Euphytica (2016) 212:229–245
DOI 10.1007/s10681-016-1763-6

The wheat ABA hypersensitive *ERA8* mutant is associated with increased preharvest sprouting tolerance and altered hormone accumulation

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Received: 23 May 2016 / Accepted: 5 August 2016 / Published online: 12 September 2016

Abstract Wheat preharvest sprouting (PHS) is the germination of mature grain on the mother plant when rain occurs before harvest. Higher abscisic acid (ABA) hormone levels and sensitivity are associated with higher seed dormancy and PHS tolerance. Consistent with this, the ABA hypersensitive *ENHANCED RESPONSE TO* ABA8 (*ERA8*) mutant resulted in increased dormancy and PHS tolerance in soft white spring wheat 'Zak'. *ERA8* seeds were initially less responsive to germination-rescue by the hormone gibberellin (GA). *ERA8* gained GA and lost ABA sensitivity more slowly than wild-type during dormancy loss through after-ripening and cold imbibition. This study examined if increased ABA sensitivity in *ERA8* likely resulted from increased ABA signaling or increased ABA hormone levels. Zak *ERA8* had higher initial grain dormancy although endogenous embryo ABA levels were similar in Zak *ERA8* and wild-type, suggesting that increased dormancy was due to increased ABA signaling rather than increased ABA accumulation. ABA levels declined with Zak *ERA8* after-ripening, suggesting that ABA turnover is not defective. Elevated *ERA8* dormancy was also associated with increased embryonic jasmonic acid-Ile and aleurone indole-3-acetic acid (IAA) levels. The possible implication that other plant hormones may influence wheat seed dormancy and germination are discussed.

Keywords Abscisic acid · Gibberellin · Auxin · Wheat · Preharvest sprouting · Seed dormancy

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Electronic supplementary material The online version of this article (doi:10.1007/s10681-016-1763-6) contains supplementary material, which is available to authorized users.

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Introduction

Preharvest sprouting (PHS) is the germination of mature seeds on the mother plant when cool, wet conditions occur before harvest (reviewed by Bewley et al. 2013). PHS susceptibility is associated with lack of seed dormancy. Dormant seeds fail to germinate even under environmental conditions (moisture,

temperature, and light) that normally induce efficient germination. Seeds can lose dormancy through cold stratification (imbibing water in the cold), seed scarification, and after-ripening during dry storage (reviewed by Finkelstein et al. 2008). While some wheat cultivars exhibit stronger seed dormancy at higher than at lower temperature, others show seed dormancy at temperatures as low as 10 °C (Nyachiro et al. 2002; Kashiwakura et al. 2016). Freshly harvested wheat is considered to have true seed dormancy over 10–20 °C (Corbineau and Come 2000). All wheat cultivars exhibit the highest degree of grain dormancy at physiological maturity, and then gradually lose dormancy through after-ripening (Paterson et al. 1989; Gerjets et al. 2010). PHS tolerant wheat lines tend to lose dormancy through dry after-ripening (AR) and cold stratification more slowly than susceptible lines (Gerjets et al. 2010; Tuttle et al. 2015). Selection for synchronous seedling emergence in the field has inadvertently led to low seed dormancy and PHS susceptibility in cereals like wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* subsp. *vulgare*), and sorghum (*Sorghum bicolor*) (Paterson and Sorrells 1990; Gualano et al. 2007; Ullrich et al. 2009). During germination, the aleurone layer releases α-amylase in order to mobilize starch granules for use by the growing embryo. The resulting starch degradation leads to poor end-use quality of baked goods made from sprouted wheat grain (Clarke et al. 1984). Farmers suffer economic losses when sprouted grain is graded as animal feed. *ENHANCED RESPONSE TO ABA8 (ERA8)* was isolated in the PHS susceptible soft white spring cultivar 'Zak' based on increased sensitivity to the dormancy-inducing hormone abscisic acid (ABA) (Schramm et al. 2013). This study characterizes the effect of Zak *ERA8* on preharvest sprouting tolerance and examines the potential mechanisms by which *ERA8* increases seed dormancy by measuring endogenous plant hormone levels and responses to applied ABA and gibberellin (GA) hormones.

Both environmental and genetic factors affect the degree of seed dormancy. Seeds that develop under cool dry conditions have higher seed dormancy (Nakamura et al. 2011). QTLs for PHS tolerance have been identified on most wheat chromosomes (Jaiswal et al. 2012; Kulwal et al. 2012). Wheat seed dormancy can result from embryo or coat-imposed dormancy (reviewed by Schramm et al. 2010; Bewley et al. 2013). Coat-imposed dormancy can be broken by cutting the seed coat, whereas embryo dormancy is not. In general, wheat varieties with a red seed coat tend to have higher seed dormancy and PHS tolerance than white wheat varieties (Paterson and Sorrells 1990). However, some red wheat cultivars lack seed dormancy and PHS tolerance, whereas some white wheat cultivars are highly dormant and PHS tolerant, such as Clark's Cream and Brevor (Walker-Simmons 1987; Upadhyay et al. 1988; Flintham 2000; Warner et al. 2000; Himi et al. 2002; Torada and Amano 2002). Thus, seed coat color is not the only factor controlling PHS tolerance. PHS tolerance of white wheat can also be associated with higher ABA signaling (Walker-Simmons 1987).

Seed dormancy is stimulated and seed germination is inhibited by the plant hormone ABA in a wide range of plant species (reviewed by Finkelstein et al. 2008). ABA induces seed dormancy during embryo maturation and maintains dormancy in mature seeds. Previous research showed that higher seed dormancy and PHS tolerance was associated with higher ABA hormone levels and ABA sensitivity in wheat embryos and intact grains (Walker-Simmons 1987; Tuttle et al. 2015). Dormancy loss through after-ripening is associated with decreasing ABA levels in wheat, barley, and Arabidopsis (Ried and Walker-Simmons 1990; Jacobsen et al. 2002; Ali-Rachedi et al. 2004; Millar et al. 2006; Barrero et al. 2009). Previous wheat mutant studies have shown that higher ABA sensitivity during seed germination is associated with seed dormancy, whereas reduced ABA sensitivity is associated with low seed dormancy (Kawakami et al. 1997; Kobayashi et al. 2008, 2010; Schramm et al. 2010, 2013). Thus, factors controlling ABA hormone accumulation likely play an important role in controlling dormancy and dormancy loss.

The semi-dominant Zak *ERA8* mutant was selected based on failure to germinate at an ABA concentration too low to inhibit wild-type seed germination (Schramm et al. 2013; Martinez et al. 2014). This ABA hypersensitive phenotype was associated with increased seed dormancy and slower loss of ABA sensitivity with after-ripening. An ABA hypersensitive phenotype can result from: (1) increased hormone levels due to increased ABA biosynthesis or decreased turnover, or (2) increased ABA response due to the loss of a negative regulator or increased function of a positive regulator of ABA signaling (Cutler et al.

1996; Ghassemian et al. 2000). This study examined whether the ABA hypersensitive phenotype in wheat *ERA8* is associated with changes in hormone accumulation or hormone sensitivity.

