materials and methods

2.1. Plant material

The *Triticum aestivum* L. Chinese Spring germplasm used in these experiments were descendents of the J. Dvorak doubled haploid line Dv418 and obtained from Warner [27]. The original Chinese Spring was a landrace and the doubled haploid selection from it is presumed to be more homozygous and homogenous. The soft white winter cultivar 'Brevor' was obtained from Walker-Simmons [51]. The hard red spring cultivar 'Scarlet' was obtained from Kidwell et al. [62].

2.2. Growth conditions

Plants were grown in the greenhouse except where otherwise stated with a photoperiod of 16 h at a light intensity of 400 μmol m^{-2} s^{-1} with a daytime temperature of 21–24 °C and a nighttime temperature of 15–18 °C. During winter months, supplemental light was provided with high pressure sodium lamps. Seeds or seedlings were planted into 3 l pots containing Sunshine LC 1 potting soil mixture (Sun Gro Horticulture). Pots were watered to saturation every two days. A nutrient solution (Peters Professional 20-10-20 Peat-Lite Special) was supplied once a week. Spikes were harvested at physiological maturity and seeds were hand threshed to avoid scarification of the seed coat. Seeds were allowed to dry at room temperature for 1 month, and thereafter stored at −20 °C to maintain dormancy unless otherwise indicated.

For evaluation of time to transition to flowering, plants were grown in 2 m^2 plots at the Washington State University Spillman farm near Pullman, WA in 2009 in four blocks each consisting of one plot per mutant and six plots of Chinese Spring wild type planted at a density of 20 g of seed per plot. For the after-ripening time course experiment (Fig. 3), M_8 mutants and Chinese Spring were grown in the field at Central Ferry, WA in 2008. Plants were irrigated early in the season as needed, and then allowed to grow on residual moisture. Spikes were harvested at physiological maturity, allowed to dry at room temperature for approximately 2 weeks, then hand threshed and stored at −20 °C until use. For the comparison of ABA sensitivity of cultivars Brevor, Scarlet, and Chinese Spring (Fig. 1), plants were grown in the field at Pullman, WA in 2000, Pullman, WA in 2005, and Central Ferry, WA in 2008, respectively. Grain was treated as described above for germination assays.

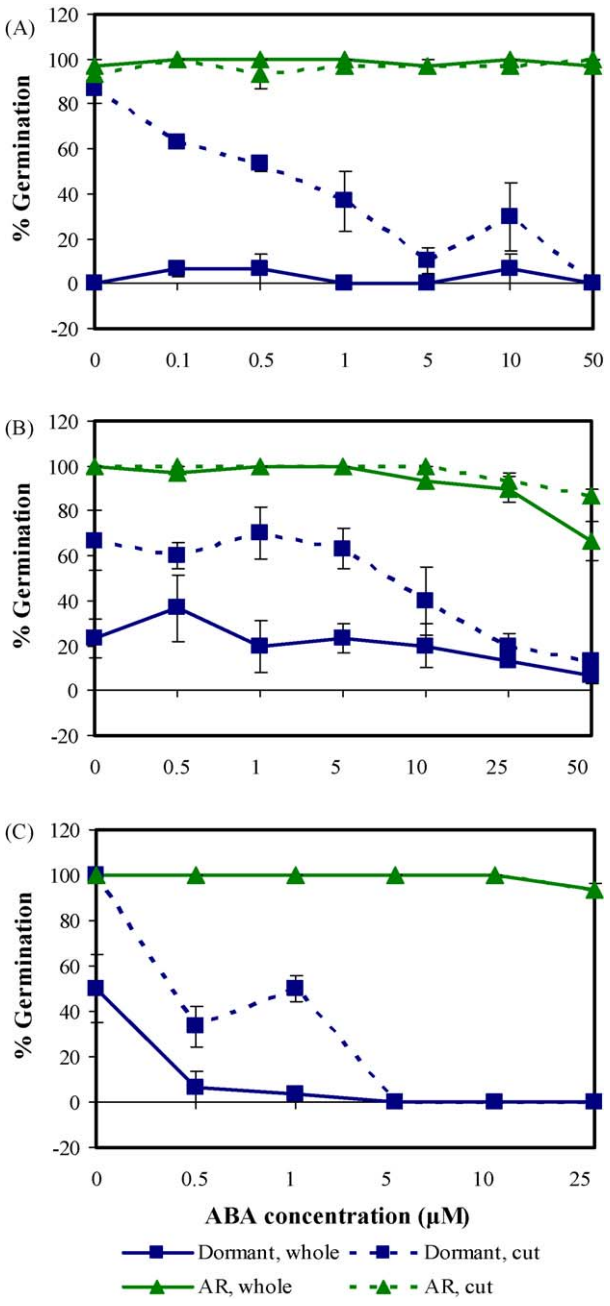

Fig. 1. ABA sensitivity of dormant and after-ripened (AR) intact (whole) and embryo half (cut) seeds of soft white winter Brevor (A), hard red spring Scarlet (B), and hard red spring Chinese Spring (C) after 72 h of imbibition. Error bars represent standard errors of three replications of 10 seeds each.