Several other hormones are associated with changes in seed dormancy and dormancy loss in wheat (reviewed by Rodríguez et al. 2015). GA stimulates seed germination and dormancy loss. Gibberellins are a large family of tetracyclic diterpenes in plants; GA_4 (gibberellin A_4) and GA_1 are the predominant bioactive forms. GA produced in the embryo/scutellum triggers the release of α-amylase from the aleurone during cereal seed germination. It appears that GA also plays a role in the germination of the embryo, because a GA biosynthesis gene, *GA 20-oxidase*, was identified as a candidate gene controlling preharvest sprouting tolerance in wheat (Appleford et al. 2006). Moreover, a *GA 20-oxidase* gene was identified as a QTL in *sd1* controlling seed dormancy in rice (Ye et al. 2013). While an increase in GA biosynthesis has been associated with dormancy loss through cold stratification of Arabidopsis, the role of GA in after-ripening has not been well studied (Yamauchi et al. 2004). Transcriptome analysis showed that while there was no induction of GA-related genes with after-ripening of barley coleorhiza, there was induction of genes involved in jasmonic acid (JA) hormone synthesis and signaling (Barrero et al. 2009). Moreover, application of methyl-JA stimulated wheat grain germination, suggesting that GA may not be the only hormone stimulating cereal seed dormancy loss (Jacobsen et al. 2013). In contrast, the hormone indole-3-acetic acid (IAA) and its precursors inhibited germination when exogenously applied to dormant but not to after-ripened wheat embryos (Morris et al. 1988; Ramaih et al. 2003). Thus, the level of grain dormancy may be influenced by JA and IAA levels, in addition to ABA and GA levels.

This study examined the hypothesis that the increased seed dormancy of Zak *ERA8* is associated with differences in ABA hormone accumulation and/or ABA sensitivity. *ERA8* had more initial seed dormancy associated with higher ABA sensitivity and lower GA sensitivity. GA sensitivity increased and ABA sensitivity decreased more slowly in *ERA8* during dormancy loss through cold stratification and after-ripening. Higher *ERA8* seed dormancy at 1 week of after-ripening was not associated with elevated ABA hormone levels, suggesting that the *ERA8* phenotype is the result of increased ABA signaling rather than ABA over-accumulation. During after-ripening, embryo ABA levels decreased and JA levels increased. Elevated *ERA8* dormancy was associated with elevated aleurone IAA levels that decreased with after-ripening. Finally, spike-wetting tests showed that field-grown *ERA8* has increased PHS tolerance.

Materials and methods

Plant materials and growth conditions

The wheat grain is a caryopsis that includes a pericarp. The word "seed" is used here in a functional sense to refer to the caryopsis or grain. *Triticum aestivum* L. cultivar 'Zak' (PI 612956) is a soft white spring wheat and Zak *ERA8* (PI 669443; BC_1F_5) is an ABA hypersensitive mutant derived from Zak (Kidwell et al. 2002; Schramm et al. 2013; Martinez et al. 2014). The F_5 plants were derived by single plant descent from F_2 with selection for increased response to ABA. Additional cultivars examined included the soft white winter 'Brevor' (Walker-Simmons 1987; Morris et al. 1991), soft white spring cultivars 'Louise' (PI 634865), 'Diva' (PI 660663), and 'Babe' (PI 656791), the club soft white spring 'JD' (PI 656790), and the hard white spring cultivars 'Otis' (PI 634866) and 'Macon' (PI 617072) (Kidwell et al. 2003, 2004, 2006a, b, 2012). Plants were grown in the greenhouse under a 16 h photoperiod and 22 °C day/15 °C night in 3 L pots containing Sunshine potting soil mix LC1. Nutrient water was applied weekly (20-10-20). Seeds were harvested at physiological maturity (PM), right after the peduncle turned yellow (seed moisture 7–12 %). All grain used for germination assays was hand threshed to prevent seed coat scarification (Paterson and Sorrells 1990).

After-ripening time courses

Grains were dry after-ripened for the indicated number of weeks under non-humid conditions at room temperature in open containers. Pooled grain from Zak *ERA8* and wild-type Zak plants grown side-by-side in the greenhouse were assayed for germination following 1, 2, 4, 6, 8, 10, 12, and 16 weeks of after-ripening (Fig. 3). Hormone profiling was performed on the same grain frozen at 1, 4, or 10 weeks of after-

ripening. A duplicate after-ripening time course was performed using Zak and Zak *ERA8* BC_2F_4 (ESM Fig. S3).

Germination and cold stratification

Seeds were sterilized in 10 % bleach/0.01 % SDS for 15 min, then rinsed 3× with sterile water. Three technical replicates of 10 grains each were plated in petri dishes containing a 9-cm blue germination disk (Anchor Paper Co) saturated with 6 mL of 5 mM MES buffer, pH 5.5 (2-[*N*-morpholino] ethane sulfonic acid, Sigma-Aldrich) containing no hormone, 5 μM (±)-ABA (PhytoTechnology Laboratories), or 10 μM GA_3 (gibberellin A_3, Sigma-Aldrich). Germination was scored daily based on embryonic root emergence. Germination index (GI), a value weighted for speed of germination, was calculated over 5 d of imbibition as $(5 \times g_{day1} + 4 \times g_{day2} \ldots + 1 \times g_{day5})/(5 \times n)$ where g is the number of additional seeds germinated on each day and n is the total number of seeds (Walker-Simmons 1987; Schramm et al. 2013). GI ranges from 0 to 1, where a GI of 1 indicates that all of the seeds germinated on day 1 of imbibition.

Cold stratification time course experiments were performed using seeds after-ripened for 1 week and thereafter stored at −20 °C. The experiment was performed using seeds from Zak and Zak *ERA8* BC_1F_5 plants grown side-by-side in the greenhouse (Fig. 2; ESM Fig. S2). Seeds were imbibed at 4 °C in the dark for 0, 6, 12, 24, 36, 48, or 72 h. Germination index was calculated over 5 days incubation at 30 °C. This temperature was selected because it was the temperature used for original isolation of the *ERA8* mutation due to the fact that differences in wheat ABA sensitivity are enhanced at 30 °C (Walker-Simmons 1988; Schramm et al. 2013).

Dormancy stages

Four stages of dormancy loss were defined based on the germination efficiency without hormone, and in the presence of 5 μM ABA and 10 μM GA (as in Tuttle et al. 2015). Comparisons of hormone levels were made between Zak and Zak*ERA8* from the same after-ripening time course, and with independent Brevor grain samples at similar stages of dormancy loss (ESM Figs. S5, S6, S7). Stage 1 or very dormant (VD) grain show reduced germination on GA (GI <0.4). Stage 2 or dormant (D) grain show GA-rescued germination (GI >0.4), but low germination efficiency without hormone (GI <0.4). Stage 3 or partially after-ripened (PAR) seeds germinate without hormone (GI >0.4), but show ABA-sensitive germination (GI <0.9). Stage 4 or fully after-ripened (FAR) seeds show ABA-insensitive germination (GI >0.9). A late stage 3 seed sample was denoted AR* (GI = 0.59 on ABA), indicating an intermediate stage between PAR and FAR.

Hormone profiling

Endogenous hormone measurements were performed using Zak *ERA8* and Zak grain in one experiment, and Brevor grain in another experiment. Field-grown Brevor grain used for hormone profiling were harvested in 2011, 2010 and 2003, and were after-ripened for approximately 1 week (VD), 2 months (D), and 8 years (FAR) before storage at −20 °C to preserve dormancy until germination experiments were performed. Thus, the Brevor grain is not a true after-ripening time course, and can only be used for comparisons with Zak and *ERA8* of similar dormancy stages. Zak and *ERA8* grain were after-ripened for 1, 4 and 10 weeks. Whole caryopses were imbibed for 8 and 18 h at 30 °C in the dark on MES-moistened filter paper. After imbibition, caryopses were dissected into (1) the embryo sample containing embryo/scutellum, and (2) the aleurone sample that consisted of a dorsal section containing pericarp, seed coat, aleurone layer, and a small amount of endosperm tissue (isolated as in Tuttle et al. 2015). The aleurone is a single cell layer that remains alive after seed maturation, unlike the starchy endosperm (Fincher 1989). Note that only ungerminated grains were used for these samples. There were five biological replicates for each treatment, consisting of pooled seeds derived from multiple plants grown side-by-side (genotype, after-ripening time point, imbibition time point, and aleurone vs. embryo sample). Dissected tissues were immediately frozen in liquid nitrogen, ground to a fine powder, lyophilized, and 60 mg dry weight used for hormone extraction. ABA, GA_1, JA, JA-Ile, and IAA plant hormone concentrations were determined by liquid chromatography tandem mass spectrometry as previously described except acetonitrile (MeCN) was used instead of methanol (Yoshimoto et al. 2009; Seo et al. 2011).

Two statistical models were used to compare hormone levels. The first was a mixed model analysis comparing Zak and Zak *ERA8* over weeks of after-ripening, and performed using the MIXED procedure in SAS/STAT software (version 9.3) with an adjusted heterogeneous autoregressive matrix with unequal variances. Least squares-means (LS-means) and standard errors (SE) were derived from the model for each treatment. Treatment comparisons were made using LS differences at $\alpha \leq 0.05$. The second model compared Zak, *ERA8*, and Brevor using dormancy stages as categories due to the fact that hormone measurements were derived from independent after-ripening experiments. In this case, the SAS/STAT MIXED procedure with an adjusted variance/covariance matrix was performed using the Toeplitz matrix. Note that both models could not use the default covariance matrix due to unequal variance. The variance/covariance matrix providing the best fit to the data was used for each model. This led to small differences in the absolute LS-means and SE values observed for Zak and *ERA8* in the two models.

Spike-wetting tests

Spike-wetting tests were performed to examine PHS tolerance based on visible sprouting as in Anderson et al. (1993). Wheat was grown in the field at Cornell University's Snyder Farm in 2008 and McGowen Farm in 2010 (Ithaca, NY, USA), and at Washington State University's Spillman Agronomy Farm in 2012 (Pullman, WA, USA). Intact spikes were hand-harvested at PM, after-ripened at room temperature for 5 days in 2008 and 2010, and 14 days in 2012. After-ripened spikes were stored at −15 °C in plastic freezer bags to preserve dormancy. In 2008 and 2010, 5 spikes from each of 3 replicate plots per genotype were analyzed. In 2012, five spikes randomly representing 3 replicated plots per genotype were analyzed over four independent experiments. Spikes were misted 6 s min^{-1} using fine spray nozzles 53 cm above the spikes (Anderson et al. 1993). After 4–5 days of misting, the spikes were scored for degree of visible sprouting on a 1 (no sprouting) to 10 (coleoptiles uniformly emerged) scale as described by McMaster and Derera (1976). Analysis of variance was performed using the MIXED procedure in SAS/STAT software (version 9.3). LS-means and SE from the model are shown in Fig. 1. Wild type and mutant comparisons were made using LS differences at $\alpha \leq 0.05$.

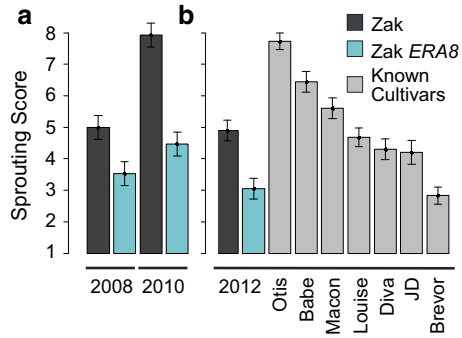

Fig. 1 The effect of *ERA8* on PHS tolerance based on sprouting score. Spike-wetting tests were performed on intact spikes of Zak (*black*), Zak *ERA8* (*blue*), and spring wheat cultivars (*grey*) grown in the Pacific Northwest. Increasing sprouting scores on a 1–10 scale indicate increasing PHS susceptibility in the indicated year. The LS-mean sprouting score and SE bars are shown. **a** Zak and Zak *ERA8* were grown in 2008 and 2010 in New York State, whereas **b** Zak, Zak *ERA8*, and indicated cultivars were grown in Pullman WA in 2012. (Color figure online)

Results

The *ERA8* mutation results in increased PHS tolerance

The *ERA8* mutation in soft white spring Zak resulted in increased seed dormancy associated with increased sensitivity to ABA during seed germination (Schramm et al. 2013). Since higher seed dormancy and higher ABA sensitivity is associated with higher PHS tolerance, *ERA8* was expected to have higher PHS tolerance than wild-type Zak (Morris et al. 1991; Gerjets et al. 2010). Spike-wetting tests were used to compare Zak and *ERA8* PHS tolerance (as in Paterson et al. 1989; Anderson et al. 1993). Because the spike-wetting tests examine visible sprouting in intact spikes, the resulting data takes into account differences in PHS tolerance resulting from both grain dormancy and spike morphology. To reduce variability due to differences in degree of after-ripening, spikes were harvested at physiological maturity (PM) and then after-ripened for the same number of days before freezing prior to conducting tests. Spikes were misted for 6 s min^{-1} over 7 days, and scored daily for sprouting using the 1–10 scale of McMaster and

Derera (1976), where higher scores indicate more sprouting. *ERA8* sprouting scores were significantly lower than those of Zak over 3 years and 3 locations (Fig. 1; ESM Table S1). In 2012, the sprouting scores of Zak and *ERA8* were also compared to other white wheat cultivars. Zak sprouting scores were similar to moderately susceptible cultivars 'Louise' and 'Diva', whereas the *ERA8* sprouting score was similar to the highly PHS tolerant cultivar 'Brevor' (Walker-Simmons 1987). Thus, the single gene *ERA8* mutation was associated with increased PHS tolerance in field-grown wheat.

The effect of *ERA8* on changes in ABA and GA sensitivity with cold stratification

PHS of wheat grain is more likely when rain is associated with cooler temperatures (Reddy et al. 1985; Biddulph et al. 2005). Thus, we examined whether the ABA hypersensitive phenotype of *ERA8* is dependent on temperature, and whether the mutation increases resistance to cold stratification. Germination of Zak WT and *ERA8* was compared with and without 5 μM ABA at 10, 15, 20 and 30 °C (ESM Fig. S1). Compared to WT, *ERA8* showed reduced germination efficiency without hormone at 20 and 30 °C. However, *ERA8* showed reduced germination on ABA at all temperatures examined. Thus, the enhanced response to ABA in *ERA8* is seen at lower temperatures, but the increased seed dormancy is observed at 20 °C and higher. The effect of the *ERA8* mutation on seed ABA and GA sensitivity was examined over a cold stratification time course (Fig. 2; ESM Fig. S2). Previous work showed that PHS tolerant cultivars gain GA and lose ABA sensitivity more slowly with cold stratification than PHS susceptible cultivars (Tuttle et al. 2015). The effect of cold stratification for 0, 6, 12, 24, 36, 48, and 72 h at 4 °C was compared in wild-type Zak and *ERA8* germinated at 30 °C in the absence of hormone and in the presence of ABA or GA_3. Based on germination without hormone, *ERA8* lost dormancy a little more slowly than Zak, showing significant differences with 24, 36, and 72 h of cold stratification ($0.0001 \leq p < 0.01$). *ERA8* was significantly more ABA sensitive than Zak with 36 h and 72 h of cold stratification ($p < 0.0001$). *ERA8* was significantly less sensitive to GA at 12 and 24 h of cold stratification ($p = 0.04$ and 0.0003, respectively).