For plant height determination, M_7 plants were grown in the greenhouse under conditions described above. M_8 plants were grown in a growth room with a 16 h photoperiod (22 °C day, 15 °C night) and light intensity of 400 μmol m^{-2} s^{-1} with a 1:1 ratio of metal halide: sodium lamps. Heights were measured from the base of the plant to the top of the spike on the tallest tiller. Data were analyzed using an analysis of variance, and tests of differences between the mutants and wild type were conducted using the Dunnett's multiple comparison adjustment in SAS/STAT software version 9.2 (SAS Institute Inc., Cary, NC).

2.3. Germination assays

The term seed is used in reference to the wheat caryopsis or grain. Germination assays were performed using either whole or cut half-grains (also referred as embryos) plated on a 9-cm petri dish lined with a single germination disc (Anchor steel blue germination blotter (SDB3.375), Anchor Paper Co., St. Paul MN) moistened with an aqueous solution of 6 mL of 5 mM MES, pH 5.7 buffer (2-[*N*-morpholino] ethane sulfonic acid, Sigma, St. Louis, MO) containing varying concentrations of optically pure (+)-ABA. (+)-ABA was obtained as a gift from S. Abrams (NRC, PBI58) and maintained as a 0.1 M stock in DMSO or methanol at −20 °C in the dark. The plates were sealed with Parafilm to prevent evaporation, wrapped with foil, and incubated at 30 °C in the dark [51]. Homozygous M_6 seeds were used to determine dose–response to the inhibition of germination by (+)-ABA with MES buffer used as a negative control. Experiments used three samples of 10 seeds for each treatment. Germination was scored based on radicle emergence every 24 h for 5 days. Germination was expressed either as a percentage germinated or as a weighted germination index (GI) calculated over 5 days of scoring as $(5 \times g_{day1} + 4 \times g_{day2} + 1 \times g_{day5})/(5 \times n)$ where g is the number of germinated seeds and n is the total number of viable seeds [51]. Using this formula, the maximum possible GI is 1.0, and the speed of germination as well as number of germinated seeds are represented by a single value. If needed, the germination of ungerminated grains was stimulated by plating in new petri dish containing a single germination disc moistened with 6 mL of 10 μM of GA_3. Stocks of 10 mM GA_3 in ethanol were maintained at −20 °C in the dark.

Arabidopsis plants were grown and germination assays performed as in [63], except that seeds were not cold stratified prior to incubation under lights at 22 °C. The seeds used were after-ripened at room temperature for either 1 week or for 7 months to determine whether differences in after-ripening show a strong effect on ABA sensitivity. Seeds were scored as germinated following radicle emergence.

2.4. Mutagenesis and mutant isolation

M_1 Chinese Spring Dv418 grain was fast-neutron mutagenized at 4 grays (R. Warner, personal communication). Fast-neutron irradiation creates random deletions or small rearrangements in the genome [64]. The M_1 grain were advanced to M_2 in the field and only one spike from each self-pollinated plant was hand-harvested in order to maximize the number of independent mutations. M_2 spikes were obtained from *R.* Warner after they had after-ripened at room temperature for 2 years. Each spike was hand threshed into pools representing 4 grains each from 10 independent M_1 spikes.

A total of 22,250 M_2 seeds were screened by plating whole grains on germination discs moistened with 6 mL of 5 μM (+)-ABA buffered. Seeds were scored for germination every 24 h until 120 h had passed. Ungerminated seeds were rescued and transferred to new petri dish containing a single germination disc moistened with 6 mL of 10 μM of GA_3 to promote germination. Mutant lines were advanced by single plant descent. Retests for inhibition of germination were performed on M_3, M_4, and M_5 grain (ESM Table 1). The original mutant isolation numbers have been converted as follows: W*arm1* is 1314-16, W*arm2* is 1314-28, W*arm3* is 1314-130, W*arm4* is 1314-45, W*arm5* is 78-15, W*arm6* is 1314-64, W*arm7* is 1314-76, W*arm8* is 1314-82, W*arm9* is 910-13, and W*arm10* is 910-22.

2.5. Gravimetric estimation of whole plant transpiration and stomatal conductance in drying soils

A gravimetric measurement of soil moisture lost through whole plant transpiration and stomatal conductance measurements were taken over a 13-day time course in drying soils. Soil moisture loss was conducted essentially according to Pei et al. [65]. Eleven M_5 plants per genotype were grown individually in 4-in. pots containing a equal amount of moistened soil by weight in a growth room until the 4-leaf stage (Zadok stage 14) [66], then allowed to acclimate to greenhouse conditions for seven days prior to the start of the experiment. At the 5-leaf stage (Zadok 15) the soil was saturated with water so that the soil would be evenly moist when the experiment began the next day. At the commencement of the experiment, pots were covered with plastic wrap to prevent water evaporation from the soil surface and watering ceased. Pots were weighed individually at noon on the first day, then every 12 h for the first 48 h at 12 p.m. and 12 a.m., and then every 24 h at 12 p.m. thereafter for until 13 days had passed. At the conclusion of the experiment the aerial portion of the plant was dried in a 70 °C oven for 48 h and weighed. The plant dry weight was subtracted from daily pot weight measurements and soil moisture loss was determined by subtracting the daily pot weight from the initial pot weight. Data were analyzed using an analysis of variance, and tests of differences between the mutants and wild type were conducted using the Dunnett's multiple comparison adjustment in SAS version 9.2. Stomatal conductance was measured every 24 h for the first 7 days of the drought experiment in mmol m^{-2} s^{-1} using a steady-state leaf porometer (Model SC-1, Decagon Devices) [67] under sunlight supplemented with sodium lamps. Because stomatal conductance relies upon stomatal density as well as stomatal aperture, the same leaf was used for each daily measurement. Two to five repeated measurements were taken on each of five plants of each genotype daily using the adaxial surface of single fully expanded leaves positioned perpendicular to the light source.

3. Results

3.1. ABA sensitivity of wheat grain during germination is dependent on dormancy status

Previous research indicated that ABA sensitivity is positively correlated with grain dormancy and PHS tolerance in wheat and in other cereals [2,51,68]. Thus, we examined whether the degree of dormancy and ABA sensitivity were correlated in three wheat backgrounds: the PHS tolerant soft white winter Brevor, the hard red spring Scarlet, and the hard red spring Chinese Spring. Field-grown grain was dry after-ripened at room temperature until it showed efficient germination in the absence of ABA. Brevor seeds were after-ripened for 3 years, Scarlet for 9 months, and Chinese Spring for 11 months. Whole and cut wheat grains were plated on increasing concentrations of ABA and germination was measured based on radicle emergence. Whole dormant Brevor seeds did not germinate within 72 h even in the absence of ABA, whereas fully after-ripened Brevor seeds were highly ABA-insensitive showing complete germination even at 50 μM ABA (Fig. 1A). Once seeds are cut in half, the germination of dormant embryos was stimulated allowing the observation of dose-dependent inhibition of germination by ABA. Similar effects were seen in all three genotypes examined, with increased efficiency of germination in cut dormant grains compared to intact dormant grains. Both Brevor and Chinese Spring were able to germinate to 100% or nearly 100% by 72 h in the absence of ABA (Fig. 1A and C), while Scarlet only reached 67% (Fig. 1B). However, the extent of ABA sensitivity in germination of cut grains varied by genotype. Brevor and Scarlet required 50 μM ABA to effectively inhibit germination of cut dormant grains (Fig. 1A and B), while Chinese Spring only required 5 μM ABA (Fig. 1C). After-ripened seeds are highly ABA-insensitive showing fairly efficient germination through 25 μM ABA, whereas dormant

Table 1
Germination phenotype of W*arm* mutants 1–6 compared to Chinese Spring wild type (CS) on 5 μM ABA.

Genotype	After-ripened whole[a] M_3 (96 h)[b] % germ[c]	Dormant embryo M_5 (120 h) % germ
W*arm1*	0	15
W*arm2*	0	10
W*arm3*	20	20
W*arm4*	0	10
W*arm5*	37.5	40
W*arm6*	0	20
CS	95	80

[a] Experimental conditions. M_3 seeds were after-ripened for 9 months and tested whole; M_5 seeds were after-ripened for 2 weeks and tested cut.
[b] Generation of seeds tested and time point shown.
[c] Percent germination of 8–20 M_3 or 10–40 M_5 seeds.

seeds are more sensitive. The ABA response of wheat grain is quite different from that seen in Arabidopsis (ecotype Columbia) where the sigmoidal ABA dose–response in seed germination is identical in 1 week old seeds and in seeds after-ripened for 7 months (ESM Fig. 1).

3.2. Isolation of ABA-hypersensitive mutants in wheat

The observation that ABA sensitivity during seed germination is dependent on the dormancy status of the grain suggested that it should be possible to isolate mutants with increased ABA sensitivity and/or grain dormancy by screening mutagenized grain for the inability to germinate on 5 μM ABA once after-ripened. M_1 Chinese Spring Dv418 seeds were mutagenized with 4 grays of fast-neutron radiation. These seeds were advanced to M_2 and then allowed to after-ripen for 2 years. 22,520 M_2 seeds were plated on 5 μM ABA and the 89 M_2 grains that remained ungerminated after 96 h incubation were considered putative ABA-hypersensitive mutants and advanced to the M_3 generation. When M_3 seeds after-ripened for 9 months were plated on 5 μM ABA, 25 independent mutants showed reduced germination capacity compared to the wild-type parent Chinese Spring (ESM Table 1). In a second experiment in the M_4, only six mutants showed increased ABA sensitivity as whole grain following 25 months after-ripening. Thus, while the original screen did detect mutants with increased ABA sensitivity, most lines do not show a reproducible phenotype with long after-ripening. Of the original 25 mutants, ten were chosen for further study based on the reproducibility of phenotype as well as fertility and viability. These lines are referred to as wheat *ABA-responsive mutants* (W*arm*). Dormant embryos of W*arm* mutants 1–10 were retested a third time in the M_5 generation (ESM Fig. 1, Table 1).

W*arm* lines have been advanced by single plant descent through the M_9 generation. Although derived from parents that showed failure to germinate on 5 μM ABA over multiple generations, M_7, M_8, and M_9 lines continue to show a high degree of variability in their germination phenotype (ESM Fig. 2A–C). On average, M_7 grain derived from M_6 mutant individuals shows reduced germination on ABA compared to wild-type Chinese Spring. However, the percent germination is highly variable. While we cannot fully rule out the possibility that this variability is due to genetic segregation, it seems more likely that this variability is due either to variability in the degree of dormancy or to differences in the degree of phenotypic expressivity in the Chinese Spring background. To try to isolate the cause of the observed variability in the M_7 generation, Chinese Spring wild type was harvested by plant when it was grown alongside plants grown for M_8 and M_9 germination tests and tested for germination of cut dormant grain on 5 μM ABA. Chinese Spring individuals also show a range of germination behaviors (ESM Fig. 2D). When averaged, these individuals show a higher germination percentage than that of many individual mutants (ESM Fig. 2B and C), but some wild-type individuals produced grain that was as sensitive, or more sensitive to ABA than some mutant individuals. This apparent variability in background dormancy may partially explain the variability of mutant phenotype expression.