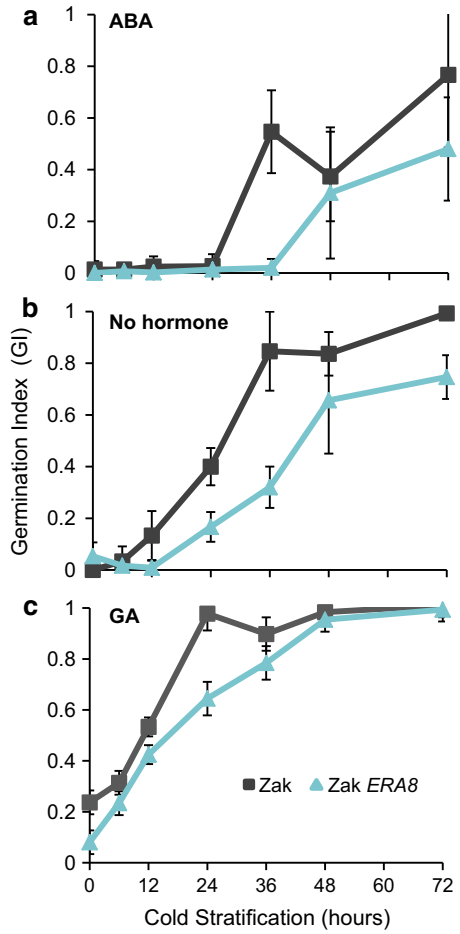

Fig. 2 The effect of cold stratification on wild-type Zak (*square*) and Zak *ERA8* (*triangle*) germination was examined over a cold stratification time course. Seed were cold imbibed at 4 °C for the indicated number of hours, and germination index calculated over 5 days imbibition in the dark at 30 °C. Seeds were plated on filter paper moistened with 5 mM MES, pH 5.5 containing: **a** 5 μM of ABA, **b** no hormone, or **c** 10 μM of GA_3. Means and standard deviation were calculated over three technical repetitions of 10 seeds each. (Color figure online)

The effect of *ERA8* on changes in ABA and GA sensitivity with after-ripening

The effect of the *ERA8* mutation on ABA and GA sensitivity was examined over an after-ripening time course. Previous work showed that *ERA8* lost seed dormancy more slowly than Zak over 6–18 weeks of after-ripening (Schramm et al. 2013). The germination of wild-type Zak and *ERA8* was compared over a 16 weeks after-ripening (AR) time course followed by imbibition in the absence of hormone and in the

presence of 5 μM ABA or 10 μM GA_3. At 1 week AR, neither Zak nor *ERA8* were able to germinate in the absence of hormone, but Zak germinated more efficiently in response to GA (GI = 0.37 vs. 0.11). This indicates that Zak was initially less dormant than *ERA8* (Fig. 3b, c; Table 1). Based on germination without hormone, *ERA8* lost dormancy more slowly with after-ripening; Zak required 10 weeks and *ERA8* 16 weeks of AR to reach a GI ≥0.9. Moreover, *ERA8* gained GA sensitivity more slowly with after-ripening. *ERA8* was consistently more ABA sensitive than wild type over 16 weeks of after-ripening. *ERA8* showed a complete failure to germinate on ABA through 8 weeks of AR, whereas Zak began to germinate on ABA with 4 weeks AR. Similar results were observed when the after-ripening time course was repeated on an independent batch of seeds (ESM Fig. S3). Thus, the *ERA8* mutation resulted in increased seed dormancy, increased ABA sensitivity, and decreased GA sensitivity compared to wild-type. Because *ERA8* had higher ABA sensitivity compared to wild type even after the mutant had after-ripened sufficiently to germinate well in the absence of hormone (16 weeks AR), we hypothesized that the *ERA8* mutation may either alter endogenous ABA hormone levels or alter ABA signaling.

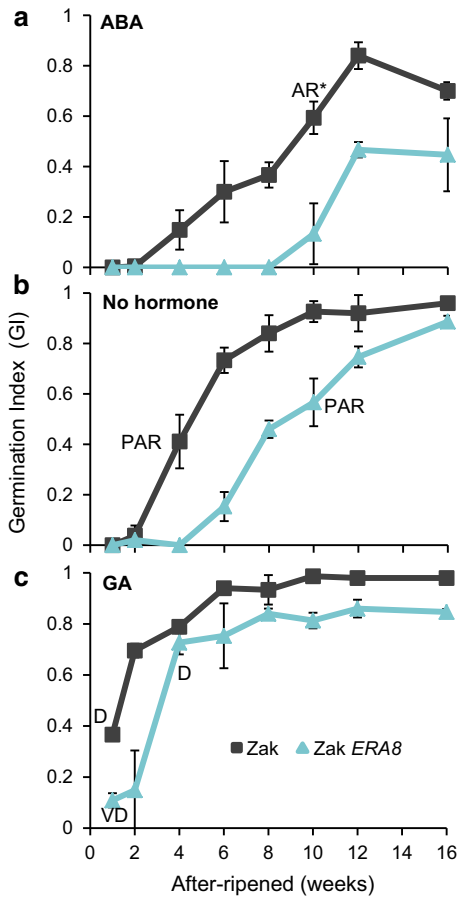

Fig. 3 Germination over an after-ripening time course of Zak (*square*) and Zak *ERA8* (*triangle*). Grain was plated at the indicated number of weeks after harvest at PM and germination index determined on media containing **a** 5 μM ABA, **b** no hormone, and **c** 10 μM GA_3. Hormone profiling was performed on 1, 4, and 10 weeks after-ripening time points labeled very dormant (*VD*), dormant (*D*), partially after-ripened (*PAR*), and after-ripened (*AR**). Means and standard deviation were calculated over three technical repetitions of 10 seeds each. (Color figure online)

Table 1 Zak and *ERA8*'s germination profiles during different dormancy stages

Genotype	Week	ABA[a]	No hormone[b]	GA[c]	Stage[d]
Zak	1	0	0	0.37	D
	4	0.15	0.41	0.79	PAR
	10	0.59	0.93	0.99	AR*
Zak *ERA8*	1	0	0	0.11	VD
	4	0	0	0.73	D
	10	0.13	0.57	0.81	PAR

[a] Germination index (GI) on 5 μM ABA

[b] GI in the absence of hormone

[c] GI on 10 μM GA_3

[d] Stages of dormancy loss

The effect of *ERA8* and of dormancy loss through after-ripening on endogenous ABA, GA_1, JA, JA-Ile, or IAA hormone levels was examined by ESI-LC–MS/MS analysis. Hormone measurements were performed using seeds from the 8 and 18 h imbibition time points of the after-ripening time course shown in Fig. 3. Hormone levels were measured in embryos, and in the "aleurone" sample consisting of a dorsal section including pericarp, seed coat, aleurone, and a small amount of endosperm tissue. The goal was to examine whether embryo and aleurone hormone levels resemble each other. Four stages of after-ripening were defined based on germination without hormone, and on ABA and GA (as in Tuttle et al. 2015). *ERA8* was very dormant (VD) and Zak dormant (D) at 1 week AR. *ERA8* was dormant (D) and Zak partially after-ripened (PAR) at 4 weeks AR, while *ERA8* was partly after-ripened (PAR) and Zak very after-ripened (AR*)

by 10 weeks AR (Table 1). Within 16 weeks of AR, neither the Zak nor *ERA8* genotype reached the fully after-ripened (FAR) stage where wheat seeds become completely ABA insensitive. Hormone levels were compared first in a model including only Zak and *ERA8* genotypes. To examine whether the hormonal differences associated with elevated *ERA8* seed dormancy could also be seen in an unrelated cultivar with similar PHS tolerance, a second model was generated comparing hormone levels in Zak, *ERA8*, and the PHS tolerant cultivar Brevor based on dormancy stages. The Brevor hormone profiling was an independent experiment examining grain at the VD, D, and FAR stages in dormancy loss (germination shown in ESM Fig. S4). In all experiments, GA_1 levels were below the detection limit. Thus, only comparisons of ABA, JA, JA-Ile, and IAA are discussed. The broad goal was to examine whether higher dormancy-promoting hormone levels (ABA and IAA) were generally associated with more dormant stages, whereas possible dormancy-breaking hormones (JA and JA-Ile) were associated with less dormant stages.