3.3. Analysis of ABA dose–response during seed germination

To further characterize the germination phenotype, the dose–response of each mutant to increasing concentrations of ABA was examined. This experiment was conducted using embryo half-grains because the ABA response is clearer using embryos than whole grain (Fig. 1). M_6 grain was first after-ripened for 1.5 months in order to obtain Chinese Spring grain that germinated well at lower concentrations of ABA. Embryos were plated on germination discs moistened with 0, 1, 5, 10, and 25 μM ABA. Germination was recorded over five days. Germination index over time is shown in Fig. 2, whereas % germination can be found in ESM Fig. 3. W*arm1* and W*arm4* showed the strongest response to ABA; however, these mutants also show reduced germination capacity in the absence of ABA suggesting that this phenotype may be due in part to increased embryo dormancy (Fig. 2A). The lines W*arm2*, W*arm3*, W*arm5* and W*arm6* showed some decrease in germination capacity at one or more ABA concentrations without showing any decrease in seed germination in the absence of ABA (Fig. 2B and C). The W*arm7*, W*arm8*, W*arm9*, and W*arm10* lines showed no apparent decrease in germination index at any ABA concentration suggesting that these lines do not have increased ABA sensitivity (ESM Fig. 4). Mutants W*arm1* through W*arm6* showed a stronger decrease in germination capacity compared to wild type at 10 μM ABA, suggesting that there is some increase in ABA sensitivity during seed germination.

3.4. The effect of Warm1 and Warm4 on dry after-ripening

We next examined whether the dormant W*arm1* and W*arm4* mutants result in an increase in the time required for seed after-ripening (Fig. 3). Dormant grain was obtained from field-grown plants and stored at −20 °C until needed. Germination of intact and embryo half-grains both in the presence and absence of 5 μM ABA was evaluated periodically until germination indices exceeded 0.8 for all genotypes tested as intact grains after 17 weeks (Fig. 3A). In the absence of ABA, intact grains of both mutants showed a decrease in germination compared to Chinese Spring from weeks 0 through 11 (Fig. 3A). When grains were cut, germination of both mutants was very similar to Chinese Spring, except for a slight decrease in germination at the first time point tested (Fig. 3B). When embryo half-grains were tested on 5 μM ABA, both mutants showed an increased sensitivity to the inhibition of germination by ABA compared to Chinese Spring at weeks 3.4 and 7.3 (Fig. 3B). Thus, it appears that the increased embryo dormancy and increased ABA sensitivity of the W*arm1* and W*arm4* lines is associated with an increase in the length of time required for seed after-ripening and loss of sensitivity to ABA. At 11 weeks of after-ripening the difference in ABA sensitivity is no longer evident. Thus, in scoring the ABA-hypersensitive W*arm* germination phenotype it is always important to choose a level of after-ripening that maximizes the difference in wild-type and mutant ABA sensitivity.

3.5. Segregation analysis

Genetic analysis was performed in the W*arm1* line because this mutant shows the strongest and most reproducible ABA-