The effect of *ERA8* on bioactive ABA levels during dormancy loss

If Zak *ERA8* is ABA hypersensitive due to the decreased ability to turnover ABA, then we would expect the ABA hypersensitive phenotype to be associated with highly elevated endogenous ABA levels as observed in the Arabidopsis ABA 8′-hydroxylase mutant (i.e. ninefold increase, Okamoto et al. 2006). At 1 week AR in embryos imbibed for 8 or 18 h, ABA levels were similar in Zak and in *ERA8* (Fig. 4a, b). However, *ERA8* had lower germination potential than Zak (GI = 0.11 vs. GI = 0.37 on GA), suggesting that the higher initial dormancy in *ERA8* may be due to increased ABA responsiveness rather than to increased ABA accumulation (Table 1). ABA levels decreased with after-ripening of both Zak and *ERA8* embryos, indicating that ABA turnover does occur during *ERA8* after-ripening (Fig. 4a, b). However, ABA levels declined more slowly with *ERA8* than Zak after-ripening, with significant differences observed at 4 and 10 weeks AR ($p \leq 0.0001$; Fig. 4b). Thus, slower dormancy loss with after-ripening was associated with a slower decrease in ABA levels. *ERA8* ABA levels were actually lower than Zak ABA levels at 10 weeks AR with 8 h imbibition ($p < 0.05$).

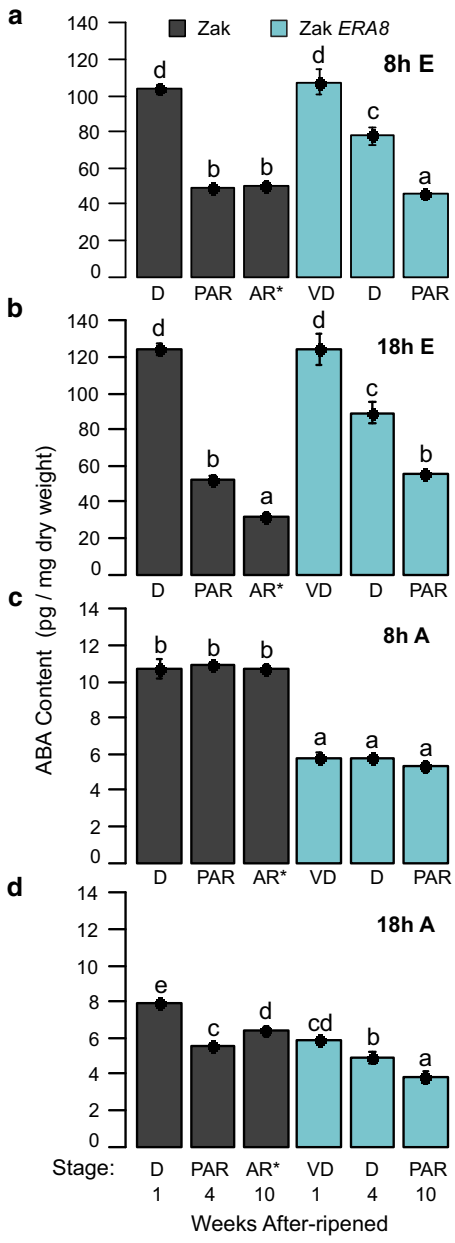

Fig. 4 The effect of after-ripening on Zak (*black*) and Zak *ERA8* (*blue*) endogenous ABA levels. ABA hormone content of Zak and Zak *ERA8* in embryo (E) (**a**, **b**) and aleurone (A) (**c**, **d**) samples imbibed for 8 h (**a**, **c**) or 18 h (**b**, **d**). *Different lower-case letters* indicate significantly ($p \leq 0.05$) different statistical classes. LS-means and SE bars are shown (n = 5). (Color figure online)

Endogenous embryo ABA content was negatively correlated with % germination on day 5 in the absence of hormone ($r = -0.79$, $p < 0.0001$) and in the presence of ABA ($r = -0.86$, $p < 0.0001$). This is

consistent with previous work suggesting that elevated ABA levels lead to higher wheat grain dormancy (Walker-Simmons 1987).

In contrast to embryos, the ABA levels in the aleurone sample were significantly lower in *ERA8* than in Zak and Brevor at 8 h of imbibition ($p < 0.0001$, Fig. 4c; ESM Fig. S5c). Thus, the increased dormancy phenotype of *ERA8* is not associated with increased aleurone ABA levels. Wild-type aleurone ABA levels decreased by 18 h, resulting in a less dramatic difference in Zak and *ERA8* ABA levels (Fig. 4d). At 8 h imbibition, ABA hormone levels in the aleurone did not change significantly with after-ripening for either Zak or *ERA8* (Fig. 4c). However, aleurone ABA levels declined with after-ripening at 18 h imbibition (Fig. 4d). ABA content was generally higher (30–135 pg mg DW^{-1}) in embryos than in aleurone (3–11 pg mg DW^{-1}) at 8 h imbibition (Fig. 4a, c).

Next, embryo ABA levels in Brevor were compared to Zak and Zak *ERA8* based on dormancy stage (ESM Fig. S5). At the VD stage, Brevor had lower ABA levels than VD *ERA8* or D Zak. However, Brevor and *ERA8* ABA levels were similar at the D stage. All three genotypes showed decreasing ABA levels with after-ripening ($p < 0.006$), and increasing embryo ABA levels with longer imbibition (8 vs. 18 h, $p = 0.0002$). Lower dormancy correlated with lower embryo ABA levels ($r = -0.82$, $p < 0.0001$). Thus, the association of higher grain dormancy with higher ABA levels is not unique to cultivar Zak.

The effect of *ERA8* on accumulation of jasmonates

Because previous work suggested that JA signaling stimulates cereal grain dormancy loss, we hypothesized that endogenous jasmonate levels should increase with dormancy loss (Barrero et al. 2009; Jacobsen et al. 2013). The precursor JA becomes biologically active upon conjugation to the amino acid isoleucine to form JA-Ile (reviewed in Browse 2009). In general, the changes in precursor JA and bioactive JA-Ile levels showed similar trends (Fig. 5). However, JA hormone levels were significantly higher and JA-Ile levels lower in the aleurone than in the embryo ($p < 0.0001$). At 8 h of imbibition, JA and JA-Ile levels both showed a tendency to increase with after-ripening; statistically significant increases in JA-Ile were observed in the aleurone but not the embryo for Zak and *ERA8* (Fig. 5a, e, g). For the Zak and *ERA8* dataset, JA ($r = 0.37$, $p = 0.003$) but not JA-Ile ($r = 0.009$, $p = 0.92$) showed a strong positive correlation with GI. This is only partly consistent with the notion that higher JA signaling stimulates wheat germination with after-ripening.

Next, we examined whether the *ERA8* dormancy phenotype was associated with decreased JA or JA-Ile levels. *ERA8* had significantly higher embryonic JA-Ile levels than Zak at 8 and 18 h imbibition (Fig. 5e, f; i.e., 1.8-fold higher at 1 week AR). This may be an attempt to overcome the increased seed dormancy rather than a cause of increased seed dormancy. Moreover, Zak showed a larger decrease in embryo JA-Ile content during imbibition than *ERA8*.

The dormant cultivar Brevor showed several similarities to *ERA8* (ESM Fig. S6, Fig. S7). Like *ERA8*, Brevor JA-Ile levels were lower in D than in VD grain with 18 h of imbibition. This decrease was not observed in Zak because Zak was already at stage D with 1 week of after-ripening. Like Zak and *ERA8*, Brevor JA-Ile levels were much lower in aleurone than in embryos ($p < 0.0001$). Thus, the observation that embryo JA-Ile levels decrease with early after-ripening was not unique to *ERA8*.