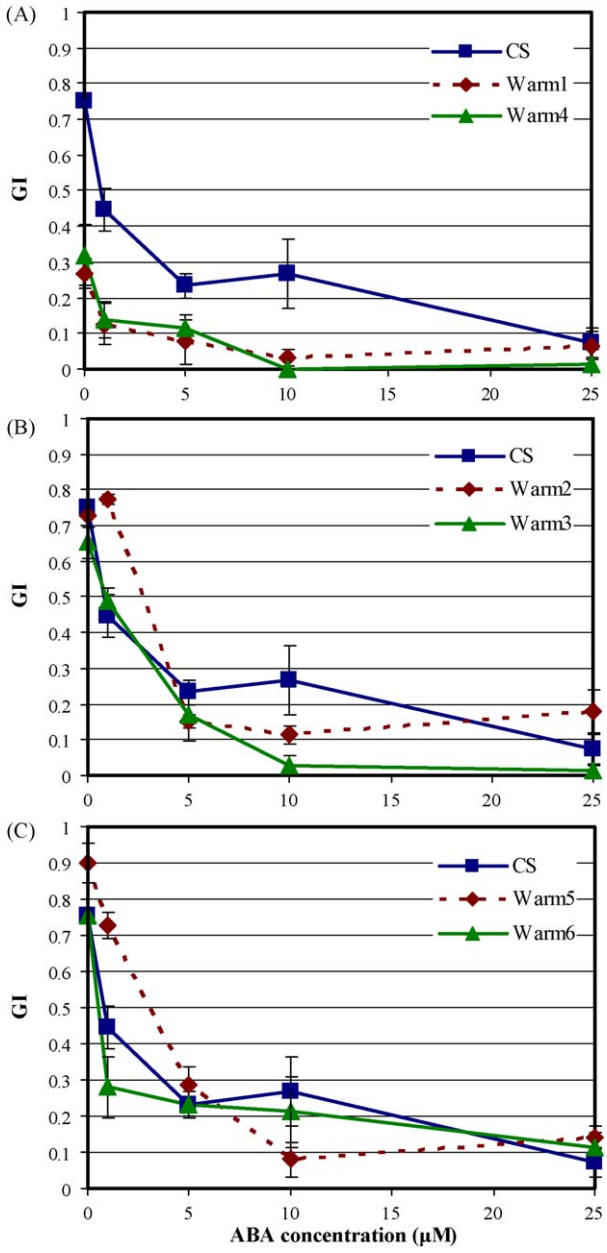

Fig. 2. ABA sensitivity of embryo half M_6 seeds of W*arm* mutants 1 and 4 (A), W*arm* 2 and 3 (B), and W*arm* 5 and 6 (C) compared to Chinese Spring wild type (CS). Seeds were after-ripened for 1.5 months before testing. Weighted germination indices (GI) are shown. Error bars represent standard errors of three replications of 10 seeds each.

hypersensitive germination phenotype. Six F_1 plants from a single cross were grown and F_2 seeds were harvested at maturity. A total of 711 F_2 seeds were tested as cut seeds on 5 μM ABA giving 18.6% germination after 1 month of after-ripening. These results suggest that the mutation in W*arm1* is dominant ($\chi^2 = 15.7$). However, the germination percentage is lower than the expected percentage for a dominant trait. Seed from a single Chinese Spring wild-type plant grown at the same time as F_1 plants gave 100% germination in this experiment. Subsequent research examining the germination of Chinese Spring seeds from multiple Chinese Spring parent plants showed a range of germination from 40% to 100% (ESM Fig. 2D) and an average germination potential of 81.7–85.3% (ESM Fig. 2A–C). Thus, we cannot rule out the possibility that some of the wild-type individuals segregating in the F2 were unable to germinate due to elevated levels of dormancy.

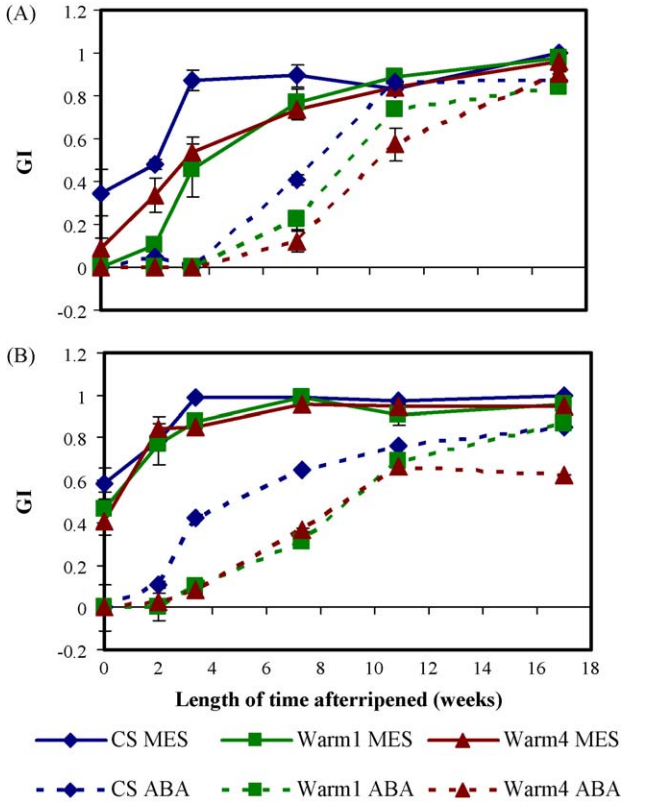

Fig. 3. Germination and ABA sensitivity of M_9 W*arm1*, W*arm4*, and Chinese Spring (CS) whole (A) and cut (B) grain over an after-ripening time course. Weighted germination indices (GI) are shown. Error bars represent standard errors of three replications of 10 seeds each.

3.6. Characterization of Warm plant height and flowering time phenotypes

It is possible that increased ABA sensitivity may result in secondary phenotypes, given that ABA regulates many important aspects of plant development in response to the environment. Thus, the effect of W*arm* mutants on plant height, heading date, whole plant transpiration, and fertility were examined. Plant height was measured using greenhouse grown plants in the M_7 and M_8 generation (Table 2). None of the W*arm* lines showed a strong dwarf phenotype with all W*arm* lines showing plant heights within 85% of Chinese Spring in the M_7 and M_8 generation. W*arm1* and W*arm6* showed small but significant and reproducible decreases in plant height (Table 2). W*arm4* also showed a decrease in plant height in the M_7 generation. The effect of mutants on the transition to flowering was examined by determining the number of days required to achieve 50% heading under field conditions. Only W*arm2* showed a delay in heading date (Table 3). A similar delay was observed under greenhouse conditions. Next, the effect of each mutant on greenhouse yield (g/plant) was examined in the M_7 generation (Table 3). None of the mutants showed a significant change in yield, although both highly dormant lines W*arm1* and W*arm4* may show a small decrease in yield.

3.7. The plant transpiration phenotype

In addition to its role in the induction and maintenance of seed dormancy, ABA also induces stomatal closure as an adaptive response to drying soils. If the ABA-hypersensitive mutants isolated in this study also alter vegetative ABA sensitivity then this should result in a change in plant transpiration in response to dry-

Table 2
Plant height of five W*arm* mutants and Chinese Spring (CS) over two generations.

Genotype	M_7[a]				M_8			
	Height[b]	Ratio[c]	SD[d]	*P* value[e]	Height	Ratio	SD	*P* value
W*arm1*	97.31	0.87	7.31	<0.001	102.55	0.95	9.97	<0.1
W*arm2*	106.73	0.96	6.11	NS	116.21	1.07	7.94	NS
W*arm3*	111.60	1.00	5.64	NS	112.58	1.04	11.03	NS
W*arm4*	103.65	0.93	2.63	<0.01	108.11	1.00	8.79	NS
W*arm6*	105.73	0.95	6.66	<0.05	93.77	0.87	5.95	<0.001
CS	111.59	–	4.65	–	108.18	–	9.21	–

[a] Generation.
[b] Mean plant height in cm.
[c] Plant heights relative to CS.
[d] SD is standard deviation.
[e] *P* values refer to significance determined by analysis of variance using the Dunnett's multiple comparison adjustment. NS: not significant.

ing soil. To examine this, a gravimetric method was employed to estimate the change in plant transpiration in drying soils of greenhouse grown plants. Pots containing an equal amount of evenly moistened soil were covered in plastic to prevent evaporation of water when plants reached the five-leaf stage. At this point watering ceased and the loss of soil moisture through plant transpiration was estimated by weighing plants in pots every 12 h for 48 h and then every 24 h until 13 days had passed.

During this experiment, W*arm1* showed a similar rate of moisture loss to wild-type Chinese Spring (Fig. 4A), whereas W*arm4* showed slower transpiration in drying soils than Chinese Spring (Fig. 4B). These results suggest that the increased ABA sensitivity in the seeds of these lines is associated with a vegetative phenotype of decreased transpiration rate in drying soils.

In W*arm4*, the decrease in the rate of soil moisture loss was correlated with a lower stomatal conductance compared to Chinese Spring during days 2 and 3 of measurements (ESM Fig. 5B). This suggests that an increase in stomatal closure during early drought stress is resulting in the reduction in soil moisture loss. In contrast, W*arm1* showed no significant difference in stomatal conductance compared to Chinese Spring (ESM Fig. 5A).

4. Discussion

This paper describes the isolation of six independent mutants showing an increased response to ABA when partially after-ripened. The fact that it is possible to isolate ABA-response mutants in allohexaploid wheat is interesting since most genes are present in multiple copies. Thus, it is likely that most mutants isolated will be dominant or co-dominant, or show phenotypes that are dependent on mutant gene expression levels. Consistent with this, genetic analysis of W*arm1* suggests that this is a dominant mutant. Two mutants, W*arm1* and W*arm4*, showed a reproducible increase in ABA sensitivity at all ABA concentrations examined. These mutants also exhibited some embryo dormancy and required more time to after-ripen than the wild-type Chinese Spring. Future research will use these mutants to examine whether increased ABA sensitivity results in increased preharvest sprouting tolerance. Previous work suggests a link between ABA sensitivity and wheat grain dormancy [43,51]. For example, mutants isolated for decreased dormancy over multiple generations tended to show either decreased ABA accumulation or ABA insensitivity [69]. In addition to increased seed dormancy, the W*arm4* mutant showed a decrease in the rate of soil moisture loss in drying soils, suggesting that this mutation may also alter vegetative water relations. Given the phenotypic characterization presented here, it appears that Wheat ABA-responsive mutants will serve an important tool to elucidate the role of ABA signaling in the control of PHS and drought tolerance.

Table 3
Time to transition to flowering and grain yield per plant for 5 W*arm* mutants and Chinese Spring (CS).

Genotype	Days to 50% headed[a]	Grain yield[b]	SD[c]
W*arm1*	66.25	4.72	0.68
W*arm2*	68.25	4.86	0.09
W*arm3*	66.25	5.01	0.23
W*arm4*	66.5	4.61	0.23
W*arm6*	66.5	5.23	0.55
CS	66.17	5.15	0.80

[a] Heading date was evaluated on M_9 plants grown in the field at Pullman, WA in 2009. Means of four plots per mutant and 23 CS plots are given.
[b] Grain yield per plant was evaluated on M_7 plants grown in the greenhouse. Means are given in grams of grain per plant and represent 6–12 plants per genotype.
[c] SD is standard deviation.