ERA8 dormancy is associated with elevated aleurone IAA levels

The most impressive differences in wild type and *ERA8* IAA levels were observed in the aleurone. The higher dormancy of *ERA8* at 1 week of after-ripening was associated with a large and significant increase in aleurone IAA levels compared to Zak at 8 and 18 h of imbibition (Fig. 6c, d); *ERA8* IAA levels were twofold higher at 18 h. Aleurone IAA levels decreased with early after-ripening in *ERA8* at 8 and 18 h (by 25–32 pg/mg at 18 h, $p < 0.0001$) and in Zak at 18 h of imbibition (by 3.7 pg/mg, $p = 0.001$). The elevated dormancy in Brevor at the VD and D stages was not associated with elevated aleurone IAA levels (ESM Fig. S8). Thus, the *ERA8* phenotype was uniquely associated with increased IAA accumulation.

Embryo IAA levels were slightly lower in *ERA8* than in Zak at 8 h of imbibition ($p = 0.048$, Fig. 6a). Embryo IAA levels sometimes increased later in after-ripening (Fig. 6a, b). These trends were observed in the Zak background, but not in Brevor. Instead Brevor showed a significant increase in IAA levels in D versus

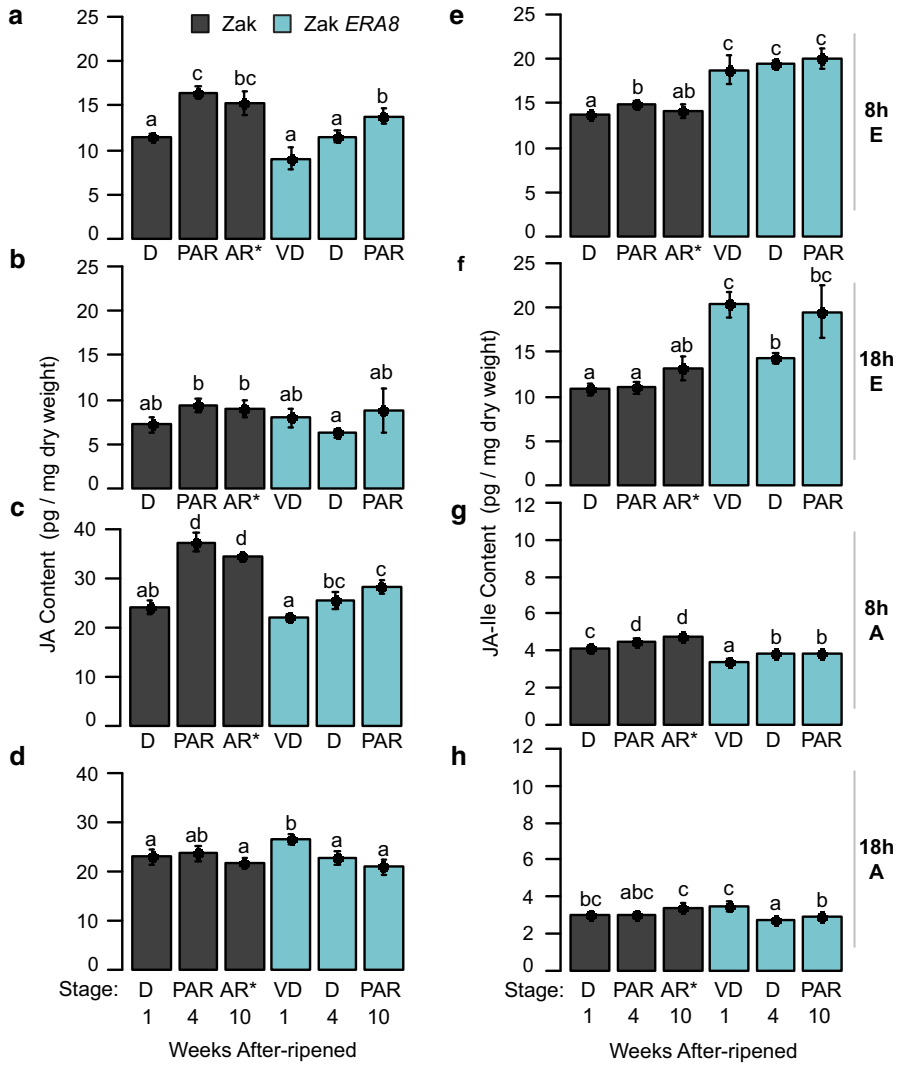

Fig. 5 The effect of after-ripening on Zak (*black*) and Zak *ERA8* (*blue*) endogenous JA and JA-Ile levels. JA (**a–d**) and JA-Ile (**e–h**) hormone content of Zak and Zak *ERA8* in embryo (E) (**a**, **b**, **e**, **f**) and aleurone (A) (**c**, **d**, **g**, **h**) samples imbibed for 8 h (**a**, **c**, **e**, **g**) or 18 h (**b**, **d**, **f**, **h**). *Different lower-case letters* indicate significantly ($p \leq 0.05$) different statistical classes. LS-means and SE bars are shown (n = 5). (Color figure online)

FAR (ESM Fig. S8). Aleurone IAA levels significantly decreased with imbibition in Zak and *ERA8* ($p < 0.0001$, Fig. 6c, d), and in D and FAR Brevor ($p < 0.0001$; ESM Fig. S8).

Discussion

The *ERA8* mutation results in a dose-dependent increase in ABA sensitivity, associated with increased seed dormancy and PHS tolerance (Fig. 1; Schramm et al. 2013). Wheat cultivars with white kernels tend to be more PHS susceptible than red. Thus, a strong single gene trait providing increased PHS tolerance may be useful in breeding white wheat. Previous work showed that dormancy loss through after-ripening and cold stratification is associated with increasing GA and decreasing ABA sensitivity over time (Tuttle et al. 2015). In general, high dormancy/PHS tolerant cultivars lose dormancy more slowly, and thus show slower changes in ABA and GA sensitivity. Consistent with this, PHS tolerant *ERA8* gained GA and lost ABA sensitivity more slowly with after-ripening and cold stratification than wild-type Zak (Figs. 2, 3). Hormone profiling showed that increased *ERA8* seed dormancy was also associated with differences in the accumulation of the plant hormones ABA, JA, JA-Ile, and IAA over an after-ripening time course.

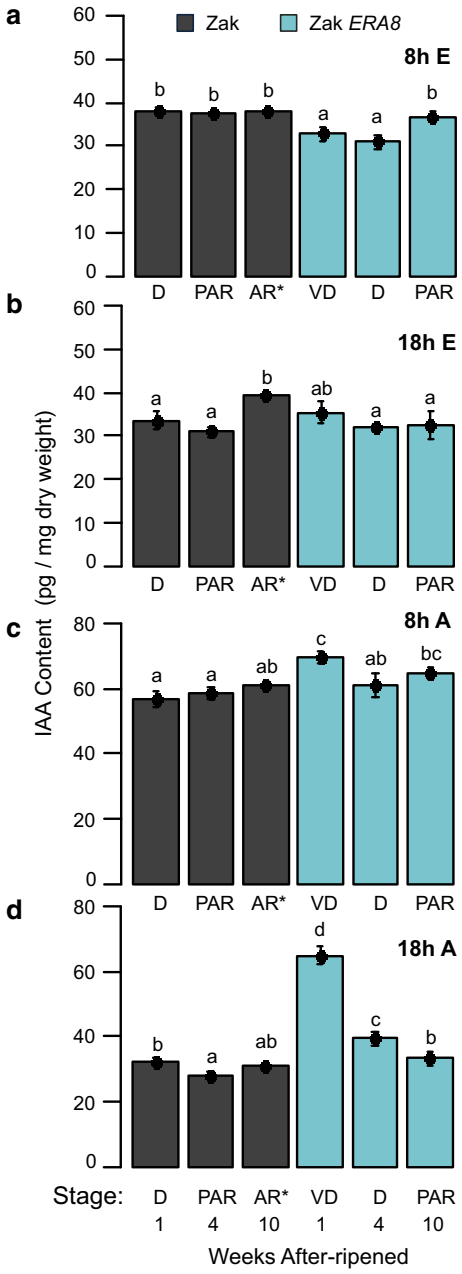

Fig. 6 The effect of after-ripening on Zak (*black*) and Zak *ERA8* (*blue*) endogenous IAA levels. IAA hormone content of Zak and Zak *ERA8* in embryo (E) (**a**, **b**) and aleurone (A) (**c**, **d**) samples imbibed for 8 h (**a**, **c**) or 18 h (**b**, **d**). *Different lowercase letters* indicate significantly ($p \leq 0.05$) different statistical classes. LS-means and SE bars are shown (n = 5). (Color figure online)