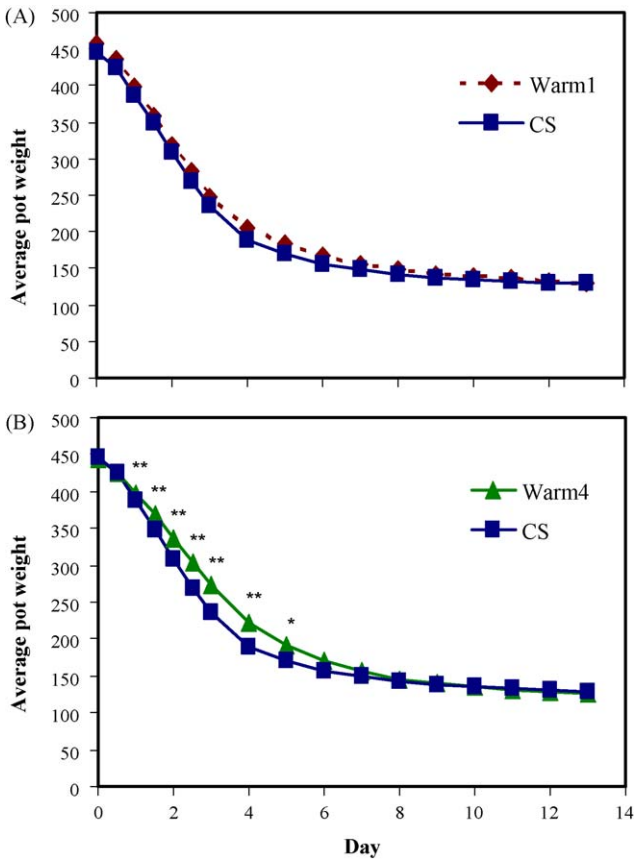

Fig. 4. Average pot weights in grams for W*arm1* (A) and W*arm4* (B) compared to Chinese Spring (CS). Symbols above data points represent significance as follows: ** is $0.01 < P < 0.05$, and * is $0.05 < P < 0.1$. Where there is no symbol, there is no significant difference between CS and the mutant at that time point.

ABA sensitivity is dependent on the dormancy status of wheat seed. Dormant wheat seeds show high sensitivity to exogenously applied ABA, whereas long after-ripened wheat seeds are extremely ABA-insensitive (Fig. 1). This is consistent with a previous observation that a more dormant red-grained wheat showed higher ABA sensitivity than a near-isogenic less dormant white grained wheat [28]. The correlation between dormancy status and ABA sensitivity is not unique to hard red wheat since a similar correlation is seen in the soft white winter Brevor (Fig. 1A). While this dependency generally holds true, the effect is stronger in some cultivars than in others. Reduced ABA sensitivity has also been observed in non-dormant embryos of Sorghum bicolor, suggesting that ABA sensitivity may correlate with dormancy status in other cereals [2]. In contrast, the dicot Arabidopsis shows the same dose–response to ABA inhibition of germination regardless of the duration of dry after-ripening (ESM Fig. 1). What is the cause of the extreme ABA insensitivity of after-ripened wheat? In barley, after-ripened grain shows lower expression of the ABA biosynthesis gene *HvNCED1* and increased expression of the ABA catabolic gene *HvABA8'OH1* (ABA 8'hydroxylase) [47]. If this mechanism is conserved in wheat, then ABA 8'hydroxylase activity in after-ripened wheat may rapidly degrade applied ABA leading to apparent insensitivity to exogenous ABA. Alternatively, after-ripening may alter ABA sensitivity directly through changes in signaling gene expression. Future work will need to examine these possibilities.

This study developed a mutant screen for increased sensitivity to ABA in seed germination that took advantage of the gradual loss of ABA sensitivity with after-ripening. The candidate ABA-hypersensitive mutants were identified based on failure to germinate on 5 μM ABA after 2 years of after-ripening. Normally, after-ripened Chinese Spring is highly insensitive to this concentration of ABA (Fig. 1C). It was expected that the resulting mutants would show either increased ABA sensitivity or a requirement for longer after-ripening to become ABA-insensitive. At least six mutants were identified showing increased sensitivity at one or more ABA concentrations (ESM Table 1, Fig. 2). Of these mutants, W*arm1* and W*arm4* embryos showed increased sensitivity at most ABA concentrations examined (Fig. 2A). While W*arm1* and W*arm4* require more time to after-ripen, they also show increased ABA sensitivity until after-ripened (Fig. 3). These mutants also sometimes show decreased germination capacity in the absence of ABA suggesting that they can cause increased embryo dormancy (Fig. 2A).

Loss of ABA sensitivity during after-ripening appears to be a gradual process that may be subject to genetic variation. During an after-ripening time course Chinese Spring wheat showed a gradual loss of ABA sensitivity, suggesting that loss of ABA sensitivity during after-ripening may always be gradual (Fig. 3). Compared to Chinese Spring, W*arm1* and W*arm4* showed decreased germination capacity as whole seeds in the absence of ABA through 7.3 weeks of after-ripening (Fig. 3). W*arm1* and W*arm4* embryos also showed increased ABA sensitivity between 3.4 and 7.3 weeks of after-ripening compared to Chinese Spring. While this may suggest that W*arm1* and W*arm4* lose ABA sensitivity more slowly than Chinese Spring, it is clear that these mutants are not immune to the effects of after-ripening. The degree of difference between wild-type and mutant strongly depended on the duration of after-ripening suggesting that the progress of after-ripening must be carefully monitored in order to score the *Warm* germination phenotype. It can be difficult to choose the right time for scoring this germination phenotype since the level of Chinese Spring dormancy is highly dependent on the environment with field, greenhouse, and growth chamber derived seeds showing markedly different degrees of dormancy [27]. The dependence of ABA sensitivity on dormancy status in these mutants may be one explanation for the variability in the W*arm* phenotypic expressivity (ESM Fig. 2). This problem may be exacerbated by the fact that red-grained wheats such as Chinese Spring and Scarlet sometimes show embryo dormancy in addition to the seed coat-imposed dormancy (Fig. 1B, Fig. 3). The soft white line Brevor shows only seed coat-imposed dormancy (Fig. 1A, [51]). The variations in seed dormancy and expressivity in the Chinese Spring W*arm* lines has led to difficulties in performing segregation analysis and determining complementation groups. It is possible that the genetic analysis of these mutants may be facilitated by moving them into a white grain background to avoid problems with embryo dormancy. A less dormant white background would also be preferable for examining the effect of mutants on PHS tolerance. Moving the W*arm* mutations into a white background will also provide opportunities to map the affected genes, thereby providing an alternate method for determining the number of altered genes.