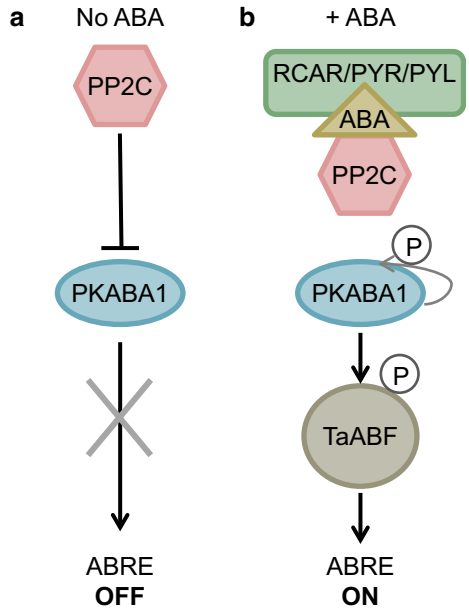

Fig. 7 Model showing positive and negative regulators of ABA signaling. **a** Without ABA, the protein phosphatase type 2C (PP2C) represses ABA signaling and ABA-responsive gene expression by inactivating the PKABA1 SNF-related kinase through dephosophorylation. **b** ABA-binding causes a conformational change in the RCAR/PYR/PYL ABA receptor, allowing interaction with and inactivation of the PP2C negative regulator. Without the PP2C, the PKABA1 kinase activates itself by autophosphorylation, and then activates ABRE-binding transcription factors (ABF) by phosphorylation. ABF activates ABA-responsive genes via binding of the ABRE promoter element. The semi-dominant *ERA8* ABA hypersensitive phenotype could result from a gain-of-function mutation in positive regulators of ABA signaling such as an RCAR/PYR/PYL receptor, a PKABA1 kinase, or an ABF transcription factor gene. Alternatively, *ERA8* could be a dominant negative allele of a negative regulator such as a PP2C phosphtase. (Color figure online)

Zak *ERA8* may be an ABA hormone response mutant

The semi-dominant *ERA8* mutation was identified in a screen for ABA hypersensitive seed germination (Schramm et al. 2013). An ABA hypersensitive phenotype could result either from a gain-of-function mutation in a positive element of the ABA signaling pathway or from a mutation resulting in an inability to turnover ABA hormone (i.e., loss of the catabolic

enzyme ABA 8′-hydroxylase, Fig. 7, Ghassemian et al. 2000; Johnson et al. 2002; Millar et al. 2006; Cutler et al. 1996). If the *ERA8* phenotype results from reduction in ABA turnover, then ABA hypersensitivity should be associated with elevated ABA levels and an inability to turnover ABA with after-ripening. Loss of the Arabidopsis ABA 8′-hydroxylase resulted in a 3- 9-fold increase in seed ABA levels (Okamoto et al. 2006). *ERA8* resulted in no change in starting embryo ABA levels, and showed no more than a 1.7-fold higher ABA levels at any after-ripening time point (Fig. 4). *ERA8* clearly did not block ABA turnover since embryonic ABA levels decreased with after-ripening. It is unclear whether slower ABA turnover is a cause or an effect of higher *ERA8* seed dormancy. Since *ERA8* has more dormancy but similar ABA levels to Zak at 1 week AR, our working hypothesis is that *ERA8* is a mutation in an ABA signaling gene (Figs. 3, 4a, b).

Based on the known ABA signaling pathway, there are multiple ABA signaling genes that could result in a gain-of-function ABA hypersensitive phenotype (Fig. 7; Fujii et al. 2009). ABA is perceived by a receptor of the RCAR/PYR/PYL (REGULATORY COMPONENT of ABA RECEPTOR/PYRABACTIN RESISTANCE/PYRABACTIN RESISTANCE-LIKE) family (Ma et al. 2009; Nishimura et al. 2009; Park et al. 2009; Santiago et al. 2009). ABA-binding causes a conformational change in the ABA receptor, allowing it to bind the negative regulators of ABA signaling, the ABI1 (ABA INSENSITIVE 1) family of protein phosphatase type 2C (PP2C) proteins. ABA receptor binding to the PP2C prevents it from cleaving the phosphate group from the SnRK kinase family member encoded by the wheat PKABA1 (PROTEIN KINASE-ABA1) gene family (Anderberg and Walker-Simmons 1992; Nakashima et al. 2009). When the PP2C is inactive, PKABA1 is able to activate itself by autophosphorylation, and then activate ABI5-family bZIP (basic leucine zipper) transcription factors by phosphorylation (Fujii et al. 2007, 2009). These ABI5-family members activate transcription by binding the ABA response promoter element (ABRE). The wheat bZIP family member, *Triticum aestivum* ABRE-binding factor (TaABF), was identified as a PKABA1-interacting protein (Johnson et al. 2002, 2008). Gain-of-function mutations in positive regulators of ABA signaling, such as the ABA receptor, PKABA1, or TaABF family members, could result in an ABA hypersensitive germination phenotype. Since both PKABA1 and TaABF are positively regulated by phosphorylation, it is possible that such an allele could result from a phosphomimic mutation, such as a serine to aspartate amino acid change.

Several loss-of-function mutations providing ABA hypersensitive germination have been cloned, such as the farnesyl transferase *ERA1*, the mRNA CAP binding protein *ABA HYPERSENSITIVE1* (*ABH1*), and the putative RNA editing pentatricopeptide repeat protein *ABA HYPERSENSITIVE GERMINATION11* (*AHG11*) (Cutler et al. 1996; Hugouvieux et al. 2001; Murayama et al. 2012). These genes are unlikely candidates for the semi-dominant wheat *ERA8* mutation, unless it is possible to recover a dominant-interfering allele. One *ERA8* candidate is the gain-of-function *AHG12* mutant conferring ABA hypersensitive germination in Arabidopsis (Murayama et al. 2012). This gene has been mapped, but not cloned.

After-ripening is associated with increasing JA hormone levels

The plant hormones GA and JA have been implicated in stimulating cereal seed germination (Barrero et al. 2009; Jacobsen et al. 2013; Tuttle et al. 2015; Xu et al. 2016). GA stimulates germination in a wide range of species including Arabidopsis and wheat. The hormone profiling approach was not sensitive enough to detect significant differences in bioactive gibberellins GA_1 and GA_4 with after-ripening and in the *ERA8* mutant. However, we were able to detect an increase in GA sensitivity with after-ripening, and the *ERA8* mutant was associated with decreased sensitivity to exogenous GA. GA-insensitive mutants, such as Arabidopsis *sly1* alleles, can result in increased ABA sensitivity associated with dwarfism (Steber et al. 1998). Given that the *ERA8* mutant is not associated with a dwarf phenotype, it is more likely the result of an ABA than GA signaling defect (Schramm et al. 2013; Martinez et al. 2014). An increase in GA levels may have been detected if more tissue had been used, or if seeds had been after-ripened and/or imbibed longer. However, long after-ripened wheat seeds sometimes germinated after only 18 h of imbibition (ESM Fig. S4). A small increase in GA_1 was detected with barley after-ripening and 30 h of imbibition (Jacobsen et al. 2002). However, after-ripening was

associated with increased accumulation of GA precursors with as little as 6 h of imbibition. Thus, future work should examine the effect of after-ripening on GA precursors, and optimize GA_1 extraction from wheat seed tissue.

If JA signaling positively regulates seed germination in wheat, then we would have expected to see increasing JA and JA-Ile levels with increasing germination capacity. There was a positive correlation between JA levels, but not JA-Ile, and germinability. This may either mean that after-ripening does not stimulate germination through increased JA-Ile levels, or it may mean that we missed a transient increase early in imbibition followed by the detected decrease (negative feedback regulation) in JA-Ile levels. In this case, increased JA levels may represent the increased potential to synthesize bioactive JA-Ile. The *decrease* in embryonic JA-Ile levels during the VD to D dormancy loss in *ERA8* and Brevor at 18 h of imbibition may result from negative feedback regulation of JA-Ile (Fig. 5; ESM Fig. S7). Moreover, the elevated JA-Ile levels in dormant *ERA8* compared to wild-type embryos at every after-ripening time point may represent positive regulation of JA-Ile biosynthesis to counteract the effects of increased ABA signaling. Thus, future work will need to examine if JA-Ile stimulation of wheat seed germination is subject to complex forward and negative feedback regulation.

Many lines of evidence support the notion that JA signaling stimulates germination. Previous work showed an increase in JA-Ile with cold stratification (Tuttle et al. 2015; Xu et al. 2016). Xu et al. (2016) also saw no increase in JA-Ile as mildly dormant grains after-ripened, and suggested that JA-Ile plays a role in stimulating germination through cold stratification, not through after-ripening. This theory is inconsistent with transcriptome analysis showing that barley after-ripening was associated with increased JA-responsive gene expression in coleorhiza (Barrero et al. 2009). It may be that the ability to detect an increase in JA levels with after-ripening depends on having very dormant grain (ESM Fig. S6a). The increase in JA early in imbibition was consistent with previous observations in barley and wheat suggesting that JA may stimulate cereal seed germination (Barrero et al. 2009; Jacobsen et al. 2013). Moreover, inhibition of JA biosynthesis using acetylsalicylic acid was associated with decreased wheat seed germination, whereas methyl-jasmonate stimulated the germination of dormant wheat grain (Jacobsen et al. 2013; Xu et al. 2016). JA may also stimulate Arabidopsis seed dormancy and germination given that the JA resistant mutants, *coi1-16* and *jar1*, show increased ABA sensitivity during seed germination (Ellis and Turner 2002; Staswick and Tiryaki 2004).

Differences in ABA and IAA levels in embryo and aleurone samples

Hormone profiling revealed that ABA and IAA respond differently to dormancy loss and *ERA8* in embryo and aleurone samples (Figs. 4, 6). Embryo ABA levels decreased with after-ripening by 8 h of imbibition, but aleurone ABA levels did not decrease until 18 h of imbibition. The higher dormancy in *ERA8* was associated with lower aleurone ABA levels, suggesting that higher aleurone ABA levels may not be needed to inhibit seed germination per se. ABA inhibits the mobilization of stored reserves in the endosperm (Gilroy and Jones 1992). It appears that ABA-inhibition of stored reserve mobilization is lifted later than inhibition of embryo germination. Similar differences in embryo/scutellum versus aleurone/endosperm ABA levels were observed in barley (Jacobsen et al. 2002). Lower embryo ABA levels correlated with increasing germination potential in all genotypes examined here (Fig. 4; ESM Fig. S5). Higher embryo ABA levels were associated with higher seed dormancy in PHS tolerant cultivars in other studies (Morris et al. 1991; Jacobsen et al. 2013), whereas whole grain ABA levels failed to decrease with after-ripening in Liu et al. (2013). Taken together, these studies suggest that endogenous embryo ABA levels determine germination capacity, and that changes in embryo ABA levels may be hard to detect in whole grain samples.

Interestingly, IAA was found to overaccumulate in *ERA8* aleurone samples. Moreover, aleurone, but not embryo, IAA levels decreased with after-ripening (Fig. 6). IAA levels also decreased with after-ripening of intact imbibing 'AC Domain' grains (Liu et al. 2013). Auxin application can inhibit the germination of dormant/PHS tolerant wheat embryos, suggesting that auxin signaling may inhibit dormant seed germination (Ramaih et al. 2003). Based on Arabidopsis, mutants resulting in IAA overaccumulation are actually somewhat rare (reviewed by Korasick et al. 2013). However, several previous studies have observed

changes in ABA sensitivity in auxin response mutants (Liu et al. 2007; Strader et al. 2008; Rinaldi et al. 2012). The *ibr5* and *iaa16-1* mutants showed increased resistance to auxin associated with ABA insensitivity. Moreover, *ARF10* mutants resistant to regulation by the microRNA160 showed an ABA-hypersensitive phenotype. Thus, future work should further explore the possible connection between *ERA8* and auxin signaling.

This study points to the importance of measuring embryo and aleurone hormone levels separately when possible. It is possible that undetected hormonal changes in specific compartments play important regulatory roles in germination. Recent progress in the development of fluorescently tagged hormones has allowed visualization of changing GA hormone levels in whole roots in response to gravity (Löfke et al. 2013). Such tools may allow future research to better address where and when hormonal changes occur in response to seed dormancy loss and germination.

Increased preharvest sprouting tolerance in Zak *ERA8*

This study used increased ABA sensitivity to overcome the low dormancy and PHS susceptibility of white-kernelled cultivar, Zak (Fig. 1). However, too much seed dormancy can result in reduced yield if seeds do not germinate well when planted in the spring. Some highly PHS tolerant lines, like Brevor wheat, can require 9–12 months to after-ripen (Schramm et al. 2013; Tuttle et al. 2015). Ideally, wheat breeders need to select wheat cultivars with sufficiently high dormancy at maturity to prevent PHS, but that after-ripen quickly enough to allow efficient germination of fall-planted winter wheat. The increased dormancy in *ERA8* directly resulted in increased PHS tolerance, but after-ripened within 8–10 weeks. When Zak and *ERA8* were plated 9 weeks after combine harvesting, Zak showed 98.2 % (SD ± 4.1) and *ERA8* 91.5 % (SD ± 8.8) germination. This supports the notion that the *ERA8* gene may improve PHS tolerance without seriously sacrificing yield (Martinez et al. 2014). Preharvest sprouting is generally more severe when the rain event in the field is associated with lower temperature (Biddulph et al. 2005). Interestingly, the fact that *ERA8* lost dormancy more slowly than wild-type was more apparent over the after-ripening than the cold stratification time course (Figs. 2, 3). Some cultivars with strong seed dormancy fail to germinate at low temperature, while others lose dormancy rapidly at lower temperature (Kashiwakura et al. 2016). At low temperature, *ERA8* showed decreased germination on ABA, but not without hormone (Fig. S1). Future work will need to investigate whether the *ERA8* mutation is more effective at preventing PHS at higher than at lower temperatures.

Acknowledgments The authors sincerely thank Dr. Mark E. Sorrells for performing spike-wetting tests in 2008 and 2010. Thanks are due to Rehana S. Parveen and Dustan Cwick for expert assistance. Thanks are also due to the USDA-ARS field crew for planting and harvesting. A special thanks to the Garland-Campbell and Steber labs for helpful feedback on the research and manuscript.

Funding This work was funded by the National Science Foundation's East Asia and Pacific Summer Institute Award grant 1209878 (to K.M.T.), the Japanese Society for the Promotion of Science Summer Internship Award (to K.M.T. and M.S.), the Washington Grain Alliance (to C.M.S.), and the USDA-ARS (to C.M.S. and K.G.C.).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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