Some of the W*arm* lines identified showed secondary vegetative phenotypes suggesting that the effect of these mutations may not be restricted to the germline. It is important to examine vegetative phenotypes because ABA signaling mutants in Arabidopsis can result in changes in seed, vegetative, or both seed and vegetative ABA sensitivity [31]. When grown in the field, only the W*arm2* line showed a delay in flowering (Table 3). When grown under greenhouse conditions, both the W*arm1* and W*arm6* lines showed a reproducible decrease in plant height compared to wild-type Chinese Spring (Table 2). Both GA and BR hormone mutants result in decreased plant height [70,71]. Future work will need to examine whether these W*arm* lines result from reduced GA or BR synthesis or response, given that such Arabidopsis GA and BR mutants show an apparent increase in ABA sensitivity during seed germination [63,72,73]. The W*arm4* line, but not the W*arm1* line showed a decrease in transpiration of soil moisture compared to wild type (Fig. 4). Future work will need to examine whether this results from an increase in stomatal sensitivity to ABA hormone. While increased ABA sensitivity in stomatal closure may result in increased drought tolerance by conserving water, it also may decrease yield due to decreased uptake of CO_2 for photosynthesis. Thus, one would like to increase vegetative ABA sensitivity—but not too much. None of the mutants showed a strong decrease in yield when grown under greenhouse conditions in the M_7 (Table 3). This preliminary data suggested that of the six lines, W*arm4* has the lowest yield on a per plant basis. In addition to its role in controlling seed dormancy, ABA also is needed to stimulate drought, cold, and salt tolerance. Previous work showed that increased ABA sensitivity can be associated with increased cold tolerance in wheat [74].

ABA-hypersensitive lines isolated in this screen may result either from increased ABA signaling or from decreased ABA turnover. For example, loss of maize *Vp1* or Arabidopsis *ABI3* function results in a seed-specific loss of ABA sensitivity [41,75]. McKibbin et al. [43] suggested that low dormancy and PHS susceptibility in bread wheat results from missplicing the ABA signaling gene *Vp1/ABI3*. Thus, it is possible that the ABA-hypersensitive lines isolated here are suppressors or modifiers of this problem resulting from mutations in genes acting either downstream of or in parallel to wheat *Vp1*. Future research should also examine whether these mutants alter ABA accumulation or sensitivity per se. For example, reduced expression of the ABA catabolic gene ABA 8′-hydroxylase could also result in increased seed dormancy and apparent sensitivity to exogenous ABA [47].

ABA-response mutants such as those identified in this study and by others will be important tools to define the role of ABA in wheat seed dormancy, dormancy loss, and PHS tolerance [69,74,76]. While ABA has long been implicated in PHS tolerance, more work is needed to establish whether known PHS QTLs are the result of mutations in ABA signaling genes. It should not be assumed that any one gene will provide the silver bullet that ends preharvest sprouting problems. It is most likely that PHS susceptibility in dif-

ferent genetic backgrounds will result from different alterations in ABA biosynthesis, catabolism, and signaling. Thus, a mutation (or transformed gene) that gives excellent PHS tolerance in one genetic background may not work as well in another background depending on epistasis. Future screens may use cultivars currently in use for wheat breeding so that this work may be more directly relevant to wheat improvement.

This study underscores the importance of mutation analysis as a tool for translational seed biology in wheat. Mutants identified by forward or reverse genetic screens are an important resource for gene discovery, elucidation of gene function, and germplasm development. Given the high degree of genetic redundancy in wheat, mutant phenotypes may show low penetrance if present on only one homeologous group. However, it might be possible to titrate a phenotype by controlling the number of mutant copies. Phenotypes showing low penetrance in allohexaploid wheat may actually be dosage-dependent or co-dominant. Their use may contribute a degree of breeding flexibility, allowing selection of intermediate phenotypes carefully chosen to suit a specific farming environment. Low penetrance of mutant phenotypes many also be one reason that breeders have historically preferred to utilize variation that has been selected over many generations by man or by nature. In using mutation breeding, especially reverse genetic strategies like TILLING [77,this issue, 78], it may be necessary to identify mutations in two or more genomes to achieve the desired phenotype.

Acknowledgements

The authors would like to thank Dr. R. Warner for the gift of mutagenized Chinese Spring grain and Dr. S. Abrams for the gift of (+)-ABA. Thanks are due to C. Hoagland, R. Parveen, A. Hauvermale, T. Ariizumi, and L. Murphy for expert assistance. Thanks are also due to M.K. Walker-Simmons for advice and encouragement. Finally, we would like to thank C. Walker for assistance with statistical analysis. This work was supported by a USDA CSREES grant to C.M.S. (2005-01099).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.06.004.

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